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Final lecture of the 10th AIEC Congress, an homage to Manuel Carrillo career by his friend Olivier Kah

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PRESENTATION

The present volume of Advances in Comparative Endocrinology collects the contributions of the participants at the 10th Congress of the Iberian Association of Comparative Endocrinology (AIEC). Eighteen years after the foundational meeting of our Association in Peñíscola, the return of this Congress to Castellón highlights the growing success of this initiative to foster the research and scientific development in the field of comparative endocrinology developed in the Iberian Peninsula. AIEC meetings have proven to be a way to keep in contact among research groups with common interests. Some of the participants in this last meeting were also present in the foundational one, others members came after and keep assisting every time. As one of the aims of AIEC has been to encourage students to participate, we are particulary proud of those young students and doctors from the first editions that have gained more permanent positions and continue participating in the AIEC meetings with new students.

A variety of studies have been grouped in the present volume, that present the latest advances of participating groups in scientific areas as reproduction, energy metabolism, wide functional analysis, stress and immune response, growth factors and molecular evolution, mineralization and pigmentation. We are grateful to all the contributors for their active participation and the interesting discussions generated during the sessions.

This 10th Congress has been also special as we celebrated the first conference held in honor to Prof. Josep Planas, presented by his foster student Prof. Joaquim Gutiérrez, which is reflected in the text that opens this book. We aim to continue this lecture in future meetings by inviting prominent scientists related to comparative endocrinology.

As editors, we acknowledge the scientific and material help of AIEC board to arrange this meeting. This is extensive to the valuable work of the members of the local organizing committee (Alicia Felip, Josep Calduch, José Miguel Cerdá, Ana Gómez, Félix Hernández, Evaristo Mañanós, Jaime Nácher and Jaume Pérez). We are also grateful to the Fundación Universidad-Empresa of Universitat Jaume I of Castellón for their valuable assistance in the organization and the excellent infrastructures holding the meeting. Finally, we would like to express our gratitude to “Publicacions de la Universitat Jaume I”, for their help in the edition of the present volume.

We hope that this publication serves as a stimulus to continue improving our research in comparative endocrinology. We wish the best for the organizers of the 11th AIEC meeting in Vigo 2017.

The Editors

Josep Calduch-Giner, José Miguel Cerdá-Reverter, Jaume Pérez-Sánchez

FROM INSULIN TO IGFs IN FISH: A SCIENTIFIC JOURNEY

J. Gutiérrez, I. Navarro, J. Fernández-Borràs, J. Blasco, J.V. Planas, E.J. Vélez, E. Lutfi, E. Capilla

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Professor José Planas should be considered one of the first comparative endocrinologists of Spain and Portugal and AIEC decided to create a lecture that will have his name and will extend during the conferences of our association. In the first of these lectures, we tried to summarize Professor Planas' main contributions to the field of fish endocrinology as well as the continuation by members of his team at the School of Biology of the University of Barcelona. It is for us an honor and a pleasure to contribute now with this chapter and we would like to express our sincere gratitude to AIEC for it.

Introduction

A lecture in honor of Professor José Planas has been created in the 10th congress of AIEC and we believe that it is fair to dedicate it to who can be considered one of the first comparative endocrinologists in Spain. For us it was a double honor to give that lecture. First because, Professor Planas was the head of the Department and the thesis director of some of the co-authors, who furthermore represent the continuity after several years of his original research line on fish insulin, at the School of Biology of the University of Barcelona (UB). And second, because we were involved in this society since the very beginning, contributing to its creation and then, occupying different positions in the direction. Thus, on behalf of the Department of Physiology and Immunology at the School of Biology at UB, our group of research and Professor Planas' family, we want to express our sincere gratitude to the AIEC association for creating this lecture.

The objective of this chapter was to review Professor Planas' contribution to understanding the fish endocrine pancreas and then to examine how his team at UB has followed this line of research during the last years.

1. First studies of fish insulin

Professor Planas presented his thesis at UB in 1955, directed by Professor F. Ponz, and proposed by Professor García del Cid. The thesis was entitled "Insulin extraction from tuna (*Thunnus thynnus*, L.)" and demonstrated his early interest in fish hormones (Figure 1). The work was focused in the localization, identification, and cytology of tuna endocrine pancreas. The study differentiated the β and α cells and the little part of exocrine pancreas. Insulin extraction from tuna pancreas and its biological activity was also established and the possibility to use it in some cases of diabetes was suggested (1, 2). Tuna glucagon was also studied showing its hyperglycemic effects (3).

Next, he obtained a position of Professor at the University of Valladolid (1958-66). Although he was trying to continue with fish endocrinology, facing real problems to obtain fish samples, he started a new line of research on iron metabolism. At that time chicken agronomy was a florescent business and there were plenty of possibilities to investigate around it. On the contrary, to obtain fish was quite hard.

Professor Planas always remembered us his visit to the Engineer in chief of the zone, looking for some kind of list of the different fish species that were present in the lakes and rivers. The Engineer answered him that they were simply fish. So, laying hens turned in a very good model and a very productive line of research, as he published four papers in Nature (4-7).

Figure 1. J. Planas sampling tuna pancreas at Almadrava, Tarragona (1951-1952).

Professor Planas returned to Barcelona continuing the line of research of iron physiology and after a period at the University of Florida (1972), he recovered the line of research in fish, directing the thesis of S. Zanuy and M. Carrillo (1975) and later those of J. Fernández-Borràs and J. Gutiérrez. So, in collaboration with Dr. E. Montoya and Dr. M. Chaves, from the Department of Biochemistry, we first obtained tuna insulin antibodies and then, we set up the specific radioimmunoassay (RIA) of insulin with T. Llorens from Hospital Clinic help. Dr. A. Thorpe and Dr. A. Duve (Queen Mary College, London), recognized experts on comparative insulin, contributed to validate the RIA. With M. Carrillo and S. Zanuy the first paper on sea bass insulin levels was then published (8).

The endogenous insulin cycles were the first studies performed and we observed diurnal rhythm of insulin with a peak at night opposite to that of glucose. The annual cycle was important in ectotherms and different fish species were studied. Sea bass presented maximum insulinaemia in summer and the inverse for glucose (9). Dogfish showed a cycle with an insulin maximum in March coincident with the lowest level of plasma glucose (10).

In 1986 the first international report on fish plasma glucagon levels was published. Based on the similarity of amino acid (AA) sequences between vertebrates, we adapted the mammalian RIA to measure plasma levels of glucagon in fish species (11). I. Navarro incorporated to the team to do the thesis and we completed the study with isolation of tuna glucagon and RIA improvement (12, 13). With these tools we did different studies in sea bass, trout and carp at UB and also in collaboration with IATS and J. Pérez-Sánchez doing the thesis. So, postprandial levels of insulin,

glucagon and glucose were described, with a first peak of insulin and then the increase of glucagon in parallel to that of glucose in sea bass (14). The post feeding profile was very similar to that in trout, where the glucagon peak was also clear and coincident with that of glucose. Fasting provoked insulin levels decrease, while glucagon presented a peak at 5 days of fasting (15). Differential effects of AA and glucose on insulin secretion were described (16). In carp, glucagon showed a clear increase when fish were exposed to 28ºC (17). The hypoglycemic effect of insulin injection in sea bass was also demonstrated (18). With all those studies a good background on insulin and glucagon function in fish was available and a new adventure was started.

2. The insulin, IGF-I and IGF-II receptors in fish

Insulin receptor research was initiated in collaboration with Dr. E.M. Plisetskaya in Seattle (1988-89). First data on insulin receptors were in salmon liver and their changes during the smoltification showed the highest binding in parallel to lowest insulin levels (19). Insulin receptor in trout muscle and its response to diet adaptation was studied, showing an increase in number and binding in response to high carbohydrate diets (20). Back to UB, we continued this line of research sharing with Professor Planas the new projects. At that time, we celebrated a party in honor of Professor Planas when he was proposed by the Rector as Professor Emeritus of UB.

To better understand insulin receptors, also in collaboration with Dr. Plisetskaya, we compared them in different fish species (21) demonstrating that trout presented low levels of insulin receptors in muscle while in carp those were significantly higher; and the receptor tyrosine kinase activity (TKA) was following the same pattern. Next, we investigated whether the insulin receptors could be regulated and we found up-regulation in number and binding in muscle and adipose tissue in response to an increase in insulin (16). An increase of insulin receptors was also observed as an adaptation to diets with high levels of digestible carbohydrates (22). Our conclusion was that the up-regulation of insulin receptors could represent a good strategy to compensate the low levels of insulin receptors when necessary. However, when comparing muscle insulin receptors in fish with rat the differences were still very important.

At that time, we were also investigating IGF-I receptors and we were the first group publishing IGF-I receptors in fish (23) characterizing IGF-I receptors in cap ovaries and their seasonal variation.

In 1994, it was organized at the University of Córdoba the 17th ESCE and during the conference the organization offered Professor Planas a special plate to recognize him as a pioneer on Comparative Endocrinology. During this period Professor Planas and the group were very active publishing an interesting series of papers on phylogenetic aspects of insulin and IGF-I receptors and their role in fish glucose intolerance. A comparison of IGF-I and insulin receptors in different fish species was done, always showing the highest abundance of IGF-I receptors (24, 25).

But when compared the IGF-I/insulin receptor ratio in the muscle of different vertebrates, we found that this was maintained through ectotherms, but it was reversed when arriving to homeotherms; so in birds and mammals, insulin receptors were more abundant than those of IGF-I (25). This was the last real paper of Professor Planas and in fact it was great because the editor of American Journal of Physiology congratulated us for the interesting study of comparative physiology and endocrinology in vertebrates and he felt very proud for it.

From these studies we summarized that from an ancestral hybrid receptor of insulin and IGF-I (Amphioxus type receptor) the evolution separated these two different receptors; but in fish insulin receptors were still poorly represented. During vertebrate evolution, insulin has acquired more relevance both in receptor number and in TKA, to reach in mammals a very important role in metabolism regulation. And this was also followed by changes in the regulative function. So, while in fish insulin and IGF-I have both growth and metabolic roles, in mammals insulin has already specialized to exert a more important function in metabolism control.

Professor Planas died in 1995 as a consequence of cancer and “in memoriam” articles appeared in General and Comparative Endocrinology and in Comparative Biochemistry and Physiology.

3. IGFs regulation of muscle and adipose tissue growth and metabolism

After Dr. Planas passed away, the group followed his line of research. Josep Planas junior, returned to Spain after his thesis and different postdocs in USA and together with E. Capilla published the first report of a fish Glut4 (26), its characterization and regulation, that helped to understand the low glucose tolerance in fish. Later, in 2001 the group published for the first time the existence of IGF-II receptors in ectotherms (27). But still we were trying to understand the respective role of IGF-I and insulin receptors in fish.

In collaboration with SCRIBE INRA we developed the primary culture of fish muscle satellite cells to study IGF-I function (28). IGFs and insulin actions were compared, demonstrating that IGFs were stronger than insulin stimulating glucose, AA, and fatty acids uptake by myocytes (29-31). These results pointed out the metabolic function of IGFs in fish muscle together with their proliferative role (29, 32).

Receptor signal transduction was also investigated and we found different responses depending on the stage of development (Figure 2). So, MAPK was very active to IGFs stimulation in myoblasts, but it was less responsive in myotubes, in comparison to AKT that even responded more at later stages (30, 33). Looking for markers for proliferation and differentiation, we focused then in regulation of muscle development in gilthead sea bream and in collaboration with international groups (J. Du and I.A. Johnston) different myogenic factors were characterized and studied through myocyte development (34). Myogenesis regulation by IGFs showed that IGF-I stimulated proliferation but at a lower level that IGF-II (35). In the same model, we found that IGF-II stimulated the gene expression of Myod and Myf5, while IGF-I did it on Myogenin, MRF4 and myosin heavy chain (36-38). So, both IGFs stimulate myogenesis, but IGF-II seems to be more involved in the regulation of proliferation and IGF-I more effective stimulating myocyte differentiation.

Moreover, TOR pathway is a key point on the regulation of protein synthesis. In gilthead sea bream myocytes, AA were able to increase TOR phosphorylation and gene expression activating its downstream molecules but IGF-I did not. Contrary, IGF-I stimulated AKT activity but not the AAs (37). However in the same *in vitro* model IGF-II increased TOR gene expression and phosphorylation, in agreement with the role of IGF-II in myocytes proliferation (38).

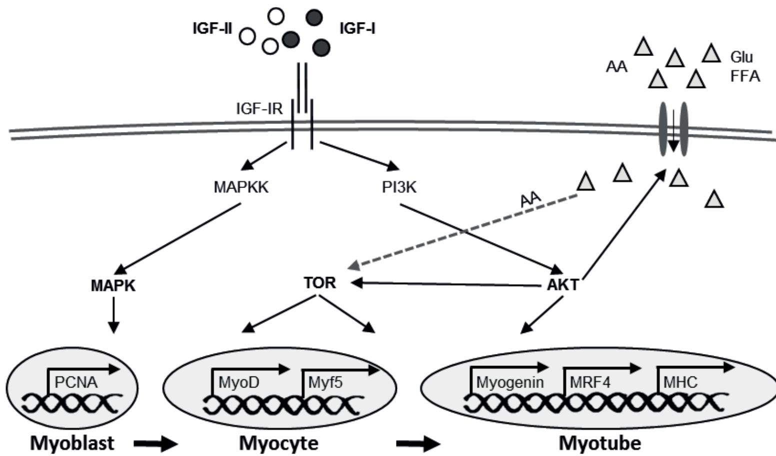

Figure 2. Summary model of the endocrine and nutritional regulation of the myogenic process. AA: amino acids; Glu: glucose: FFA: free fatty acids; PCNA: proliferating cell nuclear antigen; MHC: myosin heavy chain.

Drs. J. Fernández-Borràs, J. Blasco, and A. Ibarz have worked during the last years with a model of moderated and sustained forced swimming in trout and sea bream that resulted in improved growth without changes in food uptake (39). Modifications in metabolism, muscle structure and increase in plasma IGF-I were also observed (40, 41). In a recent experiment with 5 g sea bream, 5 weeks of forced swimming determined similarly significant higher final weigh, without change in food intake and decreasing mesenteric fat. Plasma GH levels decreased and those of IGF-I increased (42) and this was accompanied by changes in IGF-I, IGF-I and GH receptors expression in liver and muscle. Moreover, TOR increased its phosphorylation and gene expression in muscle of exercised fish (43). In recent years, Dr. Navarro and Dr. Capilla have developed an important research on the endocrine regulation of adipose tissue proliferation and differentiation, as well as the regulation on osteoblasts growth. We cannot explain these studies in this review but they represent an essential branch of the research in fish metabolism and growth that fits very well in the investigation for better nutrition and growth in fish that is being carried out in the Department of Professor Planas.

Interestingly many of the authors cited in this review have continued working in fish research and creating new teams resulting in new generations of researchers and thus, amplifying the first studies performed in tuna pancreas. However, all this research has the origin in the pioneer work of Professor Planas who was able to start it in a very difficult time. His influence has extended through different groups in Universities and Research Centers by means of his students and colleagues and over time we have been able to realize of his impressive contribution to Comparative Endocrinology and Physiology.

Acknowledgments

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ON THE SHOULDERS OF GH-IANTS: A SOMATOTROPE JOURNEY FROM COMPARATIVE TO TRANSLATIONAL ENDOCRINOLOGY

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Pituitary somatotropes provide a unique cell model to investigate the regulation of hormone secretion from an integrative viewpoint. Over the last thirty years, we have investigated multiple aspects of the biology of somatotropes, in a journey encompassing different animal species, organizational levels, and functional research targets. A retrospective view of the path covered reveals the genuine value of comparative endocrinology, and its potential to develop novel scientific knowledge. Our initial research, focused on frog pituitary, aimed at understanding the role of hypothalamic hormones on the regulation of somatotrope function. Combining in vivo and in vitro studies, immunocytochemistry and quantitative image analysis, we demonstrated for the first time that TRH is a stimulatory factor for frog somatotropes. This discovery was later extended by studies on cells from porcine and rat pituitary, and human pituitary tumors, which unveiled a multifactorial regulation of somatotropes across species by multiple neuropeptides, including GHRH, ghrelin, PACAP, TRH, somatostatin, cortistatin, kisspeptin, etc, and helped to define the common and unique signaling pathways and receptors mediating such regulation. Ultrastructural analysis of somatotropes led us to the notion of cell heterogeneity, and prompted us to investigate its biological meaning by characterizing the different subpopulations of somatotropes composing the pituitary during lifespan. Morphological and functional in vitro studies in pig and rat somatotropes unveiled a unique dynamic secretory cell cycle that includes not only hormone storage and secretion, but also the distinct ability of somatotropes to respond to regulatory cues through an integrated control of their molecular and cellular identity. Knowledge gathered in this travel led us to explore the pathological side of somatotropes and GH. First, by studying the cell biology of somatotropes from human pituitary tumors, we discovered and characterized the presence and pathological role of aberrantly spliced receptors for somatostatin (sst5TMD4) and of the In1-ghrelin variant in acromegaly patients, and examined their potential as therapeutic targets. A second area of study aims at understanding the pathophysiological importance, beyond somatic growth, of GH and somatotropes, which act as a true endo-metabolic hub, harmonically integrating multiple components of the homeostatic regulatory machinery of the glucose/insulin/IGF-I axis, whose deregulation critically influences pathologies like diabetes, obesity and cancer. Thus, departing from a comparative endocrinology onset, our journey provided us a unique opportunity to achieve scientific knowledge, enjoy discoveries, traverse fascinating translational research avenues, and treasure the privilege of receiving the wisdom from *GH-iant* mentors.

The somatotrope cell

The somatotrope is the main cell type of the anterior pituitary. Indeed, its proportion ranges 35-50% depending on the species, age, gender and physiologic conditions. It presents a round-oval shape, exhibiting the typical ultrastructure of secretory cells with numerous round secretory granules, well-developed rough endoplasmic reticulum, apparent Golgi complex and moderately abundant mitochondria. The main function of this capillary-oriented cell type is the production and secretion of growth hormone (GH).

GH (or somatotropin) is a protein hormone presents throughout the vertebrates [1] except the cyclostomes, where it has not been described [2]. Although GH is widely known by its pivotal role in the control of postnatal growth, it also contributes to regulate metabolism, reproduction, immunity, development, or osmoregulation in different species, and has been shown to be involved in the development of several types of tumors, cancers and other pathologies. GH secretion shows a pulsatile pattern in all species studied to date. Indeed, this pulsatile pattern can also exhibit a sexual dimorphism with discrete pulses and low interpeak levels in males and less pulsatility with higher interpeak levels in females [3]. It is widely accepted that this pulsatility is primarily controlled by the hypothalamus. Indeed, hypothalamic control of GH secretion in mammals has been long considered as a classic paradigm of the "dual control" system of pituitary hormone secretion. Thus, two hypothalamic peptides with opposite roles, GH-releasing hormone (GHRH) and somatotropin release-inhibiting factor (SRIF or somatostatin), directly regulate GH secretion [4]. However, the hypothalamic regulation of GH secretion is more complex and heterogeneous than initially envisioned and, additionally, this episodic secretion can be modulated by diverse factors residing in the target organ, the pituitary, other regions of central nervous system (CNS), or factors arriving from peripheral organs/tissues. Remarkably, in spite of the obvious group-specific differences, most of the main hypothalamic, pituitary, and peripheral factors involved in the control of the somatotropic function exert a comparable role in different groups across vertebrates and, therefore, although their relative importance has changed during evolution, comparative endocrinology has demonstrated a genuine value to develop novel scientific knowledge in this field.

In this regard, over the last thirty years, our group has investigated multiple aspects of the biology of somatotropes, in a journey encompassing different animal species, organizational levels, and functional research targets. Indeed, during our initial steps, the use of Basic and Comparative Endocrinology helped to more profoundly understand the intricate regulation of GH secretion and, later, the application of Translational Endocrinology and Endocrine Oncology allowed us to expand our knowledge regarding the complexity and pleiotropic roles of the components of these regulatory systems.

GH regulation: insights from comparative endocrinology

Although GH production is differentially regulated across vertebrates, with an apparent evolutionary trend to simplification, most of the main hypothalamic, pituitary, and peripheral factors involved in the control of the somatotropic function are evolutively conserved and exert a comparable role in different groups. Indeed, the constellation of GH-regulatory factors has been significantly drawn with the application of Comparative Endocrinology. The contribution of our group through this journey towards the understanding of somatotrope pathophysiology and the identification of novel, even unexpected, GH-regulators and/or mechanisms of action is illustrated by the examples listed below:

Thyrotropin-releasing hormone (TRH) regulates GH secretion: A pioneer study aimed at understanding the role of hypothalamic hormones on the regulation of somatotrope function focused on frog pituitary, and combining in vivo and in vitro studies, EM-immunocytochemistry and quantitative imaging, demonstrated for the first time that TRH is a stimulatory factor for three pituitary cell types including somatotropes, although there were temporary differences on biosynthetic and secretory response of these cell types to TRH [5-7]. This discovery was later

extended by studies on cells from porcine and rat pituitaries, and human pituitary tumors, which corroborated a relevant role of TRH on GH release.

PACAP, kisspeptins and ghrelin regulate GH secretion: Further studies implemented by our group in different species helped to expand the puzzle of factors with capacity to regulate GH secretion. In this sense, our group demonstrated that two variants of the pituitary adenylate cyclase-activating peptide (PACAP-27 and PACAP-28) regulate GH synthesis and secretion by different mechanisms [8, 9] and that the kiss1/kiss1r system is functionally expressed in rat pituitary, where it directly stimulates GH and LH secretion, likely acting as signaling integrator of metabolic, somatotropic and reproductive axes on pituitary gland [10, 11]. Similarly, our group has helped to unveil the effects and the underlying mechanisms of the ghrelin gene derived peptides (ghrelin and obestatin) on GH synthesis and secretion in several species from mouse and pigs to primates and humans [12-14].

GH plays a central role in the integration of growth, development and metabolic status: Research implemented in our group using animal models and in vitro experiments in different species has helped to define the pivotal role of GH in the integration of metabolic signals to regulate somatic growth and development. Indeed, unique mouse models developed by the group have proven the key role of GH on controlling whole body metabolism [15-22]. But, at the same time, our group has demonstrated that metabolic status [23, 24] and multiple factors related to the control of systemic metabolic balance and homeostasis such as insulin or IGF-I [23, 25, 26], leptin [27] and resistin [28] can also directly regulate GH secretion at the pituitary level.

Somatotrope population is composed by two cell subpopulations: Seeking for the identification and more detailed characterization of all these novel regulators of GH secretion in different species, our group discovered the existence of two subpopulation of somatotrope cells in porcine pituitaries [low density (LD) vs. high density (HD)], which exhibited dissimilar morphology and functionality [29]. Indeed, the response to regulators of GH secretion was markedly different depending on the somatotrope subpopulation studied, likely due to the activation of different intracellular signaling pathways [30-32]. Thus, additional studies demonstrated that these somatotrope subpopulations correspond to alternative phases of the secretory cycle and that the proportion of each cell population evolve during postnatal development [33].

Somatostatin and cortistatin play a dual stimulatory/inhibitory role in the control of GH secretion: Pioneer data generated from our group many years ago indicated that the pituitary gland exhibit a dual stimulatory/inhibitory response to somatostatin (SST) and cortistatin (CORT) in that very low doses (in the range of nanomolar) induce a stimulation of GH release, which has been proven in different species [31, 34-39]. This response to SST and CORT was markedly different depending on the somatotrope subpopulation studied, which is, likely, due to the unique distribution and abundance of SST/CORT receptors (ssts) in both somatotrope subpopulations [35]. In particular, we found that porcine sst1 and sst2 were more abundant in LD cells, whereas sst5 was more abundant in HD cells, and that this differential expression in both somatotrope subpopulations might be associated with the dual stimulatory-inhibitory response of somatotrope cells observed in response to high/low doses of SST/CORT [35].

GH regulatory systems: new players with pathological implications

Along this journey through the comparative endocrinology toward the precise understanding of the factors and mechanisms that finely regulate GH secretion, our group has identified a number of novel elements that have exhibit remarkable pathological implications.

Identification of novel sst5 isoforms: During the cloning and characterization of the ssts involved in the dual stimulatory/inhibitory response of the GH-cells to SST and CORT, we identified in several species novel variants of the sst5 receptors generated by processes of non-canonical splicing [40, 41]. These novel sst5 variant are characterized by the lack of several of the typical seven transmembrane domains observed in the ssts (and therefore designated as truncated receptors), by their particular subcellular localization (mainly found at intracellular compartments) and by their differential response to SST and CORT. In addition, these truncated sst5 variants are able to physically and functionally interact with canonical ssts (mainly sst2), to regulate their subcellular localization and response to SST, CORT or their analogs [40, 41]. Interestingly, although the tissular expression pattern of these variants is notably limited, the human sst5 truncated variants are substantially present in several tumoral pathologies [41-46], including GH-producing adenomas [41, 43, 46], where the presence of one of these variants (sst5TMD4) is associated with the reduced ability of SST analogs to inhibit GH secretion in patients [46] and with the invasion of intracranial structures [43].

Identification of alternative ghrelin gene variants (In1-ghrelin): Similarly, the study of the ghrelin gene in mouse and human lead us to the identification of a novel ghrelin variant named In2-ghrelin in mouse and In1-ghrelin in human. This splicing variant of the ghrelin gene is originated by the retention of the intron 1 (in human) or 2 (in mouse), generating a novel transcript that shares the N-terminal region with native ghrelin but presents a totally different C-terminus [47, 48]. The In1-ghrelin variant is also drastically overexpressed in several pathologies [47, 49, 50], including GH-producing adenomas [49], wherein it increases cell proliferation and hormone secretion.

Concluding remarks

The data presented herein indicate that somatotrope regulation is far more complex than originally envisioned, in that in addition to the primary hypothalamic control, other relevant factors impinge upon somatotropes to fine-tune their functional capacities. Indeed, distinct, multiple layers of regulation are superimposed and interconnected, including extrinsic, distant and proximal, as well as intrinsic factors and mechanisms. Among them, metabolism, especially as it relates to obesity and T2D emerges as a key regulatory system for somatotropes. Additionally, novel mechanisms, such as splicing of receptors and novel ligands, may help to better understand somatototrope function and pathophysiology. Altogether, the evidences presented herein demonstrate that comparative approaches substantially enrich the information and enlighten the perspective of research problems, offering valuable tools to understand and solve pathological processes.

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SPECIFIC RECOMBINANT GONADOTROPINS INDUCE SPERMATOGENESIS AND SPERMIATION FOR THE FIRST TIME IN A TELEOST FISH, THE EUROPEAN EEL

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New specific single-chain recombinant gonadotropins were tested treating European eel males. Males received recombinant FSH (rFSH) in three doses (2.8, 1.4 and 0.7 µg/fish; named high, medium and low treatments) during 3 weeks. Later, an increasing recombinant LH (rLH) dose (every 3 weeks; 1, 2, 6 µg/fish) was combined with rFSH. rFSH by itself was able to induce the spermatogenesis, causing higher androgen levels and development until the spermatogonia 2 stage in the high rFSH group. The rLH promoted further maturation, but only the highest dose of rLH induced the most advanced stage (spermatozoa 2), and significant GSI increase in all the groups. All treatments induced spermiating males, however, the best sperm quality (with ≥50% motile cells and volumes ~0.4 ml) was observed in males treated with the highest rFSH dose and a progressive increase of rLH treatment. In this way, these new specific recombinant gonadotropins have demonstrated the ability to induce the spermatogenesis *in vivo* in European eel.

Introduction

In teleosts, recombinant gonadotropins (rGTHs), both *in vitro* and *in vivo*, have been able to induce the steroidogenesis and gonad development, however the *in vivo* results have been variable [9].

In vivo, specific rGTH stimulated estradiol (E_2) production in female of species such as orange-spotted grouper (*Epinehelus coioides*; [1]) or rosy bitterling (*Rhodeus ocellatus*; [6]), achieving ovulation in the latter, in this study however, already sexually matured females were used. In males, rGTHs were also able to induce the androgenesis process e.g. in zebrafish (*Danio rerio*; [8]) and European sea bass (*Dicentrarchus labrax*; [10]). Generally, the hormonal treatment was not able to induce the spermiation *in vivo*, with the exception of goldfish (*Carassius auratus*; [6]) and European sea bass [10], where the treated fish were already sexually matured at the beginning of the study.

In Japanese eel (*Anguilla japonica*), recombinant FSH (rFSH) stimulated the *in vitro* production of E_2 and testosterone (T) in oocytes, but only at vitellogenic or further developed stages [3,7]. Also *in vitro*, both rFSH and recombinant LH (rLH) induced the germinal vesicle breakdown in mature oocytes (nuclear migration; [7]). *In vivo*, rGTHs did not stimulate an increase in the gonadosomatic index (GSI) in females, but some induction of vitellogenesis were observed [5]. In males, rGTHs expressed by baculovirus [7], yeasts [4] or Drosophila cell lines [5] were able to induce androgenesis *in vitro*. A complete spermatogenesis, with the presence of some spermatozoa in the testis, was achieved with rGTHs expressed by baculovirus [5]. *In vivo*, Japanese eel and goldfish rGTHs, expressed by baculovirus, stimulated a complete spermatogenesis in male eels, but spermiation has never been achieved [2,5].

Materials and Methods

European eel males (n=72; 100.1±1.9 g) from a local fish farm were distributed in four 150-L aquaria and progressively adapted to sea water and 20 ºC. Single-chain recombinant FSH and LH were obtained by transfection of a mammalian cell line (CHO) with further partial purification and up-concentration (Rara Avis Biotec S.L.; Valencia, Spain). Fish were submitted to three hormonal treatments administered weekly during a total of 12 weeks. Males received rFSH in three doses (2.8, 1.4 and 0.7 µg/fish; named high, medium and low treatments) during 3 weeks. After that, an increasing (every 3 weeks) dose of rLH (1, 2 or 6 µg/fish) was combined with the different rFSH doses.

Three males per treatment were sacrificed every 3 weeks. External morphometric parameters (fin color and eye index, EI) were noted. Fish and testis were weighed to calculate the GSI. Blood samples were taken and plasma levels of 11-ketosterone (11KT) and T were determined by commercial ELISA kits. A portion of testis was collected for its inclusion in 10% formalin buffered at pH 7.4 for histological determination of the stage of testis development. Histology were done following the protocol of [11], where a sections of 5 µm thickness were made and stained with haematoxylin and eosin.

When possible, sperm was collected by abdominal pressure one week after hormone administrations. Volume and density were noted, and motility was assessed in triplicate using phase-contrast microscope, video-camera (60 fps) and a computer-assisted sperm analysis (CASA) software (ISAS, Proiser R+D, S.L., Paterna, Spain).

Results and Discussion

The administration of rFSH alone was enough to initiate the spermatogenesis (week 3), inducing dark coloration of the fins, increased T levels and EI (Fig. 1A), and promote testis development until the SPG2 stage in fish reared in the high rFSH group (Table 1). However, no differences were observed in GSI. Higher 11KT values were observed in the high rFSH group relative to the lower doses, which did not show significant increases throughout the treatment.

Table 1. Distribution of stages of testis development reached by the different males through the samplings (weeks 3-12) in the three experimental groups: (●) High (2.8 µg/fish); (●) Medium (1.4 µg/fish); and (○) Low (0.7 µg/fish). Stages: SPG1: Spermatogonia 1; SPG2: Spermatogonia 2; SPC1: Spermatocyte 1; SPC2: Spermatocyte 2; SD: Spermatid; SPZ1: Spermatozoa 1; SPZ2: Spermatozoa 2.

		SPG1	SPG2	SPC1	SPC2	SD	SPZ1	SPZ2
rFSH + 6µg rLH/fish	**W12**		○	○○	●● (grey) ○	●●● ●●● (grey) ○○○	●● ● (grey)	● ● (grey)
rFSH + 2µg rLH/fish	**W9**	○		● (grey) ○○	● (grey)	●●	●	
rFSH + 1µg rLH/fish	**W6**	● (grey) ○○○		●	●● ●● (grey)			
rFSH	**W3**	● ●●● (grey) ○○○	●●					

The rLH administration promoted (week 6) additional maturation to the SPC2 stage in medium and high experimental groups, but no gonadal progression was observed in the low group. Only the high group showed darker fins relative to previous weeks. Similar maximum T levels were achieved in all the experimental groups. However, the highest 11KT values were observed in the rFSH high group (Fig. 1C).

A second increase of rLH (week 9) induced further maturation to SD-SPZ1 and SPC1 stages in the high and low groups, respectively. The medium group showed no further maturation (staying at the SPC1-2 stages), although the fish registered higher EI and darker fins. Here (week 9) the Low group fishes showed a significant increase of EI in comparison with untreated fish, for the first time. Additionally, none of the groups showed significant GSI increases. The highest dose of rLH (week 12) was necessary to mature fish to the most advanced stage (SPZ2), which also was the dose that caused the highest values of EI and the darkest fin colour. The GSI increased gradually during the treatment however, did not show significant increase before this point in any of the experimental groups. GSI increase was especially pronounced in the fish receiving the high rFSH treatment. A progressive 11KT (high group) and T (all treatments) decrease was observed from week 9 to week 12, but without significant differences in respect to week 6.

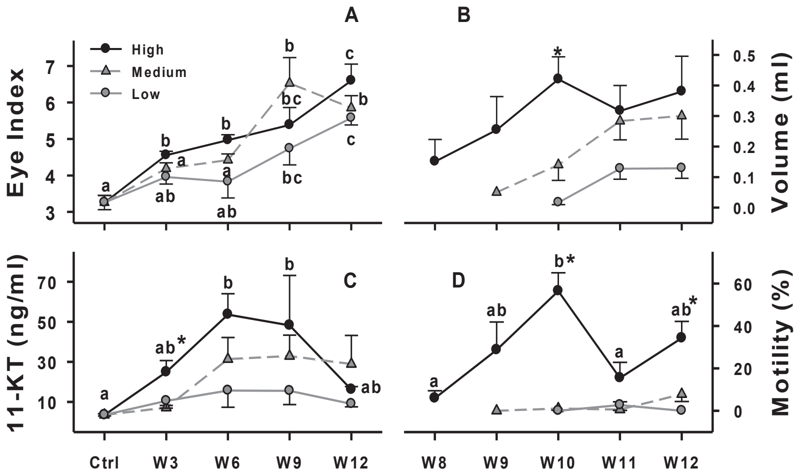

Figure 1. Sperm parameters throughout the FSH/LH treatments: A) Eye index; B) Sperm volume; C) 11KT plasma levels; D) Total motility. Different letters indicate differences during the treatment and asterisk indicates differences between treatments.

The sperm production in high, medium and low experimental groups started at 8th, 9th and 10th weeks, respectively (Fig. 1B). Although the sperm quality was variable, the fish treated with the highest rFSH dose and progressive increase of rLH yielded the best sperm quality samples, with motilities ≥50%, densities around $7*10^9$ spermatozoa/ml and volumes of approximately 0.4 ml (Fig. 1D).

By the first time in teleosts, specific recombinant gonadotropins have produced good quality sperm, demonstrating that the half-life of these recombinant gonadotropins is long enough to induce *in vivo* effects. The group treated with the highest rFSH and increasing doses of rLH provided the best sperm quality samples. However, ~20% of

non-responders were registered and the sperm quality was variable. Therefore, further experiments combining these recombinant hormones are required to improve the hormonal treatments.

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CHARACTERIZATION OF NUCLEAR AND MEMBRANE PROGESTIN RECEPTORS IN EUROPEAN EEL, AND THEIR EXPRESSION *IN VIVO* THROUGHOUT SPERMATOGENESIS

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In male teleost, the role of progestins as 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS), have been reported in the regulation of spermatogenesis. Two mechanisms of action can mediate the progestins action: the classic genomic mechanism of steroid action relatively slow involving nuclear receptors (nPRs or Pgrs), members of the nuclear steroid receptor superfamily; and the non-genomic, rapid activation of intracellular signal transduction pathways, mediated by the membrane progestin receptors (mPRs), members of the progestin and adipoQ receptor (PAQR) family.

We characterized and studied the mRNA expression of two nuclear progestin receptors (pgr1 and pgr2) and five membrane progestin receptors (mPRα, mPRγ, mPRδ, mPRAL1, mPRAL2) in brain, pituitary and gonads for the first time in a teleost, the European eel.

mPR phylogeny placed three eel mPRs together with vertebrate mPRα, and were called mPRα, mPRAL1 (alpha-like1) and mPRAL2 (alpha-like2). The two other eel mPRs (i.e.: mPRγ, mPRδ) clustered together with their respective mPR types amongst vertebrate representatives.

In *vivo* studies of mRNA expression throughout spermatogenesis suggest that progestins exert their actions in the eel brain and pituitary by both nPRs and mPRs. In the testis, the nuclear pgr2 and membrane mPRγ and mPRδ seem to be involved on the induction of meiosis, whereas mPRa, mPRAL1 and mPRAL2 seems to be involved on the process of final sperm maturation.

Introduction

In all vertebrates, progestins have a crucial function in gametogenesis. It is known that in male fish two progestins: 17alpha,20beta-dihydroxy-4-pregnen-3-one (DHP) and/or 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS) are the maturation-inducing steroids (MIS), mediating the process of sperm maturation and spermiation (1). In Japanese and European eels, DHP has been proposed to be an essential factor for the meiosis initiation at the beginning of spermatogenesis (2). Progestins can diffuse through the cell membranes (3) and bind to nuclear progestin receptors (nPRs or Pgrs). Receptor activation leads to modulation of gene transcription and translation activity (4), resulting in a relatively slow biological response. However, many progestin actions are non-genomic, and involve rapid activation of intracellular signal transduction pathways mediated by membrane progestin receptors (mPRs). The mPRs are 7-transmembrane receptors coupled to G-proteins, but they do not belong to the G protein coupled receptor (GPCR) superfamily. Instead, they are members of the progestin and adipoQ receptor (PAQR) family (5).

The European eel has complex life cycle, with a blockade of sexual maturation as long as the reproductive oceanic migration is not performed. Together with its phylogenetical position, branching at the basis of the teleosts (6), the eel is a perfect model to study the ancestral regulatory functions which are controlling reproduction.

In this study, two nuclear progestin receptors (*pgr1, pgr2*) were characterized in the European eel, and five membrane progestin receptors *(mPRα, mPRAL1, mPRAL2, mPRγ, mPRδ*) were characterized both in the European and Japanese eel. Phylogenetic analyses were performed for both nuclear and membrane progestin receptors. Also, the expression profiles of the European eel nuclear and membrane progestin receptors were quantified in the brain, pituitary and testis through spermatogenesis for the first time in a teleost.

Materials and Methods

One hundred European eel males (mean body weight 100±6 g) were gradually acclimatized during one week to sea water (37±0.3‰ of salinity) and kept at 20 °C during the whole experimental period. Once a week they were weighed and injected with human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish; Profasi, Serono, Italy) during 8 weeks (7), to induce the eel sexual maturation. 5-8 eels were sacrificed before hCG treatment, and later each week (W1-8) through the hormonal treatment. Testicular tissue samples were collected for histological analysis. Samples of brain, pituitary and testis were collected for qPCR analyses, and blood samples were collected for the analysis of DHP plasma levels. Histological analysis was performed as described by Morini et al. (8). Stages were classified as described by Morini et al. (9). Total RNA of testis, brain parts and pituitary were isolated and purified, and reverse transcripts were obtained as described by Peñaranda et al. (10). The quantitative real-time Polymerase Chain Reactions (qPCR) were carried out as described by Morini et al. (9) to determine the expression of each nPR and mPR gene, using specific European eel progestin receptor qPCR primers (7) and the Acidic ribosomal phosphoprotein P0 (ARP) as reference gene (11). Plasma concentrations of DHP were measured by mean of radioimmunoassay. Progestin receptor sequences were retrieved as described by Morini et al. (9). Phylogenetic analyses of both mPRs and nPRs are performed as described by Morini et al. (9).

Results and Discussion

One *pgr* locus is present in vertebrates, only the goldfish (*C. auratus*) (BAO48148, BAO48149, NCBI), the frog *Xenopus* (12), and both Japanese and European eels exhibit two PGRs. According the phylogenetic analyses, the frog and the goldfish PGRs may come from species specific duplication while the eel PGRs may come from the third round of whole genome duplication which occurred in early teleosts. Phylogenetic analyses were performed in order to determine the relationship of eel mPRs characterized. The resulting tree clustered the five eel mPRs in 3 groups: mPRα, mPRAL1 (alpha-like1), mPRAL2 (alpha-like2) clustered with the vertebrate mPRα/mPRβ clade, and eel mPRγ and mPRδ clustered with mPRγ and mPRδ clade, respectively.

The present study is the first to report mRNA expression of five membrane and two nuclear PRs through spermatogenesis in fish. In the brain and pituitary, mRNAs for all five mPR subtypes were constantly expressed during spermatogenesis. *mPRγ*, *mPRAL1* and *mPRAL2* showed low expression in all the brain parts and the pituitary, whereas mPRα was more highly expressed; and mPRδ showed the greatest brain expression. The presence of eel mPRs in the brain and pituitary regions may suggest a possible progestins control of functions in reproduction through a negative feedback of progesterone on GnRH secretion; however further research is required

to elucidate the specific signalling roles of mPRs in the eel brain and pituitary. Concerning the nPRs, both nPRs in the pituitary were upregulated throughout the induced-hCG maturation, showing higher expression from spermatocyte stage to spermatozoa stage, which correspond to stages from meiosis to full spermiation. Only *pgr1* mRNA expression increased in all the brain parts through spermatogenesis, corresponding with the plasma levels of DHP found in the European eel, which significantly increased during the spermatogenesis. These results highlight the implication of these nPRs controlling the spermatogenesis process; Pgr1 would be involved in the reception of DHP signal in the brain, while both Pgr1 and Pgr2 would receive the DHP signal in the pituitary, in order to regulate the spermatogenesis from meiosis to final sperm maturation.

In the testis, the three mPRalpha/alpha-like (mPRα, mPRAL1 and mPRAL2) showed the same expression pattern, increasing until the spermatozoa stage. This expression pattern agree with the increase of eel DHP plasma levels from spermatocyte to spermatozoa stage (not shown), suggesting an implication of these receptors on the regulation of the final stage of spermatogenesis in the testis, mediated by DHP. However, mPRγ, mPRδ and pgr2 showed an opposite profile, with high expression in the testis during the spermatogonia stage, and showing a fast decrease onwards, until spermatozoa stage. According to Miura et al. (2), DHP plays an important role on the initiation of the meiosis and on further spermatogenesis. From the pattern of *in vivo* expression of the different receptors through spermatogenesis in the eel, we can hypothesize that testis mPRα, mPRAL1 and mPRAL2 could be involved on the final sperm maturation, while testis mPRγ, mPRδ and pgr2 could be involved on mediating DHP effects on early spermatogenic stages.

In conclusion, we have performed the most complete description of progestin receptors in the eel, and likely in any teleost species. Two nPR and five mPR genes were identified in the genome of European and Japanese eel. All nuclear and membrane PRs were present in tissues of the BPG axis, likely involved in the eel reproductive system. Nuclear and membrane receptors seem to play different roles in reproduction. *pgr1* could be involved in the reception of DHP signal in the brain, while both *pgr1* and *pgr2* would receive the DHP signal in the pituitary, in order to regulate spermatogenesis. On the other hand, in testis, the membrane receptors *mPRa, mPRAL1* and *mPRAL2* seem to be involved in sperm maturation, while *mPRγ* and *mPRδ* could be involved in the process of meiosis.

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IDENTIFICATION OF TWO KISS RECEPTORS IN *DICENTRARCHUS LABRAX*: EVIDENCE FOR LIGAND SELECTIVITY TO KISS1 AND KISS2 PEPTIDES

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The KISS1/KISS1R system has been established as an essential modulator in mammalian reproductive function. In contrast to placental mammals, which have a single gene for the ligand (Kiss) and for the receptor (Kissr or Gpr54), some teleost species, including European sea bass, harbor two different kisspeptin coding genes (*kiss1* and *kiss2*) and two receptors. This study assesses the cloning and characterization of the two sea bass kisspeptin receptor genes (*kissr2* and *kissr3*), their tissular expression and signal transduction pathways in response to Kiss1 and Kiss2 synthetic peptides. Phylogenetic and synteny analyses indicate that these paralogs originated by duplication of an ancestral gene before teleost specific duplication. The *kissr2* and *kissr3* mRNAs encode proteins of 368 and 378 amino acids, respectively, and share 53.1% similarity in amino acid sequences. Both *kissr2* and *kissr3* genes are predominantly expressed in brain and gonads. On the other hand, *in vitro* functional analyses have revealed that sea bass Kissr signals are transduced both via the protein kinase C and protein kinase A pathways. Synthetic sea bass Kiss1 and Kiss2 peptides provoked an activation of kiss receptors with different potencies, indicating a differential ligand selectivity. Accordingly, Kissr2 and Kissr3 show a preference for Kiss1 and Kiss2 peptides, respectively, thus providing the basis for future studies aimed at establishing their physiologic roles in this teleost species.

Introduction

Kisspeptin is the peptide product of the *KISS1* gene. Studies in humans and mice have revealed that inactivating mutations of kisspeptin receptor (Kissr or Gpr54) result in the clinical syndrome of hypogonadotropic hypogonadism. It suggests that the Kiss1/Gpr54 system plays a central role in the control of mammalian reproductive function, with an essential involvement in the regulation of gonadotropin-releasing hormone (GnRH) action and gonadotropin secretion. In contrast to placental mammals, which have a single gene for the ligand (Kiss) and for the receptor (Kissr or Gpr54), evidence for two distinct *kiss* and *kissr* genes has been reported in some teleost species (1). Thus, the fishes may provide excellent animal models for the study of general principles underlying the Kiss/Gpr54 systems of vertebrates.

European sea bass has two distinct *kiss* genes, namely *kiss1* and *kiss2* (2), and two *kissr* genes (3-4). Our findings provide the cloning and molecular characterization of these two kisspeptin receptor genes (*kissr2* and *kissr3*) and demonstrate the existence of a differential ligand selectivity in sea bass.

Materials and Methods

Degenerate PCR primers were designed based on the conserved fish amino acid sequences and used to amplify fragments of sea bass kiss receptors. PCR amplifications were carried out from four sea bass genomic DNA libraries constructed with the Universal GenomeWalker Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). Specific primers were used to amplify the full-length cDNA sequences of both *kissr2* and *kissr3* genes on sea bass brain cDNA using a proofreading DNA polymerase (Pfu DNA polymerase; Stratagene). Phylogenetic analysis was

performed with the Neighbor-Joining method using the Molecular Evolutionary Genetics Analysis Software (MEGA 4.1) and full-length amino acid sequences, while synteny analysis was performed using the Ensembl Genome Browser and PhyloView of Genomicus v67.01 web site.

The complete coding sequences of sea bass *kissr2* and *kissr3* cDNAs were cloned into the pcDNA3 expression vector (Invitrogen). Receptor activation was analyzed using a luciferase (Luc) assay. Plasmids containing the *Luc* gene transcriptionally regulated by a serum response element (SRE) (pSRE-Luc) or cAMP response element (CRE) (pCRE-Luc) (BD Clontech, Palo Alto, CA, USA) were used to analyze the signal transduction via protein kinase C and protein kinase A pathways, respectively. Moreover, the pRL-TK plasmid (Promega), which constitutively expresses a *Renilla reniformis* luciferase gene, was used to control transfection efficiency. The pSRE-Luc or pCRE-Luc reporter plasmids, pcDNA-kissr2 or pcDNA-kissr3 plasmids and pRL-TK plasmid were transiently co-transfected into Chinese Hamster Ovary (CHO) cells. Two days after transfection, cells were stimulated with various concentrations (from 10^{-5} to 10^{-7} M) of sea bass Kiss1–10, Kiss2–10, Kiss1–15 and Kiss2–12 synthetic peptides. Two independent transfection experiments were performed, each conducted in duplicate.

Results and Discussion

This study reports the isolation and molecular characterization of two kiss receptor genes (*kissr2* and *kissr3*) (Table 1). The cloning was conducted using degenerate PCR primers and a genome walking approach. The full-length cDNA sequences of both genes were obtained from sea bass brain cDNA by using specific primers. The two sea bass kisspeptin receptors share 53.1% amino acid sequence identity with each other and have conserved structural elements which are considered to be involved in receptor folding, activation, signaling and internalization.

Table 1. Molecular characterization of kiss receptors in sea bass.

Gene	cDNA (bp)	5´UTR (bp)	3´UTR (bp)	ORF (bp)	Amino acids
kissr2 or *gpr54-1b* (JN202446)*	1433	107	222	1104	368
kissr3 or *gpr54-2b* (JN202447)*	2365	636	595	1134	378

*GenBank accesion numbers are indicated. ORF, open-reading frame

Phylogeny and synteny analyses of kiss receptors in vertebrates showed that they separated into four groups which were named *kissr1*, *kissr2*, *kissr3* and *kissr4*, being *kissr1* the group including the mammalian receptors as it was the first form to be described. In the case of the sea bass, the two *kissr* sequences segregated into two different groups, namely *kissr2* and *kissr3*. Furthermore, searches in the available genome databeses of Ambulacrarians pointed to the existence of kiss receptor-like genes in the sea urchin and the acorn worm. Synteny analysis revealed that the neighboring gene clusters surrounding the four *kissr* loci are highly conserved across vertebrate species, and when multiple forms are identified in the same species, they are located in different chromosomes. The sea bass *kissr2* and *kissr3* genes are

located at the linkage group (LG) LG20 and LG10, respectively. In this line, these data indicate that these paralogs, as those of their cognate ligands *kiss1* and *kiss2*, have arisen by duplication of an ancestral gene before teleost specific duplication (1, 2). On the other hand, the tissue expression of *kissr* genes in the brain and the gonads of sea bass (Fig. 1), is fully compatible with their putative roles in fish reproduction (3-5). In the testis, the expression levels of sea bass kiss receptors showed a significant variation during the reproductive cycle (data not shown). The presence of both mRNAs was also detected in a number of somatic tissues (Fig. 1).

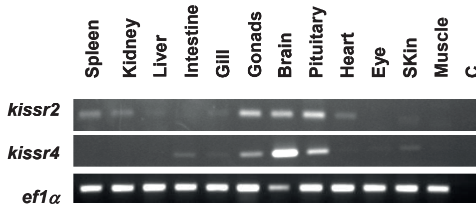

Figure 1. Tissue expression of kissr genes in sea bass by RT-PCR analysis (n=3–5 pooled tissues). The sea bass ef1α was used as an internal control to verify the correctness of the RT reaction. C: negative control for PCR which was performed using sterile water as template.

In vitro functional analyses revealed that sea bass kiss receptor signals are transduced via both the protein kinase C and protein kinase A pathways. Synthetic sea bass Kiss1–15 and Kiss2–12 peptides activated kiss receptors with different potencies, indicating differential ligand selectivity (Fig. 2).

The use of the SRE reporter system showed that sea bass Kiss1–15 was most able to activate Kissr2, while Kiss1–10 elicited an increase of luciferase activity of 4.84 in comparison to Kiss2-10 (1.6-fold) and Kiss2-12 (3.3-fold) (Fig. 2A). On the other hand, the use of the CRE reporter system exhibited that sea bass Kiss1–10 elicited an increase of luciferase activity of 9.4 compared with Kiss2–10, which showed no significant effect at all in CHO cells transfected with Kissr2 (Fig. 2B). In addition, Kiss1–15 exhibited the highest potency in activating Kissr2 (24.7-fold), while Kiss2–12 elicited only an increase of 2.49 times over control. By contrast, CHO cells transfected with Kissr3 displayed the highest response to Kiss2–12 via both SRE- and CRE-reporter constructs, with 3.3- and 3.9-fold increase compared to basal levels, respectively (Fig. 2C-D). Accordingly, our data suggest that Kissr2 and Kissr3 have a preference for Kiss1 and Kiss2 peptides, respectively, in sea bass. Future studies will be aimed at establishing their physiological roles and functionality in this species.

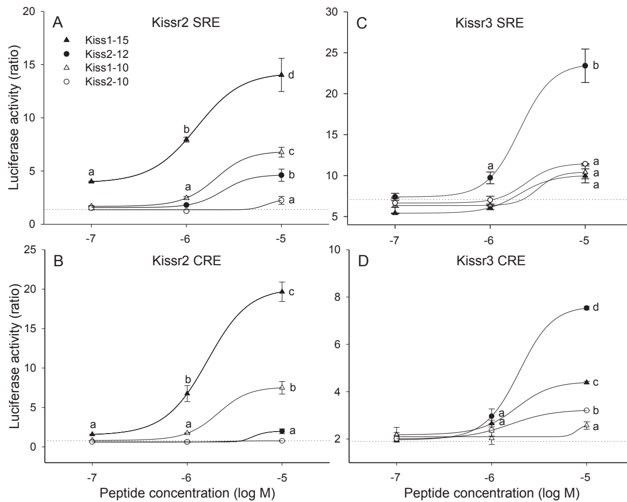

Figure 2. Differential ligand selectivity of sea bass kiss receptors. SRE- (A-C) and CRE-driven (B-D) luciferase activities in CHO cells transfected with sea bass kissr2 (A-B) or kissr3 (C-D) and stimulated with Kiss1–10, Kiss1–15, Kiss2–10 or Kiss2–12.

Acknowledgments

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EFFECT OF HIGH REARING DENSITY ON SEX DIFFERENTIATION IN ZEBRAFISH (*DANIO RERIO*): SEX RATIO AND GENE EXPRESSION ANALYSIS

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In some fish species, density is an environmental factor that can alter sex ratio. It is well-known that fish subjected to high densities increase cortisol plasma levels. Further, it has been described that cortisol can affect the process of sex differentiation, resulting an increase in the number of males. In order to better understand the effect of density and cortisol on sex differentiation in zebrafish we performed two experiments. First, we reared fish at different densities and second, we treated fish with synthetic cortisol. Sex ratio and gene expression analysis in the gonads of five canonical reproduction-related genes were performed. Results indicated that density increased the number of males and that the synthetic cortisol was able to masculinize all fish population. Molecular studies showed a significant downregulation of *nr3c1* in ovaries and testes and a significant downregulation of *cyp19a1a* in ovaries. In conclusion, when rearing zebrafish in the laboratory it is important to have in consideration the density used in order to avoid undesirable masculinization and an alteration of the gene expression in the gonad.

Introduction

Zebrafish has been used as a model organism for biomedical research and also for finfish aquaculture research (1). Although several husbandry protocols have been published so far, there is no information on the number of zebrafish that has to be reared for not altering fish welfare.

It is well-known that rearing fish (i.e., in fish farms to optimize resources) at high densities, can trigger stress situations that are in detrimental of fish survival. Consequently, stress alters the regular homeostasis and increase cortisol plasmatic levels (2). Two previous studies have described the consequences of stocking zebrafish at high density; in one, authors did not find differences in sex ratio (3) but the other, an alteration of sex ratios in some of the studied populations was observed (4).

In the present study we want to decipher if density affects sex differentiation in zebrafish not only quantifying the final sex ratios, but also studying the gonad from a molecular point of view. Within these purposes, two experiments based on different density conditions and on treatments with synthetic cortisol were performed.

Materials and Methods

Domesticated zebrafish (AB strain) were maintained in a fish facility in the “Institut de Ciències del Mar” (ICM-CSIC), Barcelona. Fish was reared with a photoperiod cycle of 12L:12D, 50% humidity, 26±1ºC water temperature. Embryo was obtained from five different pairs and the larvae were collected and fed with sera micron (Sera®, Germany). All larvae and fish were housed in 2,8 L aquarium tanks and fed *ad libitum* during all the stages of their life.

For high density experiment, four density treatments (8, 16, 33 and 66 fish/L) were designed. The density 8 fish/L was considered as the control group. The experiment started when larvae reached 6 dpf until 90 dpf. For the second experiment, fish were

exposed to different compounds; Hydrocortisone (F; H4001-Sigma); metyrapone (M; 2856525–Aldrich) and methyltestosterone (MT; M7252–Sigma). Five different treatments were established and fixed at 8 fish/L; F, M, F+M, MT and control (C, without any compound). For this second experiment, larvae received the corresponding compounds through diet from 18 to 32 dpf. From 32 dpf onwards the larvae were kept until 90 dpf and fed with commercial feed without any synthetic compound. For both experiments, when fish was sexually mature, around 90 dpf, individuals were euthanized by ice bath to extract the gonads and were kept at –80±1ºC for molecular studies. At the same time, sex was recorded for each treatment and experiment.

For molecular studies, RNA extraction and cDNA synthesis were performed with TRIzol® Reagent and Deoxyribonuclease I, Amplification Grade, respectively, following the manufactured protocols (Invitrogen, USA). Quantitative real-time PCR (q–PCR) was carried out for gene expression analysis of samples from high density experiment. Genes studied related to sex determination were: cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a,* NM_131154), nuclear receptor subfamily 3, group C, member 1 (*nr3c1*, NM_001020711), fork head box L2 (*foxl2*, XM_005157463), DNA (cytosine-5-)methyltransferase 1 (*dnmt1*, NM_131189) and anti-Müllerian hormone *(amh,* NM_001007779).

Results and Discussion

In the present study, we performed two experiments to see how density affects sex differentiation in zebrafish. Figure 1 shows sex ratio results at 90 dpf for both experiments. In figure 1A, higher number of males (p<0.05) were found in fish reared at 33 and 66 fish/L when compared to control. Figure 1B shows significant differences (p<0.05) found in F and MT treatments when compared to C. In this experiment we observed that exogenous cortisol masculinized all the fish population, whereas M compound (which blocks cortisol synthesis) inhibited this effect. The results suggested that cortisol may play an important role of masculinization process in zebrafish.

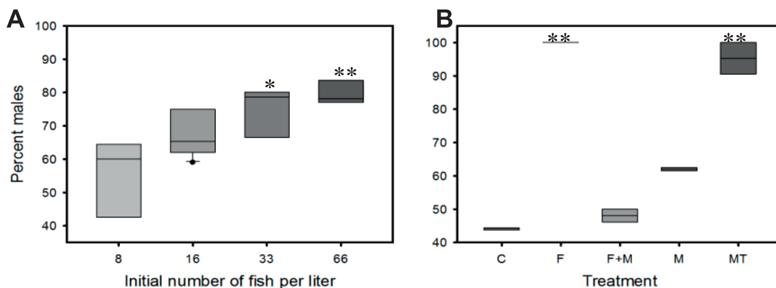

Figure 1. (A) Percent males of high density experiment. Treatments were the four density levels 8, 16 33 and 66 fish/L. The χ^2 test was significant in 33 fish/L and 66 fish/L treatments (p<0.05). (B) Percent males of compound experiment. Treatments were control (C), cortisol (F), cortisol + metyrapone (F+M), metyrapone (M) and methyltestosterone (MT). The χ^2 test was significant in F and MT treatments (p<0.05).

Cortisol is a glucocorticoid hormone release in response to stress (5). Therefore, based on our results stress generated by density due to lack of space in the tank

may increase the level of cortisol. However, further experiments are required to determine whether this hormone participate directly or indirectly to masculinization process in zebrafish.

In addition, we assessed gene expression of five genes related to sex differentiation in fish (*cyp19a1a*, ovarian aromatase), *nr3c1* (glucocorticoid receptor), *foxl2, dnmt1* and *amh*. Only *cyp19a1a* in females and *nr3c1* in males and females showed significant differences in 66 fish/L treatment (figure 2). The expression of these two genes decreased when the highest level of density was applied to zebrafish population. The enzymatic function of *cyp19a1a* is produced in the gonads and it catalyses the conversion of testosterone into estradiol (E_2), thus making possible to become female (6). The *nr3c1* gene has a transcription factor action that binds to glucocorticoid response elements (GRE) of a certain gene with the presence of ortisol (7). In some fish species it has been described different molecular mechanisms (although not exclusive) of how cortisol can masculinize gonads (8, 9, 10, 11). Unfortunately in this study we are not able to decipher this molecular mechanism for zebrafish.

In conclusion, several approaches have been performed in order to determine whether density affects sex differentiation in zebrafish. We used different density to analyze if they affect sex ratios. We found that rearing zebrafish above 33 fish/L cause masculinization. Later, we hypothesized that cortisol may play a key role affecting sex ratios. Results showed that exogenous cortisol masculinize completely zebrafish population. Gene expression analysis showed that the density inhibited *cyp19a1a* levels in ovaries of treated fish. These results, taken together, indicate that density factor has a strong effect on zebrafish sex differentiation process and researchers need to be cautious when stocking zebrafish.

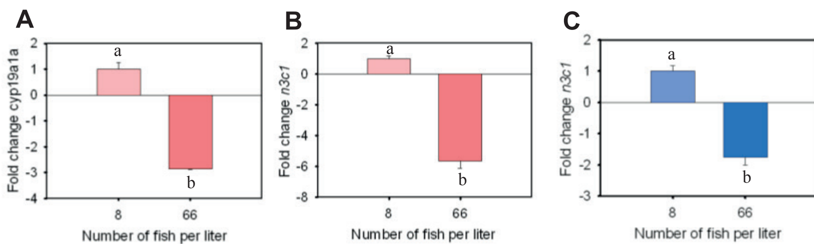

Figure 2. Gene expression results of female and male genes analyzed by qPCR. *Cyp19a1a* expression (A) and *nr3c1* expression (B) in ovaries. (C) *nr3c1* expression in testes. Letters indicate significant results *($p<0.05$)* between treatments analyzed by *t*-Student test. The number of samples per each gene was between 7–8.

Acknowledgments

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DAILY VARIATIONS OF CARBOHYDRATE AND LIPID METABOLISM-RELATED PARAMETERS IN LIVER OF RAINBOW TROUT. INFLUENCE OF LIGHT AND FOOD AVAILABILITY

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Most physiological processes, such as feeding behaviour and energy metabolism, exhibit circadian rhythmicity, thus playing a main role in the maintenance of the functional homeostasis. Light-dark and feeding-time cycles are mainly entraining such processes in order to adjust them to the chronological arrangement of the external world. The aim of the current study was to investigate in rainbow trout the existence of daily changes in activity and mRNA abundance of proteins involved in major pathways of carbohydrate and lipid metabolism in liver. The influence of both the light-dark cycle and food availability in synchronizing such rhythms was also evaluated. Four cohorts of animals previously adapted to 12L:12D (lights on at ZT0; feeding time at ZT2) were subjected to: normal conditions (LD); 48-h light isolation (DD); 96-h food deprivation (LD + Fasting); or food deprivation and light isolation (DD + Fasting) respectively. After such time periods, fish were sacrificed and sampled every 4-h on the following 24-h period (ZT/CT0, 4, 8, 12, 16, 20 and 0´). Our results reveal *gk*, *pepck*, *g6pase*, *pk*, *glut2*, *fas* and *hoad* rhythms of mRNA abundance to persist in the absence of light and/or food. Enzyme activities did not display significant daily variations although these parameters mostly fluctuated in parallel to the respective gene expression. Rhythms of mRNA abundance did mostly depend on the presence of food, but the influence of the light/dark cycle could not be discarded. The present results indicate that liver metabolism in trout is linked to a circadian system which may play a main role in generating rhythms of enzymes expression, but also influences enzyme activities. Further research must be carried out in order to understand such interaction.

Introduction

A wide variety of physiological functions, including metabolism, have been described to be under circadian control in all organisms. Daily rhythms in behaviour, physiology and metabolism are driven by cell autonomous clocks which synchronize to the environmental cycles, but maintain rarely equal 24-h rhythms, even in the absence of environmental cues (1). This clock mechanism is supposed to be driven by a canonical ‘‘body clock’’ system, but increasing reports evidence the independent action of localized ‘‘peripheral clocks’’ which provide targeted process-specific rhythmic control. An increasing number of studies carried out in mammals focused on rhythmic expression within the liver showing 8-15% mRNA to be rhythmically expressed (2). The presence of circadian oscillators has been reported in liver, where an interaction with metabolic pathways might be expected. The present study was aimed to assess in rainbow trout (*Oncorhynchus mykiss*) as model of teleost fish, the existence of daily rhythms of activity and mRNA abundance of several parameters related to major carbohydrate and lipid metabolism pathways in the liver, such as glucose transport (glucose transporter 2, GLUT2), glucose phosphorylation (glucokinase, GK), glycolysis (pyruvate kinase, PK), gluconeogenesis (phospho(enol)pyruvate carboxykinase, PEPCK), glucose release (glucose 6-phosphatase, G6Pase), lipogenesis (fatty acid synthase, FAS), and lipolysis (hydroxyacil-CoA dehydrogenase, HOAD) and how such rhythms are influenced by both the light-dark cycle, and food availability.

Materials and Methods

Rainbow trout were obtained from a local hatchery and transferred to our facilities at the ‘‘Estación de Ciencias Mariñas de Toralla’’ (ECIMAT). Fish were adapted to our laboratory conditions (13.5 ºC water temperature, LD 12:12) before any experimental procedure was performed. Animals were kept in 120 L tanks and fed once a day at ZT2 (zeitgeber time, ZT0=lights on) with a commercial dry pellet diet (1% body weight). For the study of daily rhythms in enzymatic activities and mRNA abundance, 56 animals (n = 8/group) were sacrificed every 4-h along the 24-h light/dark cycle, starting at ZT0. Thus, scheduled sampling time points were ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT0’ (following day), with trout from different tanks being sampled at each specific time point. A second set of 56 animals were maintained under the same lighting conditions and then light-isolated for 48-h and sacrificed as above. Fish exposed to constant darkness were hand-fed as scheduled. A third group of 56 trout were kept under a regular LD cycle and then food deprived for 96-h. After that, fish were sacrificed every 4-h as described above. A fourth group of 56 fish were kept at a standard LD cycle, then food deprived for 96-h, and also light isolated for the last 48-h period. After that, animals were sacrificed as indicated above. On the sampling day, fish were deeply anaesthetized with MS-222 ($50 mgL^{-1}$) and sacrificed by decapitation. Individual livers were removed under sterile conditions, divided in two portions that were placed into sterile RNase-free 1.5 ml Eppendorf tubes, and immediately frozen and stored at -80°C until assayed for enzyme activities and mRNA abundance.

Results and Discussion

We have selected several key regulatory enzymes of major metabolic pathways of carbohydrate (GK, PK, PEPCK, G6Pase and GLUT2) and lipid metabolism (FAS and HOAD) in order to assess the presence of daily rhythms in enzyme activity and mRNA abundance (Table 1). Whereas most parameters displayed significant daily rhythms of mRNA abundance, enzyme activities did not. However, rhythms of enzyme activities is not necessarily driven by oscillations of gene expression, as long as post-translational modifications, such as the acetylation of PEPCK, have also been demonstrated (3).

Regarding gene expression, all the genes herein evaluated displayed consistent daily rhythms. Dissociation between extrinsic (environmental) and intrinsic (endogenous) influences is possible as long as environmental conditions can remain unaltered over the 24-h cycle (i.e. constant lighting and food availability in the absence of synchronizing environmental cues). In these conditions, behavioral (sleep-wake and feeding/fasting), endocrine, and metabolic cycles persist in most vertebrates (4), including fish.

Food deprivation resulted in global changes in several parameters of hepatic energy metabolism, which mostly agrees with other studies in which samples were obtained only at one sampling time point (2, 5-8). We observed a clear daily rhythm of mRNA abundance for lipogenesis- (FAS) and lipolysis-related (HOAD) enzymes. Both activities were clearly dependent on feeding. In contrast, gene expression depended on light, but food (*fas*) also influenced.

Table 1. Cosinor analysis for carbohydrate-related and lipid-related genes in liver of rainbow trout exposed to normal conditions (LD), or subjected to constant darkness (DD) or food deprivation (LD + Fasted), alone or in combination (DD + Fasted).

		LD	DD	LD + Fasted	DD + Fasted
CARBOHYDRATE METABOLISM					
GK	**Mesor**	27.70	39.27	5.14	2.84
	Amplitude	27.12	38.54	4.62	1.90
	Acrophase (ZT/CT)	3.25	4.00	5.50	15.00
	R/S	0.29	0.20	0.17	0.16
PK	**Mesor**	4.14	2.23	2.15	1.31
	Amplitude	2.98	1.06	1.07	0.28
	Acrophase (ZT/CT)	11.25	12.50	6.50	15.50
	R/S	0.21	0.24	0.17	0.19
PEPCK	**Mesor**	2.56	4.35	1.95	1.18
	Amplitude	1.83	3.99	1.11	0.24
	Acrophase (ZT/CT)	1.50	0.25	5.25	3.5
	R/S	0.23	0.22	0.17	0.17
G6Pase	**Mesor**	1.87	1.79	1.68	1.24
	Amplitude	0.65	0.77	0.60	0.24
	Acrophase (ZT/CT)	8.50	15.75	16.50	13.50
	R/S	0.23	0.16	0.16	0.19
GLUT2	**Mesor**	2.17	1.76	1.72	1.62
	Amplitude	1.34	0.62	0.65	0.40
	Acrophase (ZT/CT)	6.25	5.50	8.50	11.50
	R/S	0.15	0.20	0.17	0.22
LIPID METABOLISM					
FAS	**Mesor**	6.90	2.87	6.99	2.39
	Amplitude	5.11	1.70	4.42	0.95
	Acrophase (ZT/CT)	23.25	23.25	10.50	13.50
	R/S	0.25	0.19	0.18	0.18
HOAD	**Mesor**	1.403	1.585	1.679	2.103
	Amplitude	0.402	0.604	0.619	1.137
	Acrophase (ZT/CT)	19.36	12.26	16.39	16.17
	R/S	0.76	0.17	0.15	0.15

Glucokinase (GK), phospho(enol)pyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase), pyruvate kinase (PK), glucose facilitative transporter type 2 (GLUT2), fatty acid synthase (FAS) and hydroxyacil-CoA dehydrogenase (HOAD).

Fish receiving food as scheduled (CT2) but subjected to DD, showed unaltered rhythms in parameters related to both glucose and lipid metabolism, with exceptions (*fas* amplitude and *hoad* acrophase), in contrast to that reported for lipid metabolism in sea bream liver, with photoperiod strongly influencing lipid metabolism (8). To corroborate such hypothesis, we also evaluated daily changes for all the above described parameters in fish subjected to LD but food deprived. Several parameters (GK, and HOAD) maintained comparable rhythms than those observed in fed fish under LD and DD, whereas others (PEPCK, G6Pase, PK, GLUT2, and FAS) showed altered profiles. According to that, food availability may affect the daily pattern of these parameters, thus displaying altered rhythms (PEPCK, G6Pase, PK, GLUT2, and FAS). However, the influence of light has to be taken into consideration, since some of them were affected in the absence of light. To evaluate the interaction between light and food, we finally tested whether or not all the rhythms persisted in trout subjected to both, constant darkness and food deprivation. If the daily rhythm in one parameter is only influenced by food, variations similar to those found in fasted

fish may be expected. That was observed for PEPCK, GLUT2, and FAS, but not for GK, G6Pase, PK, and HOAD, leading to speculate with the existence of any interaction between light and food in modulating those enzymes. Only *hoad* expression was mainly influenced by light. Thus, light and food availability, alone or together play a key role in modulating gene expression of metabolism-related parameters in trout liver, with light affecting HOAD, and food availability influencing PEPCK, GLUT2, and FAS. An interaction between both cues modulates GK, G6Pase, and PK.

In summary, evidence the existence of daily rhythms of gene expression for carbohydrate- and lipid metabolism-related enzymes in fish liver, with carbohydrate metabolism being assessed for the first time in fish. Rhythms mostly depended on food availability, but the light/dark cycle did also influence.

Acknowledgments

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EFFECTS OF GHRELIN ON THE RESPONSE TO OLEATE OF HYPOTHALAMIC FATTY ACID SENSORS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): INVOLVEMENT IN THE CONTROL OF FOOD INTAKE

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We have previously demonstrated the existence in hypothalamus of rainbow trout of fatty acid (FA) sensing systems responding to changes in the levels of LCFA like oleate or MCFA like octanoate. The activation of these systems induces an anorectic effect through changes in the expression of hypothalamic neuropeptides like NPY/AgRP and POMC/CART. The hypothalamic neurons possessing these sensors are known in mammals to integrate information from levels of circulating hormones like insulin, leptin or ghrelin. Ghrelin (GHRL) is a gastrointestinal hormone that plays an important role in the regulation of hormone release, energy balance, and control of food intake. We hypothesize that GHRL counteracts the response to increased fatty acid levels of hypothalamic fatty acid sensing systems. Thus, we administered ICV to 100g rainbow trout 1µl of saline-HPB (control) or containing 200 ng GHRL, 1 µmol oleate or both GHRL+oleate. After oleate treatment we observed an activation of fatty acid sensor systems, increased anorectic potential and inhibition of food intake in agreement with our previous studies thus validating the experimental design. After GHRL treatment, we observed a significant increased production of orexigenic factors (NPY, AgRP) and a significant decrease in the production of anorexigenic factors (POMC and CART) whose overall balance would be an increased orexigenic potential also in agreement with the finding of a raised food intake. The administration of GHRL+oleate resulted in a response of fatty acid sensing systems, expression of neuropeptides, and food intake similar to control group, thus suggesting that GHRL is effectively counteracting the effects induced by raised oleate levels. We therefore demonstrate, for the first time in fish, the modulatory action of GHRL of the metabolic hypothalamic integration related to the control of food intake.

Introduction

The role of GHRL in fish is far from elucidated though it has been involved in the control of food intake, glucose and lipid metabolism, and the modulation of growth hormone and prolactin release. Furthermore, there is no information available in fish regarding the possible effects of GHRL on hypothalamic fatty acid metabolism and the response of fatty acid sensing systems. Based on the anorectic actions of LCFA like oleate in rainbow trout (1), the orexigenic actions of GHRL in fish (2), and the fact that plasma GHRL levels increase in rainbow trout after a meal, especially when fish were fed with a lipid-enriched diet (3), we hypothesize that the response to raised levels of fatty acid of food intake, hypothalamic fatty acid-sensing mechanisms, and expression of neuropeptides involved in the control of food intake might be counteracted by GHRL treatment in rainbow trout. Therefore, we aimed to elucidate i) the effects of GHRL on hypothalamic fatty acid metabolism, and the response of fatty acid sensing systems involved in the control of food intake; ii) the possible modulatory effect of GHRL on the effects of raised levels of oleate. Thus, we centrally injected GHRL in the presence or absence of oleate and evaluated food intake, the mRNA abundance of hypothalamic neuropeptides involved in the control of food intake and variables related to putative fatty acid-sensing systems.

Materials and Methods

Rainbow trout (*Oncorhynchus mykiss),* after 1 month of acclimation period, were randomly assigned to 4 experimental groups in different tanks. Fish were fasted for 24h before treatment to ensure basal hormone levels were achieved. On the day of experiment, fish were anaesthetized with 2-phenoxyethanol (Sigma, 0.2% v/v), and weighed. We administered intracerebroventricularly (ICV) (4), 1μl · 100g-1 body mass of vehicle (saline-HPB), or containing 200 ng of rainbow trout ghrelin (synthesized by Bachem) (5), 1 μmol oleate (Sigma Chemical Co.), or GHRL+oleate.

In a first set of experiments, food intake was registered for 3 days before treatment (to evaluate basal level of food intake) and then 6, 24 and 48 h after ICV treatment with saline-HPB (control, n=10 for each time point) or containing GHRL (n=10 for each time point), oleate (n=10 for each time point) or GHRL+oleate (n=10 for each time point). After feeding, the food uneaten remaining at the bottom (conical tanks) and feed waste were withdrawn, dried and weighed. In a second set of experiments, fish were ICV injected with saline-HPB (control, n=15 at 2h, and n=15 at 6h) or containing GHRL (n=15 at 2h, and n=15 at 6h), oleate (n=15 at 2h, and n=15 at 6h) or GHRL+oleate (n=15 at 2h, and n=15 at 6h) with the same concentrations described above. After 2h or 6h, fish were lightly anaesthetized with 2-phenoxyethanol (Sigma, 0.2% v/v). Blood was collected and then fish were sacrificed by decapitation and hypothalamus were dissected. At each time, 9 fish per group were used to assess metabolite levels and enzyme activity whereas the remaining 6 fish were used for the assessment of mRNA levels by qRT-PCR.

Results and Discussion

The central treatment with oleate alone resulted in hypothalamic changes of parameters related to fatty acid sensing. These changes include increased levels of fatty acid, triglyceride and total lipid, decreased activities of CPT-1 and ACLY, and decreased mRNA abundance of ACLY, CPT-1c, UCP2a and Kir6.x-likle. These changes are indicative of the activation of different fatty acid-sensing systems in response to raised levels of oleate. The activation of these systems is also associated with the increased mRNA abundance of the anorexigenic peptides POMC and CART, and the decreased mRNA abundance of the orexigenic peptides AgRP and NPY (Table 1). The resultant increased anorexigenic potential agrees with the decreased food intake observed in oleate-treated fish (Table 2). All these changes are in agreement with previous studies carried out in rainbow trout (1), which validate the experimental design.

Considering the changes observed in the mRNA abundance of neuropeptides involved in the metabolic control of food intake, we observed that GHRL induced decreased expression levels of the anorexigenic peptides POMC and CART and increased values of the orexigenic peptides AgRP and NPY (Table 1). These changes are indicative of an orexigenic response in well agreement with the changes observed in food intake. GHRL treatment did not affect food intake 6h after ICV treatment but feeding levels increased 24h after treatment and this trend remained (though no significance was found) up to 48h after treatment (Table 2).

Table 1. mRNA levels of POMC, CART, NPY and AgRP in hypothalamus of rainbow trout 2 h or 6 h after ICV administration of 1 μL.100g^{-1} body mass of saline-HPB (Control) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 μmol oleate (OL), or ghrelin+oleate (GHRL+OL).

	2H			6H		
	GHRL	OL	GHRL+OL	GHRL	OL	GHRL+OL
POMC	-1.30*a	+1.45*b	+1.09b	-1.46*a	+1.98*b	+1.20c
CART	-1.33*a	+1.48*b	+1.28b	-1.23a	+1.31b	-1.24a
NPY	+1.25*a	-1.44*b	+1.00a	+1.57*a	-1.21b	-1.12b
AgRP	+1.20a	-1.38*b	+1.33a	+1.56	-1.01	+1.23

Each value is the mean + SEM of n=6 fish per treatment. Data are expressed as fold induction (+, increase; −, decrease) with respect to the control group (expression results were normalized by β-actin mRNA levels, which did not show changes among groups).* Significantly different ($P < 0.05$) from control fish. Different letters indicate significant differences ($P < 0.05$) among treatments.

Table 2. Food intake in rainbow trout 6, 24 and 48 h after ICV administration of 1 μL.100g^{-1} body mass of saline-HPB (Control) or containing 200 ng of rainbow trout ghrelin (GHRL), or 1 μmol oleate (OL), or ghrelin+oleate (GHRL+OL).

	Control	GHRL	OL	GHRL+OL
6 H	100±5.18a	88.24±5.32a	94±3.99b	42.3±3.97ab
24 H	100±5.13a	120.5±6.16b	59±4.78c	69.72±5.67ac
48 H	100±6.04a	109±4.43a	76±3.98b	90±4.89ab

Results are shown as mean + S.E.M. of the results obtained in four different tanks in which 10 fish were used per group in each tank.

Results shows differential effects depending on the fatty acid-sensing mechanism studied. The fatty acid-sensing system related to fatty acid metabolism displayed changes opposed to those previously described after central oleate or octanoate administration in the same species (5), i.e. increased ACC and FAS expression, but absence of changes in FAS and ACLY activities suggesting a desensitization of this FA sensing mechanism.

The mechanism related to binding of fatty acid to FAT/CD36 and subsequent modulation of transcription factors was also affected by GHRL treatment inducing decreased mRNA abundance of FAT/CD36 (2h) and SREBP1c (6h), i.e. changes again opposed to those described in response to raised levels of oleate or octanoate suggesting an inhibition of this fatty acid sensing mechanism.

The mechanism related to mitochondrial activity displayed few changes after GHRL treatment since no or moderate effects were observed on Kir6.x-like and UCP2a expression, respectively. In the mammalian hypothalamus, GHRL is known to

increase the production of ROS and subsequently induce UCP2a expression (6), thus suggesting again a different functional mechanism.

In summary, we have demonstrated, for the first time in a non-mammalian vertebrate, that the orexigenic actions of GHRL in fish are associated with changes in fatty acid metabolism and an inhibition of fatty acid sensing systems in hypothalamus, which ultimately lead to changes in the expression of anorexigenic and orexigenic peptides resulting in increased orexigenic potential and food intake. Moreover, the response to increased levels of oleate of hypothalamic fatty acid-sensing systems (activation), expression of neuropeptides (enhanced anorexigenic potential) and food intake (decrease) were counteracted by the simultaneous treatment with GHRL. These changes provide evidence of a possible modulatory role of GHRL on the metabolic regulation of food intake occurring in hypothalamus of fish. The mechanisms observed are, in some cases, similar to those known in mammals but not in others suggesting a new model of hypothalamic integration of peripheral signals like levels of metabolites (fatty acid) and hormones (GHRL) involved in the regulation of food intake.

Acknowledgments

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FEEDING-INDUCED CHANGES IN THE GHRELINERGIC SYSTEM IN THE PLASMA, BRAIN AND INTESTINAL BULB OF GOLDFISH (*CARASSIUS AURATUS*)

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Ghrelin is a gut-derived peptide hormone with a key role in food intake regulation and energy balance. In mammals, ghrelin secretion and expression is known to increase prior to a meal, suggesting a role for this hormone in hunger and meal initiation. However, results regarding this aspect in fish are controversial. Therefore, the aim of this study was to characterize the pre- and postprandial pattern of plasma acyl-ghrelin (AG), desacyl-ghrelin (DAG) and ghrelin O-acyl transferase (GOAT) in goldfish (*Carassius auratus*) that were either fed or unfed the day of the experiment. Moreover, the expression of *preproghrelin*, *goat* and *growth hormone secretagogue receptors (ghs-r)* was quantified in brain and peripheral tissues. Circulating hormone levels were measured by immunoassays, and gene expression was determined by real-time RT-PCR. Results show a significant preprandial rise in circulating levels of AG, but not DAG, and GOAT, supporting the role of acylated ghrelin as a meal initiator in this teleost. Consistently, *preproghrelin* and *ghs-r1a1* expression increases 1-h before scheduled feeding time in intestinal bulb, suggesting that this receptor subtype might be involved in the preprandial action of ghrelin in this tissue. Neither *preproghrelin* nor *ghs-r1a1* are observed to be modified postprandially in intestinal bulb; by contrary, *goat* and *ghs-r1a2* mRNA levels are significantly higher in unfed fish after subjective feeding time. In brain, none of the studied genes are modified preprandially, except for *goat* in the hypothalamus, whose expression is reduced 1-h before scheduled feeding time. Postprandially, a significant increase in mRNA levels of unfed fish is detected for *preproghrelin* in telencephalon, *goat* in both telencephalon and hypothalamus, *ghs-r1a1* in vagal lobe, *ghs-r1a2* and *ghs-r2a1* in hypothalamus, and *ghs-r2a2* in telencephalon and vagal lobe. Collectively, the periprandial variations observed in this study support the role of ghrelin as an orexigenic factor in fish, and show for the first time a tissue-dependent implication of GOAT and the four subtypes of GHS-R in the responses of the ghrelinergic system to feeding.

Introduction

Ghrelin is a peptide hormone identified in 1999 as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (1). It is the only biologically active peptide known to undergo a post-translational acyl modification catalyzed by ghrelin O-acyltransferase (GOAT) (1). The first physiological role described for ghrelin as a ligand for the GHS-R was the stimulation of growth hormone release from pituitary (1), but then many other functions have been attributed to this peptide (1, 2). The most important factors regulating ghrelin levels are feeding and the nutritional state. In mammals, an increase in ghrelin secretion and *preproghrelin* expression has been reported prior to a meal (1), as well as an upregulation of the system under negative energy-balance conditions such as fasting (1). In fish, several studies have reported the effects of different periods of food starvation on the ghrelinergic system (2), but few data is available on the periprandial variations of the system (3, 4, 5). In addition, these data is controversial and is restricted to some components of the system, offering only partial results. Furthermore, to our knowledge, no studies to date have investigated the relevance of the acylation process of ghrelin in appetite regulation by this hormone. With this background, this study was focused to investigate the periprandial profiles of the ghrelinergic system in goldfish, i.e. circulating acyl-ghrelin, desacyl-ghrelin and GOAT, and expression of *preproghrelin*, *goat* and the four subtypes of *ghs-r*.

Materials and Methods

Goldfish (*Carassius auratus*, 20-30 g) were housed in seven aquaria (n=7/aquarium), under a 12L:12D photoperiod, and were daily fed at 12:00 h with a commercial flake diet (1% body weight, Sera Pond). Fish from one tank were sampled at 3-h, 1-h and 0-h before scheduled feeding time. At scheduled feeding time, two of the remaining tanks were fed while food was withheld from the other two tanks. At 1-h and 3-h after scheduled feeding time, fish were sacrificed. Blood, telencephalon, hypothalamus, vagal lobe and intestinal bulb were sampled in each sampling point and stored until analysis.

Plasma levels of acyl-ghrelin, desacyl-ghrelin and GOAT were quantified using specific ELISA kits (Mybiosource, San Diego, USA), following manufacturer's instructions. Gene expression was measured by RT-qPCR, using *β-actin* and *elongation factor 1α* (*ef-1α*) as reference genes. RNA extraction, DNase treatment, cDNA synthesis and real-time PCR reactions were carried out as previously described (6). The $2^{-\Delta\Delta Ct}$ method was used to determine the relative mRNA expression. Statistical differences in circulating hormone levels and amount of mRNA transcripts were assessed using one-way ANOVA followed by Student-Newman-Keuls (SNK) multiple comparison ($p<0.05$). A t-test was performed in each post-feeding point to assess statistical differences between fed and unfed fish ($p<0.05$).

Results and Discussion

The periprandial variations in acyl-ghrelin, desacyl-ghrelin and GOAT plasma levels are shown in Figure 1. Acyl-ghrelin levels (Fig. 1A) significantly increased from -3 h to -1 h before scheduled feeding and decreased after scheduled feeding in both, fed and unfed fish. Circulating levels of desacyl-ghrelin were around 3-fold lower than detected levels of acyl-ghrelin, and were unaltered periprandially (Fig. 1B). Finally, there was a significant preprandial increase in GOAT plasma levels, although no postprandial differences were detected in none of the experimental groups (Fig. 1C). These results indicate that acyl-ghrelin might be acting as a meal initiator signal in fish, similar to what was previously described in mammals (1).

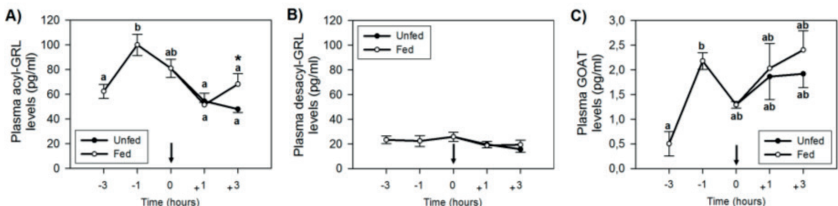

Figure 1. Periprandial changes in plasma acyl-ghrelin (A), desacyl-ghrelin (B) and GOAT (C) levels before scheduled feeding time (-3 h and -1 h), at feeding time (0 h) and after scheduled feeding time (+1 h and +3 h) in goldfish. Data are expressed as mean ± SEM (n=7). Arrows indicate scheduled feeding time. Different letters indicate significant differences among the different time points in fed or unfed groups ($p<0.05$). Stars (*) indicate significant differences between groups at the same time point ($p<0.05$).

Feeding also modified expression of the different ghrelin-related genes, as observed by their periprandial profiles (Table 1). Consistent with the proposed role of ghrelin in initiating individual meals, we observed that ghrelin precursor expression significantly rises 1-h before scheduled feeding time in intestinal bulb. This is in accordance with the reported increase in stomach *preproghrelin* mRNA levels in tilapia (4), but disagrees with the lack of effect in the gut of goldfish (5) and zebrafish (3). These discrepancies could be due to the fact that, since the stomach (or its equivalent in stomachless fish, i.e. the intestinal bulb) is the main source of ghrelin, it is plausible that if variations in expression are slight can be only detected when analysing this part of the gut separately. Two ghrelin receptor subtypes are present in intestinal bulb, but GHS-R1a1 seems the one to be involved in the preprandial action of ghrelin in this tissue, as both genes (*preproghrelin* and *ghs-r1a1*) show a similar expression profile with an important preprandial increase. The *goat* expression was unaltered in intestinal bulb before meal, but it significantly increased after feeding time in unfed fish, in agreement with previous reports in zebrafish gut (3). Postprandially, expression of *ghs-r1a2* was also significantly higher in unfed fish, but neither *ghs-r1a1* nor *preproghrelin* were modified. This observation could indicate the implication of GHS-R1a1 in habitual ghrelin responses to feeding in the gastrointestinal tract, while GHS-R1a2 might act under no available food conditions.

	Preproghrelin			***Goat***			***Ghs-r1a1***		
	Pre	**Post**		**Pre**	**Post**		**Pre**	**Post**	
		F	U		F	U		F	U
Tel	=	=	+	=	=	+	=	=	=
Hyp	=	=	=	=	=	+	=	=	=
VL	n/m	n/m	n/m	n/m	n/m	n/m	=	=	+
IB	+	=	=	=	=	+	+	=	=

	Ghs-r1a2			***Ghs-r2a1***			***Ghs-r2a2***		
	Pre	**Post**		**Pre**	**Post**		**Pre**	**Post**	
		F	U		F	U		F	U
Tel	=	=	=	=	=	=	=	=	+
Hyp	=	=	=	=	=	=	=	=	=
VL	=	=	+	n/m	n/m	n/m	n/m	n/m	n/m
IB	=	=	+	=	=	=	=	=	+

Table 1. Summary of the periprandial changes in *preproghrelin*, *goat*, *ghs-r1a1*, *ghs-r1a2*, *ghs-r2a1* and *ghs-r2a2* mRNA expression in telencephalon (Tel), hypothalamus (Hyp), vagal lobe (VL) and intestinal bulb (IB) of goldfish. Results are expressed as an upregulation (plus sign), downregulation (minus sign) or unalteration (equal sign) of the mRNA levels before scheduled feeding time (Pre, preprandial) or after scheduled feeding time (Post, postprandial) in both, fish fed at the regular feeding time (F) or unfed (U). n/m, not measured.

In the brain, we observed that the modulatory effect of feeding on the expression of the ghrelinergic system is dependent on tissue. *Preproghrelin* expression was unaltered periprandially in hypothalamus, but a significant increase in mRNA levels was detected in telencephalon of fish that missed the scheduled feeding, observations that are in accordance with previous reports in the goldfish hypothalamus (5) and the zebrafish brain (3), respectively. By contrast, there are in discordance with the observation that *preproghrelin* expression increases in the brain of tilapia before feeding (4). This discrepancy might lie on the possibility that an increase in *preproghrelin* expression is produced in a brain area different to the

hypothalamus or the telencephalon. Expression of *goat* was also affected by feeding in telencephalon, supporting the relevance of this region in the regulation of feeding responses in the goldfish brain. Concerning the receptors, our major finding is that the lack of a scheduled meal produced a significant increase in the expression of all *ghs-r* subtypes in the vagal lobe. This points out the vagal lobe, together with the telencephalon, as targets for the effects of a meal ingestion on the ghrelinergic system. It is important to note that for the three genes we found considerable differences in their periprandial expression profile in fed versus unfed fish. This suggests that most of the changes observed are likely due to the presence or absence of a meal, indicating an important modulation of the goldfish ghrelinergic system by food availability.

In conclusion, this study suggests that the telencephalon, vagal lobe and intestinal bulb are key targets for the effects of food availability on the ghrelinergic system, and demonstrates that GHS-R1a1 receptor subtype could be involved in habitual ghrelin responses to feeding while GHS-R1a2 might act in situations of food scarcity. Furthermore, differential periprandial profiles in circulating levels of acyl-ghrelin, desacyl-ghrelin and GOAT indicates that ghrelin in its acylated form is implicated in the ghrelin-induced responses to feeding, and might be acting as a meal initiator signal in goldfish.

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SERUM METABOLOME OF FASTED GILTHEAD SEA BREAM. A NON-TARGETED APPROACH FOR THE IDENTIFICATION OF ROBUST BIOMARKERS OF MALNUTRITION IN FISH

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The study assessed the metabolomic fingerprinting of fasted gilthead sea bream by means of ultra-high performance liquid chromatography (LC) coupled to quadrupole time-of-flight mass spectrometry (QTOF MS). More than 15,000 different *m/z* ions were represented in the analyzed samples and discriminant analysis (PLS-DA) allowed a clear differentiation between the two experimental groups (fed and 10-days fasted fish), explaining the first component more than 90% of total variance. The most significant metabolites (up to 45) were elucidated based on their tandem MS (MS/MS) spectra with a broad representation of amino acids, oligopeptides, urea cycle metabolites, L-carnitine-related metabolites, glutathione-related metabolites, fatty acids, lysophosphatidic acids, phosphatidylcholines, and biotine and noradrenaline metabolites. This highlighted important adaptive response in energy and oxidative metabolism, contributing this non-targeted approach to identify robust and nutritionally-regulated biomarkers of health and metabolic condition in farmed fish.

Introduction

Fish aquaculture is the sector of animal livestock production with higher growth rates at the global level. This industry highly contributes to cover the current but also the future demand of nutritious quality food for human consumption [1]. This starts with the selection of high quality raw materials to ensure that aquaculture is an efficient and environmentally sustainable sector. However, we need to refine our knowledge on nutrient requirements to produce more robust, safe and quality fish, especially with the advent of new diet formulations that are based on plant ingredients rather than marine ingredients from limited fishery resources [2]. As a result, research in fish nutrition are moving from classical methodologies to omic approaches, including transcriptomics, proteomics and metabolomics.

Unlike nucleic acid or protein-based omic techniques, metabolomics has to deal with low-molecular weight metabolic entities (< 1,000 Da) with very diverse chemical and physical properties, which can vary from millimolar to picomolar concentrations making precise quantitative determinations more difficult. Two analytical platforms are currently used for metabolomic analyses: Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) [3]. NMR sensitivity is one of its main drawbacks, making MS analyzers coupled to gas chromatography (GS-MS) or high-performance liquid chromatography (HPLC-MS) more feasible and informative techniques that have demonstrated its great potential in human metabolomic studies [4]. However, few studies have addressed in depth the metabolomic profiling or fingerprinting of body fluids of livestock animals, especially fish, with 33 results searching “MS metabolomics fish” in Scopus. Thus, the aim of this study was to demonstrate that LC-MS approaches properly describe a given metabolic phenotype, comparing the serum metabolome of well- and malnourished-fish in a short-term fasting model. The study was conceived as the proof of concept of MS approaches to identify and validate robust biomarkers of nutritional and metabolic condition in farmed fish, using gilthead sea bream (*Sparus aurata)* as a warm-water fish model highly cultured in all the Mediterranean region.

Materials and Methods

Two-year-old gilthead sea bream of Atlantic origin (average initial weight 380 g) were reared from early life stages under natural light and temperature conditions in the experimental facilities of IATS. At mid-summer (July 2014), 30 fish were randomly allocated in 500L tanks. One group continued to be fed with a standard commercial diet (Biomar) to visual satiety one time per day, whereas the other group remained unfed for a 10-days period. At the end, 10 fish from fasted and fed groups (following overnight fasting) were randomly sampled and anaesthetized with MS-222 (100 mg/L) for blood and tissue sampling. Blood was taken from caudal vessels with vacutainer tubes with a clot activator for a fast serum separation.

Prior final processing, serum samples were deproteinized with acetonitrile (1:3). A serum deproteinized aliquot was feasible to be directly analysed by hydrophilic interaction liquid chromatography (HILIC). After solvent exchange with MeOH:H_2O (10:90), a second aliquot was obtained to be analysed by reversed phase (RP) LC. Both analyses were carried out with a Waters UPLC system (Waters, Milford, MS, USA) coupled to a hybrid quadrupole-TOF high resolution mass spectrometry (HRMS) instrument. UPLC columns selected for the analysis were Acquity UPLC BEH 1.7µm particle size analytical column 100 x 2.1 mm (Waters) end-capped with C18 (RP) and HILIC. Positive and negative ionization modes were carried out for each chromatography, obtaining different analytical information related to polarity (RP vs HILIC) and ionization behaviour (positive vs negative ionization mode). A pool of all the samples was obtained and injected for chromatographic column conditioning and also to observe the goodness of the normalization process.

The LC-MS spectral data were processed with the XCMS package (R software) for peak detection, peak alignment and peak area normalization in each data set. The preprocessed metabolomics data were then uploaded into EZ-Info software for multivariate statistical analyses. The MS/MS spectra of most significant metabolites were acquired at 10, 20, 30 and 40 eV collision energy and compared with online spectra databases (Metlin, http://metlin.scripps.edu/). When it was not feasible, in-silico fragmentation software (MetFrag, http://msbi.ipb-halle.de/MetFrag/) was employed for assigning a tentative name by searching them in general chemical databases as Chemspider (http://www.chemspider.com/).

Results and Discussion

At the end of the experimental period, body weight of fed fish was 15% higher than in fasted fish. This fasting protocol also triggered a marked loss of liver and body fat mass, decreasing significantly ($P < 0.05$) the hepatosomatic index and mesenteric fat index from 1.3 to 0.9 and 1.9 to 1.6, respectively.

Data on serum metabolomics were acquired by different LC separations and ionization modes, correctly aligned and normalized to yield about 15,000 *m/z* ions in all 4 batches. All data were then merged for multivariate analysis, no outliers were found by PCA and PLS-DA allowed a clear differentiation between the two experimental groups, explaining the first component more than 85-97% of the total variance (**Fig. 1**). OPLS-DA was then performed to individually isolate the most extreme metabolites (850 m/z ions in the extreme of the plot with 0.05). Among them, up to 45 were finally elucidated: amino acids (4), oligopeptides (8), urea cycle related metabolites (2), acylcarnitines (5), glutathione related compounds (5),

lysophosphatidylcholines (10), phosphatidylcholines (4), fatty acids (5), 3-hydroxyisovaleric acid and MOPEG sulphate.

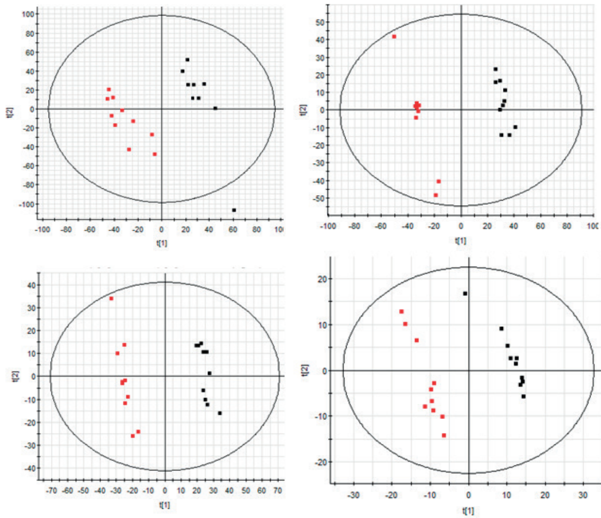

Figure 1. PLS-DA Score Plots for all four injection batches. Up, Reversed Phase plots; down, HILIC plots. Left, Positive ionization modes; right, negative ionizati0on modes. All 4 analyses explained more than 85% of total variance by the first component.

Functional analysis clearly stated an increased mobilization of body energy stores with an improved oxidative capacity of metabolic fuels, which paralleled the onset of specific nutrient deficiencies and changes in the cell redox-balance. These metabolic features are not novel at all under a negative energy balance, but it will contribute to identify highly robust biomarkers of nutritional condition in fish. In this regard, the increased mobilization of body fat stores, exemplified by the loss of liver and adipose tissue mass, was linked to increased circulating levels of sub-products of L-carnitine, a carrier of fatty acids across the inner mitochondrial membrane for their subsequent beta-oxidation. Likewise, activities of urea cycle enzymes are higher in carnivorous fish than in herbivorous and omnivorous fish species, and their regulation by fasting supports an increased muscle protein breakdown and amino acid catabolism, which was evidenced herein by the increased serum concentrations of citrulline, ornithine, argininosuccinate and arginine.

Catecholamines respond to changes in nutrient and sodium intakes, and the increased serum concentration of noradrenalin metabolites (MOPEG substrate) would reflect the activation of the Hypothalamic-Pituitary-Interrenal axis of gilthead sea bream to cope with an enhanced lipolysis and gluconeogenesis triggered by the fasting hypoglycemia. Meanwhile, the urine concentration of 3-hydroxyisovaleric is elevated in humans by the impairment of renal reclamation of biotin, and our results highlighted that this metabolite can be used as a strong fish biomarker of B7 vitamin deficiency and malnutrition in general.

Changes on glutathione (GSH) metabolism also emerged as a major fasting-mediated effect with increased levels of γ-glutamyl amino acids in combination with a depletion of GSH and its precursor gamma-Glu-Cys in the Meister´s γ-glutamyl cycle. This is indicative of changes on the equilibrium between GSH degradation and

synthesis, which is mostly limited by the availability of dietary cysteine. This, in turn, was related to low circulating levels of oxidized methionine compounds, which revealed a low risk of oxidative stress with the reduced production of reactive oxygen species (ROS) under a negative energy balance.

The metabolism of phospholipids was also largely altered under a negative energy balance, and importantly the concentration of lysophosphatidylcholines was consistently reduced in fasted fish. This might reflect a reduced fasting turnover of Land's cycle, which appears mostly regulated by Phospholipase A2 (PLA2) rather than phenylethanolamine N-methyltransferase (PNMT) in fasted fish (5).

In conclusion, MS-based metabolomics is emerging as a promising tool for the discovery and validation of robust and nutritionally regulated biomarkers in farmed fish. Besides, retrospective analysis could be carried out providing additional information about the correct identity and pathway assignation, demonstrating the power of MS faced to NMR.

Acknowledgments

The authors acknowledge the financial support of Generalitat Valenciana, as research group of excellence (PROMETEO II/2014/023, PROMETEO II/2014/085, Collaborative Research on Environment and Food-Safety, ISIC/2012/016), and the Seventh Framework European Project ARRAINA (Advanced Research Initiatives for Nutrition and Aquaculture, KBBE-2011-288925). This work has been developed in the framework of the Research Unit of Marine Ecotoxicology (IATS-CSIC; IUPA-UJI).

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ENERGY SENSING IN GILTHEAD SEA BREAM. MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF SIRTUINS

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Seven nucleotide sequences were unequivocally annotated as sirtuins (SIRTs) 1-7 after extensive searches in the Nutrigroup cDNA database of gilthead sea bream (www.nutrigroup.iat-org/seabreamdb). All isoforms conserved a characteristic enzymatic core domain and phylogenetic analysis showed three major clades according to the accepted classification of SIRTs and the present hierarchy of vertebrate species. Transcriptional studies revealed a ubiquitous SIRT gene expression that was tissue-specific for each SIRT isoform. This was evidenced by multivariate analyses, which established two main clusters corresponding to SIRT isoforms with relatively high (SIRT1, 2 and 5) and low (SIRT3, 4, 6 and 7) expression levels. A nutritional regulation was also evidenced in 10-days fasted fish, and SIRT2, 3 and 4 exhibited an overall down-regulated expression. The opposite trend was reported for SIRT1, 5, 6 and 7, although clustering analyses evidenced a tissue-specific response according to the different tissue metabolic capabilities.

Introduction

Sirtuins (SIRTs) compose the class III family of histone deacetylases (HDACs) that, unlike the class I and II HDACs, require NAD+ as cofactor substrate. This metabolic feature couples protein acetylation and its regulation by SIRTs with the energy status of the cell via the cellular NAD^+/NADH ratio, which is of importance for a number biological processes that regulate cell survival, metabolism and longevity (1). This is consistent with the observation that SIRTs are virtually ubiquitous throughout all kingdoms of life with a number of SIRT variants that ranges from only one in bacteria to seven in vertebrates (2). This offers the possibility of a complementary but also non-redundant tissue-energy sensing. Thus, in mammals, SIRT1-3 exhibit strong deacetylase activity, whereas the other isoforms have weak (SIRT5–7) or undetectable (SIRT4) protein deacetylase activity (3).

The functional diversification of SIRT homologs is also illustrated by their different cellular locations. Thus, SIRT1, 6 and 7 are localized in the nuclear compartment, although SIRT1 is known to shuttle to the cytoplasm. Meanwhile, SIRT2 is primarily cytosolic but is also present in the nucleus, whereas SIRT3-5 are mostly mitochondrial although SIRT3 is also found in the nucleus (4). Another important issue is arisen from substrate diversity. Indeed, since the reported histone deacetylase activity of yeast Sir2p (the founding member of SIRT family), the list of non-histone SIRT substrates is largely increased including a vast array of key transcription factors and enzymes of intermediary metabolism that make difficult to understand its entire complexity (5). Furthermore, the biology and functional regulation of SIRTs remains practically unexplored in livestock animals, especially farmed fish. Thus, the aim of the present study was to underline in a highly cultured fish, such as gilthead sea bream (GSB), the molecular identity and tissue-specific gene expression pattern of each SIRT variant, addressing as well their different responsiveness under a negative energy balance as a result of a short-term fasting period.

Materials and Methods

Molecular identity and phylogenetic analysis. The updated transcriptomic database of GSB (http://www.nutrigroup-iats.org/seabreamdb) was searched for automatically annotated SIRT genes and nucleotide BLAST queries, using SIRT-sequences and predictions from mammals to fish model species, respectively. The identified sequences were manually curated for frame-shifting errors and a PCR approach was used to confirm that the constructs belonged to the same gene transcript. The edited sequences were then blasted for searching conserved domains, and mitochondrial target peptides were identified by means of the on-line CBS prediction services. Amino acid sequence alignments were carried out with ClustalW and the phylogenetic tree was constructed with representative SIRT sequences of lower and higher vertebrate species in MEGA version 6.0, using the Neighbor-Joining algorithm (complete deletion) and p-distance method.

Animal care and tissue sampling. Juveniles of GSB of Atlantic origin were raised from early life stages with commercial standard diets (BioMar) in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC) under natural photoperiod and temperature conditions (40°5N; 0°10E). The oxygen content of water effluents was always higher than 85% saturation, unionized ammonia remained below toxic levels (<0.02 mg/L) and rearing density was maintained lower than 15 kg/m^3. For the tissue-specific screening of SIRT gene expression, two year-old fish (300 g) were randomly sampled and 14 target tissues, including brain, head kidney, gills, liver, skin, adipose tissue, muscle tissues, esophagus, stomach, and different intestine segments were taken and processed as described below. For unraveling SIRT energy sensing, fish of 86 g average body weight were allocated in 500 L tanks in 2 groups of 30 fish each. One group of fish continued to be fed to visual satiety (CTRL group), whereas the second group remained unfed for 10-days. At this end, eight fish per experimental condition were sampled and selected target tissues (brain, head kidney, liver, adipose tissue, skin, white muscle, red muscle, heart, anterior intestine, middle intestine, posterior intestine) were rapidly excised, frozen in liquid nitrogen, and stored at -80ºC until RNA extraction and gene expression analysis.

Gene expression analysis. Total RNA from target tissues was extracted using a MagMax-96 total RNA isolation kit (Life Technologies). Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems) using random decamers and 500 ng total RNA in a final volume of 100 µl. Reverse transcriptase (RT) reactions were incubated 10 min at 25 ºC and 2 h at 37ºC. Negative control reactions were run without RT and quantitative real-time PCR was performed using an iCycler IQ Real-time Detection System (Bio-Rad). Diluted RT reactions were conveniently used for PCR reactions in 25 µl volume in combination with a SYBR Green Master Mix (Bio-Rad) and specific primers for SIRT1-7 at a final concentration of 0.9 µm. The 96-well PCR-array layout was designed for the simultaneous profiling under uniform cycling conditions of all SIRT genes in a given sample tissue. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf) to improve data reproducibility. The fluorescence data acquired during the extension phase were normalized by the delta-delta method using β-as the housekeeping gene.

Statistics. Changes in SIRT gene expression were analysed by Student t-test or by one-way analyses of variance followed by Student-Newman-Keuls test, using the Sigma Plot software. Multivariate analysis (Principal Component Analysis, PCA; and

Hierarchical Clustering with Complete Linkage, HCL) were made by means of Genesis software.

Results and Discussion

Searches in the GSB transcriptomic database recognized seven contigs (6-120 clones in depth) as complete codifying sequences of 307-697 amino acids in length that were uploaded to GenBank with accession numbers KF018666-KF018672. Amino acid sequence alignments of these actively transcribed SIRT isoforms revealed a conserved enzymatic core domain of about 250 amino acids in length. The N- and C-terminal regions are, however, highly variable and SIRT1 was confirmed as the longest SIRT variant through vertebrate evolution (Fig. 1). Amino acid sequence identities (7-29%) and similarities (14-41%) between all the members of GSB SIRT family showed the pattern previously reported in other higher vertebrate species. Even so, the phylogenetic analysis evidenced three major clades (SIRT1-3, SIRT4-5 and SIRT6-7), which entirely agrees with the accepted classification of SIRTs and the present hierarchy of vertebrate species.

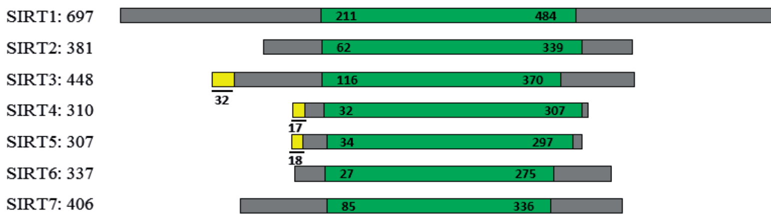

Figure 1. Graphical representation of GSB SIRT isoforms with varying open reading frames (307-697). The enzymatic core domain is labelled in green. Mitochondrial target peptides are labelled in yellow.

The tissue expression pattern of GSB SIRTs revealed a ubiquitous gene expression that was tissue-specific for each SIRT isoform. From the PCA score plot (Fig. 2A), it was possible to differentiate two main clusters corresponding to SIRTs with high (SIRT1, 2 and 5) and low (SIRT3, 4, 6 and 7) gene expression levels. HCL representation revealed that this SIRT differentiation was almost the same in all tissues, except for gills, head kidney, adipose tissue and posterior intestine (Fig. 2B). The physiological significance of this finding remains to be established, although it can be argued some association between SIRT gene expression and the inflammatory/immune response. When the effect of fasting upon SIRT gene expression was analyzed, PCA analysis identified two main clusters, reflecting the up- (SIRT1, 5, 6 and 7) or down-regulation (SIRT2, 3 and 4) by fasting (Fig. 2C). However, the HCL classification revealed a more graded response that ranges from a significant or weak down-regulation in tissues with energy-storage capacity (liver, skin and adipose tissue) to a consistent up-regulation in energy-consuming tissues (red muscle, brain, head kidney and heart), which reflects the different metabolic capabilities and energy needs of each tissue (Fig. 2D).Taken together, these findings support the usefulness of fish SIRTs as sensitive biomarkers of energy sensing and perhaps cell protection against metabolic stresses.

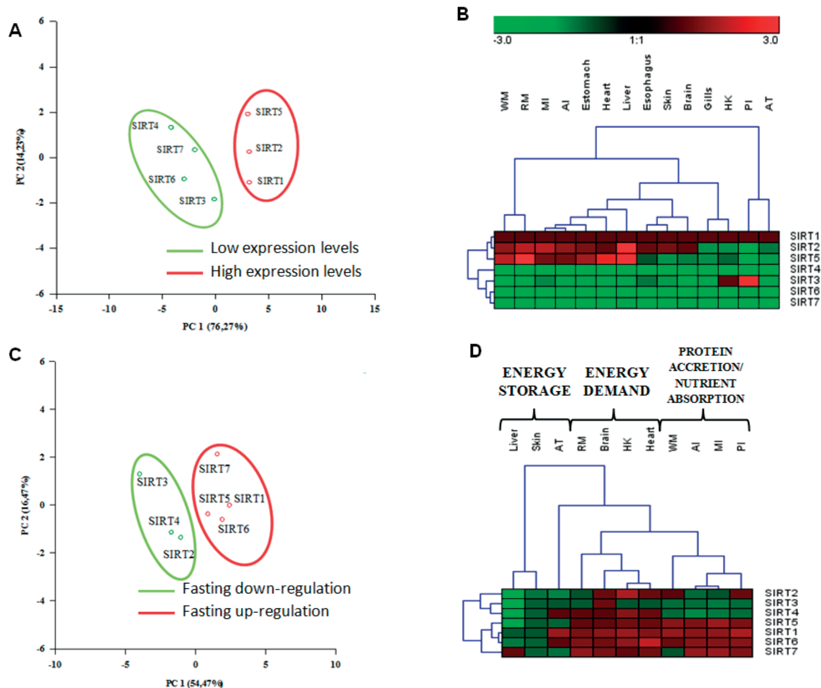

Figure 2. PCA (A) and HCL (B) of tissue-specific expression patterns. PCA (C) and HCL (D) representation of SIRT fold-changes (fasted/fed) under negative energy balance.

Acknowledgments

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METFORMIN EXERTS DIRECT ANTI-PROLIFERATIVE EFFECTS IN PROSTATE CANCER CELLS *IN VITRO* AND INHIBITS PROSTATE TUMOR GROWTH *IN VIVO* UNDER NORMAL-FED AND, SPECIALLY, UNDER HIGH FAT FED CONDITIONS

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Obesity (Ob) is a chronic endocrine-metabolic disease and one of the most serious and complex threats for the human health, which is associated with an increased incidence of some types of cancers such as prostate cancer (PC), the second most common cancer in men worldwide. Interestingly, metformin (Met), an antidiabetic drug, might represents a very promising opportunity to treat Ob and PC as some retrospective clinical studies have shown that the incidence of PC is lower in patients treated with Met. However, the endocrine-metabolic, cellular and molecular mechanisms underlying the association between Ob and higher incidence/aggressiveness of PC and the putative pharmacological effectiveness of Met in PC are still unknown. We used primary normal prostate (NP) cell cultures from mice and human PC cell lines (PC3, 22Rv1 and LNCaP) as well as, immuno-suppressed mice inoculated with PC3 cells, fed a high-fat diet (HFD) or low-fat diet (LFD), as models to test the beneficial effect of Met (in vitro: 10µM-10mM; in vivo: 250mg/Kg). Our results indicate that Met modulates key metabolic, endocrine and pathologic components (e.g. expression of components of insulin/IGF-I/somatostatin/ghrelin systems) in NP cell cultures. Interestingly, Met had no evident effect on 22Rv1 cells proliferation but significantly reduced PC3 and LNCaP cells proliferation and/or migration. Remarkably, we found that Met also have a significant beneficial in vivo effect as it reduced tumor volume and weight in mice fed a LFD and, specially, on those fed a HFD compared to their vehicle-treated control mice. Altogether, our data suggest that Met modulates NP cell function and exerts beneficial effects in the inhibition of PC cells growth in vitro and in vivo, specially, under HF conditions.

Introduction

Obesity has definitively become a worldwide health problem, affecting around 500 million adults in both high and least developed countries. Besides the well-known comorbidities associated to obesity such as insulin resistance, type 2 diabetes (T2D) or increased risk of cardiovascular and other metabolic diseases, obesity has been strongly linked with the development and/or progression of several cancer types [1]. In particular, this is of special relevance in the case of those endocrine-related cancers such as prostate cancer (PC), wherein obesity is associated not only with its development but also with its progression (e.g. obese individuals exhibit larger tumors) [2]. In addition, a recent report has indicated that obesity (particularly, BMI) is associated to the necessity of earlier androgen suppression therapy [3].

In this scenario, metformin (Met), an oral antidiabetic drug commonly used in T2D patients that decreases intestinal absorption of glucose and inhibits gluconeogenesis in the liver, has been shown to improve insulin sensitivity by increasing peripheral glucose uptake and utilization at the same time that it has minimal side effects and low cost. Interestingly, it has been recently demonstrated that metformin treatment might also decrease the incidence of some type of cancer [4]. In particular, recent retrospective clinical studies indicate that Met treatment is associated with lower incidence as well as recurrence of PC in T2D patients [5, 6]. However, to date, the

endocrine-metabolic, cellular and molecular mechanisms underlying the association between obesity and higher incidence of PC and the putative pharmacological effectiveness of metformin in PC are still unknown. Therefore, the aims of this study were to determine if there is a direct association between PC progression, obesity and Met treatment, and to determine whether metformin exerts direct *in vitro* effects in normal and tumoral prostate cells.

Materials and Methods

***In vivo* studies**: Immuno-deficient NUDE Foxn1^{nu}/Foxn1^{nu} male mice inoculated with PC3 cells were used to evaluate PC progression. The animals were housed in sterile filter-capped cages maintained under standard conditions of light (12-h light, 12-h dark cycle; lights on at 07:00h) and temperature (22–24°C), with free access to sterilized diet and water. Mice were fed a low or a high fat diet [10% or 60% Kcal fat, 70% or 20% Kcal carbohydrates and 20% Kcal proteins, respectively (n=11 per group)] for a total of 15 weeks starting at 7 weeks of age. 2 weeks after starting the diets, mice were inoculated with 2.5 million PC3 cells on each back flank. Three weeks later, both LF- and HF-diet fed groups were randomly divided in two different groups: 1) vehicle-treated, (n=5) and, 2) Met-treated (n=6) mice. Vehicle or Met treated (250mg/kg/day in drinking water) mice were maintained for 10 weeks. Tumor volume was measured weekly during this period and tumor weight was recorded at the time of sacrifice. Tumor volume was calculated as: V = area/4 × 3.141618 = (L × W) × 0.785, where L is the length and W is the width of the xenograft. In addition, histopathological and gene expression analysis by quantitative real time PCR (qPCR) were carried out in tumoral samples.

***In vitro* studies**: Human PC cell lines (PC3, 22Rv1 and LNCaP) were cultured and maintained in RPMI 1640 medium complemented with 10% fetal bovine serum, 2mM L-glutamine, and 0.2% of antibiotic (Gentamicin/Amphotericin B). The cell lines were validated by the analysis of short tandem repeat (STR) and the culture was free of micoplasma. All cell lines were maintained at 37°C and 5%CO_2, under sterile conditions. The different PC cell lines were treated with different doses of Met (10 μM-5 mM) to evaluate the effect of this drug in proliferation (n=3; using Alamar Blue assay), and in cell migration (5mM Metformin, n=3; using a wound healing assay). Prostate tissue from mice were used to obtain primary cell cultures (2-3 pooled tissues/experiment; n=3), which were treated with a single dose of Met (10mM) for 24h and then processed to analyze RNA expression levels of genes of interest by qPCR, following standard procedures previously validated by the group [7].

Results and Discussion

Firstly, our results using the *in vivo* mouse model of PC under LF and HF diet conditions revealed that mice fed a HF diet had bigger tumors than the control group fed a LF diet after 6-7 weeks of the inoculation with PC3 cells and this difference was statistical significant until the day of sacrifice (10 weeks after inoculation). These results demonstrated that a HF diet might favor aggressiveness of PC tumors. Remarkably, LF-diet fed mice treated with Met presented smaller tumors compared with vehicle-treated mice. Similarly, tumor weights were also smaller in mice treated with Met under LF-diet condition compared with vehicle-treated mice, although this difference did not reach statistical significance. Interestingly, the effect of Met was significantly more pronounced in the group of mice fed a HF diet. Indeed, tumor volume of Met-treated mice compared to vehicle-treated mice was significantly

smaller at 6-7 weeks after inoculation and was maintained until the day of the sacrifice. Of note, the tumors of HF diet-fed, Met-treated mice were 3 times smaller than that of HF diet-fed, vehicle-treated mice at the day of the sacrifice. Interestingly, comparing both groups treated with Met, the tumor volume and weight of mice fed a HF-diet were significantly smaller than the group fed a LF-diet at the time of the sacrifice. This might suggest that Met treatment could be significantly more efficient in mice fed a HF-diet. Overall, we found that both groups treated with Met have smaller tumors than the vehicle-treated groups, and that HF-diet fed vehicle-treated mice was the experimental group exhibiting the biggest tumors. In contrast, the HF-diet fed, Met-treated mice had the smaller tumors reinforcing the idea the diet and Met treatment might have a direct impact in the progression of PC.

In order to explore the putative reasons underlying these observations, histological analysis of the tumors formed were performed. Although all tumors exhibited similar histotypes, tumors of HF diet fed, Met-treated mice exhibited less necrotic cells and lower number of mitotic cells which could explain why treatment with Met could prevent from the excessive tumor growth observed in mice fed a HF diet. In addition, we observed that tumors of mice fed a LF-diet treated with Met had lower expression of IGF1-R and the Pituitary Tumor-Transforming Gene (PTTG) compared with their controls (vehicle-treated mice fed a LF diet) while mice fed a HF-diet treated with Met had higher expression of p53 and lower levels of PTTG compared with their controls (vehicle-treated mice fed a HF diet) which, altogether, could help to explain the results obtained in the tumors of these mice treated with Met (reduced tumor weight and size compared with vehicle-treated mice).

We further explored the *in vitro* effect of Met treatment on the proliferation and migration of human PC cells. First, we found that Met did not affect the proliferation rate of the 22Rv1 cells; however, in PC3 cells, Met (5mM) inhibited cell proliferation after 72h of treatment. Similarly, Met clearly inhibited cell proliferation in a dose and time dependent manner in LNCaP cells. Therefore, our data indicate that Met exerts antiproliferative effects in a dose, time and cell line dependent manner. Furthermore, we also observed that treatment with Met also inhibited other parameters associated to the aggressiveness of PC cells in that Met significantly inhibited cell migration in PC3 cells.

Finally, in order to explore the putative role of Met on normal prostate cell function, we used mouse primary prostate cell cultures and found that Met significantly altered the expression of some genes involved in metabolic homeostasis as well as in tumoral processes. Specifically, Met up-regulated the expression of IGF-I, its receptor (IGFIR), as well as Insulin-receptor; wherein all these changes might results in an improvement of insulin sensitivity at the prostate level. Furthermore, we found that Met regulated the expression of two key regulatory systems involved in the pathophysiology of different cell types including normal and tumoral prostate cells. Specifically, the treatment with Met up-regulated the expression levels of somatostatin (SST) and SST receptor subtype-2A (sst2A; the higher sst-subtype expressed at the prostate level); while it decreased the expression levels of In2-ghrelin variant. Interestingly, SST/sst2A represents a well-known inhibitory system in many pathologies and the expression of In2-ghrelin variant has been associated with a higher aggressiveness of several types of cancer. Therefore, we could speculate that all these changes might be associated with the antitumoral effect of Met on prostate cells.

Altogether, our data indicates that HF diet might favor the aggressiveness of prostate tumors; wherein Met seems to exert antitumoral effects, being this effect more dramatic under HF diet conditions compared to LF diet, which might be associated to the regulation of tumor cells mitosis and necrosis and to the expression of biomarkers associated with tumor evolution. In addition, our data indicate that Met

exerts direct anti-proliferative effects in human cancer cells which are dependent of the dose applied, the time of incubation and of the type of cell line tested and, also inhibits cell migration. Finally, our data demonstrate that Met also regulates the expression of key genes associated with metabolic homeostasis and with tumoral processes in normal prostate cells.

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WIDE-TRANSCRIPTOMIC ANALYSIS OF INTESTINE IN MEDITERRANEAN FISH

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The plasticity at the molecular level of the intestine tract of European sea bass and gilthead sea bream was studied for the assessment of new intestine health biomarkers of diagnostic and/or prognostic value. Microarray analysis of sea bass intestine highlighted a constant gene expression profile of middle (MI) and anterior (AI) intestine regions. Conversely, more than 1,800 genes were differently expressed between posterior (PI) and AI-MI. The PI resulted to be a highly immune-regulated tissue, with relevance also on vitamin B12 and bile acid metabolism. This feature was closely related to a differential expression of G-protein coupled receptor chemosensors. In gilthead sea bream, a huge (>5,000 genes) different spatial expression pattern (POS *vs* ANT) was also found, which was especially evident in active feeding periods (summer). Minor gene expression changes were detected in the intestine of fish fed balanced low fish meal/fish oil diets, and the use of feed additives reversed most of them towards the wild phenotype. All these findings contribute to identify reliable markers of intestinal health and integrity on a nutritional, seasonal, and developmental basis.

Introduction

The intestinal tract of teleosts is involved in water and electrolyte balance, immunity, and endocrine regulation of nutrient availability and metabolism. Hence, gastrointestinal syndromes due to nutritional deficiencies and infectious agents are important causes of low growth performance, poor health or serious mortalities in farmed fish. For this reason, one of the most important research lines of the Nutrigenomics group of IATS (www.nutrigroup-iats.org) is to assess the effect of new diet formulations and feed additives on the intestinal transcriptome of European sea bass and gilthead sea bream within the framework of different industry contracts and national and European projects. The aim of the present work is to summarize some of the most important outcomes, showing the plasticity of the intestine in two intensively cultured fish in the Mediterranean basin.

Materials and Methods

Animals and tissue sampling. European sea bass were reared from early life stages under natural conditions of light and temperature in the IATS experimental IATS facilities. Fish were fed commercial standard diets provided by BioMar. At midsummer (fast growing fish), overnight fasted fish of 200 g average weight were anesthetized, bled and sampled for anterior (AI), middle (MI) and posterior (PI) intestine segments for RNA extraction.

Juvenile gilthead sea bream were reared in the same experimental facilities and conditions during 20 months from 10-15 g initial body weight. Fish were fed with three experimental diets based on the formulations of the ARRAINA EU project (2). Fish meal (FM) was included at 25% in the D1 diet and at 5% in D3 and D4. Added oil was either fish oil (FO) for D1, or a blend of vegetable oils replacing 84% of FO in D3 and D4. D4 diet was supplemented with 0.4% of a commercial butyrate preparation (BP-70, NOREL). Fish were sampled for intestine tissue sections (AI and

PI) at month 8 (December 2013, 300 g average body weight) and 17 (September 2014, 800 g average body weight).

Gene expression analysis. Total RNA was isolated by means of the Ambion MagMax-96 for Microarray kit (Applied Biosystems) after tissue homogenization in TRI reagent at a concentration of 100 mg/ml following the manufacturers' instructions. RNA quantity and purity was determined by Nanodrop (Thermo Scientific) and Agilent 2100 bioanalyzer (Agilent Technologies) measurements with absorbance ratios at 260 nm/280 nm above 1.9 and RNA integrity numbers of 9.2-10, indicative of clean and intact RNA.

The updated European sea bass and gilthead sea bream nucleotide databases (www.nutrigroup-iats.org/seabassdb and www.nutrigroup-iats.org/seabreamdb, respectively) were the basis for the construction of custom high-density oligo-microarrays (8 x 15K) that were designed and printed using the eArray web tool (Agilent). The design of the microarrays is stored in the NCBI Gene Expression Omnibus (GEO) database under accession identifiers GPL18910 (sea bass) and GPL19579 (gilthead sea bream).

Total RNA (150 ng) from individuals (n = 8 for each intestine section) were labelled with cyanine 3-CTP (Low Input Quick Amp Labelling Kit, Agilent), and 600 ng of each labelled cRNA were hybridized to microarray slides that were analyzed with an Agilent G2565C Microarray Scanner. Data analysis of differentially expressed genes was carried out with the Genespring GX 13.0 software (Agilent). Functional pathway analysis of differentially expressed genes was performed with the Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com). For each gene, the Uniprot accession of the annotation equivalent for one of the three higher vertebrates model species in IPA (human, rat or mouse) was assigned.

Results and Discussion

In sea bass intestine, microarray gene expression profiling showed a gradient expression pattern with a close association of AI and MI by principal component analysis, accounting the first two components for 41% of total variance (Fig. 1). Hence, these two intestine portions were grouped (AI-MI), as no differentially expressed genes (t-test, Benjamini-Hochberg FDR) were found between them. By contrast, comparison of AI-MI *vs* PI yielded 3,654 differentially expressed genes, reduced to 1,906 (960 up-regulated in AI-MI; 946 up-regulated in PI) after filtering with a fold-change cutoff of 2.0. The magnitude of this change is similar to that reported for changes in oxidative capacity of muscle tissues when comparisons are made among cardiac muscle and aerobic/glycolytic skeletal muscle of gilthead sea bream (3).

The functional pathway analysis of sea bass intestinal transcriptome supported the differential pattern of molecular and cellular functions through the intestine. In AI-MI, there was an overrepresentation of genes related to digestion and transport of nutrients. In PI, the most significant functions were related to preservation of tissue integrity and cell-to-cell communication. Other genes overexpressed in the PI were those related to absorption of vitamin B12 (protein amnionless, cubilin, gastric intrinsic factor-like, transcobalamin-2) and bile acids (ileal sodium/bile acid cotransporter, gastrotropin). Genes related to immune or antimicrobial response were also represented in the top list of genes with a specific or up-regulated expression in the PI (immunoglobulin-like and fibronectin type III domain-containing protein 1, transmembrane and immunoglobulin domain-containing protein 1, histidine ammonia-lyase, B-L beta chain class II histocompatibility antigen).

An integrated functional pathway analysis, considering as a whole all the differentially expressed genes, revealed a highly significant network related to cell signaling that comprised a vast array of G-protein coupled receptors (GPRs). Interestingly, many of the receptors that were mainly expressed in AI-MI were related to intestine secretion and motility (GPR39; GPR112; alpha-2C adrenergic receptor; neuropeptides B/W receptor types 1 and 2), whereas those GPRs with intestinal cell proliferation and inflammatory function (GPR18; GPR63; GPR84; hydroxycarboxylic acid receptor 2-like; frizzled class receptor 10) usually had a higher expression level in PI.

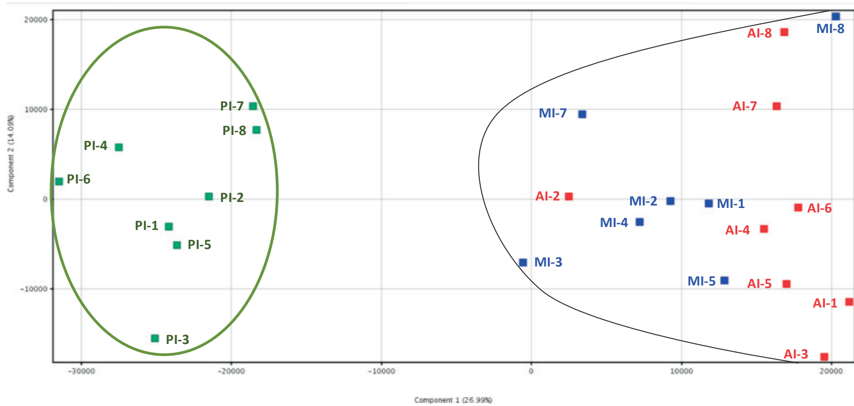

Figure 1. Principal component analysis of the gene expression profile of anterior intestine (AI), middle intestine (MI) and posterior intestine (PI) sections of European sea bass juveniles.

Regarding gilthead sea bream, for every given intestine section and sampling season minor transcriptomic differences were found among the three dietary groups. Consequently, they were very closely grouped in principal component analysis and the two first components explained 60% of total variance (Fig. 2). However, gene expression profiling of selected biomarkers by means of highly sensitive pathway-focused PCR-arrays revealed a pro-inflammatory status on the AI with the highest replacement of FM & FO in fish fed D3, which was mostly reversed with the dietary supplementation of butyrate (D4 diet). These potential benefits of butyrate were also supported by similar responses at the level of intestinal mucus proteome and gut microbiota composition (unpublished results).

On a spatial basis, transcriptomic differences between intestine segments (AI *vs* PI) were more evident in the late summer sampling point (7,303 differentially expressed genes) than in winter (4,925 differentially expressed genes). This enhanced disparity would reflect the different activity of the intestine in active and non-active feeding periods. This finding was reinforced by the observation than more than 6,500 genes were differentially expressed when comparing as a whole the AI-PI of winter and summer. This set of genes would be especially valuable to identify valuable markers of feed conversion and nutrient utilization in a temperate fish species with marked growth seasonality. At the same time, more than 5,800 genes were differentially expressed across the intestine regardless of season, which is clearly indicative of the divergent specialization of each intestine section through the evolution.

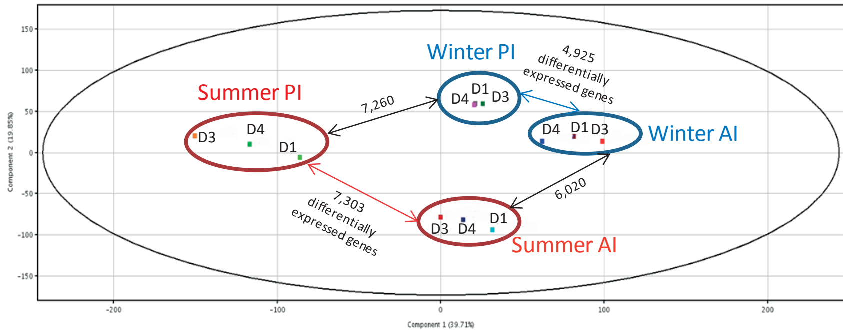

Figure 2. Principal component analysis of the gene expression profile of anterior intestine (AI) and posterior intestine (PI) sections of gilthead sea bream juveniles sampled in summer and winter.

All these results highlighted the plasticity of intestine and the convenience of wide- and targeted-transcriptomic analysis, alone or in combination with other omics approaches, to further understand how the function and integrity of the intestine can be modified and regulated by nutritional and environmental factors.

Acknowledgments

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THE SKIN AS AN ENDOCRINE ORGAN: INSIGHTS FROM THE SEA BASS SKIN TRANSCRIPTOME

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The skin in animals is an important organ for protection, sensing and communication. While in mammals it is a recognized site of hormone action and production, in fish the endocrine regulation of skin functionality is not well explored. The present study used next-generation sequencing to generate the transcriptome of sea bass (*Dicentrarchus labrax*) skin. The sea bass skin transcriptome contained 31,856 unique transcripts with a minimum and average length, respectively of 500 and 1070 base pairs. 88% of the transcripts were assigned to the sea bass genome and 95% to existing proteins or cDNAs in databases. Clustering of transcripts using general gene ontology (GO) revealed GO categories related to hormone-regulated biological processes and activities. Targeted analysis of clusters identified transcripts for hormones and receptors from several endocrine axis in the skin, supporting the hypothesis that fish skin is both a target tissue and a source of hormones. This transcriptomic survey is an important resource for future research on the impact of the environment on skin function and for the recognition of the skins endocrine contribution to whole organism physiology.

Introduction

The skin is the largest organ in the body and plays crucial roles for animal protection, sensing and communication. Fish skin has a similar structure to mammalian skin with the exception of the calcified scale layer and an outer non-keratinized epithelia, and it also has specialized roles related to osmoregulation and mineral homeostasis [1]. In mammals the skin is recognized as a site of hormone action but also of hormone production and modification [2]. In fish, some studies have characterized the effects of sex steroids and hormones on mineral homeostasis, pigmentation or regeneration of the skin or scales [reviewed in 1], but the emerging concept of skin as an endocrine organ is unstudied. The present study used RNA-seq to explore the potential endocrine function and/or regulation of fish skin, by generating and analyzing the transcriptome of sea bass skin.

Materials and Methods

Sea bass (<1g) were obtained from Ifremer, Palavas-les-flot (France) and maintained at the CCMAR Marine Station, Faro (Portugal). Fish were maintained in 500 L flow-through seawater tanks, at natural temperature and photoperiod and fed with commercial dry pellets at 1%. On the day of sampling, six immature one year-old sea bass (18.8 ± 0.3 cm; 75.8 ± 1.1g) were anesthetized in 2-phenoxyethanol (Sigma-Aldrich, dilution 1:5,000 in seawater). Skin samples were collected from the anterior region of the fish using a scalpel and carefully removing the adherent muscle, and immediately frozen in liquid nitrogen. Total RNA was extracted from frozen skin using an automated Maxwell 16 Instrument and 16 SEV total RNA purification kit (Promega) after mechanical disruption with an Ultra Turrax homogenizer (IKA). RNAs of each individual fish were pooled (0.5µg/fish, n=6), treated with DNAse (DNA-free kit, Ambion) and RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyser, which confirmed all samples had an RNA integrity number above 8. Library preparation was conducted at the Shanghai Ocean University (China)

using a TruSeq Illumina mRNA library prep kit with 0.5ug of skin RNA, followed by 100 base pairs (bp) paired-end sequencing in an Illumina HiSeq 1500. Reads were quality checked and adapters removed using fastqc and cutadapt and the filtered reads assembled with Soapdenovo.

Assembled contigs were annotated using stand-alone Blast against three different databases: i) the Swiss-Prot protein database (http://www.uniprot.org/, March 2015); ii) the annotated sea bass genome (http://seabass.mpipz.de/, June 2012 dicLab v1.0c draft) allowing a maximum distance of 1000bp to the annotated gene; and iii) the GenBank/NCBI non-redundant (nr) protein database (March 2015). The E-value threshold was set at $1 \times 10e^{-10}$ and for contigs with significant matches to more than one database, the preference order Swiss-Prot>Genome>GenBank was used for match assignment. Gene ontology (GO) categories (www.geneontology.org) were obtained from the Swiss-Prot hits. Endocrine-related transcripts were searched by name from the annotated sea bass skin transcriptome and gene networks were analyzed using STRING [3]. The RNA-seq raw reads will be available at http://www.ncbi.nlm.nih.gov/sra/ with the accession number SRP071200.

Results and Discussion

RNA-seq using Illumina technology produced 20,694,759 of 100 bp paired-end raw reads from immature sea bass skin RNA. All raw sequence reads passed the quality control threshold (mean bp quality of 35) for inclusion in filtered reads, indicating the sequencing data was of very high quality. Sequences were assembled and produced 31,856 transcript contigs, that were 500 to 11,920 bp in length with an average length of 1,070 bp. These contigs were annotated by Blast against three different databases and 55% matched the Swiss-Prot protein database, 88% matched annotated genes in the sea bass genome and 63% matched NCBI nr proteins (Table 1), with 50% (15,962 contigs) matching the three databases.

Table 1. Numbers and percentage of transcripts annotated to the different databases used for annotation.

	Number	Percentage
Swiss-prot proteins	17,573	55%
Annotated genes in genome	28,163	88%
Genbank/NCBI nr	19,991	63%
Total annotated	30,120	95%
Non-annotated	1,736	5%

Transcript contigs were preferentially annotated to Swiss-Prot proteins (55%), a curated database of known proteins, followed by 38% annotation to the sea bass genome and only 1% of the transcripts without a significant match in the preceding databases were annotated with Genbank/NCBI nr. Only 5% of the contigs remained unannotated and 30,120 contigs were annotated (Table 1), including 91% to known proteins and the remaining to predicted or uncharacterized proteins or cDNAs.

Top categories of the main biological process GO terms represented in the annotated sea bass skin transcriptome, included regulation of biological process, response to stimulus and secretion, suggesting relevant regulatory processes and possible endocrine activity occur in sea bass skin. Specific categories for biological processes included response to peptide or steroid hormone (SH) stimulus, hormone metabolic

process or secretion, thyroid hormone (TH) generation or SH metabolic process. In addition, top molecular function GO terms included binding, receptor activity and signal transducer activity and specific categories included TH receptor binding and coactivator activity or SH receptor activity.

When analyzing the annotated transcriptome for endocrine-related transcripts, several hormone receptors were identified and STRING analysis of endocrine and receptor transcripts in sea bass skin generated a network of nuclear receptors (Figure 1).

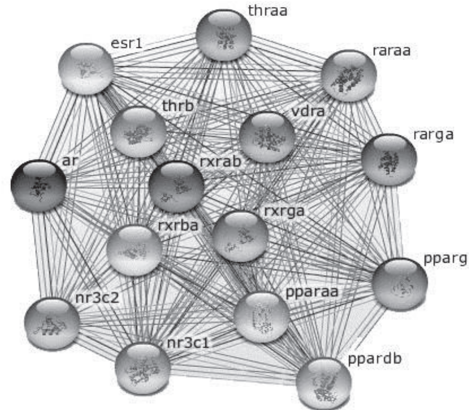

Figure 1. STRING network of nuclear receptors expressed in sea bass skin. Represented are estrogen (esr), androgen (ar), thyroid hormone (thr), vitamin D (vdr), retinoic acid and retinoid X (rar and rxr), glucocorticoid and mineralocorticoid (nr3c1 and nr3c2) and peroxisome proliferator-activated (ppar) receptors. Multiple lines represent evidence of connections (e.g. coexpression or interaction) identified by STRING [3] based on zebra fish genes.

Some of these receptors have previously been detected and related to fish skin or scales physiology. For example, we have previously detected the expression and estrogenic regulation of estrogen receptor (ER) subtypes in the skin and scales of several fish species, which may be related to estrogenic effects on calcium homeostasis and skin-scale regeneration [4-6]. Vitamin D receptors are expressed and regulated by water calcium (Ca) availability in zebrafish skin and are hypothesized to mediate vitamin D regulated Ca uptake [7]. Finally, glucocorticoid receptor expression is regulated by cortisol in rainbow trout skin, and regulates osmoregulation and immune functions in skin [8]. In mammalian skin, other functions were identified for PPARs [9] or RARs [10] but their roles remain to be investigated in fish skin.

Evidence for local hormone production was also obtained and transcripts for stanniocalcin, previously detected in fish skin and probably related to Ca homeostasis, were identified [11]. Finally, the expression of several enzymes related to the cholesterol or steroid hormone metabolism or biosynthesis were detected, including several steroidogenic acute regulatory protein (stAR)-related proteins, 3 beta-hydroxysteroid dehydrogenase 7 (HSD3B7) or cytochrome P450 1A1 (CYP1a1). It remains to be assessed if these contribute to local and systemic steroid production as reported for mammalian skin [12].

In summary, we have generated a well annotated (95%) transcriptome for sea bass skin and a targeted search for endocrine-related transcripts supported the hypothesis that fish skin, together with its calcified scale component, functions as both a target and a site of hormone production. The annotated skin transcriptome will be an

important resource for future research on fish skin physiology and its contribution to whole organism physiology and the impact of environmental stimuli on skin function.

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MULTI-CLASS SCREENING OF FEEDSTUFF CONTAMINANTS AND POTENTIAL ENDOCRINE DISRUPTORS IN FARMED FISH

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A reliable analytical strategy has been developed to evaluate current and recurrent contaminant risks in fish farming. The methodology is based on a generic extraction method followed by ultra-high performance liquid chromatography (LC) and gas chromatography (GC) both coupled to mass spectrometry (MS). In this approach, a quadrupole time-of-flight mass analyzer (QTOF MS) was used for qualitative screening, and triple quadrupole mass analyzer (QqQ) for quantitative analysis. Using this methodology, data from Atlantic salmon and gilthead sea bream trials highlighted that new diet formulations based on plant ingredients increased consumer awareness, although contaminant monitoring systems need to be continuously improved to guarantee a safe aquaculture. The incentive for this will be potentially higher prices and increased consumer demand.

Introduction

Pesticides, polycyclic aromatic hydrocarbons (PAHs), classical persistent organic pollutants (POP), mycotoxins, marker dyes, pharmaceutical compounds and personal care products are considered potential risk factors for animal welfare and food safety. Thus, in animal livestock production, including aquaculture systems, it is important to monitor the presence and carry-over of these undesirables from fish feeds to fish meat to assess the compliance with the current food legislation.

POPs have been one of the most relevant families of undesirables from years. These contaminants are associated to traditional fish feeds and are notably accumulated in fish meat with significant impact on fish welfare and final consumers. The use of plant ingredients in fish feed formulations reduces the presence of POPs in marine aquafeeds, which are usually associated with marine feedstuffs (1, 2). However, plant-derived ingredients can introduce new contaminants that have not been previously associated with fish farming (3-5). This is the case of pesticides, associated to agricultural practices, and also mycotoxins which are well-known natural contaminants that are present in plant feed materials after fungi growth during feed storage.

Participation in competitive National and EU projects (AQUAMAX, ARRAINA) has allowed us to screen a wide-range of contaminants in fish feeds and fish fillets of two main species for the European aquaculture (Atlantic salmon, gilthead sea bream). From these studies is conclusive that mycotoxins, pesticides and PAHs were the most predominant contaminants in current fish feed formulations. In some cases, detectable transfer to the edible part of the fish could happen, mainly for ethoxyquin (expected use as an antioxidant preservative) and the group of light PAHs (4). To further investigate this issue, advanced available analytical methodology based on the use of GC and LC both coupled to MS, with quadrupole-time of flight (QTOF) and triple quadrupole (QqQ) analyzers, has been used. This methodology allows the detection and reliable identification of a large list of potential undesirable compounds in wide-scope screening using QTOF MS. Subsequent quantification by QqQ was

directed towards the compounds found in the initial screening to final evaluate their occurrence and potential implications.

Materials and Methods

Feed ingredients (49 samples), fish feeds (18 samples) and fish fillets from Atlantic salmon (9 samples) and gilthead sea bream (11 samples) were studied. The list of samples covers novel fish feed formulations with plant ingredients and processed animal proteins in order to assess their feed-to-flesh carry-over through almost all the production cycles from juvenile stages to commercial size at harvest.

Samples were analysed using a comprehensive screening (i.e. qualitative analysis) followed by quantification of detected findings. Samples were processed thanks to previously validated methods for aquaculture matrices (3-5). The reliability of the methodology was assessed in every sample batch by analysis of quality control samples, consisting of different sample matrices spiked at several concentration levels with selected analytes (Fig. 1).

Detailed information about analysis by GC and LC coupled to QTOF and QqQ MS analysers can be found in Nácher-Mestre et al., 2013, 2014 and 2015 (3-5).

Results and Discussion

Based on the results of this wide-scope screening (Fig 1.), it is confirmed that POPs and mycotoxins do not represent a problem of food safety in farmed fish. POPs are mainly associated to marine ingredients, but they are finite resources and the use of marine feedstuffs in fish feed formulations has been markedly reduced in the last years by the progressive and combined replacement of fish meal and fish oil by plant ingredients, reducing also the risk of organochlorine uptake by consumers (2,6). Likewise, mycotoxins are a large group of relevant contaminants, but their presence was detected at low concentrations in feed ingredients (below maximum residue limits established by the Recommendation 2006/576/EC). Moreover, the studied mycotoxins were not deposited at detectable levels on the edible parts of sampled fish. Therefore, we concluded that the studied mycotoxins do not represent a food safety affair for public health despite of their increased levels in plant-based diets of a wide range of fish, including salmonid and non-salmonid fish. However, their presence on viscera and body fluids of farmed fish has been reported (7), and this fact has been traditionally associated to a negative impact on animal livestock production (8).

In the present study, residue levels of organophosphorus pesticides pirimiphos-methyl and chlorpyrifos were found in plant-based feeds. These pesticides may disturb steroid production and biosynthesis, but fortunately they were not detected thereafter in fish fillets. Additionally, naphthalene, phenanthrene and pyrene, besides other light PAHs, were regularly found in our analyzed samples. They are contaminants that can give (non-carcinogenic) toxic reactions in fish and might disrupt steroid synthesis and retinoid signaling (9). Likewise, it was confirmed the presence of ethoxyquin at relatively high levels in feeds but also in fish edible matter. Therefore, this synthetic antioxidant and their transformation products can reach consumers as previously reported (4).

The list of prohibited and allowed antibiotics in the EU includes many substances which may co-occur in ingredients of animal origin. In this study, after applying the

described qualitative screening to 19 processed animal proteins, pharmaceutic agents such as monensin, flumequine, enrofloxacin, trimethoprim, tylosin A, acetaminophen, salicylic acid, oxyphenylbutazone, and leucocrystal violet (metabolite of the crystal violet dye) were found. The presence of unintended background levels of illegal pharmaceutical dyes, unreported antibiotics, or unauthorized anti-inflammatory agents might have public health implications and might give cause to legal actions, making their monitoring relevant.

In summary, the developed multi-class screening helps to evaluate possible emerging risk exposition of farmed fish to more than 2000 compounds, including pesticides and POPs (1000), mycotoxins (18), PAHs (24) and pharmaceuticals (1000). The proposed strategy is presented as a useful analytical tool for risk assessment to widen the knowledge on novel ingredients and more conventional feed ingredients. As a major outcome, this exhaustive analytical approach demonstrates that aquaculture products are safe and healthly products. Therefore, the improvement of the perception of acceptance and profitability of aquaculture products will be addressed following the proposed/validated methodology.

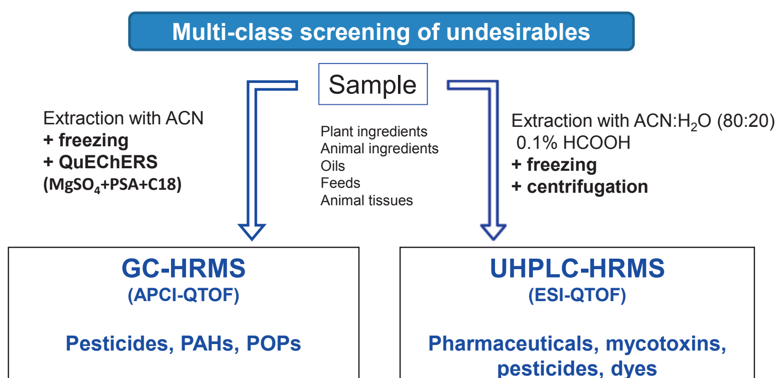

Figure 1. Comprehensive multi-class screening scheme.

Acknowledgments

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ACUTE STRESS REGULATES VASOTOCINERGIC AND ISOTOCINERGIC SYSTEM IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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The hypothalamus-pituitary-interrenal (HPI) axis is involved in stress response. Although corticotrophin releasing hormone (CRH) is considered the main hormone that activates this axis, other hormones, such as CRH binding protein (CRH-BP), arginine vasotocin (AVT) and isotocin (IT), are considered as important players in stress regulation. This study assessed, using the gilthead sea bream (*Sparus aurata*) as a biological model, hypothalamic mRNA expression changes of different endocrine factors (*crh*, *crh-bp*), as well as different neuropeptide precursors (*pro-VT* and *pro-IT*) and their specific receptors (*avtr v1a2*-type, *avtr v*2-type, and *itr*) after an acute stress situation induced by air exposition. According with our results at hypothalamic level, acute stress centrally affects mRNA levels of all precursors (*pro-VT* and *pro-IT*) and receptors (*avtr*s and *itr*), as well as hormonal factors (*crh* and *crh-bp*), by increasing and modulating their gene expression levels from the first 15 min post-stress.

Introduction

Important players in the hypothalamus-pituitary-interrenal (HPI) axis, which is involved in the regulation of the neuroendocrine stress responses, are not only corticotropin releasing hormone (CRH, generally considered as the initiator of this pathway) and CRH binding protein (CRH-BP, an antagonist of CRH function), but also the neurohypophyseal hormones arginine vasotocin (AVT) and isotocin (IT). Some studies have pointed out that synthesis and secretion into the bloodstream of those hypothalamic neuropeptides mentioned before change in response to different stress sources (1, 2, 3, 4). The specific receptors of those hormones in several target tissues are the key to produce the physiological action. However, there is partial knowledge on the functional role of the vasotocinergic and isotocinergic system throughout its receptors (AVTRs and ITR, respectively) in fish. In this work, the transcriptomic evolution of several players involved in the stress response of juvenile specimens of the gilthead sea bream (*Sparus aurata*), submitted to air exposition as an example of acute stress, was assessed.

Materials and Methods

Immature individuals of gilthead sea bream (*S. aurata*, Linnaeus 1758) (n= 64, 108.83 ± 1.47 g body mass; 17.79 ± 0.07 cm body length) were provided by "*Servicios Centrales de Investigación en Cultivos Marinos*" (SCI-CM, CASEM, University of Cadiz, Puerto Real, Cádiz). Specimens were randomly distributed and maintained in this facility in sixteen 80 L-tanks (n = 4, density 5 kg/m^3) containing flowing seawater (SW, 38 ‰ salinity), at constant temperature (18-19 °C) and natural photoperiod (February-March 2013; approximately 11 hours of light).

After 15 days of acclimation to these conditions, fish were exposed to air for 3 min by lifting the wire-net cage out of the tanks and putting back in their respective tanks after that, being sampled in a time course response (15 min, 30 min, 1, 2, 4 and 8

hours post-stress). In addition, an extra group without stress was also constituted and sampled at 0 and 8 h of the beginning of the experiment. This last group served to identify and discard possible circadian rhythms in the mRNA expression patterns of those genes analyzed that could mask our results. Plasma cortisol levels were measured by using the ELISA (Enzyme-Linked Immunosorbent Assay) as previously described by (5) for this species. Hypothalamic *crh*, *crh-bp*, *pro-VT* and *pro-IT*, as well as AVT and IT (*avtr v1a2*-type, *avtr v2*-type and *itr*, respectively) receptor mRNAs expression were carried out by total RNA extraction cDNA synthesis and qPCR with commercial kits, and following the ΔΔCT method (6) using specific primers as previously described (3, 5, 7).

Results and Discussion

In our experiment, high *pro-VT* expression levels are seen in the first hour post-stress, suggesting a role of AVT also during the acute stress processes (Fig.1a). This indicates the existence, from the beginning of the application of the stressor, of a regulation of the entire endocrine cascade triggered and mediated by different endocrine systems that are independently expressed (see below). In addition, the absence of variations in *pro-VT* expression between specimens not submitted to a stress situation by emersion, and sampled at different times (0 h and 8 h) suggest that this system, at least at hypothalamic level, does not appear to be subjected to circadian variations. Moreover, the existence of a high *pro-IT* (Fig. 1b) gene expression values also suggests the involvement of isotocinergic pathways in stress processes (4). In this regard, it has been also shown that *S. aurata* individuals held under high stocking density and food-deprivation (two conditions that can be considered as chronic stress situations) enhanced plasma cortisol and IT levels (2).

Furthermore, values of *avtr v1a2*-type (Fig.1c) mRNA expression significantly increased at 30 minutes post-stress, descending rapidly after that to similar values observed at the beginning of the trial. Moreover, *avtr v2-type* expression levels (Fig.1d) revealed a different expression pattern respect to that found for *v1a2-type* receptor, with activation of the *v2*-type just 8 hours post-stress. These results suggest that *avtr v1*-type might be involved in the control of the endocrine system during the first moments of stress, whereas *avt*r *v2-type* could control different metabolic enzymes for extra energy supply and repartitioning process to cope with this physiological action required, as has been previously demonstrated in *S. aurata* submitted to environmental salinity challenge (7). Also, down regulation of hypothalamic *itr* (Fig. 1e) gene expression indicates a possible negative feedback of enhanced plasma cortisol values (Fig. 1h) on this receptor (4).

Both HPI axis and stress system activation by air exposition has been previously demonstrated in *S. aurata* (8). Our results showed that acute stress (due to air exposition for 3 minutes) modified hypothalamic *crh* and *crh-bp* gene expression, suggesting the activation of endocrine systems as an important piece in the interrenal regulation of HPI axis. Moreover, the significant (≈ 10-fold) increase in plasma cortisol levels following exposure to air corroborates the activation of HPI axis with the existence of a primary stress response. Even so, this response can be considered temporary, since the plasma concentration of this hormone returned to baseline levels during the first hours post-stress (Fig.1h).

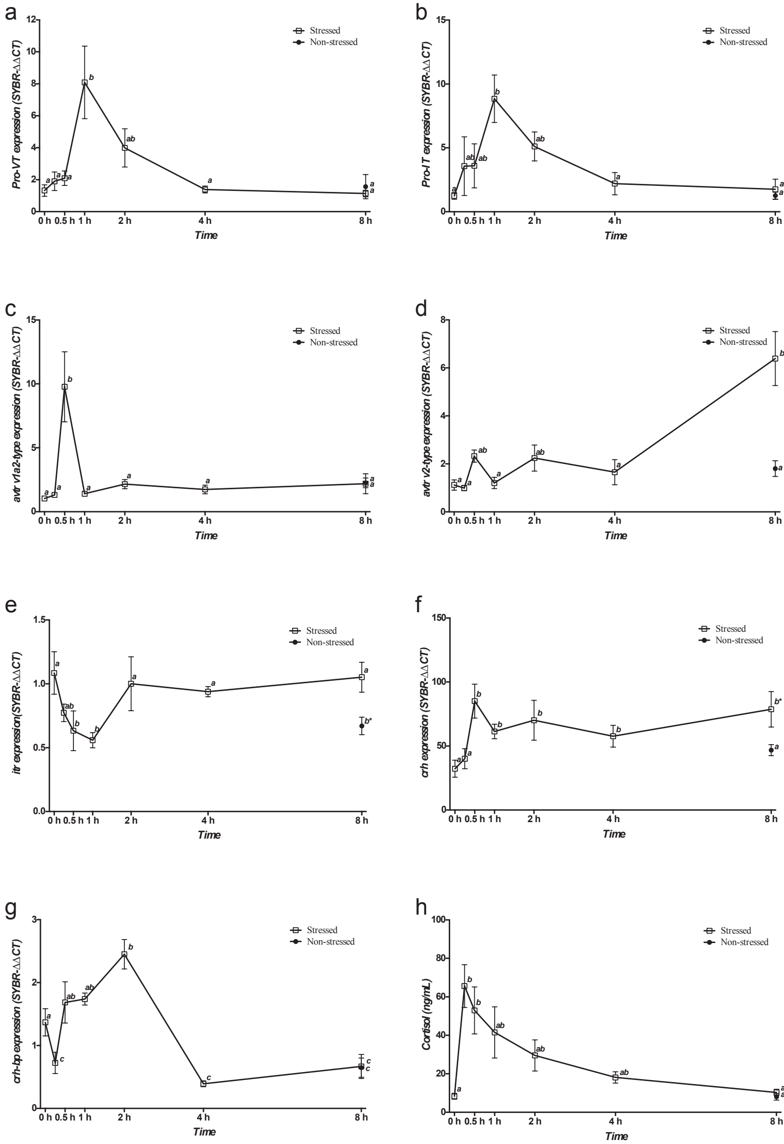

Figure 1. Time course of changes in hypothalamic *pro-VT* (a), *pro-IT* (b) *avtr v1a2*-type (c) *avtr v2*-type (d), *itr* (e), *crh* (f) and *crh-bp* (g) mRNA expression levels (relative to β- actin), as well as plasma cortisol levels (h) in specimens of *S. aurata* exposed to air for 3 min. Values are represented as mean ± S.E.M. (n = 8 fish per group). Significant differences within each group for different experimental times are identified with different letters (P<0.05, one-way ANOVA followed by Tukey's test).

In conclusion, our results show that variations in mRNA expression of those hormones analized (*pro-VT*, *pro-IT*, *crh*, *crh-bp* and cortisol) as well as specific receptors (*avtr v1a2*-type, *avtr v2*-type and *itr*) can stimulate and/or regulate the activity of HPI axis from its upstream (hypothalamus) key element.

Acknowledgments

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BRAIN MONOAMINERGIC NEUROTRANSMITTERS DURING CHRONIC STRESS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*).

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Stress is negatively affecting animal welfare in such a way that most behavioral and physiological functions, such as food intake, are jeopardized. In fish, the stress response initiates with the activation of the hypothalamus-sympathetic-cromaffin (HPC), and the hypothalamus-pituitary-interrenal cells (HPI) axes leading to increased plasma catecholamines and cortisol levels. Our previous results point to the important role played by both dopaminergic and serotonergic systems in initiating the response to acute stress. However, little is known regarding the involvement of both systems under chronic stress and their influence on food intake. To address that question an experiment was performed consisting on stressing rainbow trout (*Oncorhynchus mykiss*) by high stocking density for 3 and 10 days. Food intake was evaluated all over the experiment in non-stressed and stressed fish. Plasma levels of cortisol, glucose and lactate, the content of DA and 5HT, and their main metabolites (DOPAC and 5HIAA) in different brain regions, and hypothalamic mRNA abundance of *TH*, *TPH1* were assessed. Our results reveal that stressed animals showed reduced food intake, increased plasma cortisol levels, and enhanced dopaminergic and serotonergic activities in telencephalon, hypothalamus, optic tectum and hindbrain, independently of the stress duration. *TH* and *TPH1* mRNA abundance also increased in stressed trout. Our present results support the hypothesis of a key role played by both dopaminergic and serotonergic systems in initiating and maintaining the neuroendocrine response to stress. In addition their involvement in mediating the stress-related food intake inhibition might be taken in consideration.

Introduction

When exposed to a stressor, animals respond with a complex series of behavioral and biochemical mechanisms at different levels in order to be prepared to cope with any potential threat (1). The stress response involves the activation of the hypothalamus-sympathetic-cromaffin axis (HPC), in parallel to that of hypothalamus-pituitary-interrenal cells (HPI). Thus, plasma catecholamines and cortisol levels increase. Several biochemical factors participate in the normal function and regulation of these axes in fish. Among them, monoaminergic neurotransmitters, the catecholamines dopamine (DA) and noradrenaline, and the indoleamine serotonin (5-hydroxytryptamine, 5HT), play an important role, acting as an early signal during the initial steps of the response but also as a late response in situations of maintained stress (2). Our previous results have also demonstrated the important role played by both dopaminergic and serotonergic systems in initiating and maintaining the response to acute stress in rainbow trout (2). However, little is known regarding the involvement of both monoaminergic systems at different levels, including food intake, when the stressor lasts for longer time periods (i.e., chronic stress). To pursue this issue, we evaluated the dynamics of brain monoaminergic activities in rainbow trout during chronic stress by exposing fish to high stocking density for 3 and 10 days. The relationship with changes in food intake was also assessed.

Materials and Methods

Rainbow trout (*Oncorhynchus mykiss*) weighing 81.11 ± 5.25 g were obtained from a local hatchery (Javier de la Calle, A Estrada, Spain). Fish were acclimated in 100-L freshwater and aereated tanks under a 12L:12D photoperiod and 14 ± 1ºC water temperature. Animals were daily fed (1% body mass) with commercial dry pellets. After acclimation, four sets of animals (N=25/tank) were anesthetized (MS-222), weighed and turned back to their respective tanks. Food intake was daily assessed up to the end of the experiment, for which each tank received food (3% body mass) for 15 min. The remaining food was recovered, dried, weighed and extracted from the original food weight. Once food intake stabilized two sets of trout were subjected to mild stress by decreasing water level until reaching a stressful high stocking density (HSD; 70 kg fish mass m^{-3}). One set of fish remained for 3 days under HSD, whereas the second one lasted for 10 days before sacrificed. The remaining two sets of trout remained in normal housing conditions for 3 and 10 days before sacrificed. On the day of sacrifice fish were deeply anesthetized by addition of 0.2% 2-Phenoxyethanol to fish tank. Trout were immediately weighed, blood collected and sacrificed. Hypothalamus, telencephalon, optic tectum and medulla oblongata were dissected under sterile conditions, frozen on dry ice and stored at -80ºC until analyzed. The content of DA and 5HT, and their main metabolites (DOPAC and 5HIAA) on each brain region were HPLC assayed. The expression of hypothalamic *TH*, *TPH1* (the rate limiting enzymes of DA and 5HT synthesis) was also qPCR assayed. Plasma was obtained after blood centrifugation (6000 g for 10 min at 4°C) and divided in 50-μl aliquots and stored at -80ºC until assessed for cortisol, glucose and lactate levels. Data were analyzed by two-way ANOVA tests, with "stress condition" and "time" as main factors, followed by Tukey's post-hoc tests. $P<0.05$ was considered statistically significant.

Results and Discussion

Food intake and plasma cortisol levels are shown in Figure 1. Stress significantly inhibited trout food intake at both 3 (to 45.5% of basal values) and 10 days (to 61.4%), compared to that found in non-stressed trout. In addition plasma cortisol levels significantly increased in stressed fish at both time periods assayed, relative to the respective control groups. Increase in cortisol levels is accepted to be indicative of physiological response to acute stress in fish (3). However, a trend for such increase to attenuate under chronic stress has been described, leading to speculate with cortisol not being a good indicator under such condition. Our results reveal cortisol levels to be increased anytime, but the magnitude of such increase is lower at 10 days. This agrees with the attenuation of the stress response in parallel with the stress duration, probably due to metabolic restrictions to which animals are exposed when subjected to chronic stress. Our result showing decreased plasma glucose and lactate levels (data not shown) also support such idea.

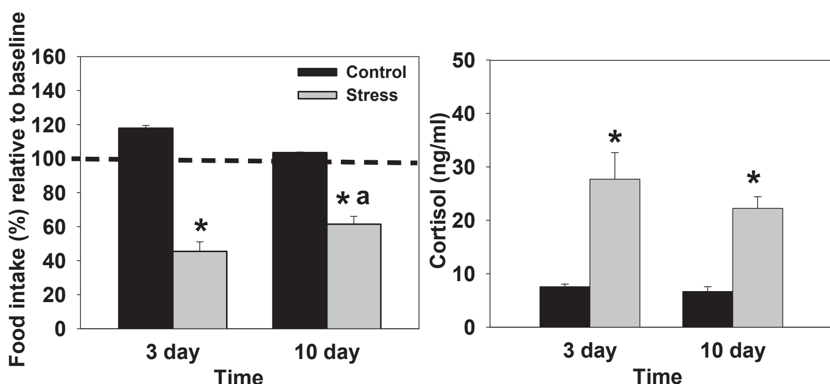

Figure 1. Food intake and plasma cortisol levels in trout exposed to high stocking density for 3 or 10 days. Data represent the average ± S.E.M. of three replicates for food intake, and 10 fish for cortisol levels. * P<0.05 relative to control group. [a] P<0.05 compared to stressed at 3 days.

It has been demonstrated that when subjected to acute stress animals show increased dopaminergic and serotonergic activities, which are reflected in increased DOPAC/DA and 5HIAA/5HT ratios (4), as our previous results carried out with rainbow trout also show (2). Our results showing monoaminergic activities in different brain regions of stressed rainbow trout are shown in Table 1. In general 3 and 10 days of stress by high stocking density induced a significant increase in both dopaminergic and serotonergic activities in all the analyzed regions, relative to that of control non-stressed groups. Such results, in addition to increased mRNA abundance of *TH* and *TPH* (data not shown, the rate limiting enzymes of DA and 5HT synthesis, respectively, are indicative of the main role played by brain monoamines in the modulation of different behavioral and physiological functions, including the regulation of the HPI axis (2,5).

In conclusion, stress by high stocking density induced plasma cortisol increase and food intake reduction in rainbow trout. Cortisol is mainly modulating the metabolic pathways in order to facilitate glucose availability all over the body in a situation in which food intake decreases. In addition, under chronic stress, brain monoaminergic activities are increased. This may be indicative of the main role played by both dopaminergic and serotonergic activities in initiating, but also in maintaining neuroendocrine response to stress.

Table 1. Changes in dopaminergic (estimated by the DOPAC/DA ratio) and serotonergic (5HIAA/5HT ratio) activities in hypothalamus, telencephalon, optic tectum and medulla oblongata of rainbow trout subjected to stress for 3 and 10 days.

	DOPAC/DA %			
	3 Day		10 Day	
	Control	Stress	Control	Stress
Hypothalamus	4.46±0.33	6.79±1.01*	4.62±0.47	6.44±0.42
Telencephalon	10.91±1.68	16.98±1.49*	11.61±0.74	15.46±1.09*
Optic tectum	142.17±7.15	195.24±12.56	153.14±4.09	208.50±20.38*
Medulla	50.13±3.29	76.47±4.35*	58.27±7.70	106.27±7.04*[a]
	5HIAA/5HT %			
Hypothalamus	4.66±0.37	6.57±0.56*	4.70±0.34	7.53±0.67**
Telencephalon	15.81±1.12	26.00±2.00**	15.80±0.89	27.58±1.31**
Optic tectum	31.29±2.24	43.33±3.04*	33.24±1.64	43.59±1.49*
Medulla	15.92±1.51	26.27±1.80*	16.44±1.11	25.99±0.94*

DOPAC: 3,4-dihydroxyphenylacetic acid; DA: dopamine; 5HIAA: 5-hydroxyindole-3-acetic acid; 5HT: 5-hydroxytryptamine (serotonin). Data represent the average ± S.E.M. (n=10 fish). ** P<0.01; * P<0.05 relative to control group. [a] P<0.05 compared to stress at 3 days.

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PLASTIC PHYSIOLOGY, SOCIAL BEHAVIOUR AND ENDOCRINE PROFILES OF AN INVASIVE CICHLID IN SOUTHERN PORTUGAL

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The chanchito, *Australoheros facetus* is a freshwater neotropical cichlid found in some streams of Southern Portugal. The ability to adapt to different habitats is key to the success of invasive species. Here, these freshwater fish were exposed to a range of salinities corresponding to those of upper estuaries, during short and long-term trials. A significant reduction in growth and in social behaviour was noticed at higher salinities. Plasma osmolality and enzymatic activity were significantly increased in higher salinities. Cortisol followed the same pattern in the short term trial, with a strong correlation to plasma osmolality in the high salinity group, but not in the long trial, where it was higher at low salinities probably due to the high incidence agonistic behaviours noticed.

Introduction

Invasive species are a growing concern for habitats worldwide and one of the challenges of understanding the ecological consequences of non-native species is the difficulty in identifying the mechanisms that allow these species to establish in the new habitat [1]. The chanchito, *Australoheros facetus*, is a neotropical cichlid which displays high tolerance to abiotic factors, such as temperature, and marked social behaviours.

Populations of these fish exist in Vascão and Foupana rivers, upstream of the Guadiana estuary and in Odelouca River, upstream of the Arade estuary. These small rivers provide habitats with striking seasonal variations in hydrological regimes, and are subject to flash floods that could displace the fish. In this way it is possible that this species can reach the estuaries downstream of its currently known settlements, crossing a range of increased salinities.

This study thus aimed to assess the chanchito's physiological potential of intrusion and establishment in brackish waters, through measurements of $Na^{+/}K^{+}$-ATPase in kidney and gills, plasma osmolality and circulating levels of cortisol in fish exposed to different salinities for different periods.

Materials and Methods

Trial 1. Fish (n=10 per group) were kept in four glass aquaria with 30 litres each were set up with an aerator and a biological filter. An aquarium was kept in salinity 0ppt (control) and the other had their salinity gradually increased in 5ppt every three days, until the values of 5ppt and 15ppt were reached. The experimental period lasted for 30 days.

Trial 2. Three groups of fish (n=32 per group) were acclimated with an increase of 3ppt each 3 days up to6ppt, 12ppt and 18ppt, while one group was maintained at 0ppt (control). The experimental period lasted for 60 days.

At the end of the trials 6-8 animals per group were anesthetized with MS-222and sodium bicarbonate, and sacrificed via rapid cervical transection. Tissue and blood

samples were collected and stored in adequate conditions until further analysis. Measurement of gill Na^+/K^+-ATPase activity followed a protocol adapted from McCormick [2] and the radioimmunoassay for cortisol followed the methodology in Scott et al, [3] and Guerreiro et al [4]. Osmolality was determined using a vapor pressure osmometer.

Results and Discussion

Trial 1. No mortality was observed. In freshwater fishes, the gill and kidney are intricately involved in ionic regulation owing to the presence of numerous ion channels, pumps, or exchangers. Gill Na^+/K^+-ATPase activity (Figure 1A) was significantly modified by salinity (one-way ANOVA, F=4.27) with elevated levels in the highest salinity that were about twice those in 0 and 5 ppt. No significant differences were found in kidney Na^+/K^+-ATPase activity (ANOVA, $p>0.05$, Figure 1B).

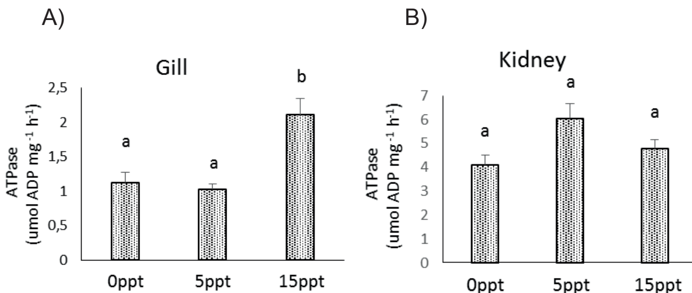

Figure 1. Activity of Na^+/K^+-ATPase in (a) gills; (b) kidney among treatments of *trial 1*. Different letters refer to values statistically different (one-way ANOVA, n=7-8; $p<0.05$, Mean±SEM).

Normal plasma is approximately isosmotic to water at 10ppt, so it is likely that only above such value external pressure becomes challenging for this freshwater species. So, similarly to gill enzymatic activity, plasma osmolality rose significantly from 312±12.3 mmol.kg^{-1} at 0ppt to 360.75±7.20 mmol.kg^{-1} at 15ppt (N=18, one-way ANOVA, F=0.81, $p<0.05$, Figure 2A), a possible signal of osmotic imbalance.

Osmoregulation in teleost is dependent on the interaction of a variety of hormones, including cortisol. Besides being a general hormone response to stress, cortisol plays an essential role in the metabolism of carbohydrates and health balance, favoring adaptation to saline environment, mainly by stimulating the Na^+/K^+-ATPase, thereby decreasing the pressure osmotic blood and lowering the internal osmolality [5, 6]. This may be an explanation for the observation that circulating cortisol levels were markedly increased (one-way ANOVA, F=7.97), up to five–fold higher in fish exposed to 15ppt than in the lower salinities, a pattern that resembled both osmolality and branchial Na^+/K^+-ATPase activity.

Although, the individual osmolality values returned only a weak, but positive, correlation with gill Na^+K^+/ATPase activity (r^2=0.24; $p<0.05$), no significant correlation occured for the Na^+K^+/ATPase activity in the kidney (r^2= 0.03; $p>0.05$). However, the correlation between individual osmolality and cortisol levels was stronger (r^2= 0.44; $p<0.05$).

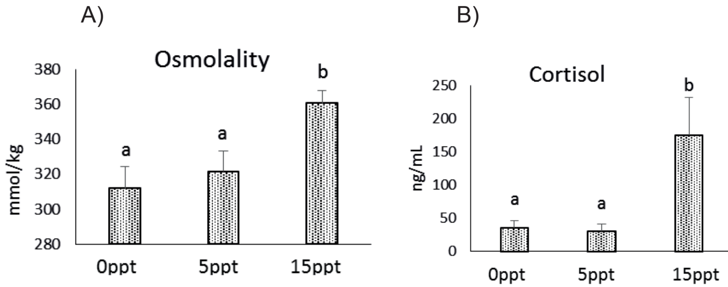

Figure 2. Values of plasmatic osmolality (A) and cortisol (B) among treatments of *trial 1*. Different letters refer to values statistically different (one-way ANOVA, n=7-8; p<0.05, Mean±SEM).

Trial 2. At the end of 60 days, growth rate was significant lower in fish at 18ppt, while in freshwater the fish experimented sustained growth (ANOVA, F=24.74, p<0.01, Table 1). In this trial it also was observed that salinity had a strong influence on social behavior. While in the lower salinities (0, 6 and 12ppt) the fish formed hierarchies through agonistic interactions, no such observations were made at 18ppt. This incidence of aggressive behaviour could be the cause for the high levels of plasmatic cortisol measured in fish at lower salinities, contrary to the first trial, and not evident in the higher salinities.

Table 1. Differences of specific physiological and behavioural features observed among treatments.

	0ppt	6ppt	12ppt	18ppt
Growth	+++	++	++	0
Osmolality	0	+	+	+++
Cortisol	++	+++	++	+
Aggressiveness	+++	+++	++	0
Reproduction	+++	+++	+	0

Many African cichlids show remarkable eurihalinity, being able to reproduce in freshwater and seawater [7], and with high invasive potential [8]. However not much is known about this feature in neotropical species, and data on their salinity tolerances can provide some information about the potential distribution or abundance of these fish as invasive species.

In conclusion, these results suggest that chanchito can intrude in brackish waters, at least for a short period of time, at the expense of increased energy expenditure. Increased cortisol levels in relation to salinity show up-regulation of this endocrine pathway as an initial effort to keep the balance between external and internal ion concentrations. However its limited osmoregulation may be an impediment for the establishment of the species in lower estuaries, as fish in high salinities showed reduced growth and little social behavior. In this way, when at higher salinities the chanchito may be not able to form hierarchies, a crucial step to reproduction. Further studies will determine how abiotic factors impact other endocrine axis controlling hierarchy formation and mating behavior.

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ACTIVATION OF SOMATOSTATIN RECEPTOR 3 REDUCES CELL VIABILITY AND HORMONAL SECRETION IN NON-FUNCTIONING PITUITARY ADENOMAS THROUGH MAPK SIGNALING

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Non-functioning pituitary adenomas (NFPA) are characterized by the lack of hormone hypersecretion and are associated to severe comorbidities related to mass effects. Surgical resection of NFPAs is generally needed because pharmacological treatments using currently available somatostatin (SST) analogs that preferentially bind to SST-receptor subtypes 2 and 5 (sst2 and sst5) are ineffective and therefore, identification of novel therapies deem necessary to manage this pathology. One of the reasons of this lack of response to SST-analogs might be related to the fact that NFPAs express high levels of sst3 and low sst2 and sst5 levels. Thus, the aim of this study was to determine the effect of an sst3-specific non-peptidic analog (L-796,778) on key functional parameters (cell viability, apoptosis/necrosis, hormone secretion and intracellular signaling) in primary human NFPA cell cultures. Firstly, we analyzed the expression profile of a series of 41 NFPAs and 10 normal pituitaries (NPs) by quantitative real-time PCR (qPCR). We found a clear alteration in the expression of key hormones (increased FSH levels) and receptors (increased sst3 and truncated sst5TMD4 levels) in NFPAs compared to NPs. Remarkably, *in vitro* treatment of NFPA cultures with L-796,778 significantly reduced cell viability and chromogranin-A secretion and increased necrosis by a Ca^{2+}-independent mechanism. These direct effects of L-796,778 might be mediated by the inhibition of MAPK signaling (ERK1/2-phosphorylation). In conclusion, our study provides new evidence regarding the role and potential clinical implications of sst3 in the pathophysiology of NFPAs.

Introduction

The pituitary is a key neuroendocrine gland located at the base of the brain (in a depression of the sphenoid bone), just below the hypothalamus. In mammals, it is constituted by two lobes, the neurohypophysis or posterior pituitary, and the adenohypophysis or anterior pituitary, which is mainly composed of five types of hormone-secreting endocrine cells (1). Pituitary hormones play crucial roles in the regulation of whole body homeostasis and the control of the precise synthesis and release of all these hormones is extremely complex, integrating both stimulatory and/or inhibitory mechanisms.

Recent studies suggest that specific genetic alterations that may drive to the malignant transformation of a single pituitary cell, together with the dysregulation of stimulatory and/or inhibitory signals, could trigger a tumorigenic cascade leading to the development of a pituitary adenoma (1,2). Among them, non-functioning pituitary adenomas (NFPAs), which constitute 30% of all pituitary tumors, are characterized by the lack of hormone hypersecretion, but are associated to severe comorbidities related to mass effects. In these adenomas, transsphenoidal surgery is the only curative approach and, unfortunately, tumoral relapses are frequent (1,3).

Pharmacological treatments currently available to treat other pituitary adenoma subtypes, such as somatostatin analogs (SAs) are normally ineffective in these tumors (3). This is likely due to the fact that SAs preferentially bind to somatostatin receptor (sst) sst2 and sst5, which are expressed at low levels in NFPAs (4,5). For

these reasons, identification of novel therapies deem necessary to manage this pathology.

In this scenario, previous studies have shown high and predominant expression of sst3 in NFPAs compared to sst2 and sst5 (4,5). In addition, sst3 has been associated to apoptotic and antiproliferative actions in heterologous models using HEK-293 and CHO-K1 cell lines (6,7). Based on all these data, the specific objectives of the present work were: 1) to perform a systematic analysis of the expression profile of key receptors and hormones in NFPAs and normal pituitary (NPs) samples and, 2) to determine the functional role of sst3, by studying the direct effect of a sst3 selective analog in NFPAs primary cultures, on key functional parameters as intracellular signaling pathways, hormone secretion, mRNA expression, cell viability and apoptosis/necrosis, as well as to determine the role of sst3 silencing on cell viability.

Materials and Methods

Human samples: 41 NFPA samples resected during transsphenoidal surgery and 9 NP samples obtained from autopsies were included in the study. Gene expression profile of the samples was determined by qPCR. Additionally, 13 NFPAs were successfully dispersed to establish primary cultures and perform *in vitro* studies using the non-peptidic analog L-796,778 (provided by Merck).

Cell viability and measurement of chromogranin A (CgA) secretion. Cell viability was estimated using Alamar-blue reagent (Biosource International). CgA concentrations were measured in the culture media derived from NFPAs using a commercial ELISA (EIA-4937, DRG).

Measurement of necrosis and apoptosis rate. Cells were plated and incubated for 12h with L-796,778 or vehicle. Then, samples were stained with Annexin-V-FITC/propidium iodide following manufacturer´s instructions (Bender Medsystems) and measurement were carried out by flow cytometry.

Changes in mRNA expression in response to sst3 analog. RNA extraction, quantification, reverse-transcription as well as the development, validation and application of qPCR to measure the expression levels of the genes of interest in the present study have been previously reported elsewhere by our group (8).

Determination of intracellular signaling pathways. As previously reported, changes in $[Ca^{2+}]_i$ in response to L-796,778 were measured using fura-2AM dye and using an inverted microscope Eclipse TE2000-E (Nikon) coupled to a digital camera ORCA II BT (Hamamatsu Photonics).

Furthermore, activation of ERK1/2 signaling pathway was determined by western blotting. Briefly, cells were cultured in 12-well plates and incubated 8 minutes with L-796,778 or vehicle. Proteins were extracted and separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Finally, membranes were incubated with primary antibodies for ERK1/2 (Santa Cruz), p-ERK1/2 (Santa Cruz) and the appropriate secondary antibody (Cell Signaling).

- Proliferation rate in response to sst3 mRNA silencing using specific siRNAs. For silencing experiments, two different siRNAs were designed to specifically target sst3 mRNA sequence. 200,000–500,000 cells were seeded on 12-well plates. Then, cells were incubated with lipofectamine RNAiMAX (Life Technologies) and sst3 siRNAs (100nM) for 24h. Finally, cells were detached and seeded in 96-well plates for cell viability measurements as indicated above. Additionally, the remaining cells were collected to validate the transfection efficiency (measured by qPCR).

Results and Discussion

Expression profile of NFPAs and NPs. As expected, the hormone expression profile revealed a clear differential pattern between NFPAs and NPs at mRNA level. Specifically, NFPAs expressed higher levels of FSH and lower levels of LH compared to NPs. In addition, a not significant decrease in the expression of the alpha subunit of the glycoproteins was observed. The analysis of the expression pattern of ssts revealed a clear overexpression of sst2 and sst3 in NFPAs compared with NPs, being sst3 the most abundantly expressed receptor. Interestingly, in our cohort, sst5 expression levels were lower in NFPAs compared to NPs. In fact, we found that sst5 was the most expressed receptor in NPs followed by sst2.

Direct effects of L-796,778 on cell viability and CgA secretion. Cell viability is a key parameter in NFPAs inasmuch as these tumors normally grow and proliferate (although at a low rate), being most of them diagnosed as macroadenomas (3). The treatment with sst3 agonist was able to significantly reduce cell viability in NFPAs primary cultures reaching ~20-25% of reduction. However, treatment with sst3 analog was ineffective in reducing cell proliferation in other types of pituitary adenoma primary cultures, such as somatotropinomas or corticotropinomas, probably due to the low expression of sst3 in these tumors.

Additionally, sst3 agonist tended to reduce CgA secretion [a glycoprotein widely expressed in neuroendocrine cells considered a useful marker in NETs and NFPAs (9,10)] after 4- and 24-h of incubation, although this difference did not reach statistical significance, likely due to the low number of samples analyzed at this time.

Direct effects of L-796,778 on apoptosis/necrosis rate. Although we found that the effect of L-796,778 on apoptotic rate differed among the analyzed cases (stimulatory, inhibitory or no effect), sst3 analog consistently induced a clear and reliable increase of necrosis after 12-14h of incubation in NFPAs. Currently, additional measurements are being implemented to elucidate the causes of this variability in apoptotic rate in response to sst3 analog.

Direct effects of L-796,778 on mRNA expression. We also studied the changes on the mRNA expression of relevant pituitary markers in response to sst3 analog. Interestingly, a 24h treatment with the sst3 analog induced a significant increase in sst3, FSH and LH expression. In addition, treatment with sst3 analog increased mRNA levels of p53 and ERK1/2, but did not affect Akt1 levels.

Direct effects of L-796,778 on intracellular signaling. We next explored several signaling pathways involved in the response of sst3 analog in NFPAs. Specifically, we firstly studied the effects of sst3 analog on free cytosolic calcium kinetic, but we did not observe any alteration of calcium influx after sst3 analog treatment. Then, we studied the MAPK pathway since it is widely known to be associated with cell proliferation (11). We observed that incubation with sst3 agonist was able to significantly reduce the phosphorylation levels of ERK1/2 compared to controls, suggesting that the antiproliferative effects observed in response to sst3 analog might be associated to the inhibition of ERK signaling.

Effects of sst3 silencing on cell viability. Finally, we aimed to assess the role of endogenous sst3 in NFPAs using sst3 specific siRNAs. Specifically, we compared the effect of two different siRNAs, which induced a similar silencing effect when validated by qPCR. Interestingly, silencing of sst3 expression increased cell viability in 50% of NFPAs analyzed (2 cases out of 4), which might suggest a constitutive activity of this receptor in this pathology.

Altogether, our study provides novel evidence regarding the role and potential clinical implications of sst3 in the pathophysiology of NFPAs, suggesting that pharmacological treatment specifically targeting this receptor could be a promising therapeutic approach for these tumors.

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LACK OF CORTISTATIN OR SOMATOSTATIN PLAYS DIFFERENTIAL ROLES IN THE CONTROL OF MAMMARY GLAND TUMORIGENESIS IN LEAN AND OBESE MICE

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Somatostatin (SST) and cortistatin (CORT) are two peptides that exert common biological actions, including the suppression of tumor cell proliferation, offering a potential therapeutic target. However, attempts to apply SST analogs in the treatment of breast cancer have been controversial and the specific role of SST and/or CORT in mammary gland tumorigenesis remains uncertain, especially in obesity. We studied the role of endogenous SST and CORT on carcinogen-induced mammary gland tumorigenesis under normal-weight and obesity conditions, by treating SST- and CORT-knockout (KO) and littermate-controls mice, fed a low fat (LF) or a high fat (HF) diet, with 7, 12-dimethylbenzaanthracene (DMBA). Lack of SST did not impact DMBA-induced tumor incidence under lean conditions, while lack of CORT aggravated DMBA-induced tumorigenesis in LF-fed mice. These differences were not attributable to altered mammary gland development or changes in relevant, circulating hormones. Interestingly, HF-diet increased the sensitivity to DBMA-induced carcinogenesis in control and SST-KO mice while, tumor incidence was not statistically altered in HF-diet compared with LF-diet CORT-KO; however, tumor incidence was already significantly elevated in LF-fed CORT-KO, which might preclude a further elevation in obesity. Therefore, our data indicate that endogenous SST and CORT distinctly contribute to the control of DMBA-induced tumorigenesis, which is strongly influenced by the metabolic/endocrine milieu, suggesting that CORT, rather than SST, might represent a key inhibitor factor of mammary tumorigenesis. Thus, these data invite to suggest that CORT-like rather than SST-like molecules could be more promising tools for the treatment of breast cancer tumors.

Introduction

SST [1] and CORT [2] are two peptides evolved from a common ancestral gene that share a high structural homology but are produced from different cell types, as SST is mainly produced by the stomach and hypothalamus whereas CORT is mainly produced in the cerebral cortex. Both peptides exhibit functional homology; indeed, they share similar inhibitory functions including the regulation of hormonal secretions from the pituitary gland as the inhibition of GH release [3]. However, our group and other collaborators demonstrated that both peptides can also exert unique functions, even opposite, including the regulation of prolactin release [4]. These differences in the regulation of hormonal secretion could be associated to their slightly different pharmacology; specifically, although SST and CORT can bind with comparable affinity to sst1-5 receptors; CORT but not SST can also bind other receptors as the ghrelin receptor or the MrgX2.

Besides the ability to inhibit pituitary secretions, nowadays several biological functions have been described for SST and CORT, including inhibition of other hormonal secretions and the regulation of the response to metabolic challenges such as fasting or obesity. Interestingly, SST and CORT can also regulate differentiation and proliferation of normal and tumoral cells, which have paved the way to explore

the possibility of using SST/CORT analogs in the treatment of tumoral pathologies where ssts are present, such as pituitary and neuroendocrine tumors. In this sense, ssts have been shown to be expressed in many other tumoral pathologies and, therefore, it has been suggested that SST, CORT or their analogs could be used as potential therapeutic options to combat these pathologies.

Of particular relevance for our group is breast cancer, which is the second leading cause of cancer worldwide, being the most frequent cancer in women with 1.7 million cases and 500.000 deaths per year [5]. This is a complex and heterogeneous disease with variable clinical prognosis and strongly influenced by sedentary life and obesity. In line with this, breast cancer development and progression is under the tight control of several endocrine systems (GH, IGF, estrogens), where SST/CORT could play a relevant role. Indeed, ssts have been shown to be expressed in breast cancer samples where they exert antiproliferative effects *in vitro* [6]. However, *in vivo* therapy with SST/CORT analogs, which is commonly used in the treatment of pituitary and neuroendocrine tumors, has generated inconsistent clinical results in breast cancer patients [7], suggesting that more studies are necessary to fully elucidate the exact role of SST and CORT in breast cancer. Given that SST and CORT play important roles in the control of metabolic deregulations such as obesity [8], as well as in the development and progression of several types of cancers, the hypothesis of our work was that SST and CORT could play a pivotal role in the pathological interaction between obesity and the development and progression of breast cancer. Based on this hypothesis, the main aim of this study was to determine the influence of endogenous SST and CORT and diet-induced obesity on tumor development and progression, using a classic *in vivo* breast cancer model (DMBA-treated mice).

Materials and Methods

Female SST- and CORT-knockout (KO) FVB/N mice and their respective littermate controls (WT) were fed a low fat (LF) or high fat (HF) diet starting at 8 weeks of age. Subsequently, animals were injected at 20 weeks of age with an intragastric carcinogenic compound (DMBA; 0.5mg/10g body weight in olive oil) once a week for 3 weeks. Tumor incidence was monitored for additional 25 weeks and mice were sacrificed at 47 weeks of age or after the observation of a tumor bigger than 1 cm^3. Palpable mammary tumors were fixed for hematoxylin-eosin analysis, while inguinal mammary glands were processed to analyze non-palpable mammary tumors and ductal hyperplasias by whole mount. In addition, trunk blood of DMBA-treated mice was collected the day of sacrifice to measure glucose and circulating hormones [leptin, GH, IGF-I, prolactin (PRL), insulin and corticosterone]. Finally, mammary development studies were performed at 8 and 20 weeks (in two additional groups of mice) and in DMBA-treated mice.

Results and Discussion

Firstly, confirming the obese phenotype induced by the HF diet, growth curves indicated that HF-fed mice had higher body weight that those fed a LF diet in all the experimental groups, while glucose levels were not altered by HF diet. Further confirming the obese phenotype, plasma leptin levels were significantly increased in HF diet animals compared to lean mice and the percentage of fat mass showed a significant increase in obese mice. Once we confirmed the effectiveness of HFD, we

aimed to explore the effect of DMBA treatment and found that obese WT mice did not exhibit significant differences in the incidence of tumors, tumor latency, multiplicity or burden compared to controls. However, the analysis of the MGs by whole mount revealed that the presence of ductal mammary hyperplasias significantly increased with HF feeding. Interestingly, lean mice lacking CORT showed a dramatic increase in tumor incidence compared to lean WT mice. Also, tumor multiplicity and percentage of non-palpable tumor were higher in LF-fed CORT-KO mice compared to lean controls. Of note, HFD did not exacerbate DMBA effect observed on CORT-KO mice as LF and HF mice showed similar tumor incidence, although HF fed mice exhibited more hyperplasias. Surprisingly, lack of endogenous SST did not impact DMBA-induced tumorigenesis of LF-fed SST-KO mice, which was similar to that observed in LF-fed WT mice. However, under obese conditions, SST-KO significantly showed an increase in tumor incidence, tumor multiplicity and hyperplasia compared to lean SST-KO. These results were further supported by the analysis of tumor development over time; specifically, CORT-KO mice showed faster tumor development than WT mice or than SST-KO mice and, in addition, SST-KO obese mice developed tumors faster than SST-KO lean mice. Despite the obvious changes in tumor development in the different experimental groups, histopathological analysis of a subset of these tumors showed that all samples were adenocarcinomas, squamous carcinomas or undifferentiated solid tumors without apparent effect of diet or genotype on tumor histotype.

To further explore the putative causes underlying the differences observed in DMBA-induced tumor incidence, we firstly investigated the effect of lack of CORT or SST in the mammary gland development. Specifically, we analyzed the mammary gland complexity (by counting extent of ductal branching) and the number of terminal end buds (TEBs) by whole mount. These analyses indicated that mammary gland complexity and TEBs number were not different between 8 weeks old WT, CORT-KO and SST-KO mice suggesting that lack of CORT of SST did not impact MG development. Furthermore, a second group of mice was fed a LF or HF diet at 8 weeks of age and followed by 12 weeks. As expected, HF diet feeding promoted an elevation of the mammary gland complexity and TEBs number in HF-diet mice compared to LF-fed animals, while the genotype did not significantly influence these morphological parameters. Similarly, after DMBA treatment, whole mount analyses reinforced this idea showing that obese mice presented a higher development of their mammary glands at complexity level and TEBs formation, while the lack of CORT and/or SST did not influence the mammary development. Therefore, changes in MG development did not seem to be able to explain the differences found in tumor incidence between genotypes. Finally, in order to identify circulating factors that could be involved in the differential incidence of DMBA-induced tumors in these models, plasma levels of several hormones with potential to influence tumor development and/or progression were determined. Measurements of circulating levels at the end of the study revealed that plasma levels of GH were slightly elevated in LF-fed CORT-KO group compared to LF-fed WT; however, plasma levels of IGF-I, insulin, PRL and corticosterone did not statistically differ among the different experimental groups.

Altogether, our data demonstrated that endogenous SST and CORT could distinctly contribute to the control of DMBA-induced mammary gland tumorigenesis. Specifically, CORT, rather than SST, might act as a key inhibitory factor of mammary gland tumorigenesis in mice. However, although the underlying factors of these different responses of CORT and SST still need to be fully elucidated, the results seem to suggest that CORT-like molecules, rather than SST-like molecules, could be more promising tools for the medical treatment of breast cancer. Finally, these data also suggest that more efforts should be done to identify the mechanisms of action of

CORT towards the development of more useful diagnostic or therapeutic targets in breast cancer.

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THE TRUNCATED SOMATOSTATIN RECEPTOR SST5TMD4 IS OVEREXPRESSED IN PROSTATE CANCER AND INCREASES AGGRESSIVENESS FEATURES THROUGH REGULATION OF ANGIOGENIC FACTORS AND WNT/B-CATENIN PATHWAY

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Somatostatin is a highly conserved, pleiotropic neuropeptide that binds to a family of G protein-coupled receptors with seven transmembrane domains (TMD), called somatostatin receptors (sst1-5), to exert its biological function. Our group has identified and characterized in several species additional sst5 variants originated from alternative splicing that lack several TMDs. Particularly, the human sst5TMD4 variant, a receptor with four TMDs, seems to have pathological consequences since it is abundantly expressed in a subset of pituitary tumors and breast cancer samples, wherein it could exacerbate the malignant phenotype. Here, we analyzed the presence and role of sst5TMD4 in human prostate cancer (PC). We found a clear overexpression of sst5TMD4 in PC samples compared to control tissues and, its expression was higher in tumors from patients with metastasis compared to those without metastasis. Thus, the *in vitro* functional role of sst5TMD4 was explored by using androgen dependent (VCaP) and independent (PC-3) PC cell lines. Specifically, sst5TMD4 overexpression increased cell proliferation and migration in both cell lines, while its silencing by siRNA decreased basal cell proliferation. Moreover, several important pathways were deregulated after overexpression of sst5TMD4, including a dramatic downregulation of two tumor suppressor proteins from the Wnt/B-catenin pathway (APC and SFRP1) and an increase of angiogenic factors such as VEGF and ANG1. Altogether, our results suggest a relevant role of sst5TMD4 in PC where is associated to enhanced features of aggressiveness through regulation of angiogenic factors and Wnt/B-catenin pathway.

Introduction

Somatostatin (SST) and its receptors are expressed in a wide variety of tissues where they play multiple, relevant roles in regulating body homeostasis [1]. Specifically, SST is well-known for playing an inhibitory role in regulating certain endocrine secretions including the inhibition of several pituitary hormones as growth hormone (GH). Twenty years after the discovery of SST, a new SST-related peptide encoded by an independent gene was identified and named cortistatin (CORT) [2]. Both peptides, SST and CORT, exhibit a strong structural, functional and pharmacological similarity, which may help to explain their similar role regulating common endocrine and non-endocrine relevant processes [2]. To exert their biological actions, SST and CORT bind to a family of G-protein coupled receptors with 7 transmembrane domains (TMD), which are encoded by five different genes (sst1-5). Classically, it was accepted that these genes encode for 6 different SST/CORT receptors, including a carboxiterminal spliced variant of the sst2, named sst2B. However, our group has demonstrated the existence of two additional truncated spliced variants of the human sst5 that display 4 and 5 TMDs, respectively, and are consequently named sst5TMD4 and sst5TMD5 [3]. In fact, over the last years our group has demonstrated that the shorter truncated sst5 receptor,

ss5TMD4, is normally absent in normal cells but highly expressed in a broad variety of tumours (i.e. pituitary tumours, breast cancer, thyroid cancer), wherein it is associated with poor prognosis and increased malignancy [4-6]. However, to date, there is no available information on the role of sst5TMD4 in prostate cancer (PC), one of the most common and lethal tumoral pathologies in men [7]. Therefore, the aim of the study was to investigate the presence and functional role of the truncated receptor sst5TMD4 in PC using human samples and *in vitro* PC cell lines as models.

Materials and Methods

1. *Human prostate biopsies*: Human prostate biopsies were collected at Hospital Universitario Reina Sofia of Cordoba (HURS) and placed at -80C. Each sample was identified as normal tissue or carcinoma by an experienced pathologist. RNA was extracted using AllPrep DNA/RNA/Protein Mini Kit (Qiagen). Total RNA (2 µg) was reverse transcribed (RT) using the cDNA First-Strand Synthesis kit with random primers according to the manufacturer's instructions (Fermentas). A complete expression profile of the SST/CORT system [ligands (SST and CORT) and receptors (sst1-5 and sst5TMD4/sst5TMD5)] was determined by quantitative PCR (qPCR).

2. *Formalin-fixed, paraffin-embedded (FFPE) prostates*: 25 FFPE samples from PC with their corresponding non-tumoral adjacent regions were collected at HURS. Each tumoral and non-tumoral region was independently extracted using RNeasy FFPE Kit (Qiagen) according to the manufacturer's instructions. In all cases, total RNA concentration and purity was assessed using Nanodrop 2000 spectrophotometer (Thermo Scientific), and subsequently retro-transcribed using random hexamer primers and the cDNA First Strand Synthesis kit. cDNAs was amplified with the Brilliant III SYBR Green Master Mix (Stratagene) using the Stratagene Mx3000p system and specific primers for each transcript of interest. A complete expression profile of the SST/CORT system comparing the tumoral vs. non-tumoral adjacent regions was determined by qPCR.

3. *PC derived cell lines*: Both androgen dependent (VCaP) and androgen independent (PC3) PC derived cell lines were used to analyse the function role of sst5TMD4 in different stages of the disease.

4. *Measurements of cell proliferation and migration*: Each PC cell line was transfected with an sst5TMD4-containing plasmid and proliferation rate was measured at different times (24, 48 and 72h) using MTT or Alamar Blue methods. The absorbance of this coloured solutions was measured by the FlexStation System 3 (Molecular Devices). In addition, cell migration was measured by Wound Healing assay in PC3- and VCaP-sst5TMD4 stably transfected cells or mock (empty vector; as a control) at 8h or 12h, respectively.

5. *Silencing of the endogenous expression of sst5TMD4*: An sst5TMD4 specific siRNA was designed and validated in our laboratory. To test the functional consequence of sst5TMD4 silencing, we measured cell proliferation by Alamar Blue technique at 24-72h in sst5TMD4 siRNA transfected cells as compared with control siRNA transfected cells.

6. *RT^2 Prostate Cancer PCR Array*: To analyse the molecular alterations triggered by sst5TMD4 overexpression, a RT^2 Prostate Cancer PCR array was used in order to simultaneously examine the mRNA levels of 84 genes associated with PC development, including five "housekeeping genes" in 96-well plates following the manufacturer's protocol (cat. number 330231 PAHS-135ZA, Quiagen). Briefly, RNA from 4 consecutive passes of stably transfected sst5TMD4- or mock-PC3 cells were

isolated with Absolutely RNA RT-PCR Miniprep Kit (Agilent, La Jolla, CA, USA) and first-strand cDNAs was synthesized using RT2 First Strand kit (Quiagen) while qPCR was performed using the Brilliant III SYBR Green Master Mix (Stratagene) and the Stratagene Mx3000p system.

Results and Discussion

1. *sst5TMD4 was overexpressed in PC samples*: Firstly, we determined the expression levels of sst5TMD4 in PC tissues (biopsies and FFPE samples compared with normal prostates or non-tumoral adjacent tissues, respectively) by qPCR. We found that sst5TMD4 expression was higher in the tumoral region of the prostate compared with the non-tumoral adjacent region (FFPE samples) and, interestingly, that the expression of sst5TMD4 was increased in patients with metastasis compared with non-metastatic and controls (biopsies). Therefore, these results are in agreement with data previously reported from our group in pituitary adenomas, breast cancer or thyroid cancer [4-6] and indicate that sst5TMD4 is up-regulated in PC, wherein its expression is associated to worst outcome.

2. *Functional consequences of sst5TMD4 overexpression in PC cell lines*: We next analyzed the functional role of sst5TMD4 in PC using PC cell lines (VCaP and PC-3). Specifically, the overexpression of sst5TMD4 clearly increased the proliferation rate of both cell lines at 48-72h. Moreover, sst5TMD4 overexpression also promoted cell migration in PC3 and VCaP by wound healing assay. These results suggest that sst5TMD4 overexpression in PC cells could be promoting malignancy features such as cell proliferation and migration, which has also been previously shown in breast cancer cell lines with *in vitro* an *in vivo* approaches, and also, in pituitary adenomas [4, 5]. Additionally, we found that the expression of two pro-angiogenic factors such as VEGF and ANG2 were increased in cells overexpressing sst5TMD4, which might be associated to the capacity of sst5TMD4 bearing tumors to promote the formation of new blood vessels.

3. *Functional consequences of sst5TMD4 silencing by specific siRNA:* Since sst5TMD4 is up-regulated in PC samples and seems to promote malignancy features, we hypothesized that sst5TMD4 silencing could be a new therapeutic tool in clinical practice. Accordingly, we designed and validated a specific sst5TMD4 siRNA and measured the functional consequence of sst5TMD4 silencing in the cell proliferation of PC3 cells. We observed that sst5TMD4 endogenous silencing decreased cell proliferation growth at 72h. Therefore, these data, together with the increase in proliferation in sst5TMD4-stably transfected cells, highlights the key role of sst5TMD4 in promoting cell proliferation and invites to suggest that sst5TMD4 targeting could be a new promising approach in clinical treatment of PC.

4. *sst5TMD4 overexpression altered the expression of key genes involved in PC development and progression:* We also analyzed the effect of sst5TMD4 overexpression on 84 key genes associated to PC. We found that several genes involved in PC progression were up-regulated (CAV1, CAV2, IL-6, DAXX), while the expression of genes that have a tumor suppressor role in PC were down-regulated (i.e. APC, PTEN, CDKN2A, NRIP1, ZNF185, LOXL1 and SFRP1). These changes were further validated by qPCR and suggest the putative molecular basis underlying the sst5TMD4-associated effects.

In conclusion our results showed that *sst5TMD4* could represent a new relevant player in PC, wherein it is up-regulated and promotes malignancy features such as cell proliferation or migration through the deregulation of key PC progression genes (CAV1, CAV2, DAXX, IL-6) and tumor suppressor genes (APC, PTEN, CDKN2A,

NRIP1, ZNF185, LOXL1 and SFRP1). Further approaches are warranted to firmly determine if sst5TMD4 could represent a novel target in PC clinical assays.

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IN1-GHRELIN INCREASES MALIGNANCY FEATURES OF BREAST CANCER CELL LINES

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Aberrant alternative splicing is a hallmark of tumoral process that can worsen cancer outcome by inducing the appearance of alternative proteins, which can increase malignancy features of the tumoral cells. Our group has recently discovered a splicing variant of the ghrelin gene, named In1-ghrelin, which results from the retention of intron 1. In1-ghrelin variant is overexpressed in breast cancer, and its expression correlates with proliferation markers, suggesting the involvement of In1-ghrelin in the development and/or progression of this pathology. The aim of this study was to determine the functional role of In1-ghrelin (and native-ghrelin) in two breast cancer derived cell lines: MDA-MB-231 and MCF7. Particularly, treatment with In1-ghrelin peptides or native-ghrelin stimulated proliferation and migration in both cell lines. Similarly, overexpression of native-ghrelin and In1-ghrelin increased the proliferation rate, while only In1-ghrelin overexpression was able to increase migration ability in MDA-MB-231 and MCF7 cells. These results were further corroborated by silencing endogenous In1-ghrelin in MDA-MB-231 cells. In addition, In1-ghrelin, but not native-ghrelin, overexpression induced an increase in pERK1/2. We then analyzed dedifferentiation processes in response to native-ghrelin and In1-ghrelin by studying the percentage of cells with mesenchymal-like phenotype and found that In1-ghrelin, but not ghrelin, increased epithelial to mesenchymal transition in MDA-MB-231 cells. In addition, we studied the capacity to form mammospheres and found that only In1-ghrelin overexpression and treatment led to the formation of more and bigger mammospheres. In summary, our data demonstrate that In1-ghrelin splicing variant increases malignancy features of breast cancer cells from two different cell models, suggesting a relevant role of In1-ghrelin in breast cancer development and/or progression.

Introduction

Ghrelin gene (GHRL) is comprised of 6 exons encoding the pre-pro-ghrelin peptide, which can undergo a proteolytic processing to generate ghrelin and/or obestatin. This pre-pro-peptide can be modified by the addition of an octanoyl group at Ser3, which is catalyzed by the GOAT enzyme. This process generates the acyl-ghrelin, which is able to bind to its canonical receptor (GHSR-1a) to exert its functions [1]. Alternative splicing of ghrelin gene can also originate In1-ghrelin, a splicing variant discovered by our group [2] and generated by a process known as intron retention, wherein intron 1 is included in the mature mRNA altering the reading frame and modifying the C-terminal end of the generated pre-pro-peptide. In1-ghrelin is expressed in different tissues, specially, thymus and testis [2] and has been observed to be involved in the development and/or progression of a number of tumoral pathologies such as pituitary [3] and neuroendocrine tumors [4] or breast cancer [2]. In the latter, In1-ghrelin is overexpressed in tumoral samples where its expression correlates with tumoral markers as compared with normal breast samples. Finally, In1-ghrelin transient overexpression increases proliferation rate in the breast cancer cell line MDA-MB-231. However, the specific role of In1-ghrelin in breast cancer malignancy and progression remains to be fully elucidated. For this reason, the aim of the present study was to investigate the role of In1-ghrelin splicing variant on malignancy-associated features, such as proliferation, migration and dedifferentiation of breast cancer derived cell lines (and compares it with the role exerted by native ghrelin).

Materials and Methods

Cell lines. MDA-MB-231 and MCF-7 cell lines (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 4.5g/L glucose (Lonza) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (100X solution, 10,000u/ml penicillin, 10mg/ml streptomycin and 25μg/ml amphotericin B, Sigma), and 2mM L-glutamine at 37ºC and 5% CO2. Adding 1% geneticin in stably transfected cell lines cultures.

Plasmid and siRNA transfection. In1-ghrelin and ghrelin sequences inserted in pCDNA3.1 vector were transfected in MDA-MB-231 and MCF-7 breast cancer cell lines using Lipofectamine-2000 (Gibco), following the manufacturer's recommendations. pCDNA3.1 empty plasmid was used as control (mock cells). Specific In1-ghrelin siRNAs were designed in collaboration with IPSEN and transfected with RNAiMax Lipofectamine (Gibco) using a commercial scramble siRNA as negative control according to the manufacturer's instructions.

Pharmacological treatment. Acylated In1-ghrelin derived peptides, acyl-ghrelin, and paclitaxel treatments were administered at a final concentration of 10^{-7}M. IGF treatment was used at a final concentration of 10^{-8}M.

RNA isolation and reverse transcription. Nucleic acids were isolated with Trizol (Invitrogen) following the manufacturer's instructions and, subsequently, treated with DNAse for removing genomic DNA. Total RNA (1μg) was reverse transcribed (RT) using the cDNA "First-Strand Synthesis Kit" and random primers according to the manufacturer's instructions (Fermentas).

Quantitative real-time PCR (qPCR). All qPCRs were run with 25ng of complimentary DNA using a 2x IQ-SYBR-green mastermix (BioRad) and were run in an iCycler IQ thermal cycler (BioRad).

ERK and AKT phosphorylation levels were analysed by Western blot technique. Proteins were extracted from 6-well plate confluent cell cultures using SDS-DTT buffer (62,5mM Tris-HCl, 2% SDS, 20% glicerol, 100mM DTT and 0,005% bromophenol blue). Primary antibodies for ERK1/2 (Santa Cruz Biotech), p-ERK1/2, p-Akt, or Akt (Cell Signaling) and the appropriate secondary antibodies (Anti Rabbit IgG HRP-linked, Cell Signaling) were used.

Proliferation assay. Proliferation studies were carried out using two complementary approaches named MTT assay (Sigma Aldrich) and Alamar blue technique (Thermo Scientific) following manufacturer's recommendations. Assay measurements were performed by the FlexStation system (Molecular Devices).

Migration assay. Migration capacity was determined by wound healing technique. Specifically, a wound was made in confluent cell cultures and the area re-covered was monitored after 24 hours incubation. Wound healing was calculated as the area of a rectangle centered in the picture 24h after the wound vs. the area of the rectangle just after doing the wound

Epithelial-to-mesenchymal transition (EMT) analysis. Mesenchymal phenotype of MDA-MB-231 and MCF-7 transfected cells was analysed by counting the percentage of mesenchymal- and epithelial-like cells.

Mammospheres formation assay. The ability to form mammospheres in response to In1-ghrelin or ghrelin overexpression or treatment was evaluated by mammospheres formation assay. Cells were seeded in low-adherent plates and incubated for 7 days at 37ºC and 5% CO_2. Subsequently, mammospheres were counted under an inverted microscope considering cells clusters bigger than 50μm of diameter.

Results and Discussion

To analyze the effects of In1-ghrelin and ghrelin overexpression on breast cancer, MDA-MB-231 and MCF-7 cell lines were stably transfected with both sequences generating monoclonal cell lines. The effect of the overexpression of each ghrelin gene variant in the proliferation rate was evaluated by two different techniques (Alamar Blue and MTT assays). Our results indicated that overexpression of both variants increased cell proliferation in MDA-MB-231 cells. However, only In1-ghrelin increased proliferation in MCF-7 cells. These results were further confirmed by treatment with ghrelin and In1-ghrelin derived peptides (paclitaxel and IGF-I were used as negative and positive controls in proliferation assays, respectively). Similarly, migration studies by wound healing technique showed that In1-ghrelin overexpression and treatment increased migration abilities in both cell lines. Interestingly, ghrelin peptide only induced an increase in migration rate in MDA-MB-231 cells, suggesting a cell line specific effect.

As a proof of concept, we performed downregulation studies in MDA-MB-231 cell line with two In1-ghrelin specific siRNAs that demonstrated significant reduction in In1-ghrelin expression levels. Remarkably, transient transfection with both siRNAs reduced proliferation and migration rates in this cell line, which, altogether, support the role of In1-ghrelin expression on breast cancer cell malignancy.

In order to further explore the signaling pathways implicated in these functional changes, we analyzed the basal phosphorylation levels of two important cancer-related pathways as ERK and AKT [5] in MDA-MB-231 cells. We found that overexpression of In1-ghrelin, but not ghrelin, induced an increase in the phosphorylation of ERK signaling pathway.

Additionally, we sought to explore the putative role of In1-ghrelin on dedifferentiation processes such as presence of cancer stem cells (CSCs) and epithelial to mesenchymal transition (EMT). Regarding the EMT, we analyzed the percentage of mesenchymal-like cells and found increased levels in In1-ghrelin, but not ghrelin, transfected cells. Then, we studied the formation of mammospheres in response to In1-ghrelin or ghrelin overexpression and treatment and found a clear increase in the number of mammospheres in both cell lines transfected or treated with In1-ghrelin, but not ghrelin. Moreover, MDA-MB-231 cells transfected with In1-ghrelin showed mammospheres with higher size, suggesting a possible proliferative effect of In1-ghrelin on CSCs.

Finally, we analyzed the expression levels of key elements of signaling pathways involved in dedifferentiation processes. This approach showed that both ghrelin and In1-ghrelin overexpression increased TGF-β1 and JAG1 mRNA expression, but only In1-ghrelin was able to increase the expression of β-catenin, the principal effector of Wnt signaling pathway, representing a possible specific signaling cascade that could explain the differential effects of In1-ghrelin variant.

In summary, our results indicate that:

1. Overexpression and/or treatment with In1-ghrelin increases the malignant phenotype of MDA-MB-231 and MCF-7 cells by promoting:
    a. Cell migration and proliferative capacity
    b. The percentage of mesenchymal-like phenotype cells in MDA-MB-231 cells
    c. The number of mammospheres and therefore CSCs.
2. In addition, endogenous In1-ghrelin down-regulation seems to inhibit proliferation and migration abilities.
3. The effect of In1-ghrelin is clearly different to that exerted by ghrelin, indicating differential functions of two products of the same gene in breast cancer cells.

Altogether, our data demonstrate that In1-ghrelin variant increases malignancy features in breast cancer cells, suggesting that this variant could play a relevant functional role in the regulation of this pathology.

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RESPONSIVENESS OF SEA BASS SCALES TO ESTRADIOL AND GENISTEIN

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Phytoestrogens are plant-derived compounds with estrogen-like activity, to which fish may be exposed either in the wild or in aquaculture when they are fed plant-based feeds. The disruptive effects of phytoestrogens on fish reproductive function have been described, but relatively few studies of the impact of these compounds on mineralized tissues exist. This study characterized the responsiveness of European sea bass scales to estradiol (E_2) and the phytoestrogen genistein (Gen). E_2 and Gen had significant but different effects on the circulating levels of calcium, phosphorus (increased by E_2 but not Gen after 5 days) and vitellogenin (increased by both after 1d but only by E_2 after 5d). E_2 and Gen did not affect the activity of alkaline phosphotase (ALP) or tartrate-resistant acid phosphatase (TRAP) or their transcript abundance. Gen increased the circulating levels of E_2 after 1d, raising the possibility that part of its effects may be due to this induction. Sea bass scales expressed *aromatase B* at low levels and it expression slightly changed in response to E_2 and Gen, opening the hypotheses of local E_2 synthesis and regulation. In addition, the mRNA expression of the estrogen receptor and selected estrogen-responsive genes was also affected by both compounds in a similar manner. Overall, this study shows that estrogens and phytoestrogens both have actions on mineralized tissues modifying their homeostasis.

Introduction

Scales act as a mechanical barrier and mineral reserve in fish, and tissue turnover involves cycles of tissue formation/resorption mediated by osteoblasts (OSB) and osteoclasts (OSC) [1]. E_2 is an hypercalcaemic hormone in fish and appears to increase calcium mobilization from scales and other mineralized tissues [2, 3] and regulates calcium deposition [4]. Most estrogen actions are mediated by nuclear estrogen receptors (ERs) and to a lesser extent by the recently identified membrane estrogen receptors (GPERs). We previously reported the three ER subtypes (ESR1, ESR2a and ESR2b) in fish scales and other mineralized tissues [5]. Recently two GPER subtypes were also detected for the first time in scales [6]. This suggests that the reported E_2 effects [3, 4] result from a direct action on fish mineralized tissues, particularly the scales. Phytoestrogens such as Gen are potential disruptors of the endocrine system as they have chemical structures similar to E_2 and have been shown to have estrogen-like activity and to bind to nuclear estrogen receptors in vertebrates [7, 8]. In this study the impact of E_2 and Gen on European sea bass (*Dicentrarchus labrax*) mineral homeostasis and on scale estrogenic responses were compared.

Materials and Methods

Immature sea bass (60g) were obtained from local fish farms and maintained at Ramalhete Marine Station (CCMAR, Faro, Portugal) in 500 L flow-through seawater tanks, at natural temperature and photoperiod for winter and fed with commercial dry pellets at 1% wet weight. Fish were randomly subdivided into three groups, one was injected with coconut oil alone (control), another with coconut oil containing 5 mg/kg of E_2 (Sigma-Aldrich, Madrid, Spain) and the third with the same dose of Gen (AbCam). After 1 and 5 days treatment, n=10 fish per group were anesthetized with 2-phenoxyethanol (Sigma-Aldrich, 1:5,000), washed with clean seawater, measured,

weighted and blood and scales collected. E_2 plasma levels were measured using a specific radioimmunoassay, total plasma Ca and P were determined using colorimetric assays and vitellogenin (Vtg) by SDS-PAGE. The activities of tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) were determined in scales using a colorimetric assay by measuring the amount of p-nitrophenyl-phosphate (pNPP) converted into p-nitrophenol (pNP). Transcript levels of *trap* and *alp*, *aromatase B (aroB),* nuclear and membrane estrogen receptors and several known-estrogen responsive genes were determined by quantitative real time RT-PCR as described in [6]. Reactions were carried out using EvaGreen chemistry and the relative standard curve method. Normalization was performed using the geometric mean of the transcript abundance of 18S ribosomal RNA sub-unit (*18s*) and elongation factor 1α (*ef1α*).

Results and Discussion

In immature sea bass injected with E_2, plasma levels of E_2 and Vtg significantly ($p<0.001$) increased after both 1 and 5 days (Table 1), confirming the effectiveness of the treatment. Gen induced a significant increase ($p < 0.001$) in plasma Vtg and E_2 after 1 day of treatment. Since Gen did not cross-react in the E_2 RIA the rise in plasma E_2 levels detected in this group indicates that it stimulated E_2 synthesis. The effect of Gen on E_2 synthesis may be via increasing testosterone synthesis and/or by increasing aromatization, in line with previous reports of the effects of Gen on fish aromatase activity *in vitro* [9]. The tissue(s) responsible for the increased circulating E_2 after Gen treatment were not determined but potential sources in immature fish are the gonads or the brain, that have high levels of aromatase B (*aroB*) that has previously been found to be affected by endocrine-disrupting chemicals [10]. In the scales, *aroB* expression (but not *aroA*) was detected at low levels in all experimental groups and significant up-regulation occurred after 5 days of treatment with Gen (Table 2). This reveals for the first time evidence of local E_2 synthesis in fish scales, as well as its possible regulation by Gen.

Table 1. E_2, Vtg, Ca and P plasma levels detected in control, E_2 and Gen treated groups, 1 and 5 days after intraperitoneal injection. *p<0.001

	E_2 (ng/ml)		Vtg (band volume)		Ca (mM)		P (mM)	
	1 d	**5 d**	**1 d**	**5 d**	**1 d**	**5 d**	**1 d**	**5 d**
Control	0.56±0.06	0.27±0.06	0.08±0.02	0.24±0.03	3.04±0.14	2.67±0.15	3.56±0.13	3.18±0.29
E_2-treated	**60.36±7.57***	**4.84±0.83***	**0.34±0.04***	**0.46±0.04***	2.58±0.08	**4.01±0.20***	3.92±0.23	**6.86±0.45***
Gen-treated	**1.11±0.10***	0.40±0.05	**0.41±0.07***	0.18±0.02	2.92±0.16	2.62±0.10	3.84±0.28	3.40±0.12

E_2 treatment also caused a significant ($p<0.001$) increase in Ca and P plasma levels after 5 days (Table 1), which may have resulted from changes in body calcium influx or calcium mobilization from mineralized tissues [2, 3], while in contrast Gen did not affect circulating mineral levels (Table 1).

Table 2. ALP, TRAP and aromatase B transcript expression levels in scales from control, E_2 and Gen treated groups, 1 and 5 days after intraperitoneal injection. *p<0.05

	alp mRNA expression (fold relative to control)		*trap* mRNA expression (fold relative to control)		*aroB* (fold relative to control)	
	1 d	**5 d**	**1 d**	**5 d**	**1 d**	**5 d**
Control	1.00±0.09	1.00±0.09	1.00±0.10	1.00±0.18	1.00±0.24	1.00±0.24
E_2-treated	1.77±0.36	**2.10±0.25***	1.77±0.40	1.26±0.11	0.93±0.15	1.50±0.26
Gen-treated	1.42±0.17	**1.96±0.31***	1.32±0.17	1.10±0.18	0.92±0.139	**2.10±0.30***

The enzymatic activities of TRAP and ALP, markers of OSC and OSB, respectively, were significantly ($p<0.05$) increased by Gen 1 day after injection, while no differences were found for E_2 possibly due to the high inter-individual variation (Table 3). The mRNA levels of these enzymes were not affected in the short term but after 5 days of exposure to E_2 and Gen an up-regulation of *alp* transcript expression was detected. These results suggest that Gen and E_2 have a similar effect on calcium turnover in sea bass scales, and confirms previous studies reporting an increase in alp activity in response to E_2 [3, 4, 6].

Table 3. ALP and TRAP scale enzymatic activity determined in control, E_2 and Gen treated groups, 1 and 5 days after intraperitoneal injection. *p<0.05

	ALP (nmol pNP/min/mg)		TRAP (nmol pNP/min/mg)	
	1 d	**5 d**	**1 d**	**5 d**
Control	0.22±0.06	0.34±0.05	0.66±0.03	0.79±0.12
E_2-treated	0.31±0.08	0.46±0.16	1.19±0.41	1.09±0.23
Gen-treated	**0.44±0.08***	0.58±0.19	**0.99±0.13***	0.67±0.04

Finally, the effects of E_2 and Gen were also analyzed on the expression of other known estrogen-responsive genes (described in detail in [6]). The results from that study suggest that the response of the scales to E_2 and Gen was similar and higher at 1 day compared to 5 days, with most genes being up regulated. However, a recent transcriptomics study detected a higher number of genes being regulated by E_2 or Gen in scales after 5 compared to 1 day and also allowed identification of a sub-group of genes that were down-regulated and specifically regulated by Gen (*Pinto et al.*, unpublished results). This highlights the power of whole transcriptome studies to identify novel responsive genes in specific tissues, like the scales, rather than the candidate gene approach based on the regulation in other tissues. In addition, we have shown that sea bass scales express the mRNAs of two nuclear (*esr2a* and *esr2b*) and one membrane (*gperl*) estrogen receptor subtypes, with *esr2a* and *gperl* being up regulated by E_2 and Gen after 1 day and *esr2a* up regulated by E_2 after 5 days [6].

In summary our study indicates that mineral turnover and homeostasis of scales can be influenced by both E_2 and the phytoestrogen Gen. The responses included a rapid, 1 day response, and a longer, 5 days, response evident as a change in gene expression, enzymatic activities and circulating mineral levels. *esr2a* and *gperl* appear as good candidates to mediate, respectively, classical genomic estrogenic

actions and alternative rapid modes of action, like those mediated by membrane receptors in OSCs and OSBs in mammals [6, 11]. Gen treatment caused similar but also non-overlapping effects compared to E_2, increased plasma E_2 levels and scale *aroB* expression. This indicates that the Gen effects observed in sea bass scales *in vivo* may include direct actions via ERs/GPERs in OSCs/OSBs, indirect effects as a consequence of its actions in other tissues and/or indirect effects resulting from an increase in circulating or locally-produced E_2.

Acknowledgments

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AN APPROACH TO STANNIOCALCIN SECRETION IN GILTHEAD SEABREAM (*SPARUS AURATA*)

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Calcium balance is of vital importance for vertebrates. In teleost fish, the most important endocrine modulators of calcium homeostasis are stanniocalcin (STC) and the parathyroid hormone-related protein (PTHrP), which act as antagonists. In turn, both hormones are apparently regulated by a calcium-sensing receptor (CaSR), and no other regulators are yet described for either hormone. In this sense, teleost fish provide a unique model to study STC regulation, as they have a specialized STC-producing gland called the corpuscles of Stannius (CS). Here, using the seabream as a model, we have developed an *ex vivo* assay to culture CS of fish and a competitive ELISA method to measure STC concentrations. STC release from the CS is mediated by a CaSR, as indicated by the effects of incubation with different calcium levels, and the use of calcimimetic and calcilytic compounds. Moreover, we describe for the first time the presence of two PTHrP receptors in the seabream CS *i.e.* PTHR1 and PTHR3. Thus, ex vivo incubations revealed a dose-response inhibitory effect of PTHrP on STC secretion under basal calcium concentrations. This inhibition is carried out through specific and reversible second messenger pathways *viz.* transmembrane adenylyl cyclases and phospholipase C. Together, these results revealed a fascinating novel process of endocrine modulation between two antagonist hormones, involved in calcium regulation that resembles the terrestrial regulation of calcium balance in the parathyroid gland.

Introduction

Calcium balance results critical in vertebrates and is under endocrine regulation (1). Calcitonin and parathyroid hormone are the main controllers of plasma calcium homeostasis in humans. Similarly, teleost fish present STC and PTHrP acting as hypo- and hypercalcemic hormones, respectively (2). Stanniocalcin is secreted by specific glands named the corpuscles of Stannius, which are exclusive of bony fish. However, this hormone has been also described in higher vertebrates (3), where is ubiquitously expressed. In addition to its hypocalcemic action, STC has also being related to other processes such as oncogenesis (4). The importance of this hormone is therefore relevant. A few studies have pointed to a CaSR as a mediator of STC production (5), but the presence of other putative controllers is still unknown. To date, no useful models are available to unreel STC production and secretion agents. The aim of this study was to establish an *ex vivo* culture of CS of a teleost fish and develop a series of methodological tools to study the regulation of STC secretion.

Materials and Methods

Immature juveniles of gilthead seabream (*Sparus aurata*) (84.2 ± 1.9 g body weight) were obtained from commercial resources in the Algarve (Portugal) and transported to Centre of Marine Sciences (CCMAR, Faro, Portugal) facilities. Fish were maintained for at least 10 days in 550 L tanks in a recirculated-close circuit at 36-37 psu, 23-25 ºC and natural photoperiod (September-December) at a density of 4.5 kg m^{-3} and fed twice daily 1% body weight (Sorgal S.A., Portugal, Balance 3). Animals were fasted 24 h before the experiments. All handling protocols complied with the guidelines of the European Union Council (86/609/EU) and conducted by staff possessing the appropriate permissions (“Group-1” license from the Direção-Geral

de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal). Euthanasia was performed by a lethal dose of 2-phenoxyethanol (0.1% v/v; P-1126, Sigma-Aldrich) and subsequent beheading. The corpuscles of Stannius were then collected for *ex vivo* culture in plasma-like buffer following the protocol described before for seabream pituitaries (6). An ELISA method was specially validated to measure STC quantity in this species. Culture volume and incubation times were previously tested to ensure the analysis of the secreted STC in single incubated CS. Calcium was tested under physiological concentrations (1). Calcimimetics, used as positive modulators of the CaSR, doses were obtained from literature: 500 μM Gadolinium (Gd^{3+}) (G-7532, Sigma-Aldrich), 200 μM Neomycin (N-1876, Sigma-Aldrich), 500 μM Spermine (S-4264, Sigma-Aldrich) and 1 μM R-568 (3815, Tocris Bioscience, UK). A selective antagonist of the CaSR (a calcilytic drug), the NPS 2143 hydrochloride, was also used at a concentration of 0.1 μM (3626, Tocris Bioscience, UK). The antagonistic hormone of the STC, the PTHrP was tested under the physiological range of the hormone in this species (1). The putative control of STC release by second messenger pathways was tested by the use of the adenylyl cyclase (AC) inhibitor 100 μM SQ-22536 (S-153, Sigma-Aldrich) and phospholipase C (PLC) inhibitor 10 μM U-73122 (U-6756, Sigma-Aldrich).

Results and Discussion

The 6.5-fold increase in STC secretion by high levels of calcium (Fig. 1) coincides with previous studies describing the mediation of a CaSR in the release of the hormone (5). Moreover, the use of different agonists and an antagonist of the CaSR (Table 1) confirmed this line of control in STC secretion.

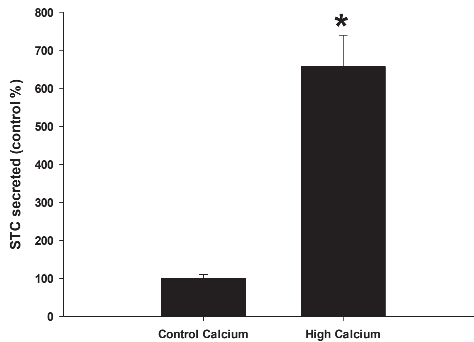

Figure 1. Effects of normal (1.46 mM) and high (2.92 mM) calcium levels on STC secretion in gilthead seabream´s *ex vivo* incubated corpuscles of Stannius. Values are mean ± SEM (n=19). * indicates statistical differences ($P<0.0005$, Student´s t-test).

Table 1. Effects of calcimimetics (Neomycin –Neo-, R568, Gadolinium –Gd^{3+}- and Spermine) and calcilytics (NPS 2143 –NPS-) on STC secretion in gilthead seabream´s *ex vivo* incubated corpuscles of Stannius. Values represent % to control groups: 1.46 mM calcium for the calcimimetics and 2.92 mM calcium for the calcilytic NPS. Values are mean ± SEM (n=6).* indicate significant differences from control groups (P<0.05, Student´s t-test).

	Neo	R568	Gd^{3+}	Sperm	NPS
Control %	381±104*	260±56*	166±6*	93±22	50±10*

The novelty of the present study appears when single CS are incubated with the antagonist hormone of the STC, PTHrP. STC secretion is inhibited by PTHrP in a dose-response manner (Fig. 2A). By PCR techniques we have described the expression of at least two PTHrP receptors, PTHR1 and PTHR3, in the CS of the seabream (data not shown), which substantiates this inhibition. Moreover, these results highlight the heterogeneity of fish species endocrinology, as the lack of PTHrP receptors was recently proposed in the CS of the Japanese eel (7).

Further characterization of PTHrP regulation of STC secretion shows that it is carried out through specific second messenger pathways (Fig. 2B). PTHrP effects are mediated by the activation of a transmembrane adenylyl cyclase and a phospholipase C. The combined use of specific inhibitors of both enzymes rescued STC secretion to basal levels and abolished the inhibitory effects of the PTHrP. The CS present at least two types of differentiated secretory cells (8). It remains to be confirmed if PTHrP acts separately in different pools of STC secretory cells, or in the same.

In conclusion, by the use of an *ex vivo* approach, we have demonstrated consistent STC release from the CS of gilthead seabream controlled by plasma calcium levels, but also by its endocrine antagonist hormone, PTHrP. Different types of membrane receptors are mediating this action: CaSR and two PTHrP receptors. The present study has thus described that fish could act as excellent models to investigate calcium-related endocrine processes.

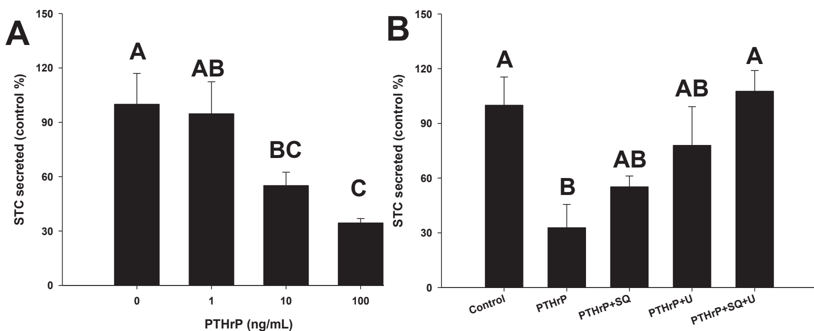

Figure 2. (A) Dose-response curve of PTHrP on STC secretion. (B) Use of 100 ng PTHrP mL^{-1} with or without specific inhibitors of the adenylyl cyclase (SQ) and phospholipase C (U). Values are mean ± SEM (n=6). Different letters indicate significantly different groups (P<0.05, I-way ANOVA, Tukey post-hoc test).

Acknowledgments

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ABLATION OF PTH4 NEURON CELLS IMPAIRS SKELETAL MINERALIZATION IN ZEBRAFISH

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Bone formation and mineralization are dynamic processes that occur during the embryonic development but also along adult life through remodeling. In our previous work in zebrafish we identified a novel hypothalamic neuropeptide, named parathyroid hormone 4 (Pth4), which is involved in bone mass accrual in adult fish. To further evaluate the function of Pth4 during larval stages, we first characterized Pth4 expressing cells and abolish them using laser ablation technique. Results revealed Pth4 cells as post-differentiated neurons which appears to be involved in bone mineral regulation in zebrafish.

Introduction

Bone metabolism has conventionally been characterized by an intricate regulation among multiple local and systemic molecules but also by neuronal factors that have drawn particular attention during the last decade. Thus, research in neuro-skeletal physiology has revealed that the central and peripheral nervous system is also a pivotal modulator of bone homeostasis (1). Particularly, the hypothalamus has been documented as an important center regulating the skeletal mass through efferent neural discharges and neuroendocrine signals (2,3). Mineralization is the most distinctive feature of the bone since not only confers the protective and supporting function but also turns the skeleton into a great reservoir of calcium, phosphate and magnesium in order to contribute to the mineral homeostasis through ion mobilization into the bloodstream (4). In vertebrates, PTH family members like PTH (parathyroid hormone) and PTHLH (parathyroid hormone like hormone) are known regulators of bone mineral homeostasis (5). In addition, PTHLH participates in the regulation of embryonic development of the skeleton (6).

We have recently identified a novel PTH family neuropeptide, Pth4, involved in bone metabolism in adult zebrafish. Here, we characterized the Pth4 expressing cells located in the hypothalamic area. In addition, we show how neural cell ablation can be used for loss-of-function experiments to study the role of Pth4 in the initiation of skeletal mineralization.

Materials and Methods

Pth4:EGFP transgenic line were generated using the Tol2 transposon system (7). Stable transgenic offspring were used in all described experiments. Ethical approval (Ref: AGL2014-52473R) was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the Spanish Authority (RD53/2013) and conformed to European animal directive (2010/63/UE) for the protection of experimental animals. 2dpf embryos were fixed in 4%

paraformaldehyde and washed in PBST. Whole mount antibody staining was performed using anti-GFP, anti-HuC/D primary antibodies followed by fluorescence-labeled secondary antibodies.

Targeted cell ablation was done in embryos at early stages with a two-photon laser on a Zeiss LSM 710 inverted confocal microscope as described in Saxena *et al.*, (2013) (8). Afterwards, 7 dpf Larvae were euthanized with a lethal dose of tricaine (MS222) and fixed in 2% buffered paraformaldehyde at 4°C overnight. Subsequently, no acid bone and cartilage stain was adapted from Walker and Kimmel (2007) (9) and performed in larvae. 7dfp Larvae were washed in 100 mM Tris pH 7.5/10 mM MgCl2 for 20 min, stained in 0.04% Alcian blue/10 mM MgCl2/70% EtOH pH 7.5 for 1h and 30min, taken through graded EtOH series (80% EtOH/100 mM Tris pH 7.5/10 mM MgCl2; 50% EtOH/100 mM Tris pH 7.5; 25% EtOH/100 mM Tris pH 7.5). Then, they were washed twice in 25% glycerol/0.1% KOH for 10 min each, stained in 0.01% Alizarin red/25% glycerol/100 mM Tris pH 7.5 for 1 h, and de-stained with two washes of 50% glycerol/0.1% KOH. Finally, specimens were stored in 85% glycerol at 4°C.

Results and Discussion

Pth4:EGFP transgenic line revealed Pth4 expressing cell grouped in two main clusters and localized in the ventral part of the periventricular hypothalamus in zebrafish embryos. A double immunostaining was performed at 2dpf transgenic embryos in order to investigate the nature of these hypothalamic cells. HuC/D is a marker for differentiated neurons as the Hu-proteins are expressed in cells that have left the mitotic cycle (10). The results showed a total overlapping between EGFP positive and HuC/D positive cells in this particular hypothalamic area (Fig.1). Therefore, Pth4 is expressed in two clusters of post-mitotic neurons.

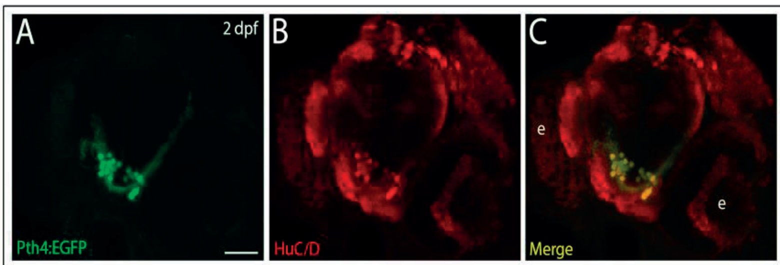

Figure 1. Pth4 cells localized in the periventricular hypothalamus are HuC/D positive neurons. Inmunostaining at 2 dpf embryos using anti-GFP antibody (A) and anti-HuC/D antibody (B) indicates the overlapping between both markers (C). Abbreviation: e, eye. Scale bar: 50 μm.

Subsequently, we chose two photon laser cell ablation technique in order to remove the Pth4 neurons and evaluate the possible effects on bone development related to the deficiency of neuropeptide. Targeted Pth4 neuronal ablation was performed in 1 and 2 dpf double transgenic zebrafish embryos (Pth4:EGFP and βactin2:H2A-mCherry). EGFP-positive and H2A-mCherry positive cells were ablated based on βactin2 marker. βactin2 is a ubiquitious nuclear marker which allowed the specific and precise single cell ablation without damaging surrounding structures. In 7 dpf ablated larvae, EGFP signal was restricted to few cell bodies compared to control (data not shown). Skeletal staining in ablated larvae did not show any visible change

in the cartilage morphology. However, in the absence of Pth4, decreased mineralization were observed in the most craniofacial structures such as otolith, notochord tip, operculum, ceratobranchial arch 5 and cleithrum (Fig.2). Alteration of skeletal formation and impaired calcification was also reported by loss of function studies of duplicated co-orthologs *Pthlha* and *Pthlhb* in zebrafish (6).

In conclusion, our results suggest that Pth4-expressing hypothalamic neurons may play a key role in the regulation bone mineral homeostasis.

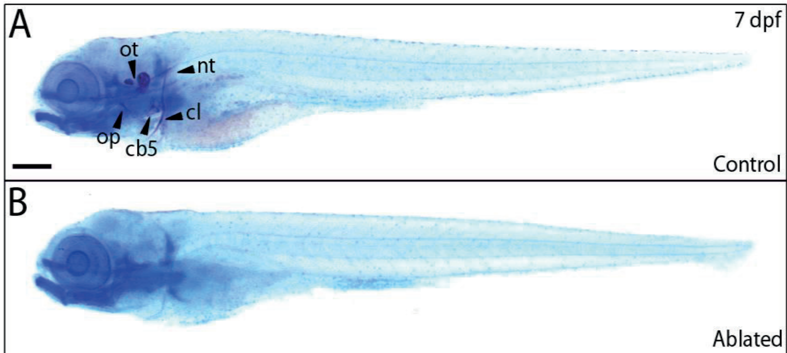

Figure 2. Alizarin red and alcian blue double staining for bone and cartilage, respectively, in control (A) and ablated larvae (B) at 7 dpf. Skeletal staining analysis shows defective mineralization in craniofacial bones. Abbreviations: nt, notochord tip; op, operculum; ot, otolith; ceratobranchial arch 5 (cb5); cl, cleithrum. Scale bars: 200 μm.

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THE GENE REGULATORY NETWORK UNDERPINNING DORSAL–VENTRAL PIGMENTATION PATTERNING IN FISH. ANALYSIS OF *AGOUTI* CIS-REGULATORY LANDSCAPE

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Dorso-ventral pigment pattern differences are the most widespread pigmentary adaptations in vertebrates. In mammals, agouti-signaling protein (ASIP) have been reported as the key factor in this process by regulating melanin types and levels in melanocytes. In fish, studies of pigment patterning have focused on stripe formation, identifying a core striping mechanism dependent upon interactions between different pigment cell types. We have recently demonstrated thatzebrafish utilize two distinct adult pigment patterning mechanisms -an ancient dorso-ventral patterning mechanism, and a more recent striping mechanism based on cell-cell interactions; remarkably, the dorso-ventral patterning mechanism also utilizes Asip. We hypothesize that Asip have a conserved role in the countershading pigment pattern formation in vertebrates. However, the molecular and cellular mechanisms underlying this dorso-ventral *asip1* expression gradient remain largely unexplored. We report here the *asip* expression in the holostei spotted gar (*Lepisosteus oculatus*), one of Darwin's defining examples of 'living fossils'. Using phylogenetic footprinting and transgenesis in zebrafish, we examine the conserved cis-regulatory elements surrounding *agouti* gene.

Introduction

Pigment pattern establishment is a really complex mechanism in vertebrates. In mammals it depends upon the regulation of the production of melanin types and levels in melanocytes. So far, the most important regulator of skin and hair pigmentation in mammals is the agouti signaling protein (ASIP)-melanocortin receptor subtype 1 (MC1R) system. ASIP antagonizes the effect of α-melanocyte stimulating hormone (α-MSH) binding to MC1R on the follicular melanocyte, resulting in a decrease in the production of eumelanins (dark pigments) and an increase in pheomelanins (red/yellow pigments)(1). Therefore, *ASIP* differential expression drives the dorso-ventral pigment pattern establishment by higher expression in ventral skin (2). In fish, *asip1* also shows a higher expression in ventral skin and lower in the dorsal, although the pigmentation mechanism is very different with independent cell-types differentially distributed, each expressing chemically distinct pigments. Recent studies have also shown that the *agouti* gene (*asip1* in fish) and its dorso-ventral gradient expression, are greatly conserved in fish (3,4). It is therefore of exceptional interest to establish whether Asip1 might also function in regulating fish pigment pattern formation.

We hypothesize that the conserved expression of *agouti* may reflect a conserved role in pigment pattern formation in vertebrates, and specifically that the pale ventrum of all vertebrates depends upon elevated levels of asip expression. Consistently , recent studies have shown graded *agouti* expression in adult zebrafish, with higher expression levels in ventral regions (5). Furthermore, another and more evolutionarily recent mechanism, likely independent of agouti expression was suggested that acts in parallel to impose the striped pattern on the dorsal body (5).

Zebrafish belong to group of rayfinned fish, which underwent an additional round of polyploidization in contrast to most of vertebrates, the teleostei genome duplication

(TGD)(6). Recently, it has been shown that holostei and teleost lineages diverged before the TGD. Therefore, the spotted gar (*Lepisosteus oculatus*) genome is not duplicated (7). Thus, these molecular and physiology characteristics of gar would facilitate the functional characterization of *agouti* gene. Therefore, we report the *asip* expression pattern in spotted gar skin. Furthermore, we have generated a transgenic zebrafish line expressing eGFP under the control of spotted gar *asip* regulatory elements in order to determine the conserved cis-regulatory elements surrounding the agouti gene in vertebrates.

Materials and Methods

Dorsal and ventral skin samples were extracted from four 1 year-old spotted gar. Before manipulation, they were anesthetized with tricaine methasulfonate (MS-222) 0.02% and sacrificed by rapid decapitation. Animals were provided for the Institute of Neuroscience (Oregon,EEUU). RNA was extracted by Tri-reagent method and the retrotranscription was performed following the manufacturer's manual. The expression was analyzed by qRT-PCR.

A reporter transgenic zebrafish line with the *Loc.asip* was generated by transposon-mediated BAC transgenesis(8). *Loc.asip* is included in a BAC from the VRMC56 (unpublished) library, (Postlethwait laboratory (Institute of Neuroscience, University of Oregon)).

Results and Discussion

To determine the asip dorso-ventral expression in spotted gar, we measured *asip* transcript levels in skin from dorsal and belly regions of 1 year old fish (Fig. 1). Dorso-ventral differences in *asip* expression levels reached significant relevance (Fig.1) with the highest and lower levels in the belly and dorsum, respectively. This expression pattern correlates with the dorso-ventral pigment pattern that shows darkly and light colored skin in the dorsal and ventral areas, respectively. Our data thus show that *asip* expression pattern in the skin of the spotted gar is identical to that found in zebrafish (5), further, supporting that *agouti* function is crucial for the development of the dorso-ventral counter-shaded pattern in vertebrates. In order to determine the conserved cis-regulatory elements surrounding the agouti gene in vertebrates, a reporter BAC (Loc-*asip-iTol2-eGFP-BAC*) transgenic zebrafish line was created and analyzed. eGFP expression was compared with zebrafish endogenous *asip1* expression. Loc-*asip-iTol2-eGFP-BAC* reporter Tg line shows eGFP expression in zebrafish ventral skin at 20dpf (Figure 2.A', 2.B'). Additionally, eGFP expression was also found in the pectoral and pelvic fins and the lower jaw (Figure 2 A', 2B'). At 30dpf, the eGFP expression still appears in jaws, pectoral and pelvic fins, and strongly in ventral skin (Figure 2C', 2D'). The dorso-ventral gradient of eGFP expression is clearly perceptible through metamorphosis process and at post-metamorphic stages. Therefore eGFP reporter construct containing spotted gar genomic sequence surrounding *asip* gene drove eGFP expression in a pattern that recapitulated the onset and the locations of endogenous zebrafish *asip1* transcript (5). Consequently, supporting the hypothesis of conserved cis-regulatory elements surrounding the agouti gene in vertebrates.

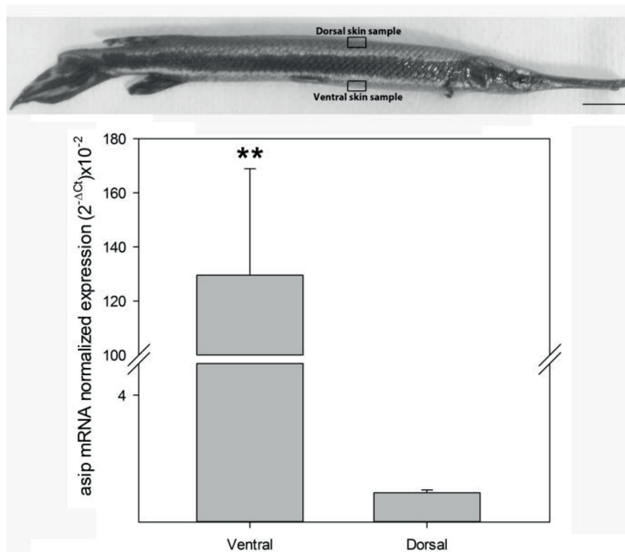

Figure 1. Analysis of *asip* expression in dorsal and ventral skin samples of Spotted gar. (A) Spotted dorso-ventral pigment pattern showing darkly coloured skin in the dorsal area and light ventrum. (B) Normalized gene expression levels of spotted gar *asip* in ventral and dorsal skin samples. The expression of *asip* mRNA was quantified by real-time qRT-PCR. Shown are log10 transformed ΔCt values of *asip* relative to *β-actin*. Data are the mean ±SEM from four samples after triplicate qRT-PCR analysis. Comparisons of numerical data were made by paired Student t-tests. Asterisk ** indicates significant differences ($P<0.05$) in gene expression levels between dorsal and ventral skin regions. Scale bar: 1 cm.

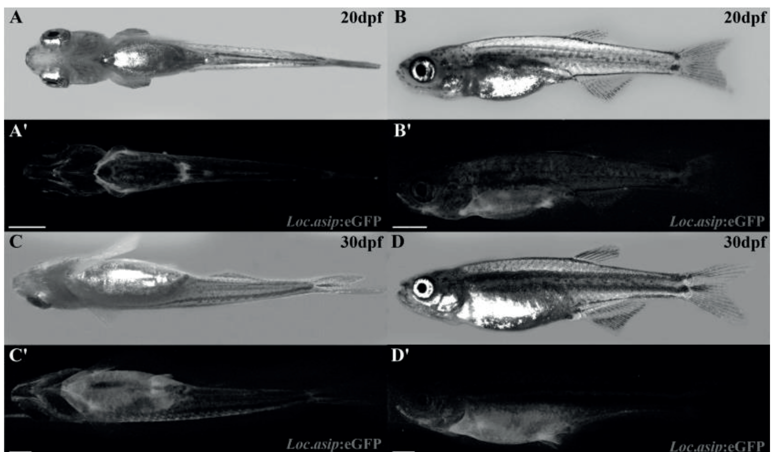

Figure 2. *eGFP* expression in zebrafish carring a modified BAC construct (*Loc-asip-iTol2-eGFP-BAC).* Expression of *eGFP* (white) was analyzed at the indicated developmental stages 20dpf (**A', B'**) to 30dpf (**C', D'**). At 20dpf and 30dpf eGFP was detected in the operculum (**A'C')**, , jaws (A'C'), base of fins (A'C') and ventral skin (**B',D'**). Scale bars: 1 mm.

Acknowledgments

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INFLUENCE OF AVT AND CORTISOL TREATMENT ON STRESS AND THYROID PATHWAYS IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*)

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The present study was designed to investigate the possible interaction of stress, thyroid and vasotocinergic pathways at hypothalamic level in the gilthead sea bream (*Sparus aurata*) after an intraperitoneal administration with slow release implants of coconut oil alone (control), or containing different doses of AVT (0.5 or 1.0 µg/g body weight) or cortisol (50 µg/g body mass) in a time-course response. Thus, both hormones significantly decreased hypothalamic corticotrophin releasing hormone (*crh*) mRNA expression from 12/24 hours onwards, while CRH-binding protein (*crh-bp*) mRNA expression did not alter its values in any of the experimental groups during the time that the experiment lasted. Moreover, AVT and cortisol administration independently produced a significant decrease in thyrotropin-releasing hormone (*trh*) gene expression from 12 h post-treatment, whereas an up-regulation in thyroid receptor β (*trβ*) mRNA expression was observed just 12 h post-injection. Our results clearly suggest that both AVT and cortisol hormones independently co-regulate the functions of these endocrine systems.

Introduction

The endocrine system could be considered as a complex net of pathways in which the interconnection between some of them must not be ruled out. Cortisol, the main corticosteroid in teleosts, is the final step on the hypothalamic-pituitary-interrenal (HPI) axis, and its functions are related to intermediary metabolism, osmotic and ionic regulation, growth, stress and immunity. In turn, the vasotocinergic, homologous to the mammalian vasopressinergic pathways, and thyroid systems also present an important role in several physiological processes in which cortisol plays a role (e.g. osmoregulation, metabolism or stress). Moreover, the physiological actions of each one will depend on the distribution of their receptors along the different target organs and tissues where they are present. The aim of this study was to evaluate the possible interaction between different endocrine axes (stress and thyroid pathways) in the gilthead sea bream (*Sparus aurata*), through assessing the effects of exogenous arginine vasotocin (AVT) and cortisol administration.

Materials and Methods

Experimental design: Juvenile gilthead sea bream acclimated to seawater (38 ‰ salinity) were intraperitoneally implanted with vegetable oil (mixture 5:1 coconut and seeds oils) alone (control) or containing different doses of AVT (0.5 or 1.0 µg/g body mass) or cortisol (50 µg/g body mass) and sampled at 12 hours, and 1 and 3 days after injection. Complete hypothalami were put in a 1/10-relation w/v of RNA*later*™ stabilization solution (Ambion®) for 24 hours at 4 ºC and then stored at -20 ºC.

Quantification of mRNA expression levels (qPCR): Total RNA was extracted using the NucleosSpin® RNA kit (Macherey-Nagel). After reverse transcription (qSCRIPT™cDNA Synthesis Kit; Quanta BioSciences) qPCR reactions were carried out with the Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep

realplex^{2} S). Results were normalized to *S. aurata* β-actin (*actb*, X89920) owing its low variability (less than 0.5 CT) under our both experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method.

Results and Discussion

CRH stimulates the release of adrenocorticotropin hormone (ACTH), which activates cortisol synthesis in the steroidogenic cells of the interrenal gland of teleosts. In addition, its regulation by other different neuropeptides (e.g. AVT/AVP hormones) and factors (TRH) has also been suggested since their hypothalamic-produced neurons directly innervate hypophyseal ACTH cells in fish (1, 2). Our results clearly demonstrate that both exogenous cortisol and AVT hormones did not produce changes in *crh-bp* levels (Figures 1B, 2B) together with a down-regulation of *crh* mRNA expression (Figures 1A, 2A), suggesting that mechanisms other than the HPI-axis are able to carry out their regulation.

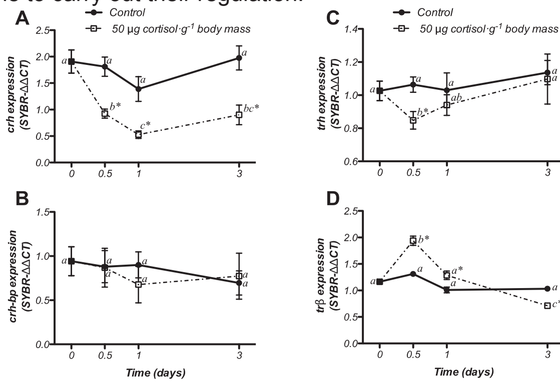

Figure 1. Time course of changes on hypothalamic *crh* (A), *crh-bp* (B), *trh* (C) and *trß* (D) mRNA expression in SW-adapted sea breams implanted with vegetable oil (mixture 5:1 coconut and seeds oils) alone (control) or containing 50 µg cortisol·g^{-1} body mass. Values are represented as mean ± S.E.M. (*n* = 7-8 fish per group). Significant differences among sampling points at the same treatment (control or cortisol) are identified with different letters, whereas different symbols showed differences between groups at the same time (*P*<0.05, two-way ANOVA followed by Tukey's test).

The endocrine control of hypothalamic-pituitary-thyroid (HPT) axis in lower vertebrates, as fish, remains unclear. Thus, HPT could be mediated and affected by TRH (3), CRH and cortisol (4), and may be by other hormones or factors. Our results showed that both AVT and cortisol hormones inhibit the mRNA expression of the putative key upstream element of the HPT axis (*trh*) (Figures 1C, 2C), being *trß* up-regulated at least during the firsts hours post-administration and also in a dose-dependent manner (Figures 1D, 2D), suggesting that a positive feed-back of this axis is produced to regulate those physiological functions mediated by the interconnection between the endocrine pathways studied.

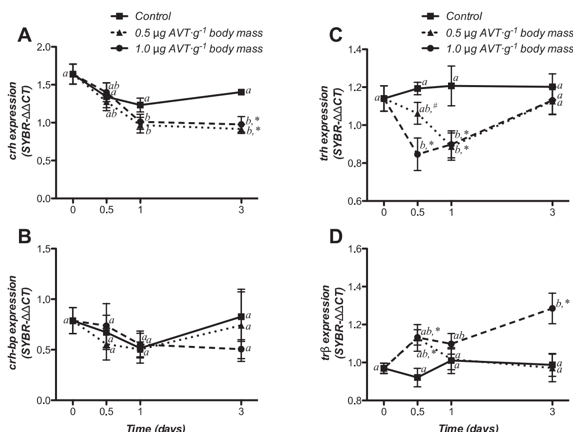

Figure 2. Time course of changes on hypothalamic *crh* (A), *crh-bp* (B), *trh* (C) and *trß* (D) mRNA expression in SW-adapted sea breams implanted with vegetable oil (mixture 5:1 coconut and seeds oils) alone (control) or containing different doses of Arg^8-vasotocin (AVT, 0.5 or 1 µg $AVT·g^{-1}$ body mass). Further details as described in the legend of Figure 1.

In conclusion, using an *in vivo* approach in the gilthead sea bream as a biological model, we have demonstrated that both stress and thyroid pathways are centrally and independently regulated by two different hormones produced at hypothalamic (AVT) and interrenal (cortisol) levels.

Acknowledgments

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STUDY OF THE EXPRESSION OF SOMATOTROPIC HORMONES DURING THE EARLY DEVELOPMENT OF THICK-LIPPED GREY MULLET (*CHELON LABROSUS*)

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Mullets are considered as potential species for sustainable aquaculture due to their omnivorous/detritivorous feeding characteristics. Among all the mullets found in the Iberian Peninsula, the thick-lipped grey mullet *Chelon labrosus* has a higher commercial interest because of its faster growth rate. During growth and development, the recently hatched larvae go through a series of molecular, biochemical and morphological changes which are controlled by a sequentially secretion of hormones by the endocrine system. With the purpose of brodening the knowledge of the possible factors involved in the early development and differentiation in this species, expression of the two somatotropic genes, *gh* and *igf1*, was studied during the first 92 days of life using real time PCR. Completely different expression patterns were observed for these genes. *gh* expression increased significantly, reaching to its maximum at 43 days post hatch (dph), and decreased drastically afterwards, maintaining relatively low levels during the rest of the studied period. However, *igf1* showed a gradual raise, peaking at 50 dph, and thereafter maintained these relatively high levels. The maximum growth rate, between 22 and 54 dph, coincides, to a large extent, with the elevated expression levels of *gh* and the beginning of the *igf1* rise. This can suggest that *gh* and *igf1* are expressed in an independent manner during the first 50 days of life, while the GH/IGF-I axis starts to be functional afterwards.

Introduction

Thick-lipped grey mullet (*C. labrosus*) is an interesting candidate for sustainable aquaculture especially due to its omnivorous/detritivorous feeding habits. In fish, as in mammals, the endocrine control of growth is exerted through the Growth Hormone (GH)/Insulin-like Growth Factor I (IGF-I) axis, both of which are known to play a critical role in early life stages (1, 2). However, generally there are very few studies on the role of these factors in embryonic and larval development, and specifically no data has been reported in *C. labrosus* or other mullets. Therefore, the aim of this study is to investigate the ontogeny of the expression of the somatotropic factors *gh* and *igf1* during the first 92 days post hatch (dph) using real time PCR.

Materials and Methods

Biological samples were taken at 14 points along the development. A total of 5 to 10 larvae were processed depending on the age of the specimens. Total RNA was extracted from the whole larvae fixed in RNA*later*®. Subsequently, specific primer pairs were designed for real-time PCR using the *gh* (KC195966) and *igf1* (KC195967) sequences available in the GenBank. Quantification was performed using SYBR green and $\Delta\Delta C_T$ method. β-actin was used as an internal control. A pool of cDNA from all the samples was used as an inter-assay calibrator. Significant differences between sampling points were analyzed by one-way ANOVA followed by the post-hoc Tukey test. Data were considered statistically different when $p<0.05$.

Results and Discussion

In our study, *gh* transcripts were detected from 3 dph (Fig. 1). Early appearance of GH, which has been previously observed by other authors, demonstrates the important role of this hormone during larval development (1, 2). In the present study, high *gh* expression (till 43 dph) was followed by a significant decline and these relatively low levels were maintained until the end of the studied period (Fig. 1). Similar pattern have been obtained in other fish species (2, 3). In fish, the first weeks of larval development are characterized by organogenesis and high growth rate. Therefore, it can be suggested that GH requirement in association with organogenesis and fast growth might be fulfilled by its endogenous formation. Interestingly, in *C. labrosus* maximum growth rate was observed at 22-54 dph (4) which coincides with the high *gh* expression levels (21-43 dph) and the beginning of the rising trend of *igf1* (50 dph).

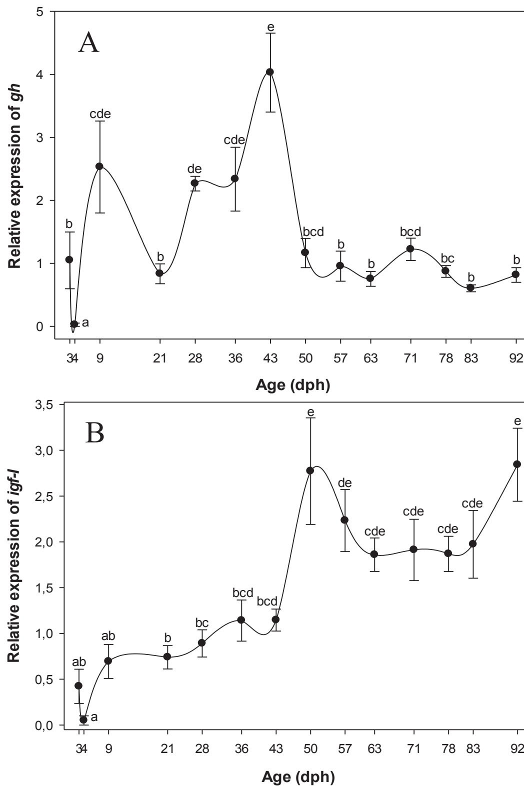

Figure 1. Transcriptional changes of *gh* (A) and *igf1* (B) in *C. labrosus* whole-body larvae during development. Sampling points not sharing the same letter show statistically significant differences (mean ± standard error of the mean).

On the other hand, a completely different expression pattern was obtained for *igf1*. For this gene, mRNA expression was relatively low during the initial stages. It increased gradually, reaching to the highest level at 50 dph and was maintained for

the rest of the studied period (Fig. 1). Early detection and increasing trend of *igf1* in the late larval stages has been described in other teleosts (1, 2, 3). These authors propose that this increasing pattern is linked to the numerous processes involved during developmental changes to the juvenile stage. The fundamental role of IGF-I on growth, differentiation, and morphogenesis has been described in different vertebrate systems (5). Interestingly, according to the histological study performed in the thick-lipped grey mullet, metamorphosis takes place between 23-78 dph (4).

Today, there is solid evidence for existence of a functional GH/IGF-I axis in adult fish, which is regulated through negative feedback (5). However, whether such an axis is functional in developing larvae is unknown. In the present work, *igf1* showed a significant up-regulation approximately in the middle of the period of metamorphosis (50 dph) and maintained these high levels till the end of the experiment (Fig. 1). This up-regulation of *igf1* appears to be concurrent with the decrease in *gh* levels. This evidence is in agreement with the hypothesis that IGF-I synthesis acts as a mediator of a negative feedback by inhibiting GH expression. Nevertheless, there is not any clear relevance between their expression patterns before this sampling point (Fig. 1). Therefore, we can suggest that before 50 dph they are expressed in an independent manner and the GH/IGF-I axis starts to be functional afterwards.

Acknowledgments

This study has been partially funded by a grant for applied innovation and transfer of knowledge in the professional formation of the educative system from the Spanish Ministry of Education, entitled “Optimización del Proceso de maduración y desove del mugílido *Chelon labrosus*”, and co-financed by ESF. The authors acknowledge the IES Els Alfacs (Sant Carles de la Rápita, Tarragona, Spain) for providing the fertilized eggs.

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LONG-TERM EFFECTS OF rBGH TREATMENT ON GH/IGFs AXIS IN FINGERLINGS OF GILTHEAD SEA BREAM (*SPARUS AURATA*)

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In the present study, the effects of an rBGH treatment in fingerlings of gilthead sea bream were analyzed. After six weeks post-injection the rBGH-treated fish grew significantly more than control fish. Moreover, gene expression analysis indicated the increased expression of several genes of the GH/IGFs system (i.e. IGF-I, IGF-II, IGFBP-2, IGF-IRb, GHR-I and GHR-II) in the liver of rBGH-treated fish compared to control fish. Although only minor differences were observed in bone and muscle tissues, the expression of both IGF-I receptors (IGF-IRa and Rb) in rBGH-treated fish was down-regulated in comparison with control fish. These findings reveal the main effects of long-term GH treatment in this species through hepatic IGFs, and provide new insights to better understand the endocrine regulation of fish somatic growth, which may contribute to improve aquaculture production.

Introduction

Fish growth is mainly regulated by the growth hormone (GH)/insulin-like growth factors (IGFs) axis. GH is secreted by the pituitary and stimulates the hepatic synthesis of IGF-I, which is the largest growth inducer in muscle and bone tissues. Secreted IGFs can be modulated by different binding proteins (IGFBPs) that can inhibit or potentiate their actions by regulating bioavailability, plasmatic half-life or facilitating the subsequent binding to membrane receptors (IGF-IRs). Binding to the receptors activates different signaling pathways to regulate metabolism and to promote growth. In the last decade, a sustained-release formulation of recombinant bovine GH (rBGH, Posilac®) treatment has been proved very useful to increase IGF-I levels and consequently to enhance the growth rates in several fish species (1-5). This work aimed to study the long-term effects of rBGH in gilthead sea bream fingerlings, in order to improve knowledge on GH/IGFs axis and the growth potential of this species.

Materials and Methods

Fingerlings of gilthead sea bream (initial weight of 1±0.05 g) were randomly distributed into eight tanks and maintained within a temperature-controlled system (23±1ºC) and 15 h light/9 h dark photoperiod. For the experimental trial, fish of four tanks each were injected with a dose of 4 mg rBGH/g of body weight (rBGH group), or with the equivalent volume of sesame oil (Control group). Fish were fed *ad libitum* with a commercial diet five times a day. At 6 weeks post-injection, the weight of all the fish was determined and 12 fish per group (3 fish per tank) were anesthetized, sacrificed and samples of liver, white muscle and bone tissue were collected, frozen in liquid nitrogen and stored at -80ºC until further analysis. Next, total RNA was extracted from 100 mg of tissue and its concentration, purity and integrity were determined. After that, DNase I was used to remove all genomic DNA of 1 µg of total RNA, that then was reverse transcribed. Finally, the mRNA transcript levels of IGF-I, IGF-II, IGFBP-2b, IGFBP-4, IGFBP-5b, IGF-IRa, IGF-IRb, GHR-I and GHR-II, plus three reference genes (β-actin, RPS18 and RPL27) were examined in a CFX384™

Real-Time System as previously described (6). The expression level of each gene analyzed was calculated relative to the geometric mean of the most stable reference genes.

Results and Discussion

After 6 weeks of intraperitoneal injection of rBGH, the fingerlings of gilthead sea bream grew significantly more than Control fish (8.84 ± 0.18 g *vs.* 8.07 ± 0.25 g, respectively), similarly to that observed in previous experiments in other species (1-5). Moreover, gene expression results indicated an up-regulation on the GH/IGFs system in the liver, where the mRNA levels of both peptides (IGF-I and IGF-II) and the GH receptors (GHR-I and GHR-II) were significantly increased. Contrarily, no differences were observed in these molecules in the other tissues studied (Table 1). In concordance with our results, previous studies found that an rBGH treatment in rainbow trout and channel catfish increased the hepatic gene expression of IGF-I and IGF-II, associated with higher plasma levels of IGF-I, while no changes in muscle gene expression were observed (1,2,5).

Table 1. Relative mRNA expression normalized to reference genes of IGF-I and IGF-II, IGF binding proteins (IGFBP-2/BP-4/BP-5), IGF-I receptors (IGF-IRa/Rb) and GH receptors (GHR-I/II) in liver, white muscle and bone tissue in fingerlings of gilthead sea bream after 6 weeks of rBGH injection. Data are presented as mean of fold change relative to Control group (n=12). Significant differences are indicated with * (P<0.05), † (P<0.01) or †† (P<0.001). N.D.: non-detected.

	LIVER	WHITE MUSCLE	BONE
IGF-I	+0.612†	+0.077	-0.026
IGF-II	+0.757*	-0.200	-0.165
IGFBP-2	+0.581††	N.D.	N.D.
IGFBP-4	N.D.	-0.205	+0.052
IGFBP-5	N.D.	+0.096	+0.021
IGF-IRa	N.D.	-0.056	-0.307*
IGF-IRb	+0.929*	-0.202†	-0.361*
GHR-I	+0.865*	+0.165	-0.100
GHR-II	+0.648*	-0.212	-0.226

Regarding IGF-IRs, rBGH-treated fish showed increased transcription of IGF-IRb in liver, whereas in white muscle this receptor was down-regulated. Furthermore, in bone tissue the expression of both IGF-IRs was also diminished, suggesting a decrease in sensitivity in response to IGF-I in this condition. In this sense, Biga et al (1) did not found differences in the expression of IGF-IRa in cardiac muscle of rainbow trout. With respect to the IGFBPs, only IGFBP-2 showed enhanced expression in the liver of rBGH-treated fish, while IGFBPs 4 and 5 remained stable in both muscle and bone tissues (Table 1).

Overall, these results are in concordance with previous studies in rainbow trout (1-3), coho salmon (4) and channel catfish (5), highlighting the important role of liver

coordinating the GH related-growth effects in fish through the GH/IGFs axis (Fig. 1). Furthermore, these findings provide new insights to better understand the role of GH and IGFs function in gilthead sea bream, and help to know how far we are from its potential maximal growth.

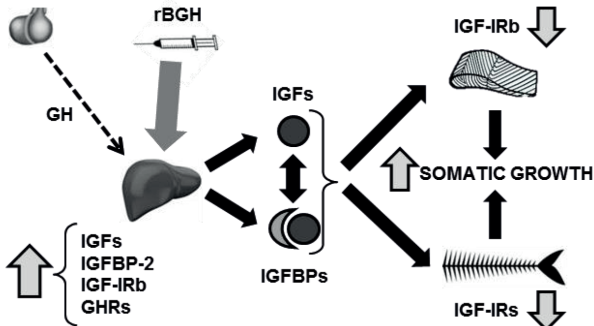

Figure 1. Proposed model of GH/IGFs axis-regulation in response to rBGH in gilthead sea bream fingerlings.

Acknowledgments

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HYPER- AND HYPO-OSMOTIC CHALLENGES MODIFY HORMONAL PATHWAYS IN THE GILTHEAD SEA BREAM (*SPARUS AURATA*): A MICROARRAY APPROACH

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Gills, liver and hypothalamus play an important role in the osmoregulatory processes due to their implications in ion and water exchange, metabolism and endocrine regulation, respectively. Transcriptomic analysis in these tissues by means of a specific oligo-microarray revealed new aspects related to the osmoregulatory processes mediated by different endocrine pathways after hypo- (38‰ → 5‰) or hyper- (38‰ → 55‰) osmotic challenges in juvenile of gilthead sea bream (*Sparus aurata*). Our results point out the importance of liver, gills and hypothalamus in the response to osmolality change, showing the differentially regulated endocrine components and pathways that process the harmful metabolites related to osmotic stress.

Introduction

Euryhaline teleosts can live in a wide range of environmental salinities, maintaining the osmolality of their internal fluids between certain values regardless of the external osmolality (1, 2). This ability is carried out by different osmoregulatory and non-osmoregulatory tissues, being gills, liver and hypothalamus very important due to their implications in ion and water exchange, acid-base regulation and excretion of nitrogenous wastes (3), metabolism (4) and endocrine regulation (2), respectively. However, the molecular phenotyping of osmotic-stress response remains still far to be resolved, especially when it is considered in a tissue-integrated manner. The aim of the present study was to describe new aspects related to endocrine osmoregulatory processes, analyzing the effects of hyper- and hypo-osmoregulatory challenges in different tissues of gilthead sea bream (*Sparus aurata*) using a custom oligo-microarray.

Materials and Methods

Immature specimens of *S. aurata* (80-100 g body mass) were acclimated during 10 days in an open circuit of SW (38‰ salinity), under constant temperature (18-19ºC) and natural photoperiod (April-May 2009, 11 hours of light). After that, specimens were transferred to different experimental conditions (LSW: 5‰; SW; and HSW: 55‰) in 400-L tanks with a water recirculation system. Specimens were sampled at day 7 post-transfer. After blood extraction, hypothalamic lobules and representative biopsies of liver and gills were taken for total RNA extraction using a commercial kit. The design of the oligo-microarray used in this experiment is stored in the NCBI Gene Expression Omnibus (GEO) database under accession number GPL13442. Microarray hybridizations were performed as previously described by (5). Functional analysis was performed by the Ingenuity Pathway Analysis software (IPA, www.ingenuity.com).

Results and Discussion

Table 1. List of differentially expressed genes (P < 0.05, one-way ANOVA with corrected p-value, Tukey´s HSD post hoc test, Benjamini-Hochberg multiple testing correction) with fold-change values after hypo- (Hypo) and hyper- (Hyper) osmotic transfer referred to seawater acclimated fish (control, 38‰). Numbers highlighted, in bold and brackets represent the canonical pathway in which each gene is involved.

TISSUE, CANONICAL PATHWAY / GENE NAME	SYMBOL	Hypo (38→5 ‰)	Hyper (38→55 ‰)
LIVER: ***Aldosterone signaling in epithelial cells (1)***			
DnaJ (Hsp40) homolog, subfamily C, member 8	*DNAJC8*		1.34
DnaJ (Hsp40) homolog, subfamily C, member 11	*DNAJC11*	1.55	2.33
DnaJ (Hsp40) homolog, subfamily C, member 17	*DNAJC17*	1.40	1.43
DnaJ (Hsp40) homolog, subfamily C, member 30	*DNAJC30*	1.33	1.31
Heat shock protein 90kDa alpha (cytosolic), class B member 1	*HSP90AB1*	1.55	
Heat shock 70kDa protein 14	*HSPA14*	1.53	
Heat shock 60kDa protein 1 (chaperonin)	*HSPD1*		2.80
Heat shock 10kDa protein 1	*HSPE1*	2.72	3.48
Kirsten rat sarcoma viral oncogene homolog	*KRAS*	-1.54	-1.56
Phosphatidylinositol 3-kinase, catalytic subunit type 3	*PIK3C3*	-1.47	
Protein kinase C, alpha	*PRKCA*	-1.72	
GILLS: ***Serotonin degradation (2)*** ***Thyroid hormone metabolism (3)*** ***Melatonin degradation (4)***			
Cytochrome P450, family 1, subfamily A, polypeptide 1 **(*4*)**	*CYP1A1*	7.57	
Cytochrome P450, family 1, subfamily B, polypeptide 1 **(*4*)**	*CYP1B1*		3.43
Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1 **(*2*,*3*,*4*)**	*SULT1A1*	1.91	
UDP glucuronosyltransferase 1 family, polypeptide A1 **(*2*,*3*,*4*)**	*UGT1A1*	2.02	
UDP glucuronosyltransferase 2 family, polypeptide A3 **(*2*,*3*,*4*)**	*UGT2A3*		1.52
HYPOTHALAMUS: ***Melatonin degradation (4)***			
Cytochrome P450, family 1, subfamily B, polypeptide 1	*CYP1B1*	3.21	
Cytochrome P450, family 2, subfamily J, polypeptide 2	*CYP2J2*	2.77	
Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	*SULT1A1*	4.69	
UDP glucuronosyltransferase 1 family, polypeptide A8	*UGT1A9*		-1.76

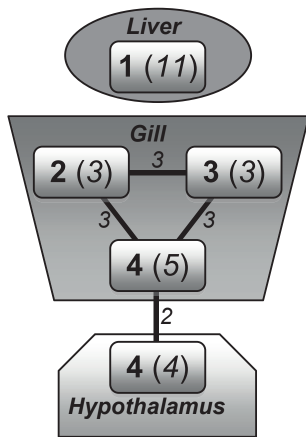

figure 1. Overlapping network of hepatic, branchial and hypothalamic response to hyper- and hypo-osmotic challenges in *S. aurata* specimens 7 days post-transfer. Solid lines show a direct connection between canonical pathways, indicating the number of common genes presented between them. In bold, the number assigned to each canonical pathway corresponded to the name described in Table 1.

The functional analysis of the differentially expressed genes involved in the four endocrine pathways assessed in liver, gills and hypothalamus are established in a major cluster related to cellular stress, being the liver the most responsive tissue to salinity changes. Interestingly, all common genes affected by both salinity transfers induced the same type of response in terms of magnitude and direction, suggesting that putative candidate genes for the control of the osmoregulatory processes and the disequilibrium caused by them could be proposed.

Taking together, these results suggest that osmoregulatory (gills) and non-osmoregulatory (liver and hypothalamus) organs can be considered as important tissues regulated by endocrine systems, assisting with reactions that break down harmful metabolites synthetized due to osmotic stress.

Acknowledgments

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CLONING, SEQUENCING AND EXPRESSION OF GENES *BRDT* AND *TERT* IN GILTHEAD SEABREAM (*SPARUS AURATA*): POTENTIAL NEW SEX MARKERS IN FISH?

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Applying molecular amplification and cloning techniques obtained cDNA sequences of *Brdt* and *Tert* genes, which has allowed to analyze levels of differential expression in gonads, brain and somatic tissues, by q-PCR quantitative, comparatively, using two reference genes (β-actin and 18S rRNA). At the same time, its cellular-tissue distribution has been analyzed, and phylogenetic analyses have been performed. The results for both genes showed relative expression levels significantly higher in testis, in relation to ovarian, and other tissues, corroborating their great importance during the reproductive process in general, and in the spermatogenesis in particular. Expression was shown in postmeiotic stages, as in spermatocytes and spermatids, and no signals have been reported in gametes.

Introduction

Although the basic mechanisms of reproduction of gilthead seabream have been extensively studied, application of genetic, molecular and cellular markers will allow us to advance in the knowledge of the molecular mechanisms that can be involved the genetic regulation of the reproductive process in fish. There are multiple genes and transcription factors (*Cyp19a, Amh, vasa, dmrt*…) involved in the regulation and control of sex determination and differentiation and gametogenesis in fish species. Currently, other genes are analyzed for their involvement in the cell proliferation process *versus* apoptosis, such as *Brdt* and *Tert* genes, which in mammals regulates spermatogenesis, male fertility, and cell aging. *Brdt* gene belongs to the bromodomain-extraterminal domain (BET) family of transcriptional coregulators. In mammals, *Brdt* is nearly exclusively expressed in the testis, and its protein is involved in elongating spermatids by binding to acetylated histones, and thereby *Brdt* is required for proper spermatogenesis and male fertility. On the other hand, *Tert* gene encodes the telomerase enzymatic subunit, which is one of the components of telomeres in mammalian germ cells, tumors and neoplastic cells, although it seems be ubiquitous in fish. For this purpose, we have isolated and cloned the mRNA and cDNA of *Brdt* and *Tert* genes in gilthead seabream, and have characterized the quantitative and qualitative gene expression patterns in different organ systems (gonads, brain, hypophysis, heart, kidney, spleen among other somatic tissues).

Materials and Methods

The full-length complementary DNA sequences from *Brdt* and *Tert* genes were obtained from gonadal RNA by 5’ and 3’ rapid amplification of cDNA ends (RACE). The phylogenetic analysis of the putative amino acid sequences were performed with MEGA 6.06 software. The relative expression levels of both genes, *Brdt* and *Tert*, were analyzed by real-time PCR, using the method described by Pfaffl (1) and two reference genes (18S rRNA and beta actin). *In situ* hybridization was performed according to Úbeda-Manzanaro *et al*. (2).

Results and Discussion

Two *Brdt* transcripts and two *Tert* transcripts were isolated from gonads of gilthead seabream: *SaBrdt-a* (3930 bp), *SaBrdt-b* (2319 bp), *SaTert-a* (4473 bp) and *SaTert-b* (3429 bp). These products encode 978 aa, 662 aa, 1107 aa and 1084 aa, respectively. Each pair of transcripts reported for both *Brdt* and *Tert* genes differs in their 3'- ends and in their 3'-UTR. These two seabream BRDT protein products contain the consensus motifs of the BET protein family, according to Paillisson *et al*. (3), whereas the two TERT products showed the functional motifs highly conserved in other vertebrate TERT sequences (4).

The phylogenetic tree analysis constructed with amino acid sequences for BET proteins family showed a strong evolutionary conservation of BET sub-family proteins in fish. As it was expected, both BRDT and TERT protein sequences from seabream were clustered with other BRDT and TERT orthologs of the Perciformes order, respectively.

Real-time PCR revealed ubiquitous expression of *Tert* mRNA in all organs and tissues examined in adult seabream specimens, as in other fish species (5,6) where the highest expression level was observed in testis. Moderate *Tert* expression was detected in ovary, muscle and heart from seabream.

In mammals, *Brdt* is almost exclusively expressed in the testis (7), although only some authors have reported low expression in oocytes (3) and brain (8) from mice. Whereas in seabream, *Brdt* mRNA is mainly expressed in testis, a moderate *Brdt* expression was observed in ovary, and was very weakly detected in other organs or tissues.

In ovary (Fig. 1A), *Brdt* expression was evidenced in the cytoplasm of late previtellogenic (LPO) or transitional oocytes, and a weak *Brdt* expression was detected in early vitellogenic oocytes (EVO). In testis (Fig. 1B), was observed in spermatids (Sd) and spermatocytes II (St II), but no signal was evidenced in spermatocytes I (St I). The *Brdt* gene is preferentially expressed in gonads and particularly in certain germ cells, which may suggest an important role in gametogenesis and germ cell development of fish species.

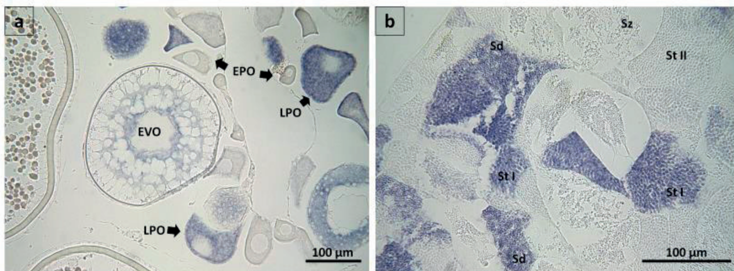

Figure 1. *In situ* hybridization using DIG-labeled *Brdt* antisense probes.

Tert mRNA in ovary *in situ* hybridization signals (Fig. 2A) were localized mainly in both early (EPO) and late previtellogenic oocytes, decreasing in early vitellogenic oocytes. *Tert* mRNA in testis (Fig. 2B) was located in both types of spermatocytes (I and II) and in spermatids. No signal was evidenced in spermatozoa (Sz) for both genes. Although *Tert* gene are expressed ubiquitously in most organ systems

tissues, the highest expression levels have been reported in gonads and particularly in immature or maturating germ cells, which could suggest an important role in gametogenesis and cell-tissue development of fish species.

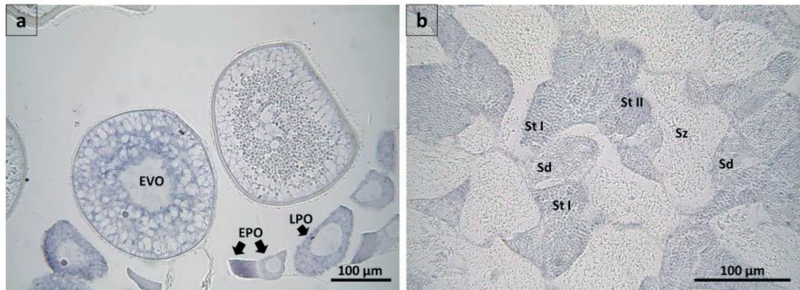

Figure 2. *In situ* hybridization using DIG-labeled *Tert* antisense probes.

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AVT AND IT SYSTEMS MEDIATED METABOLIC EFFECTS INDUCED BY AIR EXPOSITION IN THE GILTHEAD SEA BREAM (*SPARUS AURATA)*

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Arginine vasotocin (AVT) and isotocin (IT) are considered as important players in stress regulation. The present study assessed the response of both vasotocinergic and isotocinergic systems in the gilthead sea bream (*Sparus aurata*), through their specific receptors in liver (*avtr v1a2*-type, *avtr v2*-type and *itr*), as well as their possible implications in metabolism after an acute stress situation induced by air exposition for 3 minutes. Acute stress response involved changes in gene expression of specific hepatic receptors, which are differentially expressed in a time course response. Our results suggested that activation of both vasotocinergic and isotocinergic systems is important during metabolic mobilization and reorganization induced by an acute stress situation, which is stated, at least, through changes in mRNA expression levels of these analyzed genes.

Introduction

An important role in acute stress has been proposed for the vasotocinergic and isotocinergic systems in different fish species, including the gilthead sea bream (1,2). Some studies refer that synthesis of hypothalamic neuropeptides arginine vasotocin (AVT) and isotocin (IT), as well as secretion of both mature hormones into the bloodstream, change in response to acute stress. The specific receptors of these hormones in several target tissue are the key to produce its physiological actions. Nevertheless, there is only partial knowledge on the functional role of the AVT and IT systems throughout its receptors (AVTRs and ITR) in marine fish. In this work, the hepatic changes in AVT and IT receptor genes, as well as the metabolic challenges in plasma and liver, were assessed in juvenile specimens of *S. aurata* submitted to air exposition as an example of acute stress.

Materials and Methods

Immature specimens of gilthead sea bream (*S. aurata*, Linnaeus 1758) (n= 64, 108.83 ± 1.47 g body mass, 17.79 ± 0.07 cm body length) were provided by "*Servicios Centrales de Investigación de Cultivos Marinos*" (SCI-CM)" (CASEM, University of Cadiz, Puerto Real, Cádiz). Then, fish were randomly distributed and maintained in this facility in sixteen 80 L-tanks (n = 4, density 5 kg/m^3) containing seawater (38 ‰ salinity), in open circuit, at constant temperature (18-19 °C) and natural photoperiod (February-March 2013; approximately 11 hours of light).

After the acclimation period, animals were exposed to air for 3 min by lifting the wire-net cages out of the tanks and putting them back in their respective tanks after that, being sampled in a time course response (15 min, 30 min, 1, 2, 4 and 8 hours post-stress). In addition, a non-stressed group was also sampled at 0 and 8 h to identify and discard possible circadian rhythms in the levels of metabolites and expression patterns of those parameters analyzed that could mask the results. The analyses of

all metabolites were performed with specific commercial kits from Spinreact, adapted to 96-well microplates. Hepatic *avtr*s (namely *v1a2*-type and *v2*-type) as well as *itr* mRNA expression were carried out by total RNA extraction with a commercial kit, cDNA synthesis and qPCR following the $\Delta\Delta C_T$ method (3).

Results and Discussion

Our results showed a pattern of changes in metabolic parameters at both plasma and hepatic levels that agreed with the previously reported for *S. aurata* submitted to the same experimental design (4). Air exposure induces a strong and rapid increase of both glucose and lactate, which point to a sympathetic activation of the chromaffin cells in head kidney with a release of catecholamines (data not showed). Moreover, this acute stress situation triggers a metabolic reorganization in fish to cope with induced hypoxia condition. Thus, it has been shown that metabolic changes involved: i) an increase in hepatic glycogenolysis and lactate production, ii) high levels of glucose and lactate available in plasma, and iii) a decrease in free plasma proteins.

In teleost, liver is the major organ responsible for energy storage (mainly in the form of glycogen/glucose) as well as for fatty acids synthesis and the processes of aminogenesis and gluconeogenesis. The presence of AVT and IT receptors in liver suggest a putative role of both hormones in the control of energy metabolism due to the important metabolic role of this organ (5). Our results (Figure 1) showed a clear and rapid mRNA up-regulation in the expression of both AVT receptors, already in the first hour of the experiment and 4 hours post-stress for *avtr v2*-type. AVTR V2-type function is mediated by PKA activation, suggesting that the metabolic actions of AVT are controlled by this receptor in hypoxia condition due to the higher energy requirement. Furthermore, stress caused after air exposition also enhanced hepatic *avtr v1a2*-type mRNA levels, which could be associated with the incorporation of inorganic phosphate in phosphatidyl inositol routes, acting as a substrate for phospholipase C, which is the intracellular pathway for this type of AVT receptor (6). These results suggest that hepatic gene activation of *v1a2*-type and/or *v*2-type receptors is related to the control of the energy supply due to the higher requirements produced by the air exposition challenge, both regulating those enzyme controlled by the same intracellular pathways (5). Regarding *itr* mRNA expression, our results indicated a rapid enhancement of this gene in the first hour post-emersion and a subsequent increase 4 hours after stress (in a way similar to what was seen for *avtr v2*-type). These results corroborate a role of this hormone in the regulation of the stress response, which may trigger processes of metabolic reorganization at hepatic level in *S. aurata*. Even so, this response seemed to be stressor-dependent, since *itr* expression appeared to be undisturbed by osmotic challenge, suggesting a minor role (if any) for IT in *S. aurata* during a stress situation induced by salinity challenge (5).

The metabolic reorganization observed at hepatic level seems to be regulated firstly by both AVT (*v1a2* and *v2* types) and IT receptors, and later on by *avtr v*2-type and *itr*. The increase in the expression levels of the three receptors analyzed (*avtr v1a2*-type, *avtr v2*-type, and *itr*) seems to suggest a role of all of them associated with stressful situations that involve high levels of cortisol, with the subsequent need of metabolic activation for energy release into the internal medium of the animal and its control by different endocrine axes.

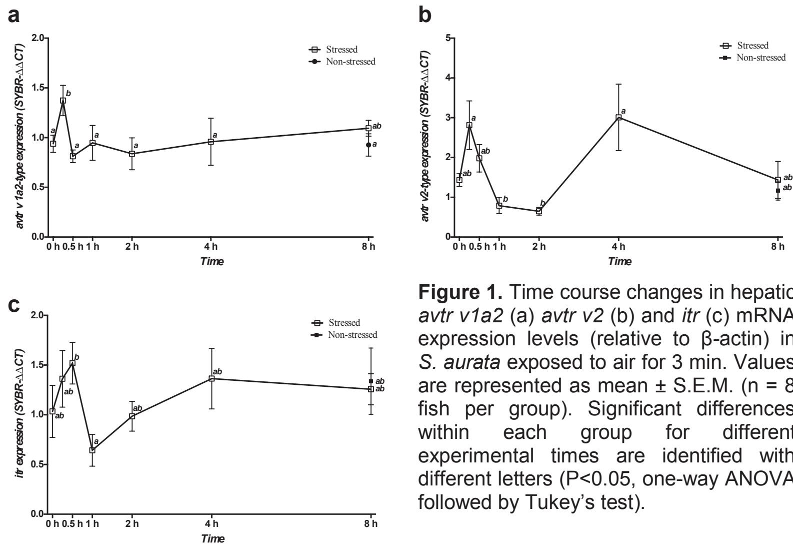

Figure 1. Time course changes in hepatic *avtr v1a2* (a) *avtr v2* (b) and *itr* (c) mRNA expression levels (relative to β-actin) in *S. aurata* exposed to air for 3 min. Values are represented as mean ± S.E.M. (n = 8 fish per group). Significant differences within each group for different experimental times are identified with different letters (P<0.05, one-way ANOVA followed by Tukey's test).

Acknowledgments

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INFLUENCE OF GLUCOSE LEVELS ON LACTATE METABOLISM IN BRAIN GLUCOSENSING AREAS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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There is no direct evidence in fish brain demonstrating the existence of changes in lactate metabolism in response to alterations in glucose levels to support the existence of an astrocyte-neuron lactate shuttle (ANLS) in these areas. Therefore, we induced in rainbow trout, through IP treatments, hypo-glycaemic or hyper-glycaemic changes to assess the response of parameters involved in putative ANLS in glucosensing areas like hypothalamus and hindbrain. To distinguish those effects from those induced by peripheral changes in the levels of metabolites or hormones, we also carried out ICV treatments with 2-deoxy-D-glucose (2-DG, a non-metabolizable glucose analogue thus inducing local glucopenia) or glucose. Finally, we also incubated hypothalamus and hindbrain *in vitro* in the presence of increased glucose concentrations. As a whole, the induction of changes in glucose levels in hypothalamus and hindbrain of rainbow trout resulted in the activation of the glucosensing mechanism dependent on glucokinase (GK) in these areas in agreement with similar previous studies. However, when we assessed in these areas the response of parameters related to lactate metabolism, the results obtained were contradictory. The increase in glucose levels did not produce in general the expected changes in those pathways with only a minor increase in their capacity of lactate production. The decrease in glucose levels was however more clearly related to a decreased capacity of the pathways involved in the production and use of lactate, and this was especially evident after ICV treatment with 2-DG in both areas. In conclusion, the present results partially support the existence of an ANLS in hypothalamus and hindbrain of rainbow trout relating glucose availability to lactate production and use.

Introduction

The transfer of glucose-derived lactate from astrocytes to neurons is referred to as the astrocyte-neuron lactate shuttle (ANLS) hypothesis. Briefly, blood-borne glucose, which is the major energy substrate for the brain, enters the brain parenchyma via glucose facilitative transporter type 1 (GLUT1) located on endothelial cells forming capillaries. It is provided both to neurons and astrocytes in which it will be taken up via specific glucose facilitative transporters (GLUT1, GLUT2 and GLUT3). In astrocytes, glucose is stored as glycogen or converted into pyruvate through glycolysis, which is metabolized to lactate by the reductase isoform of lactate dehydrogenase (LDH-R) formed by LDH-A subunits. Lactate is then released via monocarboxylate transporter 1 (MCT1) into the extracellular space, where an extracellular lactate pool is maintained. Meanwhile, in addition to glucose, activated neurons will take up lactate from the extracellular pool via monocarboxylate transporter 2 (MCT2). Once in neurons, lactate will be converted to pyruvate by the oxidase isoform of lactate dehydrogenase (LDH-O) formed by LDH-B subunits before entering in the tricarboxylic acid cycle. The activity of ANLS is essential in areas involved in glucosensing dependent on glucokinase (GK), GLUT2, and ATP-dependent inward rectifier potassium channel (K_{ATP}) where lactate can replace glucose as a fuel recognized by glucosensing.

In fish, there are not direct studies demonstrating the existence of ANLS. However, there are several indirect evidences allowing to suggest its presence. These include: (1) Lactate levels in fish brain are approximately five times higher than those in mammals; (2) Rainbow trout brain uses lactate as fuel in the absence of glucose at

rates comparable to those of glucose and higher than those described in mammals; (3) The expression in bran of rainbow trout and zebrafish of MCT-1, MCT-2 and MCT-4; (4) MCT-2 mRNA abundance increased in brain by exercise in rainbow trout and cold acclimation in zebrafish; and (5) The presence in zebrafish brain of LDH isoforms displaying changes in response to cold acclimation or hypoxia. To assess ANLS presence, we induced in rainbow trout, through *ip*, *icv* and *in vitro* treatments hypoglycaemic or hyperglycaemic changes to assess the response of parameters involved in lactate metabolism.

Materials and Methods

Rainbow trout obtained from a local fish farm (A Estrada, Spain), were maintained for 1 month under laboratory conditions and were fed once daily to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 55/2013) for the use of animals in research.

Experiment 1. Intraperitoneal administration: 15 fish per group received *ip* 5 mL Kg^{-1} injection of saline solution alone (normoglycaemic treatment) or containing insulin (hypoglycaemic treatment, 4 mg Kg^{-1} body mass of bovine insulin, insulin from Sigma Chemical), or D-glucose (hypergycaemic treatment, 500 mg Kg^{-1} body mass).

Experiment 2. Intracerebroventricular administration: 15 fish per group received ICV 1 µl/100 g body mass of saline alone (control) or containing 30 µg 2-deoxy-D-glucose (2-DG, Sigma Chemical Co.) or 40 µg D-glucose (Sigma Chemical Co.).

Experiment 3. In vitro incubation: the tissues were incubated at 15 ºC for 6 h with 250 µl of modified Hanks´ medium per well containing 25 mg of tissue and 2,4 or 8 mM D-glucose.

Results and Discussion

The treatments *ip*, *icv* and *in vitro* resulted in the expected changes in glucose levels in both assayed tissues (hypothalamus and hindbrain). These results correlated with changes in parameters related to the glucosensing mechanism based on GK, GLUT2, and K_{ATP}. The results obtained of parameters related to lactate metabolism were contradictory. The increase in glucose levels did not produce in general the expected changes in those pathways with only a minor increase in their capacity of lactate production. The decrease in glucose levels was more clearly related to a decreased capacity of the pathways involved in the production and use of lactate, especially after ICV treatment with 2-DG in hypothalamus and hindbrain *(Table 1)*. In conclusion, the evidence obtained to demonstrated the presence of ANLS in fish brain is enough to justify further research, such as cellular distribution of MCT and LDH isoforms in neurons and astrocytes or pharmacological disruption of metabolic coupling between neurons and astrocytes followed by histological analysis of neural activation.

Table 1. Activities of LDH-R and LDH-O,and mRNA abundance of LDH-A, LDH-B, MCT-1 and MCT-2 in hypothalamus (*left column*) and hindbrain (*right column*) of rainbow trout. Effects of *ip administration* of 5mL Kg^{-1} injection of saline alone (C) or containing insulin (Hypo, 4 mg bovine insulin Kg^{-1} body mass), or D-glucose (Hyper, 500 mg Kg^{-1} body mass); *icv administration* of 1 µl 100 g^{-1} body mass of saline alone (C) or containing 30 µg 2-deoxy-D-glucose (2-DG) or 40 µg D-glucose; and *in vitro incubation* at 15 ºC in modified Hanks' medium containing 2, 4, or 8 mM D-glucose for 6 h. In the *ip* and *icv* treatments, data represent mean±SEM of ten measurements whereas in the *in vitro* treatment each value is the mean±SEM of four independent experiments carried out with pools of tissues from 3 to 4 different fish. *Different letters* indicate significant differences (*P<0.05*) within each treatment.

Hypothalamus				Hindbrain			
ip administration	C	Hypo	Hyper	*ip administration*	C	Hypo	Hyper
LDH-R activity	4.07±0.67	3.34±0.46	3.94±0.56	LDH-R activity	4.17±0.40	4.56±0.54	4.09±0.28
LDH-O activity	1.90±0.01 a	0.52±0.09 b	0.35±0.02 c	LDH-O activity	0.08±0.01 a	0.12±0.01 b	0.14±0.01 b
LDH-A mRNA	1.00±0.24 a	1.73±0.25 b	0.75±0.17 a	LDH-A mRNA	1.00±0.16 a	0.38±0.06 b	0.39±0.06 b
LDH-B mRNA	1.00±0.32 a	3.27±0.52 b	2.08±0.27 c	LDH-B mRNA	1.00±0.20 a	1.10±0.16 a	0.39±0.05 b
MCT-1 mRNA	1.00±0.26	1.06±0.30	0.85±0.13	MCT-1 mRNA	1.00±0.24	0.99±0.18	0.90±0.27
MCT-2 mRNA	1.00±0.14 a	1.06±0.20 a	1.57±0.18 b	MCT-2 mRNA	1.00±0.07 a	1.43±0.16 b	1.23±0.18 ab
icv administration	C	2-DG	Glu	*icv administration*	C	2-DG	Glu
LDH-R activity	1.05±0.10 ab	0.83±0.08 a	1.07±0.06 b	LDH-R activity	0.53±0.04	0.53±0.04	0.65±0.08
LDH-O activity	0.07±0.009 a	0.005±0.0007 b	0.07±0.009 a	LDH-O activity	0.09±0.01 a	0.07±0.01 a	0.03±0.007 b
LDH-A mRNA	1.00±0.16 a	1.20±0.14 ab	1.73±0.26 b	LDH-A mRNA	1.00±0.12	0.72±0.10	0.90±0.09
LDH-B mRNA	1.00±0.14 ab	0.78±0.07 a	1.13±0.09 b	LDH-B mRNA	1.00±0.12 a	0.62±0.09 b	0.73±0.07 b
MCT-1 mRNA	1.00±0.17 a	2.52±0.34 b	3.03±0.26 b	MCT-1 mRNA	1.00±0.07 a	1.75±0.18 b	0.76±0.14 a
MCT-2 mRNA	1.00±0.10 ab	0.85±0.04 a	1.18±0.09 b	MCT-2 mRNA	1.00±0.12 a	0.47±0.05 b	0.67±0.10 b
in vitro incubation	2 mM Glu	4 mM Glu	8 mM Glu	*in vitro incubation*	2 mM Glu	4 mM Glu	8 mM Glu
LDH-R activity	18.23±0.86 a	13.94±2.34 b	10.25±1.19 b	LDH-R activity	9.40±0.59	8.49±0.98	8.86±0.56
LDH-O activity	0.43±0.11	0.55±0.12	0.48±0.02	LDH-O activity	0.13±0.03	0.14±0.03	0.19±0.06
LDH-A mRNA	1.00±0.17	1.27±0.06	1.40±0.15	LDH-A mRNA	1.00±0.17 a	1.27±0.08 a	2.43±0.26 b
LDH-B mRNA	1.00±0.10	0.82±0.11	0.71±0.13	LDH-B mRNA	1.00±0.14	0.99±0.14	0.97±0.10
MCT-1 mRNA	1.00±0.10 a	0.43±0.11 b	0.37±0.005 b	MCT-1 mRNA	1.00±0.18	1.07±0.17	1.33±0.16
MCT-2 mRNA	1.00±0.15	1.03±0.12	0.76±0.09	MCT-2 mRNA	1.00±0.24 a	1.14±0.11 a	1.73±0.22 b

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ORGANOTIN ENDOCRINE DISRUPTORS ACT AS OBESOGENS IN RAINBOW TROUT CULTURED ADIPOCYTES

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In the present study, the effects of two organotin endocrine disruptors (TBT and TPT) were analyzed in rainbow trout cultured adipocytes. Results obtained by oil red O staining indicated that TBT and TPT induce only slightly lipid accumulation compared with the differentiation medium in the absence of lipid mixture; but when present, lipid accumulation was significantly higher with and without organotins. Moreover, triglyceride content measurement revealed that TPT produces stronger lipid accumulation than TBT, although the highest accumulation of triglycerides was observed when adding lipid mixture. Furthermore, results showed that TBT and TPT activate PPARγ and CEBPα protein expression; however, the stronger effect was observed again when lipid mixture was added to the differentiation medium. Overall, the activation of PPARγ and CEBPα, together with the increase of triglyceride levels inside the cells confirmed that TBT and TPT enhance adipocyte differentiation and act as potential obesogens in rainbow trout. Moreover, our results highlighted that lipid mixture is essential to achieve complete cell differentiation in cultured adipocytes from this species.

Introduction

In recent years, several environmental pollutants have been identified to act as obesogenic compounds affecting endocrine signalling and lipid homeostasis. Among them, organotins such as tributyltin (TBT) and triphenyltin (TPT) have been the most widely studied in mammalian species and aquatic environments. *In vitro* and *in vivo* studies have demonstrated that TBT and TPT promote adipogenesis through peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptor (RXR) activation. The aim of the present study was to investigate *in vitro* the effects of TBT and TPT on the development and lipid metabolism of rainbow trout (*Onchorynchus mykiss*) primary cultured adipocytes.

Materials and Methods

1. Cell culture and treatments. Independent primary cultures of adipocyte cells were performed following the method previously described (1). After counting, cells were seeded to a final density of $0{,}2\text{-}0{,}26 \cdot 10^3$ cells/cm^2 in both pretreated six-well or twelve-well plates and kept at 18°C. At day 7, cells were incubated using adipogenic media (10 µg/ml insulin, 0,5 mM 3-isobutyl-1-methylxanthine (IBMX) and 0,25 µM dexamethasone) with the following treatments: 1. Control, 2. TBT (100 nM), 3. TPT (100 nM), 4. lipid mixture (5 µl/ml - containing cholesterol, cod liver oil FA, polyoxyethylene sorbitan monooleate and D-α-tocopherol acetate), 5. TBT+lipid mixture and 6. TPT+lipid mixture.

2. Lipid staining assay (oil red O) and quantitative determination of triglycerides. Oil red O (ORO) assay was performed after 72 hours of incubation as previously reported (2). Additionally, after 72 hours of incubation, total triglyceride

extraction was done as described elsewhere (3). Following the extraction, triglyceride content was determined using a triglyceride assay kit (Spinreact, Spain) and total protein was evaluated according to the Bradford method.

3. Immunofluorescence. Immunofluorescence assay was performed based on the protocol previously reported (4) with minor modifications. After 72 hours incubation, cells were fixed and incubated with primary antibodies PPARγ (1:50) or C/EBP (1:100) during 1 h at room temperature and labeled with the secondary antibody Alexa Fluor® 488-conjugated goat anti-rabbit antibody (1:500), in combination with Hoechst (1:1000).

4. qPCR. Total RNA was extracted using the triReagent according to the manufacturer's protocol. cDNA and qPCR assays were performed as described previously (5).

Results and Discussion

Total triglyceride quantification and ORO staining results revealed that TBT and especially TPT induce an increase of triglyceride accumulation in cells, indicating that these compounds have obesogenic effects in rainbow trout adipocytes in culture. Nevertheless, treatments with lipid mixture, produced a stronger obesogenic effects, increasing triglyceride content; compared to TBT and TPT single treatments (Fig. 1). Furthermore, the immunofluorescence analyses showed that TBT and TPT enhance PPARγ and C/EBPα protein expression (data not shown). However, again, treatments with lipid mixture presented higher both PPARγ and C/EBPα protein expression compared to TBT and TPT single treatments. These results suggest that addition of lipid into the media is essential to achieve complete cell differentiation and lipid droplet formation in rainbow trout adipocytes in culture.

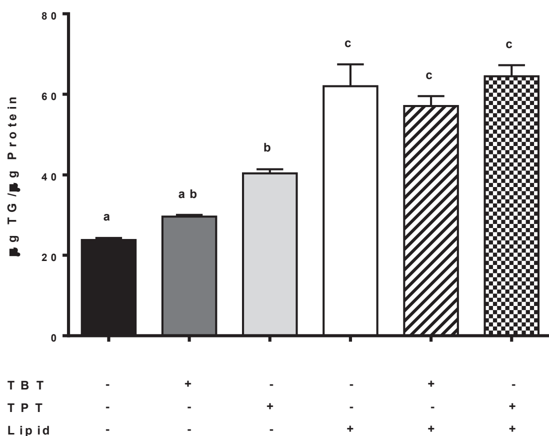

Figure 1. Triglyceride accumulation of rainbow trout cultured adipocytes incubated with TBT, TPT, lipid mixture and the combination of TBT or TPT with lipid mixture during 72h (day 10). Data are shown as mean values ± SEM (n=3). Significant differences are shown as different letters (P<0.05).

Gene expression results showed that TBT and TPT produce an increase of FAS as previously described in trout liver cells RTL-W1 (6). The addition of lipid mixture also down-regulates PPARβ gene expression, whereas TBT and TPT show no effect.

Moreover, TBT and TPT together with lipid mixture synergistically enhanced C/EBPα gene expression (Fig. 2). These data suggest that TBT and TPT appear to induce adipocyte differentiation by increasing the expression of adipogenic genes as described in mammals. Overall, the current findings demonstrate the effects of TBT and TPT on fish adipogenesis by the increase in gene and protein expression and lipid accumulation, contributing to improve our knowledge on the role of organotins, as obesogens, in rainbow trout adipocyte metabolism.

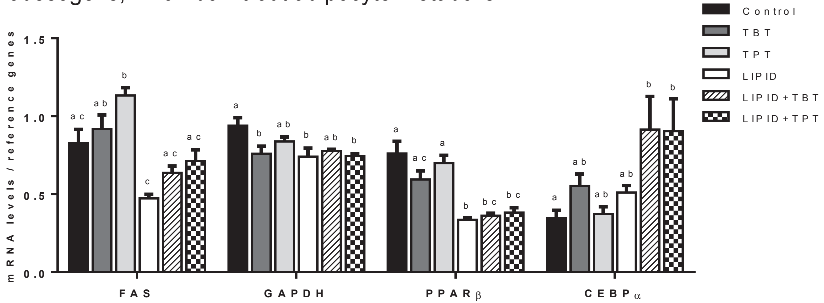

Figure 2. Relative gene expression of lipid metabolism-related genes in rainbow trout cultured adipocytes incubated with TBT, TPT, lipid mixture and the combination of TBT or TPT with lipid mixture during 24h (day 8). Data are shown as mean values ± SEM (n=5-6). Significant differences are shown as different letters (P<0.05). Abbreviations. FAS, fatty acid synthase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; PPARβ, peroxisome proliferator-activated receptor beta; CEBPα, CCAAT/enhancer-binding protein alpha.

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EFFECTS ON FEEDING RAINBOW TROUT WITH A LIPID-ENRICHED DIET ON FATTY ACID SENSING, FOOD INTAKE REGULATION AND CELLULAR SIGNALING PATHWAYS IN HYPOTHALAMUS AND LIVER

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In previous studies carried out in rainbow trout, we have characterized in hypothalamus and liver the presence and functioning of fatty acid-sensing systems whose activation ultimately lead to decreased food intake. Since a reduced food intake has been observed after feeding fish with lipid-enriched diets, changes in fatty acid-sensing systems are expected in fish fed with diets containing different lipid levels that have not been assessed yet. There is evidence in rainbow trout for the presence and functioning of AMPK and proteins involved in cellular signaling like mTOR and Akt but to date there is no information in any fish tissue regarding the response of these sensors and proteins to changes in the levels of nutrients like fatty acids. The aim of this study in rainbow trout fed with low fat or high-fat diets was to determine if the response of food intake, mRNA abundance of hypothalamic neuropeptides, and fatty acid sensing systems in hypothalamus and liver is similar to that previously observed when levels of specific fatty acid were raised by injection and to determine if the phosphorylation state of AMPK, Akt and mTOR display changes in hypothalamus and liver in response to changes in dietary lipid levels.

Introduction

A reduced food intake has been observed after feeding fish like rainbow trout with lipid-enriched diets (1). Therefore, changes in fatty acid (FA) sensing systems are expected in fish fed with diets containing different lipid levels that have not been assessed yet. In fish, there is evidence in rainbow trout for the presence and functioning of AMPK in liver and muscle (2,3) but to date there is no information in any fish tissue regarding the response of AMPK to changes in the levels of nutrients like fatty acids. Furthermore, proteins involved in cellular signaling like target of rapamycin (mTOR) and protein kinase B (Akt) have been also suggested to be involved in the nutritional regulation of carbohydrate and lipid metabolism in fish. However, there are no available studies in fish assessing the response of these proteins to changes in levels of circulating nutrients like FA. The aim of this study in rainbow trout fed with low fat (LF) or high-fat (HF) diets was 1) to determine if the response of food intake, mRNA abundance of hypothalamic neuropeptides involved in the metabolic regulation of food intake, and FA sensing systems in hypothalamus and liver is similar to that previously observed when levels of specific FA were raised by injection; and 2) to determine if the phosphorylation state of intracellular energy sensors (AMPK), and proteins involved in cellular signalling (Akt and mTOR) display changes in hypothalamus and liver in response to changes in dietary lipid levels that could be related to changes observed in parameters related to FA-sensing and the control of food intake.

Materials and Methods

Two fish meal based diets were formulated to be isonitrogenous, but to contain two different levels of crude lipid. LF diet contained 1.6 % oil blend, whereas HF diet

contained 16% oil blend. Each of the two experimental diets was fed by hand (twice per day) to visual satiation to five replicate groups of fish for 4 weeks and food intake was assessed every day. After 4 weeks of feeding the experimental diets, 6 fish per diet were sampled from different tanks 1, 3 and 6 h after the meal to assess changes in the levels of proteins involved in cellular signalling. In a second set, 15 fish per diet were sampled from different tanks 6h after the meal to assess changes in mRNA abundance (6 fish per diet) and metabolite levels (9 fish per diet). We used 6h in this second set because changes in gene expression are expected at the same time or later than those of cell signalling.

Results and Discussion

Changes in cumulative feed intake did out display significant differences between diets (data not shown).

Free FA levels in plasma, hypothalamus, and liver increased in the group of fish fed with HF diet compared with the group fed with LF diet. Triglyceride levels increased in plasma of fish fed with the HF diet but there were no significant differences in hypothalamus and liver (data not shown).

In hypothalamus, the ratios for Akt increased 6h after the meal in fish fed with the HF diet compared with fish fed LF. The ratios of AMPK in fish fed the HF diet decreased 3h after the meal and increased 6h after the meal compared with fish fed the LF diet whereas in fish fed the HF diet the ratio observed 3h after the meal was lower than that observed after 1 or 6h. Finally, the value of mTOR increased 3h after the meal in the fish fed the HF diet compared with fish fed the LF diet. No differences with time were noted for Akt and mTOR whereas for AMPK values in fish fed HF diet were lower 3h after the meal compared with those observed after 1 and 6h. In liver, the ratio for Akt was higher 6h after the meal in the fish fed with the HF diet. No significant changes were noted between groups for the ratio P-AMPK/AMPK. The ratio P-mTOR/mTOR increased 3 and 6h after the meal in fish fed with HF diet compared with fish fed the LF diet whereas values in fish fed the LF diet were higher 1h after the meal than after 3 or 6h (data not shown).

Changes in mRNA abundance of transcripts assessed in hypothalamus and liver 6h after the last meal, are shown in Table 1. In hypothalamus, values of FAT/CD36, CPT1c, LXRα, PPARα, SREBP1c, CART, NPY and POMC-A1 were higher in the group fed with the HF diet than in the group fed with the LF diet. No significant changes were noted for mRNA abundance of ACC, ACLY, FAS, HOAD, UCP2a, Kir6.x-like, AMPKα1 and AgRP. In liver, ACLY mRNA abundance in the group fed with the HF diet was lower than the group fed with the LF diet whereas ACC, CPT1a, HOAD, UCP2a, PPARα, and AMPKα1 mRNA levels were higher in the group fed with HF diet than in the group fed with LF. No significant changes were noted for levels of FAT/CD36, FAS, Kir6.x-like, LXRα, and SREBP1c.

In conclusion, the FA-sensing systems characterized in rainbow trout whose activation in response to increased levels of oleate or octanoate has been found to result in decreased food intake did not respond in the same way when fish were fed for 4 weeks with an lipid-enriched diet. The increased levels of FA in hypothalamus and liver of rainbow trout fed the HF diet only partially activated FA-sensing systems, suggesting that FA-sensing response in fish to increased levels of FA is more dependent on the presence of specific FA. AMPK , mTOR and Akt in hypothalamus and liver were generally activated in fish fed the HF vs LF diet suggesting the activation of these cellular signaling pathways in response to the increased availability of FA.

	Hypothalamus		Liver	
	LF	HF	LF	HF
FAT/CD36	1.00±0.10	2.20±0.22*	1.00±0.10	0.79±0.09
ACC	1.00±0.07	0.85±0.09	1.00±0.09	1.41±0.09*
ACLY	1.00±0.10	1.19±0.09	1.00±0.09	0.40±0.02*
CPT-1c	1.00±0.08	1.82±0.24*	-	-
CPT-1a	-	-	1.00±0.22	3.59±0.55*
FAS	1.00±0.08	0.86±0.06	1.00±0.09	0.90±0.02
HOAD	1.00±0.19	1.24±0.20	1.00±0.14	1.43±0.21*
UCP2a	1.00±0.06	0.8±0.09	1.00±0.07	1.56±0.20*
Kir6.x-like	1.00±0.05	0.91±0.09	1.00±0.08	1.11±0.09
LXRα	1.00±0.11	1.83±0.18*	1.00±0.13	1.32±0.10
PPARα	1.00±0.10	1.44±0.14*	1.00±0.11	1.69±0.24*
SREBP1c	1.00±0.09	1.66±0.23*	1.00±0.09	1.32±0.21
AMPK-α1	1.00±0.14	1.37±0.12	1.00±0.08	1.50±0.07*
AgRP	1.00±0.10	0.76±0.08	-	-
CART	1.00±0.08	1.97±0.27*	-	-
NPY	1.00±0.19	1.96±0.28*	-	-
POMC-A1	1.00±0.08	2.43±0.44*	-	-

Table 1 mRNA levels in hypothalamus and liver of rainbow trout 6h after the last meal with the LF and HF diet.
*, Significantly different ($P<0.05$) from fish fed with the LF diet.

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REGULATION OF ZEBRAFISH GAMETOGENESIS: A TRANSGENIC APPROACH

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Regulation of pubertal timing remains unresolved in most species including humans. However the first sexual maturation in *Xiphophorus* is regulated by the *locus P, locus* occupied by multiple copies of the melanocortin receptor 4 (MC4R). To demonstrate the involvement of melanocortins in the first sexual maturation, we generated a transgenic zebrafish line ovexpressing agouti-signaling protein (ASIP), an endogenous antagonist that competes with the melanocortin peptides by binding to MCRs. Our results demonstrated that ASIP males reach puberty earlier than wildtype fish. In addition, we demonstrate the presence of MC4R in ovary and testes by *in situ* hybridization (ISH). In conclusion, we demonstrated that the inhibition of melanocortin signaling by ASIP may modulate to the zebrafish gametogenesis.

Introduction

Early puberty affects growth, food efficiency conversion and market values adversely. Delayed puberty can also be a problem for the aquaculture by increasing costs and health risk to maintain stocks of fish. In Poecilid fish, the first sexual maturation is regulated by a unique sex-linked Mendilian locus named *locus P* (1). Recent experiments have demonstrated that *locus P* is filled by multiple copies of the melanocortin receptor 4 (MC4R) (2). Melanocortins are small peptides encoded by a complex precursor called proopiomelanocortin (POMC). These peptides include the melanocyte-stimulating hormones (MSHs) and the adrenocorticotropic hormone (ACTH). Five different receptors (MC1R-MC5R) mediate their physiological actions. Melanocortin signaling is regulated also by endogenous antagonists, agouti-signaling protein (ASIP) and agouti-related protein (AGRP) that compete with the melanocortin peptides by binding to MCRs. AGRP is mainly expressed in the hypothalamus and works as an inverse agonist and/or competitive antagonist at MC4R (3). ASIP is mainly expressed in the ventral skin and binds MC1R to inhibit ventral melanogenesis but also binds MC4R (4). We have developed a new transgenic zebrafish line ovexpressing ASIP. This fish line exhibits profuse alteration of the dorso-ventral pigment pattern (4). In addition, transgenic zebrafish growth longer, heavier and faster (5). The present work evaluated the puberty onset in zebrafish using same-aged embryos from different ASIP and wild type breeding mates.

Materials and Methods

Wild type TU and transgenic ASIP strains were maintained in the same experimental conditions and fed with the same quantity of food. During the experiment, fish were sampled at 30-34-38-42-46-60-75 days post fertilization (dpf). Samples from 50 over-anaesthetized live animals/sampling time were fixed with 1% glutaraldehyde and embedded in methacrylate resin**.** Sections at 2 µm were cut and stained with toluidine blue for morphological analysis of the gonads (6). For determinate the zebrafish pubertal process, samples were classified according to the testicle (7) or ovarian (8) development stages.

The full coding region of the zebrafish MC4R gen was obtained from public databases and subsequently cloned in pGEM-T easy vector (Promega)(9). Nonisotopic riboprobe for full-length zfMC4R was synthesized using a digoxigenin RNA labeling mix (Roche Diagnostics), according to the manufacturer's instructions. Testicles and ovaries from adult zebrafish were fixed with 4% paraformaldehyde. The *in situ* hybridization (ISH) procedure was according to the methodology described by (9).

Results and Discussion

Following previous studies (10), four stages of testicular development were identified: 1. Immature (cysts are formed by type A undifferentiated and A differentiated spermatogonia), 2. Early maturing (cysts become filled with B spermatogonia), 3. Mid-maturing (mainly cysts containing primary and secondary spermatocites) and 4. Maturing (all types of cells can be recognized, predominantly cysts filled with spermatids and sperm). The first testicular structures were identified at 30 dpf in transgenics fish but wild type counter side reached this state at 34 dpf (Fig.1). In both cases cysts contained spermatogonia type B. The presence of this cell type in the cysts is considered as the beginning of the testicular maturation. Therefore, maturing stage started at 34 dpf in the transgenics 38 dpf in the wild type fishes. Subsequently the proportion of cysts in stage 4 increased in both strains however transgenic fish exhibited cysts containing mostly sperm at 46 dpf. On the contrary, trasngenic fish were fully mature at 60 dpf.

In relation to females six stages of ovarian development were recognized, based from (8): 1. Primary growth (perinucleolar oocyte), 2. Previtellogenic (cortical alveolar stage), 3. Early vitellogenic (initiation of exogenous vitellogenesis), 4. Mid vitellogenic, 5. Late vitellogenic and 6. Full grown (completion of oocyte growth). At 30 dpf ovarian structures were seen in fish from both strains (data not shown). Previtellogenic stage started at 34 dpf in the wild type and 38 dpf in the transgenics fishes. Preliminary studies suggest that transgenic female puberty is delayed compared with wild type strain. We further demonstrate the expression of MC4R gene in previtellogenic follicles and spermatocytes by *in situ* hybridization (ISH), suggesting that melanocortin systems is involved in the gonad development.

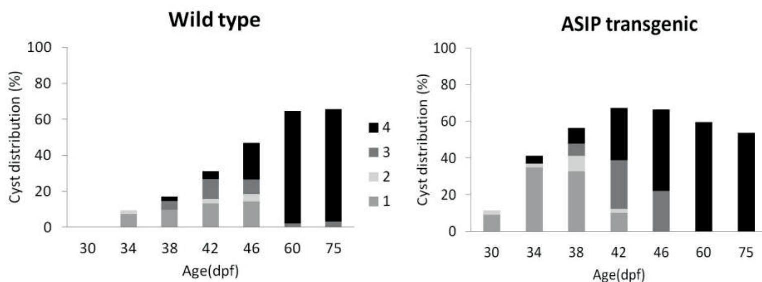

Figure 1. Testicle development stages. 1: immature, 2: early maturing, 3: mid-maturing, 4: maturing.

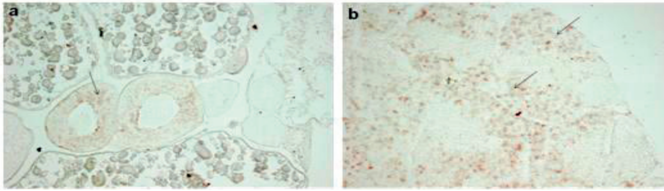

Figure 2. MC4R expression by ISH in ovary (a) and testes (b) at 10x magnification.

Acknowledgments

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MELANOCORTIN RECEPTOR ACCESORY PROTEINS (MRAPs) PARTICIPATION IN THE FISH MELANOCORTIN SYSTEM

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Our previous studies have demonstrated the presence of three melanocortin receptor accessory proteins (MRAPs) in the zebrafish genome (MRAP1, MRPA2a and MRAP2b) and function of MRAP2s is unknown. Here, we demonstrate that both MRAP2s are coexpressed with MC4R in same neurons. Immunoprecipitation studies show also a physical interaction between MC4R and MRAP2s that is able to change pharmacological properties of the receptor. In addition, we have demonstrated that intraperitoneal $ACTH_{1-24}$ reduces food intake levels via MC4R. Using CRISPR/Cas9 system we have successfully deleted MRAP2a from zebrafish genome and we have refused the involvement of MRAP2a in the larval growth as previously suggested.

Introduction

Melanocortin system is composed of hormones derived from proopiomelanocortin precursor (POMC), including adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormones (MSHs), melanocortin receptors (MC1R-MC5R), and endogenous antagonists, i.e. agouti-signaling protein (ASIP) and agouti-related protein (AgRP) (1). Recently, a new member of melanocortin system has been added, the melanocortin receptor accessory protein (MRAP) (2). The main role of MRAP is to traffic the MC2R to the plasma membrane thus allowing its functional expression (2). Once on the membrane, MC2R is able to respond to the extracellular ACTH by triggering second messenger pathway.

Our studies have reported the presence of MRAP1 paralogues in the fish genome as it happens in tetrapod species. Therefore, zebrafish genome contains two related genes named MRAP2a and MRAP2b. The objective of this work was to characterize the MRAP2 involvement in the melanocortinic response in zebrafish. We demonstrate that both MRAP2a and b are mainly expressed in the brain and colocalize with MC4R. MRAP2a enables MC4R response to ACTH whereas MRAP2b decrease MC4R constitutive activity. The ACTH administration inhibits food intake but a functional MC4R is required.

Materials and Methods

Real-time quantitative (qPCR) was carried out according to "MIQE protocol" (3). Double *in situ* hybridization (ISH) were carried out according to the methodology described previously (4). For pharmacological experiments, transient transfections were performed in HEK-293 cells using Lipofectamine LTX (Life Technologies). The methodology used to quantify the response of the receptor to stimulation with α-MSH and ACTH is based on the colorimetric assay described by Chen *et al*., (5) and modified by Sánchez *et al,.* (6). Co-immunoprecipitation and immunofluorescence experiments were performed using MCRs and MRAPs transiently transfected in HEK-293 cells and subsequently detected using specific antibodies. For immunofluorescence, cells were examined with a confocal laser scanning

microscope (Olympus FV1000). For the food intake experiment, $MC4R^{-/-}$, mutant *sa0122* strain on Tupfel long fin background was used. Fish obtained from the Zebrafish Mutation Project (Sanger Institute). Fish ($MC4R^{+/+}$ and $MC4R^{-/-}$) were randomly housed in aquariums and food consumption was recorded daily for 4 consecutive days. On the fifth day, 20 fish of each genotype (n= 10 per treatment) were injected intraperitoneally with saline (control) or 10 µg of human $ACTH_{1-24}$ (Bachem) and after 15 minutes post injection, the fish were fed. The food intake levels were calculated as the percentage of the average of the 4 previous days. Production of fish defective of MRAP2a ($Mrap2a^{-/-}$) gene was carried out following the methodology described by (7). Guide RNA was performed using a free program (https://chopchop.rc.fas.harvard.edu).

Results and Discussion

The expression of MC4R and MRAP2 studied by qPCR were observed in brain (data not shown). Double ISH demonstrated MRAP2a/MC4R co-localization in same neurons of the parvocellular preoptic nucleus, anterior part. On the contrary, MRA2b and MC4R were co-localized in neurons of the lateral tuberal nucleus. Both areas have been considered as homologous to the NPV and arcuate nucleus of tetrapods, respectively (8). These areas have been involved in the control of energy homeosthasis in mammalian species. Accordingly, immunoprecipitation studies showed that both MRAP2s physically interact with the MC4R (data no show). Pharmacological experiments demonstrated that co-transfection of MRAP2a/MC4R transforms zebrafish MC4R, a canonical MSH receptor, into an ACTH receptor whereas MRAP2b/MC4R interaction results in a reduction of the constitutive activity of the receptor (Fig. 1A and 1B). Zebrafish $MC4R^{+/+}$ injected with ACTH show a decrease in food intake levels, but this inhibitory effect was absent in $MC4R^{-/-}$ fish (Fig. 2).Using CRISPR/Cas9, we have develop a MRAP2a knockout zebrafish line. Ongoing experiments have demonstrate that MRAP2a is not involved in the control of developmental growth (Fig 3), as previously reported using morpholino knockdown (9). We are currently studying the effects of MRAP2a in the control of food intake and growth of adult zebrafish.

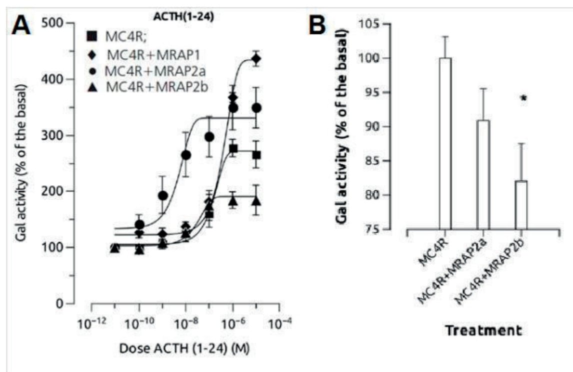

Figure 1. Pharmacologic properties of melanocortin agonist $ACTH_{1-24}$ at HEK-293 transiently expressing MC4R and different MRAPs (A). Effects of MRAP2a and MRAP2b on MC4R-induced galactosidase basal activity in HEK-293 cells stably expressing MC4R but transiently expressing MRAP2s (B) (Data are mean ± SEM of the 3 independent experiments; * show significant differences after Student's t test ($P < 0.05$). Adapted from Agulleiro et al., 2013.

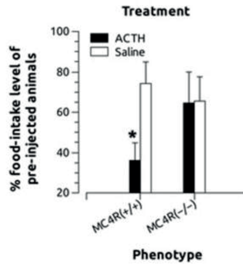

Figure 2. Effects of $ACTH_{1-24}$ on zebrafish sa122 food intake levels. Data are mean ± SEM; * show significant differences after one-way ANOVA and Tukey's method (P< 0.05). Adapted from Agulleiro et al., 2013.

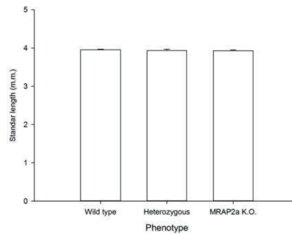

Figure 3. Standard length (mm) of larvae at 5 days post fertilization. No significant differences were observed (n = 96).

Acknowledgments

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POSSIBLE ROLE OF CENTRAL MELANOCORTIN SYSTEM IN THE CONTROL OF CIRCADIAN LOCOMOTOR ACTIVITY RHYTHMS

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The melanocortin system integrates peptides derived from proopiomelanocortin that show melanocortinic and/or corticotropic activity. Endogenous melanocortin antagonists, agouti-signaling protein (ASIP) and agouti-related protein (AGRP) compete with melanocortin peptide for binding to five different melanocortin receptors (MC1R-MC5R). In zebrafish, AGRP and MC5R are duplicated. AGRP1 is specifically expressed in the hypothalamus but AGRP2 is expressed in the pineal. AGPR2 modulates the expression of the melanin-concentrating hormone (MCH) that is involved in the background adaptation. The expression of AGRP2 in the zebrafish pineal gland suggests the involvement of the melanocortin system in the regulation of circadian rhythms. We use a zebrafish transgenic strain overexpressing ASIP (Ag zebrafish) to study the role of melanocortin antagonist in the circadian structure of fish. We demonstrate that both diurnal and nocturnal melatonin levels in Ag zebrafish are statistically similar. Accordingly, comparison of brain transcriptome between Ag and wild type (WT) zebrafish revealed a significant enrichment of tryptophan and circadian rhythms go terms. Finally, the analysis of daily locomotor activity exhibited significant differences between Ag and WT fish further supporting a role for melanocortin system in the activity rhythms of zebrafish.

Introduction

The melanocyte-stimulating hormones (MSHs) and adrenocorticotropic hormone (ACTH) are the main melanocortin peptides. All of them are encoded in the proopiomelanocortin (POMC) precursor, which is expressed mainly in the pituitary. Five receptors (MC1R-MC5R) mediate melanocortin signaling but two endogenous antagonists, agouti-signaling protein (ASIP) and agouti-related protein (AGRP) compete with melanocortin peptides by binding to MCRs. ASIP is mainly produced in the ventral skin and it is a potent melanocortin antagonist at MC1R and MC4R. In contrast, AGRP is mainly produced in the brain and it is potent in inhibiting signaling at MC3R and MC4R (Cerdá-Reverter et al., 2011; Cortés et al., 2014).

The structure of the melanocortin system in fish diverges as the genome of the teleost antecessor doubled once more (3R). Zebrafish genome has two POMC, AGRP paralogue genes (AGRP1 and AGRP2), a single copy of ASIP gene and six different MCRs since MC5R duplicated. AGRP1 is mainly expressed in the tuberal hypothalamus ans its involved in the control of linear growth and energy balance via binding to MC4R (Guillot et al., in press). On the contrary AGRP2 in mainly expressed in the pineal and its involved in the background adaptation by modulating melanin-concentrating hormone (MCH) expression. Pharmacological profile demonstrate that AGRP2 is a competitive antagonist at MC1R and MC4R both expressed at the zebrafish hypothalamus Pineal expression suggest that AGRP2 may be involved in the control of circadian rhythms In recent experiments, we

generated a transgenic zebrafish strain over-expressing goldfish ASIP that also potently antagonizes MSH effects on MC1R/MC4R (Ceinos et la., 2015). Therefore this zebrafish strain supposes an excellent model for the study of melanocortin involvement in the regulation of circadian activity. In this paper, we demonstrate ASIP fish exhibit a different locomotor circadian pattern than WT fish. Trascriptomic data reveal an enrichment of gene ontology (GO) terms related to circadian rhythms but also ASIP fish exhibit no increase in nocturnal melatonin levels as regularly occurs in WT fish.

Methods

To evaluate nocturnal/diurnal melatonin levels, forty WT (n=20) or ASIP (n=20) adult zebrafish were placed separately in two 20-liter tanks under a 14h light/10h dark cycle for 15 days. After the acclimation period, brains from 5 females and 5 males from each genotype were sampled at 2.00 h (n=10) and 14.00 h (n=10). Central melatonin levels were then assayed by HPLC according to Muñoz et al. (2009).

Microarray analysis description, full transcriptome comparison as well as qPCR validation were previously published (Guillot et al., 2016).

To evaluate circadian activity patterns, female/male ASIP or WT fish (n=4/tank) were placed into four six-liter aquaria provided with four infrared sensors (860nm) set 2cm under the water surface in the opposite corners and over tank bottom. Sensors connected to a computer system that recorded the date, the time and the probe from which each locomotor activity was originated Data were collected using AUTOMATA software. Sensor detection range was between 5 and 100 mm. ANOVA test multiple dependent variable (MANOVA) will be used for statistical analysis. Differences were considered at $p<0.05$.

Results and Discussion

WT zebrafish exhibited higher melatonin levels at night than during the photophase. No statistical differences were found when comparing diurnal and nocturnal melatonin levels in ASIP zebrafish (Fig. 1). Transcriptome comparisons reveled a significant enrichment of the tryptophan metabolism (*aanat1*, *acat2*, *aldh2*, *cyp1a*, *ddc*) and the proper circadian rhythm GO terms (*aanat1*, *clock3*, *ddc*, *homer1b*, *nr1d2a*, per3, *5ht-7*) only in males. Additionally, the hidroxindol O-metiltransferasa (HIOMT), last enzyme in the synthetic pathway of melatonin, was dowregulated in transgenic fish (data not shown) thus supporting the observed reduced levels of central melatonin in ASIP fish.

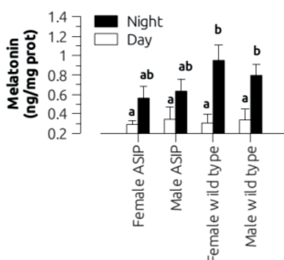

Figure 1. Brain melatonin levels of male and female ASIP and WT fish sampled during the light (14.00) and dark phase (2.00) of the photoperiod. Adapted from Guillot et al., 2016.

All fish, independently of genotype, were mainly active during the light phase of the photoperiod supporting the existence of a circadian locomotor activity pattern with the acrophase during the light phase of the photoperiod. ASIP males were more active than both WT males and females during the whole daily cycle. During the light phase WT females were more active than males but this pattern was inverted in ASIP fish. More interestingly, transgenic fish exhibited always significant enhanced locomotor activity during the night (Fig.2). The enhanced activity during the dark phase in ASIP males is in well agreement with reduced nocturnal melatonin levels, reduced HIOMT central expression and transcriptomic enrichment of Go terms related to circadian rhythms in transgenic males. In summary, data suggests that the involvement of melanocortin in the regulation of circadian activity and/or sleep by modulating central melatonin synthesis in zebrafish.

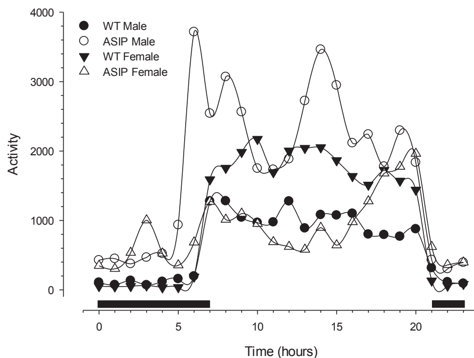

Figure 2. Circadian locomotor activity patterns of ASIP and WT zebrafish. Black bar shows dark phase of the photoperiod.

Acknowledgments

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THE INVOLVEMENT OF GALANIN IN THE REPRODUCTIVE CYCLE OF SEA BASS (*DICENTRARCHUS LABRAX*)

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This study was conducted to assess the putative involvement of galanin in the regulation of the reproductive cycle of sea bass, an economically important aquaculture species. Cultivated sea bass males present high rates of precocious puberty which takes to economical losses. Thus, the identification of putative regulators of the onset of puberty in sea bass would be highly important. Galanin (GAL) is a hypothalamic neuropeptide with orexigenic properties and a potential candidate to be involved in modulating the neuroendocrine control of puberty. The current work has evaluated GAL receptors expression in pituitary and the effects of GAL on FSH and LH release by cultivated pituitary cells from male sea bass at different stages of the reproductive cycle. Results showed that GAL receptors are expressed in the pituitary and that pituitary cells respond to GAL by increasing FSH and LH release. Furthermore, FSH and LH release depends on the reproductive stage of fish. These results support the involvement of galanin in the regulation of male sea bass reproductive cycle.

Introduction

In commercial aquaculture systems, male sea bass (*Dicentrarchus labrax*) consistently presents high rates of precocious puberty, a problem that negatively affects productivity [1]; thus, the identification and characterization of puberty regulators in sea bass could be highly beneficial to commercial aquaculture systems. In fish, the onset of puberty seems to depend on genetic, physiological and environmental factors [1]. The acquisition of reproductive capacity requires the activation of the hypothalamus-pituitary-gonadal axis; gonadotropin-releasing hormones (GnRH) secreted by hypothalamic neurons stimulate the pituitary secretion of gonadotropins, which stimulate the production of sex steroids and supports gametogenesis in the gonads [2]. Pulsatile GnRH secretion at the onset of puberty requires the occurrence of a series of neuroendocrine events which combine genetic, internal and environmental signals that influence the timing of puberty [1]. Galanin (GAL) is a multi-functional neuropeptide widely expressed in the central and peripheral nervous system [3], which seems to mediate multiple physiological processes in vertebrates such as nociception, feeding and reproduction [4]. However, studies on the involvement of GAL in fish reproduction are still limited. The main objective of this work was to evaluate the putative involvement of GAL in the reproductive cycle of male sea bass, by assessing the *in vitro* effects of GAL on pituitary cells and the expression of GAL receptors (GalRs) at different stages of the reproductive cycle.

Materials and Methods

Male European sea bass were obtained from AtlantikFish (Castro Marim; Portugal) and maintained at the Ramalhete experimental station (Faro-south of Portugal) in 1000L flow-through tanks under natural photoperiod and temperature conditions and fed with commercial pellets. The stage of testicular development (STD) was based on the time of the year and confirmed by histology. Pituitary cell cultures were

performed with young fish (2 years old) at different reproductive stages: prepubertal (September-STD I); pubertal (November-STD I to V) and early postpubertal (January-STD V). Animals were sacrificed and pituitaries quickly extracted and placed in ice-cold dispersion medium L-15 with Hanks Balanced Salt Solution (Sigma-Aldrich, Portugal), 25 mM Hepes, 0.5% BSA, 1% Penicillin-Streptomycin, 0.1 $mgmL^{-1}$Gentamicin pH=7.4. All pituitaries (n=5) were washed with dispersion medium at RT and sliced into 1 mm^3 fragments [5]. Pituitary cells were dispersed using a trypsin digestion method according to Cerdá-Reverter *et al.* [6]. Following enzymatic treatment, fragments were vigorously pipetted, favoring mechanical dispersion. Dispersed cells were filtered through a filter with a pore size of 100 µm. Cells were harvested by centrifugation at 500 x g for 15 min and resuspended in 5 ml of dispersion medium containing 0.1% BSA. Dispersed pituitary cells were cultured in 96-well culture plates (Sarstedt -Portugal) at a density of 2.5 x 10^5 cells/ml. Cells were precultured overnight in L-15 medium containing 10% FBS at 20ºC prior to treatments. Cells were incubated with rat/ mouse Galanin (1-29) (Tocris) at 1pM, 100pM and 10nM. 10nM of Luteinizing Hormone Releasing Hormone analogue (LHRHa) (Sigma-Aldrich), was used as positive control. After 1hour incubation the media were collected and stored at -80ºC until hormone analysis. Each treatment was repeated 6 times in different pituitary cell preparations (3 wells/treatment per experiment).

FSH release was analyzed by competitive ELISA for sea bass FSH; using a specific antiserum (AbFSHβ-2) and recombinant FSH heterodimer produced in a baculovirus expression system, as previously described [7]. LH release was analyzed by competitive ELISA for sea bass LH, using a specific antiserum (AbLHβ). Polystyrene ELISA 96-well microplates were coated with recombinant European sea bass LH heterodimer solution overnight at 4°C. Before distribution into the wells, standard and samples were preincubated with 100 µl AbLHβ overnight at 4°C. All standards, samples and the antiserum solution were diluted in PBST buffer containing 2% normal goat serum (NGS, Sigma–Aldrich). After preincubation, samples and standards were dispensed in duplicate into the coated wells and incubated for 24 h at 4°C. The non-specific binding wells and five LH coated wells (maximum binding, B0) received only AbLHβ solution. Both for FSH and LH assays the antigen–antibody complexes formed were detected by incubation with GAR-HRP, diluted in PBST- 2% NGS buffer for 1 h at 37°C. The presence of enzyme complexes was detected by the addition of TMB reagent (Bio-Rad). For data calculations, sigmoid curves were linearized using the logit transformation (logit (Bi/B0) = ln (Bi-NSB/B0-Bi)), where Bi represents the binding of each point, B0 is the maximum binding and NSB the non-specific binding. Data are presented as mean ± SEM. Differences between groups were determined by one-way ANOVA followed by Dunnett's multiple comparison test.

Transcript expression of the four described galanin receptors (GalRs) were determined by semi-quantitative real time RT-PCR [8] using pituitary cDNAs from fish at same testicular stages. The 18S ribosomal RNA was used as internal control.

Results and Discussion

Transcript expression analysis showed that Galanin receptor subtypes 1a, 1b, 2a and 2b are all expressed in the pituitaries of male sea bass at the three sampled reproductive periods (Fig. 1).

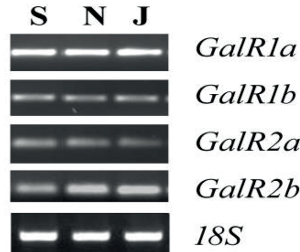

Figure 1. mRNA expression of galanin receptor (GalR) genes in pituitaries of 2-year old male sea bass collected at different periods of the reproductive cycle: September (STD-I), November (stages I to V) and January (STD-V), determined by RT-PCR (n=3-6 fish/sampling point). The 18S ribosomal RNA was used as internal control.

Pituitary cells from fish in STD I (September) responded to GAL (1pM) with a slight (but significant) increased of FSH release, not observed for higher GAL concentrations (Fig. 2). No effects of GAL were observed in LH release in September or November or FSH in November. In contrast, GAL incubation of male sea bass pituitary cells sampled in January (STD-V) induced a significant increase in FSH and LH release at all tested concentrations.

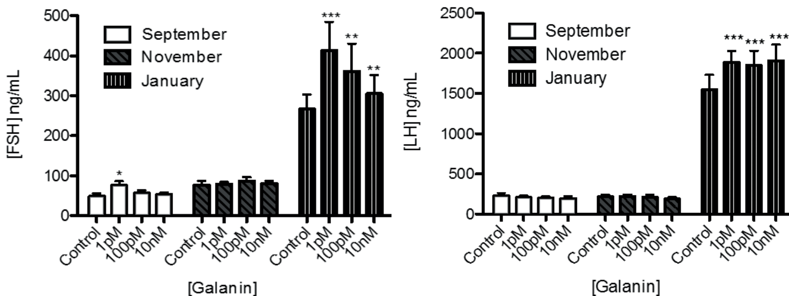

Figure 2. *In vitro* effects of rat/mouse 1-29 galanin in FSH (left) or LH (right) release from dispersed pituitary cells from 2 year-old male sea bass at different points of the first reproductive cycle. Each value is the mean ± SEM for n=6 independent experiments. *** , ** and * indicates significant differences compared to the control (p<0,0001, p<0,005 or p<0,05, respectively).

These results suggested the involvement of GAL in the regulation of the male sea bass reproductive cycle, as previously hypothesized [1], as it was shown to regulate the release of both FSH and LH at particular stages of the reproductive stage. These effects appear to be direct and may involve one or more GalR subtypes, whose transcript expression was demonstrated in pituitaries of all tested reproductive stages. Additional studies will be required to evaluate possible regulation of GalR

pituitary transcript levels across the reproductive cycle as well as to detail their localization in male sea bass pituitaries.

In summary, this study suggested that galanin, a neuropeptide involved in feeding in fish, also appears to be involved in the control of the reproductive cycle in male sea bass. Cultured pituitary cells from male sea bass responded to GAL by increasing FSH and LH released *in vitro*, a response which was dependent on the animal's reproductive stage. Future studies will be required to confirm these effects in male sea bass *in vivo*, at several points of the reproductive cycle, as well as in early pre-pubertal fish. This would permit to test the hypothesis that GAL could be a link between energy related signals and the control of puberty onset.

Acknowledgments

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SEASONAL SYNCHRONIZATION TO ENVIRONMENTAL CUES IN SALMONIDS: ARE PHOTOPERIOD AND TEMPERATURE ACTING THROUGH A COMMON PATHWAY?

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Global warming is currently impacting numerous processes in poikilotherms, including the time-keeping system, which controls metabolism, physiology and behavior. Indeed, while photoperiod remains constant, temperature is increasing, challenging the production of timed signals such as melatonin (MEL) in vertebrates. In fish, the duration of the nocturnal MEL secretion relies on photoperiod while temperature controls amplitude. Accordingly, the shape of the MEL oscillations, which allow fish to orientate in daily and calendar time, is likely to change if warming does not stop. The secretion of MEL is a Ca^{2+} dependent process that takes place in the pineal and retinal photoreceptor cells. While the mechanisms of its regulation by the light/dark cycle are quite well understood, those by temperature remain unelucidated. We recently reported that thermoreception might be mediated by transient receptor potential (TRP) channels in trout. Here we report on Atlantic salmon (*Salmo salar*), which life cycle is highly dependent on precise timed processes, the first of these being smoltification (*i.e.*, the timing of downstream migration from fresh to sea water). We quantified the expression of two receptors from the vanilloid subfamily (TRPV1 and TRPV4) in the retina, gills and pituitary of salmon in the course of smoltification. For this purpose fish were raised under different photoperiod and temperature conditions and sampled every two months from December to June. We found significant tissue-dependent effects of photoperiod and/or temperature on TRPVs' expression levels. The results open a new path in the investigation of the possible role of TRPV in fish thermoreception.

Introduction

The life cycle of Atlantic salmon (*Salmo salar*) involves two migratory processes: a first one downstream from the mother river to feeding zones in the sea, and a second one upstream to the original river for reproduction. The timing of these processes is triggered by a combination of photoperiod and temperature [1]. Downstream migration needs a set of adaptation processes, the smoltification (physiological adaptation towards sea water tolerance) [2], in the course of which fish display morphological and behavioral changes [1]. The timing of smoltification is being compromised by the temperature rise caused by the global climate change. In teleost, photoperiod and temperature dictate, respectively, the duration and amplitude of the nocturnal secretion of the time-keeping hormone, melatonin (MEL), in retinal and pineal photoreceptors. MEL is responsible for the synchronization of many biological events to the variations of the cyclic environment. The light/dark regulation of MEL secretion is a Ca^{2+} dependent process, but the mechanisms of temperature action remain unknown. We reported recently that Transient Receptor Potential Channels from the vanilloid family (TRPV1 and TRPV4) could mediate the effects of temperature on trout pineal MEL secretion [3] and proposed that light and temperature act through different mechanisms to modulate the calcium-dependent production of MEL by teleost photoreceptors cells. Here we provide information concerning abundance of TRPV1 and TRPV4 mRNA in the Atlantic salmon retina, gills and pituitary in the course of smoltification.

Materials and Methods

Pre-smolt Atlantic salmon (23.76 g ± 0.65) were raised at the *Conservatoire National du Saumon Sauvage* (CNSS, France). At time zero (2014, December 15th), animals were indoors in open circuit of river water under a photoperiod mimicking the natural one. From winter solstice, fish were distributed in four different tanks (100 fish/m^3): two tanks followed the natural (N) photoperiod and the other two were kept under winter (W) photoperiod. In each group one tank was supplied with water at natural temperature (named N or W) while the temperature of the other was elevated up to 5°C (named N+5 or W+5). Fish (n = 8) were sampled before, during and after the smoltification (as indicated below), and anesthetized with Eugenol (Sigma Aldrich). Retina, gills and pituitary glands were sampled for automated RNA extraction (Maxwell®, Promega) and qPCR analysis as detailed elsewhere [3]. The relative expression (ΔΔCT) of TRPV1 and TRPV4 was performed according to the Pfaffl method [4]. The elongation factor 1 alpha (EF1α) was used to normalize the results. Statistical analyses (two-way ANOVA) followed by Holm-Sidak or Bonferroni post hoc tests were performed using SigmaPlot®11.

Results and Discussion

The abundance of TRPV1 and TRPV4 mRNA was affected by photoperiod and/or temperature, in a tissue-specific and time-dependent manner.

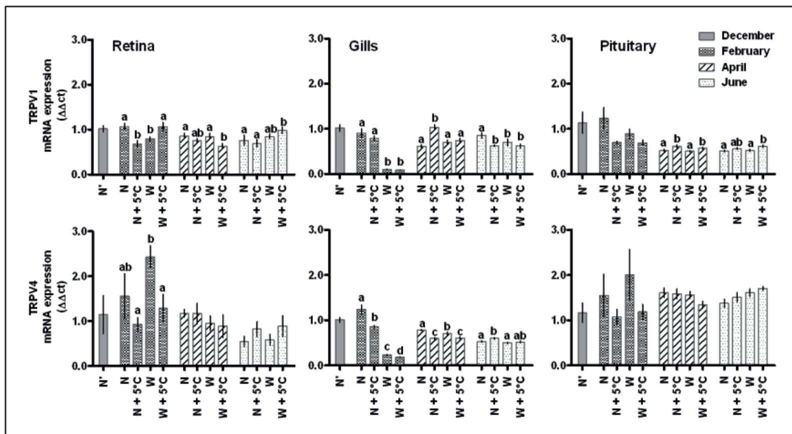

Figure 1.TRPV1 and TRPV4 mRNA abundance (mean ± SEM) in retina, gills and pituitary from *S. salar* throughout smoltification. Different conditions are indicated as follows: N', natural conditions at time zero; N, natural conditions at each time point; N+5°C, natural photoperiod with temperature elevated in 5°C; W, winter photoperiod; W+5°C, winter photoperiod elevated in 5°C. Different superscript letters indicate significant differences between conditions ($p<0.05$; two-way ANOVA) in each sampling time (months shown in the top right). The ΔΔct was calculated using Δct values of December as reference.

The more dramatic effects were observed in February, where the winter photoperiod induced inhibition of expression for the two TRPV subtypes in gills, and the temperature induced reduction of TRPV4 expression in the retina. In gills, the activity of the enzyme NaK-ATPase increases during smoltification, up-regulating ion extrusion as an adaptation to sea water tolerance [2]. The important inhibition of TRPVs expression in this tissue under extended winter photoperiod might suggest a general delay of physiological adaptation associated to smoltification. In this sense, TRPV4 could also be involved in sea water tolerance mechanism, since it is also described as an osmotic sensor [5, 6]. The present study is a first approach in the investigation on the roles TRPVs play in the different tissues of *S. salar.* TRPV may have multiple functions, one being thermosensitivity as shown for TRPV1 in the trout pineal gland [3]; therefore it is possible that the roles they fulfill in fish depend on the tissue where they are expressed. How this relates to salmon smoltification awaits for further functional investigations.

Acknowledgments

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QUANTIFICATION OF PLASMA STEROIDS IN SOLE (*SOLEA SENEGALENSIS)* BY ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

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The study of steroid hormones is crucial to understand reproduction related processes (i.e., sex differentiation, puberty, gonad maturation, reproductive cycles). Steroids are present in biological matrices at low levels (in plasma at ng mL^{-1} to pg mL^{-1}) and thus, a robust and sensitive analytical method is required. Liquid chromatography coupled to tandem mass spectrometry is a suitable technique for the simultaneous determination of several hormones. In this work, an ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) method was developed for the analysis of 14 of the most relevant fish steroids, including progestogens (pregnenolone (Preg), progesterone (P), 17α,20β-dihydroxyprogesterone (17,20β-P), 17α,20β,21-trihydroxyprogesterone (20β-S)), androgens (androstenedione (A4), 11β-hydroxyandrostenedione (11βOHA4), 11-ketoandrostenedione (11KA), testosterone (T), 5α-dihydrotestosterone (DHT), 11-ketotestosterone (11-KT)), estrogens (estrone (E1), estradiol (E2)) and corticosteroids (cortisol (F), 11-deoxycortisol (S)). The method was developed and validated for plasma samples of the flatfish Senegalese sole. Sample treatment was based on protein precipitation with acetonitrile and subsequent clean-up with OASIS HLB SPE cartridge. Target compounds were determined in selected reaction monitoring mode, using a triple quadrupole analyzer with a rapid chromatographic separation (6 min). The method was validated by recovery experiments, obtaining satisfactory results of accuracy and precision. Lowest level validated was 0.1 ng mL^{-1}. Limits of detection (LODs) were in the range of pg mL^{-1}.

Introduction

The culture of sole is regarded as an excellent option for the economic diversification and expansion of European aquaculture. However, despite scientific and technological efforts in the last decade, problems to reproduce hatchery-produced cultured soles (F1 generation) still remains. The study of steroid hormones is crucial to understand the different processes of reproduction during fish development (ontogeny, gonad differentiation, puberty and reproductive periods). Steroids are present in biological matrices at low levels and thus, robust and sensitive analytical methods are required (1). Immunoassays are the preferred technique for steroid determination due to its high sensitivity; however, they require processing one sample for each analyte. Liquid chromatography coupled to tandem mass spectrometry is the alternative technique which allows a sensitive and reliable multiclass determination (2). In this work, an ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) method was developed and validated for the analysis of 14 steroids in plasma samples of sole at sub ng mL^{-1} levels.

Materials and Methods

<u>Sample preparation:</u> Blood from Senegalese sole was collected on ice-cold heparinized tubes. Plasma was obtained by centrifugation (3000g, 4ºC, 15 min) and stored at -20ºC. Acetonitrile (1 mL) and an isotopically labeled hormones mix (0.1

mL), used as surrogate, were added to 0.5 mL of the sample and left stand in the freezer for at least 3 h, to boost protein precipitation. Mixture was centrifuged (12000 rpm, 5 min) and supernatant collected and diluted with 8 mL Milli-Q water before its purification in a polymeric Oasis HLB 60 mg 3cc cartridge. The cartridge was pre-conditioned with 3 mL of methanol (MeOH) and 3 mL of H_2O. After sample passing, cartridge was washed (2 mL H_2O), dried and target compounds eluted with 2 mL of MeOH. The eluates were evaporated to dryness at 40ºC under gentle N_2 stream and subsequently reconstituted with 100 µL of 30% aqueous methanol. Finally, 10 µL of the final extract were injected into the UHPLC system.

UHPLC-MS/MS: Determination was performed by means of an UPLC™ system (Acquity, Waters) interfaced to a triple quadrupole mass spectrometer (TQS, Waters Micromass) equipped with an electrospray ionization source (ESI). Chromatographic separation was achieved with an UPLC Acquity BEH C18 analytical column (50×2.1 mm, 1.7µm; Waters) employing as mobile phase water (A) and methanol (B). In positive ionization mode, 0.5 mM ammonium acetate (NH_4Ac) and 0.01% formic acid (HCOOH) were added as modifiers in both phases; in negative ionization mode 0.025% NH_3 was used. In both cases, flow rate was 0.3 mL min^{-1}. A linear gradient program was set up as follows: min 0, 30% B; min 4, 90% B and maintained during 1 min. Finally, the gradient was held to initial conditions (30% B) to re-equilibrate the column. The total run time was 6 min. ESI experiments were performed in negative ionization mode for E2 and E1 and in positive ionization mode for the other steroids. Target compounds were monitorized in selected reaction monitoring mode (SRM), selecting 3 SRM transitions per compound. Cone and collision energy were optimised by acquisition of full-scan MS and MS/MS spectra of targeted hormones from chromatography of 1 µg mL^{-1} reference standard in MeOH/H_2O (50:50,v:v).

Results and Discussion

A sensitive and reliable method was developed for steroid analysis in sole (*Solea senagalensis*) at sub ng mL^{-1} levels. For method development, it was necessary the optimization of spectrometric and chromatographic conditions for the selected compounds. In general a better ionization was observed in positive ionization mode, except for E1 and E2. Chromatographic conditions were studied to enhance the ionization of selected hormones. The addition of the modifiers HCOOH and NH_4Ac into the mobile phase improved the sensitivity of several compounds. However, when NH_4Ac was present in mobile phase, E1 and E2 showed a poor ionization. As a result, it was not possible to determine all selected hormones using the switching positive/negative ionization at the desired levels. For this reason, samples were injected twice, one in positive ionization mode and another in negative mode with the optimized conditions for each chromatography.

The methodology was validated by means of recovery experiments (n=5) at two different levels (0.1 and 1 ng mL^{-1}) for each compound. Accuracy, precision, linearity, specificity, and limits of detection and quantification were studied. Quantification was performed using external calibration (standards in solvent). Matrix effects were studied and compensated with a correction factor (F) or isotopically labeled internal standards when available. Recoveries between 70-120% with relative standard deviations (RSDs) below 15% were obtained for 12 steroids (Figure 1).

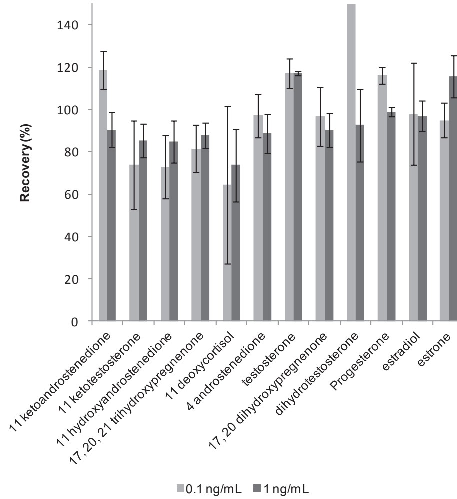

Figure 1. Mean recoveries and RSDs (n=5) calculated from spiked samples

Work is underway to solve problems related to the validation for pregnenolone, due to its poor ionization and for cortisol, due to the high concentration of this hormone in samples used for spiking experiments. The acquisition of three SRM transitions for each compound allowed an unequivocal confirmation of detected steroids by the accomplishment of their ion ratios when compared with reference standards. Preliminary analysis of plasma samples from sole breeders, using the newly developed UHPLC-MS/MS method, showed that E2 and E1 were only detectable in females, 11-KT only detectable in males and the other 9 steroids present in both sexes.

Acknowledgments

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THE ROLE OF LIPIDS AND FATTY ACIDS THROUGHOUT SPERMATOGENESIS OF EUROPEAN EEL (*ANGUILLA ANGUILLA*) AND EFFECT OF DIETS ON REPRODUCTIVE PERFORMANCE

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The present study describes the results obtained in two experiments on European eel males. Results of the first experiment showed ARA, EPA and DHA maintenance in the testis during eel spermatogenesis, while all other fatty acids decreased. ARA and EPA maintenance may have a physiological function, i.e. as prostaglandins precursors, while the maintenance of DHA levels may have a structural role for spermatozoa membrane formation. On the other hand, EPA and DHA acted as modulators of androgens synthesis particularly during the final phase of the sperm maturation, and ARA had an effect on sperm velocity. A second experiment was carried out in which we designed feeds with different fatty acid percentages in order to evaluate the influence of these diets on sperm quality. Together, this work demonstrated higher sperm motility and increased milt volume from eels fed balanced diets.

Introduction

The life cycle of the European eel is quite complex including transoceanic migration where native conditions are still unknown and the natural spawning process has never been observed. Eels cease feeding during migration and that is why their energy reserves, such as lipids, play a crucial role in obtaining good quality gametes (eggs and sperm). In the last years, efforts have been directed at studying the influence of fatty acids on the reproductive performance of females, while less effort has been devoted to males. The first experiment focused on the effect of fatty acids throughout sexual maturation, their relationship with steroid hormones, and their effect on sperm quality parameters. The second experiment compared the effects of different dietary regimes on sperm quality traits.

Materials and Methods

First experiment. European eel males (n=317; 100±2 g) from a local fish farm were distributed in six 200 L aquaria equipped with separated recirculation systems, thermostats and coolers and covered to maintain constant shade. Fish were progressively adapted to sea water and 20 ºC. The fish underwent three thermal regimes: T10, (10 ºC for the first 6 weeks, 15 ºC for the next 3 weeks and 20 ºC for the last 6 weeks); T15, (15 ºC for the first 6 weeks and 20 ºC for the last 9 weeks); and T20, (20 ºC throughout the whole experiment). For 13 weeks, the males were hormonally treated to induce maturation and spermiation through weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU g-1 fish) as previously described by Pérez et al. (1).

Different spermiation patterns were observed depending on the initial water temperature. The samples used to determine the relationship between the fatty acid levels and the sperm quality parameters were collected once sperm production had

been achieved, independently of the initial temperature. Sperm volume and concentration were evaluated following the methods described by Gallego et al. (2). The evaluation of sperm motility and velocity parameters followed the standardized methodology described by Gallego et al. (3). Between 5 and 8 fish per thermal regime were sacrificed each week, after anesthetize them. Only spermiating males were sampled and fatty acids from muscle, liver and testis were correlated with the sperm parameters. The muscle was crushed in a meat grinder and homogenized before storage. All the samples were stored at -80 ºC until lipid extraction and fatty acid quantification. Lipid and fatty acid analysis of tissues were carried out as described by Baeza et al. (4,5). In order to evaluate the different dynamics of the use of fatty acids, a categorization of the results from each sperm quality parameter (volume, concentration, motility and velocity) was performed (6).

Second experiment. Male broodstock were reared in four separate freshwater recirculation aquaculture systems and fed during 38 weeks three “enhanced” diets (PRO-EEL1, 2 & 3) and one commercial diet (DAN-EX; BioMar A/S, Denmark). PRO-EEL1 and 2 had relatively higher ARA levels (2.31 and 3.21%, respectively) than PRO-EEL3 (0.52%) and the DAN-EX diet (0.45%). Furthermore, PRO-EEL2 had the highest levels of EPA and DHA, followed by intermediate levels in PRO-EEL3 and the DAN-EX diet; lowest levels were detected in PRO-EEL1 for these two essential fatty acids. All fish were fed the DAN-EX diet until the start of the feeding experiment. Male fish (n = 71; in the range of 83-178 g) and transported to an experimental facility of the Technical University of Denmark (DTU), where they were housed in 300 L tanks equipped with a closed re-circulation system. Acclimatization to saltwater took place over a 14-day period. After acclimatization, 32 males (8 per diet) were sacrificed for morphometric records, histology, and lipid analyses. The remaining male eels received weekly injections of 1.5 IU hCG /g fish. During maturation, male eels were kept at a density of ≤30 kg/m^3. Salinity and temperature ranged from 36.7 to 37.3% and 19.5 to 20.5 °C.

After the 11th injection, milt was sampled for analyses of lipid composition as well as sperm performance and males were subsequently sacrificed for analyses of morphology and histological testes development.

Results and Discussion

Low and moderate correlations were observed between muscle tissue and some sperm quality parameters but no high correlations were found. EPA in the liver seems to have a role in determining the volume of sperm produced. This can be explained by the fact that EPA is a major requirement in the early phases of sperm production (probably as a component of the spermatozoa membrane). In addition, the levels of α-linolenic acid and linoleic acid in the liver decreased when sperm motility increased. In all the tissues, a negative correlation was observed between ARA and the different sperm velocity parameters. The fact that an increase in the consumption of ARA coincides with an increase in the speed of spermatozoa, highlights the important role that this fatty acid plays not only in sperm production, but also in sperm velocity.

The second experiment assessed the impact of dietary fatty acids on muscle, liver, and milt composition, as well as sperm production and motility in male European eel fed four different diets. Neutral lipids from the muscle and liver incorporated the majority of the dietary fatty acid profile, while phospholipids incorporated only certain fatty acids. Independent of dietary contribution, the substantial high levels of PUFAs in the phospholipid faction of the tissues, indicates that these fatty acids are preferentially esterified on phospholipids. Diet had an effect on the majority of sperm

fatty acids, on the total volume of extractable milt, and on the percentage of motile sperm. Here, our results suggest that the total volume of extractable milt is a DHA-dependent process, as we found the diet with the highest DHA level induced the most milt while the diet with the lowest DHA level induced the least amount of milt. The diet with the highest level of ARA, induced medium milt volumes but the highest sperm motility, suggesting that metabolites of this fatty acid, i.e. eicosanoids and prostaglandins, may be involved in sperm maturation. EPA also seems important for sperm quality parameters since diets with higher EPA percentages had a higher volume of milt and higher sperm motility. In conclusion, dietary fatty acids had an influence on fatty acids in the tissues of male eel and this impacted sperm performance traits. All this information could prove useful in the development of suitable broodstock diets to improve eel sperm quality and larval development.

Acknowledgments

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EXPOSURE OF SOLE (*SOLEA SENEGALENSIS*) LARVAE TO DIFFERENT ENVIRONMENTAL CONDITIONS AFFECT THE SYNTHESIS OF REPRODUCTIVE HORMONES

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In fish, environmental factors during early life have profound effects on many physiological and reproductive processes. To study the effects of temperature and light on the early establishment of the reproductive axis, in this work sole larvae were reared under routine conditions (constant temperature and white light -BL-) or under conditions that were previously shown to influence positively larval development (daily thermocycle and blue light -AZ-). The “optimal” conditions were applied in different time frames, covering pre-metamorphosis (0-12 dph), metamorphosis (0-30 dph) and post-metamorphosis (0-100 dph), to determine a potential sensitive period. Larva samples were analyzed for steroid (estradiol, testosterone, 11-ketotestosterone and 17,20β-P) levels and GTH subunits (*α*, *fshβ*, *lhβ*) mRNA levels (qPCR). Results showed differences in steroid synthesis and GTH expression between the BL and AZ groups. No critical differences were found within the AZ groups, suggesting an early action (first 12 days) of the environmental factors. These results provide endocrine evidences that larval culture conditions may affect the reproductive axis early in development.

Introduction

The sole has consolidated as a priority species for diversification of European aquaculture, but production is still limited by reproductive problems (i.e. the absence of fertilized spawning from cultured soles). We have hypothesized that reproductive dysfunctions could come from an abnormal establishment of the reproductive system during early development, caused by negative inputs in larval rearing. In previous works, we showed that daily thermocycles, compared to constant temperatures, affect growth, development and sex ratio (Blanco-Vives *et al*., 2011). Moreover, blue illumination, compared to other light spectra, reduce stress and deformities, increase growth and affect, probably positively, the synthesis and secretion of critical hormones of the circadian, stress and reproductive axes (Blanco-Vives *et al*., 2010). In these works, conditions were applied all along the rearing period and thus, it is unknown if the influence of environmental factors is exerted similarly through development. To investigate this aspect and to deepen into the effects on the reproductive axis, this work studied the effect of the “optimal” conditions when applied at different time frames, by analyzing the synthesis of critical reproductive hormones, from early development (0-20 dph) to gonad differentiation (GD) (100-150 dph).

Materials and Methods

Sole eggs collected from broodstock tanks were maintained in the dark for 24 h and then incubated whether with white or blue light, until hatching. Larvae were transferred to 8 culture tanks (4 groups; duplicate) and reared under: 1) constant temperature (19.5 ºC) and white light (0-170 dph; BL group), 2) daily thermocycle (18-21 ºC) and blue light (AZ conditions) for 0-12 dph (AZ12 group), 3) for 0-30 dph

(AZ30 group) and, 4) for 0-100 dph (AZ100 group); each AZ group was transferred to BL conditions until 170 dph, after the corresponding exposure time. Photoperiod was 12L:12D and intensity of the white and blue (LED, 460 nm) lamps adjusted to $1.5x10^{18}$ photons m^{-2} at water surface. Larvae were sowed at a density of 30 larvae L^{-1} and weaned at 60 dph. Steroids were analyzed by specific ELISAs (Guzman et al., 2009) and GTH subunit mRNA levels by qPCR (Guzmán *et al.*, 2009). Data were analyzed by One-way ANOVA, followed by a multiple comparison Tukey test.

Results and Discussion

Time of metamorphosis (14-20 dph) was not affected by the treatments. Body weight was similar in all groups until 30 dph and higher in the AZ groups than in BL from 30 to 100 dph (not shown). Time of GD occurred at 100-130 dph in all groups (35% and 90% of fish differentiated at 107 and 128 dph, respect.).

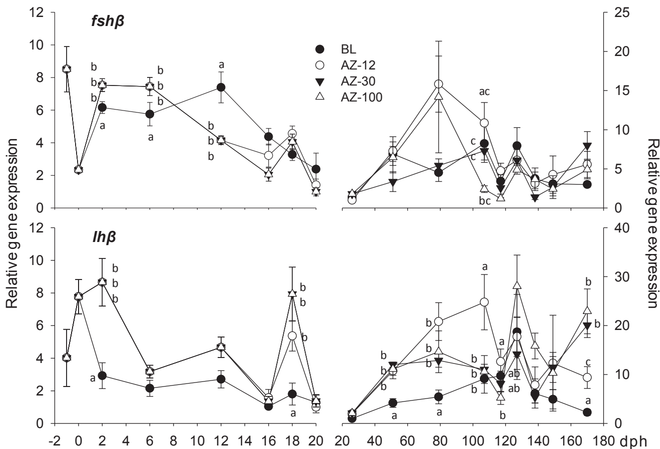

Figure 1. mRNA levels of gonadotropin subunits (*fshβ* and *lhβ*) in whole larva (left graphs) or head (right graphs) homogenates. Different letters indicate differences ($p<0.05$) between groups for each point. Data given as mean ± SEM (n=6).

Gene expression of all GTH subunits (*α* (not shown), *fshβ*, *lhβ*) was detectable since -1 dph, with high mRNA levels around hatching (0-2 dph), were levels in the BL group were lower than in AZ´s (fig. 1). *fshβ* and *lhβ* mRNAs peaked at full metamorphosis (18 dph) and again *lhβ* mRNAs were lower in the BL group, indicating a quick effect of temperature/light. At GD, GTH mRNAs were high at the initiation (80-100 dph) and the end of the period (128 dph), with *lhβ* mRNA levels in the BL group lower than in AZ groups in some points. Levels in all AZ groups were similar during this period or even higher in the AZ12 group in some points, suggesting a similar effect of the duration of the treatment.

Levels of T (not shown) and E2 showed highly similar profiles (fig. 2). Levels were first detectable at 12 dph and peaked at the end of metamorphosis, were levels in BL were lower than in AZ´s. Levels of 11-KT were low during early development, contrary to E2 and T and peaked, together with E2 and T, at late GD (149 dph). The progestin 17,20β-P was first detectable at 6 dph and maintained high levels through development, with a slight increase at late GD (149 dph). Effect of treatments on

steroid levels was mostly observed during metamorphosis, where lowest levels for the BL group.

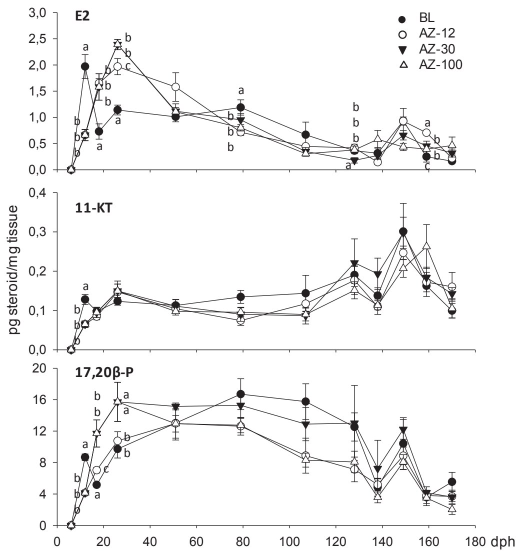

Figure 2. Levels of estradiol (E2), 11-ketotestosterone (11KT) and 17,20β-P. Different letters indicate group differences ($p<0.05$) for each point. Data are mean±SEM (n=6).

The different profiles suggest a relevant role of E2 and T in early development and 11-KT later in development (GD); all of them probably playing critical roles on driving sex differentiation (Guiguen *et al.*, 2010). The biological actions of 17,20β-P should be further investigated, mostly on its potential role on cell proliferation and differentiation in the developing gonads.

Acknowledgments

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EXPRESSION OF NUCLEAR AND MEMBRANE ESTROGEN RECEPTORS IN THE EUROPEAN EEL THROUGH THE SPERMATOGENESIS

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Estrogens can bind to nuclear estrogen receptors (ER) or membrane estrogen receptors (GPR30 or GPER). For the first time in a teleost, we analysed the expression of 3 nuclear ERs (ESR1, ESR2a, ESR2b) and 2 GPERs (GPERa, GPERb) in the brain-pituitary-gonad (BPG) axis, through the spermatogenesis. The expression of eel estrogen receptors resulted being tissue-specific (with different patterns in brain, pituitary and testis), as well as phase-specific through the testis development. Testes nuclear ERs seem to play a role in the spermatogonia renewal, while testes GPERs seem to be involved in final spermatogenesis. The change of water salinity induced an increase in the ERs expression along the BPG axis, together with the GPERs in pituitary and an increase of estradiol (E_2) plasma levels, suggesting an osmolality effect on the expression of reproductive genes along the BPG axis.

Introduction

Estrogens are small lipophilic steroid hormones, which play significant roles in the control of spermatogenesis. The sex steroid estrogens can bind nuclear receptors (ER) or membrane receptors (GPR30 or GPER) to elicit a slow genomic action by modulating the gene transcription and translation activity, or a rapid, non-genomic activation of intracellular signal transduction pathways. The nuclear ER includes ESR1 and ESR2, with at least three distinct subtypes, ESR1, ESR2a and ESR2b in the teleosts species (1). Two membrane GPERs are present in most teleosts, and are called GPERa and GPERb (2).

Due to their phylogenetical position, branching at the base of teleosts, and their complex life cycle, with a blockage in pre-puberty until its oceanic migration, the European eel (*A. anguilla*) is a powerful model to understand the ancestral regulatory functions of reproduction in teleosts, the largest group of vertebrates. In this study, we measured for the first time in a teleost the expression *in vivo* of the three nuclear and two membrane estrogen receptors simultaneously in the tissues of the BPG axis, through the spermatogenesis.

Materials and Methods

One hundred European eel males (BW 100±6 g) were gradually acclimatized during one week to sea water (37±0.3‰ of salinity) and kept at 20 °C during the experiment. Once a week they were weighed and injected with human chorionic gonadotropin (hCG; 1.5 IU g^{-1} fish) during 8 weeks (3), to induce the sex maturation. Groups of 5-8 eels were sacrificed in freshwater (FW) and sea water (SW) conditions (before hCG treatment), and later each week through the hormonal treatment. Testis samples were collected for histological analysis. Brain, pituitary and testis samples were collected for qPCR analyses, and blood samples for the analysis of E_2 plasma levels. Histological analysis was performed as described by Morini et al. (4). The stages were classified as described by Morini et al. (5). Total RNA of testis, brain parts and

pituitary were isolated and purified, and reverse transcripts were obtained as described by Peñaranda et al. (6). The qPCRs were carried out as described by Morini et al. (5) to determine the expression of each gene, using specific European eel estrogen receptor qPCR primers (2) and ARP as reference gene (7). Plasma concentrations of E_2 were measured by RIA.

Results and Discussion

Before hormonal treatment, E_2 plasma level of immature European eels showed a sharp increase with the change of FW to SW condition, suggesting an effect of the salinity on the steroidogenesis. These results agree with previous studies where the increase of salinity augmented E_2 plasma levels in hypophysectomized or intact silver European eel (8), suggesting that the transfer to SW modulate the E_2 level, through an extra-pituitary mechanism. These results can be explained by the catadromous life cycle of European eel, which must respond to salinity changes, attending to its reproductive migration. Furthermore, the change to SW induced an increase of the 3 nuclear ERs expression in all the tissues of the BPG axis, as well as *GPERa* and *GPERb* in the pituitary, and seem to be induced by the E_2 increase in the plasma.

In the pituitary, the nuclear ERs expression remained stable or decreased until the end of the testis development. The up-regulation of ESR1 observed in European (2) and Japanese (9) eel females is missing in the male eel. In the brain, the nuclear ERs expression remained stable during the maturation, except in the mes-/diencephalon, where the 3 nuclear ERs expression increased until the spermatozoa stage. In the female European eel, only ESR1 increased in the forebrain with the maturation (2), suggesting a different regulation of the nuclear ERs between male and female eels. The up-regulation by E_2 of ESR1 suggested in female brain Japanese eel (9) probably also occurs in the males, inducing an up-regulation of the 3 nuclear receptors.

In the testis, nuclear ERs are highly expressed at Spermatogonia A (SPGA) stage, then sharply decreased in Spermatogonia B/Spermatocytes (SPGB/SPC) stage, and remain very low expressed until the end of the spermatogenesis. In the Japanese eel, Miura et al. (10) showed that E_2 plays an important role in spermatogonial renewal. The very high expression of all nuclear ERs at SPGA stage that we found is in accordance with the proposed role of estrogens as a spermatogonial renewal factor. In the European eel, the parallel regulation of the 3 nuclear ERs suggests that the E_2 role as a spermatogonial renewal factor is mediated by ESR1, ESR2a and ESR2b.

Concerning the membrane estrogen receptors, GPERs are low expressed at SPGA stage, and progressively increased until the end of the spermatogenesis. These results suggest a potential role of both GPERa and GPERb in the final sperm maturation process, after the action of nuclear ERs.

In conclusion, the nuclear ERs in the testis and in the neuroendocrine system, as well as the membrane GPERs in the pituitary, seems to play a critical role in the eel osmoregulation. Testes nuclear ERs are likely to play a role in the spermatogonia renewal, while testes GPERs seems to be involved on the final spermatogenesis.

Acknowledgments

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INFLUENCE OF EXOGENOUS MELATONIN ON GROWTH, GONADAL MATURITY AND *kiss/gnrh* GENE EXPRESSION PATTERNS IN THE BRAIN OF MALE SEA BASS (*DICENTRARCHUS LABRAX*)

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Effects of melatonin implants on growth and reproductive performance of adult male sea bass were investigated. Fish were organized into two groups: the control group (C, empty implants) and the melatonin-treated group (M, melatonin implants). Melatonin reduced the fish weight and condition factor and also affected testicular maturation. The gonadosomatic index decreased after 150 days of treatment and a lower percentage of spermiating males was observed in this species. Melatonin also resulted in lower plasma levels of T and 11-Kt during the reproductive period, and showed a significant decrease of circulating Lh and Fsh levels after 30 and 60 days of treatment, respectively. Furthermore, the hypothalamic expression of *kiss1* was significantly higher in group M than in group C after 30 days of treatment, while a significant increase in *kiss2* expression was detected on day 90 of treatment. By contrast, melatonin showed a significant decrease in kisspeptin expression in the dorsal brain on day 150 of treatment and affected the expression of *gnrh-1* and *gnrh-3* as well as *gnrhr-II-1a and 2b* and the *fshβ* gene in the pituitary. These data reveal that melatonin presumably induces the downregulation of kisspeptin-gnrh members on the dorsal brain thus affecting *fshβ* transcription during early gametogenesis and altering spermatogenesis.

Introduction

Melatonin may drive the seasonal changes in expression of kisspeptin cells and GnRH/gonadotropin secretion in mammals, thus modulating their reproductive activity. The influences of melatonin on several daily and annual processes have been also widely investigated in teleost fish, although very few studies have explored the neuronal networks through which melatonin acts to influence the activity of the reproductive axis (1-4). European sea bass is a highly valued fish that, in the Mediterranean area, reproduces under short-photoperiod regimes. Melatonin administration in this teleost fish is known to inhibit nocturnal mRNA expression of *gnrh-1*, *gnrh-3* and gnrh receptors (4). Interestingly, changes in kisspeptin expression in the brain of adult sea bass during different gonadal stages suggest a potential implication of kisspeptins in the seasonal control of its reproduction (5). In this work, we studied the brain expression changes of the kisspeptin and gnrh systems in response to long-term melatonin administration, as well as its potential effects on certain biometric parameters and gonadal maturity in male sea bass.

Materials and Methods

Seventy 2-yr-old male sea bass were organized into two groups (35 fish each): in the first group, animals were administered empty implants (control group, C), whereas in the second, animals were administered melatonin implants (M) (i.e., a first implant containing a melatonin dose of 3.81 mg/g of fish body weight in October and a second implant 60 days later in December) (6). All fish were periodically weighed and their length, condition factor and the gonadosomatic index (GSI) were calculated. Gonads were collected for histological analysis and the stages for testicular development were assessed. The percentage of maturing males was also periodically evaluated (January-April). Blood samples were collected during the light

phase and plasma was stored at -20ºC until hormonal analysis. The pituitary was separated from the brain, and the brain was dissected in order to remove the hypothalamus and dorsal brain. All these tissues were frozen on dry ice and stored at−80 °C until later use for total RNA extraction and qRT-PCR analysis (5-6). Data are represented as the mean ± SEM and were analyzed using a two-way ANOVA followed by all pairwise multiple comparison procedures (Tukey´s test).

Results and Discussion

Melatonin had an anorexic action, thus affecting the performance of male sea bass. Melatonin reduced the fish weight and condition factor, with the mean weight of melatonin-treated fish amounting to only 77.7% as compared to controls (Fig. 1A). Melatonin also affected testicular maturation in adult sea bass. A decrease in the GSI after 150 days of treatment (29% lower than in controls) and a lower percentage of running males during the spermatogenesis and full spermiation stages (January-March) was observed (Fig.1B).

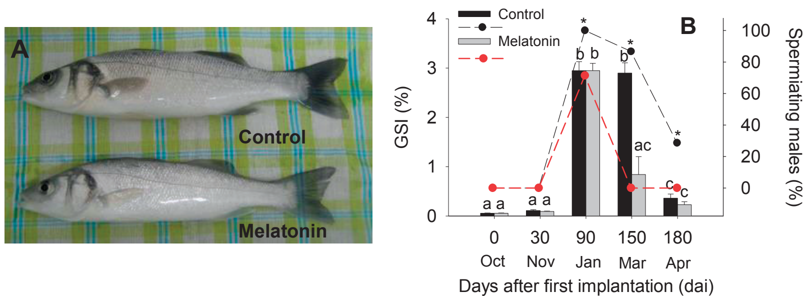

Figure 1. Representation of how size of melatonin-treated fish (A), the gonadosomatic index (GSI; in bars) and percentage of spermiating males (in circles) (B) were affected as compared to controls. Different lowercase letters indicate significant differences (P<0.05) either between groups or within the same group. Asterisks in the percentage of spermiating males indicate differences between groups during the same month.

The plasmatic levels of T and 11-Kt were low in group M. It could explain the reduced gonadal growth and altered spermatogenesis in this species. Melatonin treatment also induced a significant decrease of plasma gonadotropins during the early gametogenesis, probably contributing to the reduced sexual maturation observed in this group. Furthermore, those individuals treated with melatonin showed an altered expression pattern of some elements of the kisspeptin and gnrh systems with respect to the controls. Melatonin apparently exhibits two distinct effects at the central level: a transient stimulatory effect on the mRNA expression of *kiss1* and *kiss2* on the hypothalamus at 30 and 90 dai, respectively, and an inhibitory effect on the mRNA expression of *kiss1*, *kiss2*, *gnrh-1* and *gnrh-3* genes on the dorsal brain and also in the hypothalamus although changes in the expression levels were more modest here. Accordingly, melatonin appears to evoke a decrease of transcriptional activity of the expression of *kiss* genes in the dorsal brain (Fig. 2A), which might decrease the expression of hypophysiotropic *gnrh-1* and *gnrh-3* forms (Fig. 2B) and, in turn, the expression profiles of *gnrhr-II-1a* and *gnrhr-II-2b* in the pituitary. This could be part of a neuroendocrine regulation of the gnrh system in this teleost fish (4). No

effect of melatonin on *gnrh-2* mRNA levels was observed as previously reported (4). The variability of the impact of melatonin on brain and gonad observed among vertebrates, including fish (1-4), indicates the existence of species-specific responses to this hormone. In the case of sea bass, present data indicate that melatonin evokes seasonal changes in key reproductive elements that affect spermatogenesis in this teleost species.

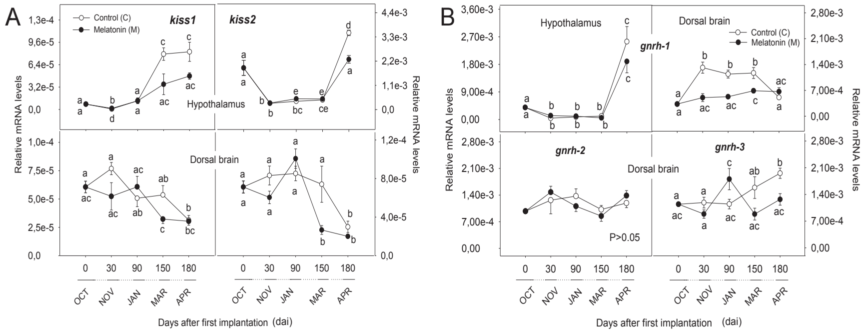

Figure 2. Relative mRNA levels of *kiss* (A) and *gnrh* (B) genes in the brain of control (white circles) and melatonin-treated (black circles) sea bass. Different lowercase letters indicate significant differences (P<0.05) either between groups or within the same group.

Acknowledgments

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VASOTOCINERGIC AND ISOTOCINERGIC CO-REGULATION IN STRESS RESPONSE OF COMMON CARP (*CYPRINUS CARPIO L.*)

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Under stress conditions, the hypothalamic-pituitary-interrenal (HPI) axis is stimulated to cope with the situations that could disturb the homeostasis of the fish. Endocrine regulation of this axis is controlled at different levels, involving several pathways that activate the synthesis and release of cortisol. This picture becomes more complex when other players as arginine vasotocin (AVT) and isotocin (IT) systems, homologous to mammalian vasopressin and oxytocin pathways, have been demonstrated both in central and peripheral stress response regulation. The aim of this study was to assess the effect of AVT and IT on the release of melanocyte stimulating hormone (MSH) and cortisol in common carp (*Cyprinus carpio* L.). With this purpose, we performed an *in vitro* superfusion setup for pituitary gland and head kidney, incubated with different combinations either corticotropin-releasing factor (CRF) or adrenocorticotropic hormone (ACTH), with AVT and IT. Our results suggest the enhancement of the stress response by AVT and IT, stimulating the release of cortisol in co-operation with ACTH in head kidney, whereas no effects were found in pituitary CRF releasing owing to these peptides in common carp.

Introduction

The stress response in teleostean fish is regulated by several elements at different levels, depending on the grade and nature of the stressors. In order to cope with those distress situations, HPI is activated aiming the synthesis and release of cortisol as final product. This axis starts with the integration of the stressors perception by the brain and the release of CRF from the hypothalamic nucleus preopticus (NPO). Neurons from this nucleus synthesizing CRF innervate both ACTH- and MSH-cells in pituitary *pars distalis* and *pars intermedia* respectively (1). CRF stimulates mainly the synthesis of ACTH from pituitary *pars distalis* and, as final result, the production and release of cortisol by the interrenal cells in the head kidney. Furthermore, those NPO terminations that inervate also *pars intermedia* cells produce acetylated endorphins and MSH. The role of MSH is not yet clear in the stress response, being either associated with pigmentation and food intake, but also with cortisol levels regulation. In addition, AVT and IT peptides are involved in many aspects of fish physiology including the stress response in both central and peripheral pathways (2). Effects on these peptides' synthesis and release have been described in fish depending on the type of stress, demonstrating changes in hypothalamic, pituitary and plasma AVT and IT levels under different stress conditions such as stocking density, food deprivation, confinement or osmotic disturbance (3,4). In order to appraise the effect of these endocrine pathways in the stress response in common carp (*C. carpio*); we performed a superfusion setup for pituitary and head kidney to assess the effect of AVT and IT on the release of MSH and cortisol.

Materials and Methods

In vitro pituitary and head kidney from *C. carpio* specimens (*N* = 25) were incubated in a superfusion setup with different combinations of either CRF or ACTH with /

without AVT or IT (Table 1). Incubation time and solutions were performed as Metz et al. (1) previously described.

Table 1. Peptide combinations and concentrations in pituitary and head kidney of *C. carpio* assessed in the superfusion setup.

PITUITARY	HEAD KIDNEY
DA (10^{-7} M)	ACTH (10^{-7} M)
DA (10^{-7} M) + CRF (10^{-7} M)	ACTH (10^{-7} M) + AVT (10^{-6} M)
DA (10^{-7} M) + CRF (10^{-7} M) + AVT (10^{-6} M)	ACTH (10^{-7} M) + IT (10^{-6} M)
DA (10^{-7} M) + CRF (10^{-7} M) + IT (10^{-6} M)	ACTH (10^{-7} M) + AVT (10^{-6} M) + IT (10^{-6} M)
DA (10^{-7} M) + CRF (10^{-7} M) + AVT (10^{-6} M) + IT (10^{-6} M)	AVT (10^{-6} M) + IT (10^{-6} M)

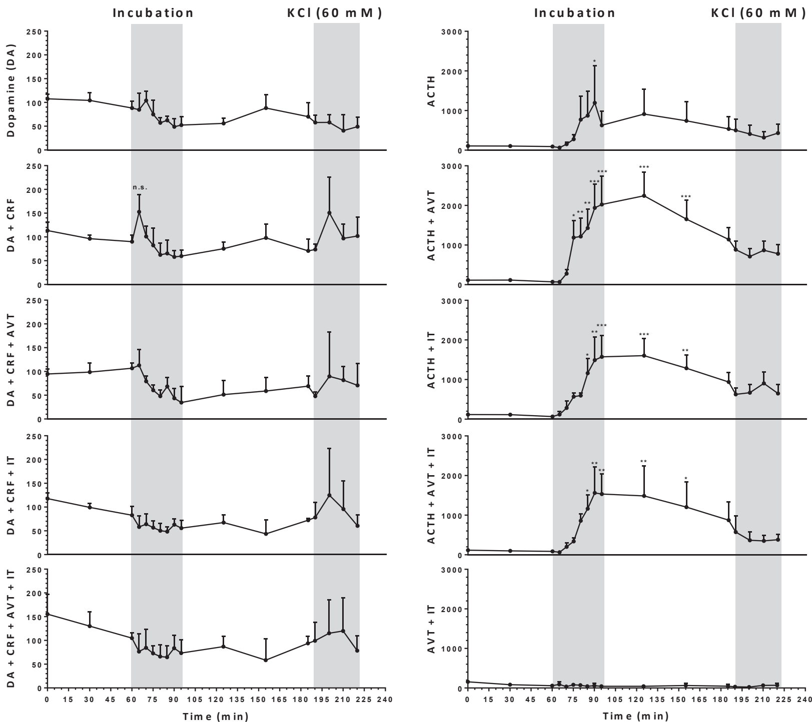

Figure 1. *In vitro* MSH and cortisol (left and right, respectively) release from carp pituitary and head kidney in the superfusion setup (n = 5). Concentrations used during the incubation period are described in table above. Asterisks indicate statistical differences with basal release: (n.s.) not significant; (*) $p < 0.05$; (**) $p < 0.001$; (***) $p < 0.0001$.

Results and Discussion

MSH release did not show any variation induced by either AVT or IT incubation in combination with CRF, even with only CRF. This results confirm the unclear role of α-MSH in the stress response (5), being either associated with pigmentation, food intake and lipolysis, and depending on the species in the regulation of cortisol levels under chronic stress situations. In contrast, cortisol release was significantly increased due to AVT and IT in co-operation with ACTH, demonstrating the important role of these neuropeptides on the interrenal cells and the peripheral stress response as Martos-Sitcha et al. (6) described for *Sparus aurata*. Finally, our results suggested the necessity of further studies involving the use of molecular biology tools in order to define how changes in cortisol and α-MSH release are induced by both vasotocinergic and isotocinergic systems in *Cyprinus carpio*.

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CHANGES IN PLASMA STRESS-RELATED PARAMETERS IN RAINBOW TROUT EXPOSED TO CONSPECIFIC CHEMICAL CUES

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It has been demonstrated that chemical cues from conspecifics are involved in the alarm response in fish. However, it is still unclear whether an activation of the neuroendocrine stress pathways participate in this reaction in fish. In the present study we evaluated the physiological response of rainbow trout (*Oncorhynchus mykiss*) to the presence in water of blood and skin extracts coming from conspecifics. We exposed trout to four different scents: ‘stressed blood’ and ‘unstressed blood’ (blood obtained from stressed and unstressed trout, respectively), ‘unstressed skin extract’ or distilled water (control). Trout from all groups were sacrificed avoiding handling stress, and blood samples were obtained and assayed for plasma levels of stress-related hormones (catecholamines, cortisol) and metabolites (glucose, lactate). Trout exposed to blood from a stressed conspecific or to skin extracts showed increased plasma catecholamines and cortisol levels which were moderate as compared to typical response to other acute stressors. At the metabolite level, we found increased plasma lactate content in fish exposed to stressed blood and unstressed skin extracts. Altogether, these data indicate that different chemical cues from blood and skin of conspecifics can induce a mild but detectable stress response in trout, thus demonstrating that these cues, which were already known to induce behavioral alterations in fish, could also have a metabolic cost.

Introduction

Many fish species use chemical cues for predator recognition and defensive behavior. These chemical signals are generally produced, or contained, within the skin or in body fluids, and are only released through mechanical damage or injuries, eliciting alarm reactions on conspecifics that comprise behavioral and physiological changes (1,2). In addition, social communication from stressed conspecifics has been reported to be also involved in the alarm response (3). However, it is still unclear whether an activation of the neuroendocrine pathways that are common in the stress response participates in this reaction to chemical alarm cues in fish.

Most fish species respond to a variety of stressors with a coordinated set of behavioral and physiological changes. At the organism level, physiological responses are primarily mediated by the neuroendocrine system and are characterized by an increased release of catecholamines and cortisol from head kidney to blood. These stress hormones have been shown to induce cellular and metabolic effects, the latter being characterized by rapidly increased glucose concentration in blood through activation of glycogenolysis and gluconeogenesis in liver (4). At the central level the stress response is mediated by increased monoaminergic neurotransmitter activity, which has been proposed to play a role in the nervous system integration of the alarm cues and the initiation of the neuroendocrine response (5). Although changes at neuroendocrine and metabolic levels were demonstrated for a variety of physical and chemical stressors, the effect of alarm substances released from conspecifics were less studied. Therefore, the aim of this study was to evaluate the physiological stress response of rainbow trout (*Oncorhynchus mykiss*) to the presence in water of blood and skin extracts coming from unstressed and stressed conspecifics.

Materials and Methods

Rainbow trout were obtained from a local hatchery and transferred to our facilities at the ECIMAT (Estación de Ciencias Mariñas de Toralla, Vigo) where the fish were acclimated to laboratory conditions (water temperature 13.5 ± 1 ºC; 12L:12D photoperiod) before any experimental procedure was performed. Animals were divided in experimental 100 L tanks and fed once per day two hours after the onset of the light period with a commercial dry pellet diet (1% body weight). In an initial experiment, groups of trout were exposed for 3 min and 15 min to water-dissolved blood (1 ml per tank) obtained from stressed and unstressed trout or a distilled-water control. In a second experiment, fish were exposed for similar times to skin extracts obtained from unstressed trout or a distilled-water control. Skin samples were obtained from fish and homogenized in a 1 ml water volume before included in the tanks. Trout from all groups were captured avoiding handling stress and anesthetized (2-phenoxyethanol 0.2% v/v). Blood obtained by venous puncture was centrifuged and plasma samples separated and frozen until metabolites and hormones assays. Plasma cortisol levels were measured by an EIA kit, whereas catecholamines were determined by HPLC with coulochemical detection as previously described (Gesto et al., 2015). Plasma glucose and lactate levels were determined by using specific kits.

Results and Discussion

It has been suggested that blood cues induce antipredator behavior in teleost fish (Barreto et al., 2013). In the present study we also showed that chemical cues present in the blood of conspecifics were able to moderately trigger the release of stress-related hormones (catecholamines, cortisol) (Figure 1). Thus, trout exposed to blood obtained from both unstressed and stressed trout showed significant but moderate (2-4 fold) increases in plasma catecholamines (in particular noradrenaline, 3 min after exposure to unstressed blood and 15 min after exposure to stressed blood). Cortisol levels were also higher (2-3 fold) in trout exposed to blood cues with significant increased levels after 15 min exposure to unstressed blood, and 3 and 15 min exposure to stressed blood. No changes were observed in plasma glucose levels, whereas lactate content was increased in fish exposed (15 min) to stressed blood (data not shown).

Trout also responded with moderate hormonal increases to skin extracts obtained from a conspecific (NA levels and cortisol were higher after 15 min of exposure). Similarly to that found with stressed blood, increased lactate values were found 15 min after fish exposure to skin extracts. These data agree with several studies suggesting the existence of chemical cues from epidermal 'club' cells that elicit alarm reaction in fish (Brown and Smith, 1997; Ide et al., 2003).

Altogether, these data indicate that different chemical substances from blood and skin of conspecifics can induce a mild but detectable stress response in trout, thus demonstrating that these cues, which were already known to induce behavioral alterations in fish, could also have a metabolic cost. The relevance this kind of stressor may have in fish farms, where fish often suffer skin lesions, is yet to be determined.

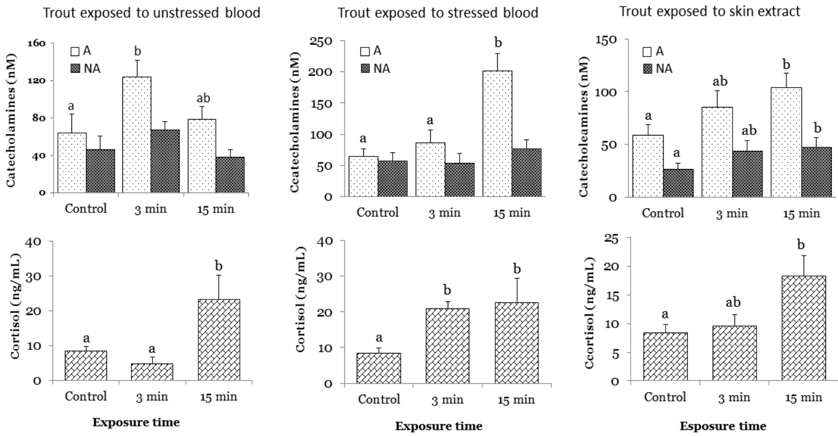

Figure 1. Plasma levels of catecholamines (A: adrenaline; NA: noradrenaline) and cortisol in rainbow trout exposed to chemical cues from blood and skin of conspecifics. Different letters indicate significant differences among groups.

Acknowledgments

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GHRELIN MODIFIES CLOCK GENES EXPRESSION IN THE LIVER OF GOLDFISH VIA PLC-PKC PATHWAY

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Ghrelin is a peripheral orexigenic peptide hormone which main role is related to the energy balance regulation. In addition, recent studies of our group show that an intraperitoneal injection of ghrelin modifies clock genes expression in the liver of goldfish. The objective of this work was to investigate if ghrelin acts directly on the liver by modifying clock genes expression and the underlying intracellular pathway. The direct effects of ghrelin on clock genes expression were studied using an *in vitro* culture of goldfish liver and then, the possible involvement of PLC-PKC pathway was investigated. Ghrelin induced (4-6 fold) the expression of *gper1a*, *gbmal1a* y *grev-erb*α after 1 h, and this stimulatory effect was decreased or completely lost after 5 h. The effects elicited by ghrelin at 1 h were either partially or completely blocked by the ghrelin antagonist (D-Lys^3]-GHRP-6), and by inhibitors of PLC (U73122) and protein kinase C (chelerythine). Present results show for the first time in teleosts a direct action of ghrelin on the hepatic oscillator by modulating clock genes expression via the PLC-PKC pathway. Taken together, these results evidence that ghrelin may be a link between food intake/energy balance and the circadian system in teleosts.

Introduction

The circadian system controls the rhythmic physiological processes that let organisms to anticipate cyclic environmental changes. In vertebrates, these endogenous timing systems are organized by multiple coupled central and peripheral oscillators that are entrained by different environmental cues (1). Among all the oscillators, the liver seems to be the most important link between the circadian system and metabolism in mammals (1), and in goldfish it works as an endogenous oscillator that is regulated by different external and also endogenous signals (2). Ghrelin (GRL) is an orexigenic peptide synthesized by the gastrointestinal tract in vertebrates, that has been related to circadian system in goldfish (3). This hormone acts through specific membrane receptors, being GHS-R1a the most studied (4). The objective of the present study was to investigate the possible role of GRL in the direct regulation of the hepatic oscillator functioning in goldfish by using an *in vitro* approach.

Materials and Methods

Goldfish (*Carassius auratus*; 7.2 ± 0.5 g) were maintained under a 12 h light: 12 h dark photoperiod and fed (1% body weight) 2 h after lights on. The day of the experiment, fish were fasted, anesthetized in MS-222 (Sigma) and sacrificed to quickly dissect the liver, which was immersed in Dulbecco's Modified Eagle Medium (DMEM, Sigma) in sterile wells. One portion of liver (~15 mg) from at least 6 different fish was employed in each experimental group. Liver samples were cultured in 1 ml DMEM for stabilization (1-2 h) before *in vitro* treatments. Then, medium was replaced by control medium (containing the respective vehicle) or medium containing the drug. The liver cultures were performed under constant dim light and temperature (21 ± 1°C).

First, a concentration-response study of GRL (ghrelin-17, GTS(octanoyl)FLSPAQKPQGRRPP; Bachem) was performed and clock genes expression (*gper1a, gbmal1a* and *grev-erbα*) was quantified. Then, GRL effects were assessed in the presence of a GRL receptor antagonist ([D-Lys^3]-GHRP-6), a phospholipase C (PLC) inhibitor (U73122), and a protein kinase C (PKC) inhibitor (chelerythine, CHEL). The antagonist and the inhibitor were added to the culture medium 15 min before GRL addition.

Clock genes expression was measured by RT-qPCR using *gβ-actin* as a reference gene as previously described (3). RNA extraction (TRI® Reagent method, Sigma), DNase treatment (Promega), cDNA synthesis (Invitrogen) and real-time PCR reactions (iTaqTM SYBR® Green Supermix, Biorad) were carried out as previously described (3). The relative mRNA expression was determined by ΔΔCt method (3). Data were analysed by one-way ANOVA or two-way ANOVA (when both, GRL and an inhibitor drug were used) followed by a SNK post-hoc test ($p<0.05$).

Results and Discussion

All the clock genes studied were induced by GRL at all the concentrations tested (1-10 nM) after 1 h of treatment (Fig. 1). This effect of GRL on clock genes expression is fast and transient, as it disappeared 5 h later. This is in accordance with a previous *in vivo* study carried out in goldfish where an acute intraperitoneal injection of GRL induces *per* genes in the liver at 1-h post-injection, with no observable effect 3-h later (3). Such characteristics (rapidity and transience) found for the effect of GRL on the induction of clock genes expression, are essential properties for hypothetical synchronizing agents. We propose that this could be the role of GRL on liver oscillator, to act as an energy status input of the clock.

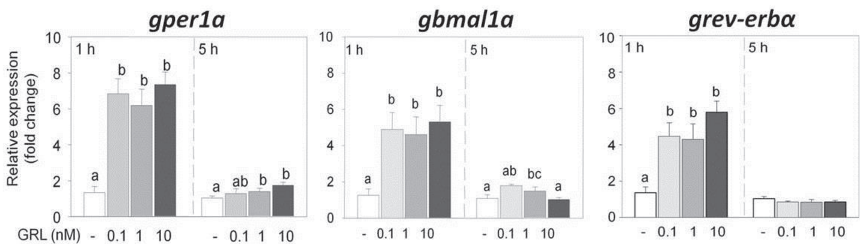

Figure 1. Concentration-response effects of ghrelin (GRL) on clock genes expression in goldfish cultured liver. Data are shown as mean ± s.e.m. (n=6). Different letters indicate significant differences among groups ($p<0.05$, one-way ANOVA followed by SNK test).

The specificity of the GRL-evoked clock genes induction was tested by using the specific antagonist [D-Lys^3]-GHRP-6 of GRL receptors (Fig. 2A). Preincubation for 15 min with this antagonist abolished GRL effects on the expression of clock genes after 1-h of exposure. These results confirm the involvement of a specific GRL receptor in the *in vitro* action of this hormone as modulator of clock genes expression in goldfish liver.

The GRL receptor GHS-R1a, described in the hepatocytes of goldfish (own data not shown) is classically linked to the PLC-PKC intracellular transduction pathway (4). To analyze the possible involvement of this intracellular pathway in the GRL signaling, we studied the effect of a pretreatment with either a PLC inhibitor or a PKC inhibitor on the *in vitro* GRL action.

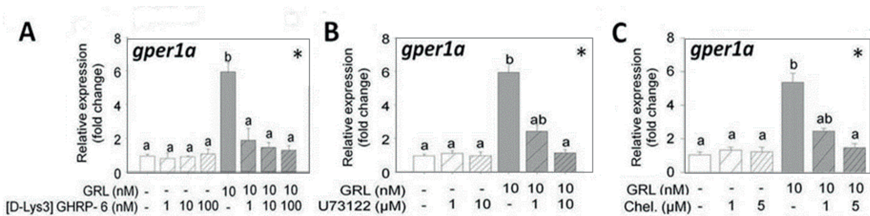

Figure 2. Relative expression of *gper1a* in cultured liver treated with GRL for 1 h and preincubated with A) GRL receptor antagonist ([D-Lys3] GHRP-6), B) PLC inhibitor (U73122) and C) PKC inhibitor (chelerythine). Data are shown as the mean ± s.e.m. (n=6). Asterisks indicate significant interference ($p<0.05$) between GRL and the drug (two-way ANOVA). Different letters indicate significant differences among groups (SNK test).

The preincubation with PLC or PKC inhibitors partially or completely abolished the GRL induction of *gper1a* (Fig 2B and C), *gbmal1a* and *grev-erb*α (data not shown) at 1-h of treatment. Present results demonstrate that the induction of clock genes in the goldfish liver by GRL is transduced via PLC-PKC intracellular pathway. It is reported that this intracellular pathway underlies the clock functioning, but present results are the first ones that show its involvement in the ghrelin mechanism of action on clock genes expression (5).

In conclusion, present results demonstrate for the first time in any vertebrate a direct effect of ghrelin modulating the molecular functioning of the hepatic oscillator by inducing the expression of some clock genes. Taking into account the role of GRL as an indicator of the energetic status and as a modulator of the hepatic oscillator, we suggest that GRL in teleosts is a good candidate as a link in the regulation of both, energy balance and circadian system.

Acknowledgments

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PROLACTIN IN THE MEAGRE (*ARGYROSOMUS REGIUS*): MOLECULAR CLONING AND EXPRESSION ANALYSIS UNDER DIFFERENT ENVIRONMENTAL SALINITIES

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Prolactin (PRL) plays an important role at fresh-water adapting process in many species of perciforms, although it remains unveiled in the meagre *Argyrosomus regius.* Degenerated primers were designed using available cDNA prolactin sequences from others species, which were used to clone and amplify a partial cDNA sequence of PRL in *A. regius* and design specific oligos to be used in semi-quantitative expression (qPCR) in specimens submitted at four different environmental salinities (5, 12, 38 and 55 ‰). Our results showed that *prl* mRNA expression levels are significantly affected by environmental salinity degree, indicating an inverse pattern between this expression and environmental salinity.

Introduction

The meagre (*Argyrosomus regius*) demand has been growing in the late years, due to it rapids growth rates, ease in processing and low fat content (1). This species presents a good euryhalinity capacity (2) but, to our knowledge, there is no information on the endocrine control of this capacity. Prolactin (PRL) plays an important role at fresh-water adapting process in many species of perciforms, promoting water removal and preventing ion loss (3), although the role of PRL in this process has not been investigated in *A. regius*. Thus, understanding of this role will provide knowledge assisting in the development of molecular strategies to identify osmotic stress and optimal environmental salinity to improve the culture of this species. To aim for this goal, a partial *prl* CDS was sequenced and *prl* mRNA expression level was assessed in specimens acclimated to various environmental salinities.

Materials and Methods

Juvenile specimens of *A. regius* were divided in four groups (n = 8) and transferred to 400 L tanks containing different environmental salinities: 5 ‰ (hypoosmotic environment), 12 ‰ salinity (nearly isoosmotic environment), 38 ‰ salinity (nearly sea water) and 55 ‰ salinity (extremely hyperosmotic environment). After 21 days of acclimation in their respective salinity, the entire pituitary gland was retrieved and a total RNA extraction was performed with NucleoSpin® RNA XS kit (Macherey-Nagel). Prolactin degenerated primers were designed from published cDNA prolactin sequences, especially in perciforms, using Oligo Analyzer 1.1.2. A mixture of total RNA samples from meagre individuals acclimated to different salinities was used as template for cDNA synthesis. Retrotranscription (RT) was carried out with *SuperScript® III First-Strand Synthesis System for RT-PCR* (Invitrogen|Life Technologies). Amplified RT-PCR product with degenerated primers was cloned into pJET 1.2/blunt and sequenced by STAB VIDA Lda (Caparica, Portugal).

After that, 250 ng of each total RNA samples were retrotranscribed using qScript™ cDNA Synthesis Kit (Quanta BioSciences), in order to synthetize cDNA for quantitative PCR (qPCR) assay. The qPCR primers were designed based on the cDNA sequence obtained. All the qPCR assays were performed using PerfeCTa™ SYBR®Green FastMix™ (Quanta BioSciences). Different pituitary cDNA template concentrations were applied in triplicate to check the assay linearity and the amplification efficiency. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (4), choosing beta-actin (*actb*) as the internal reference gene for normalizing mRNA expression data. Each reaction was run in triplicate, non-template controls and non-reverse transcribed RNA were used as negative control in every experiment. Generated data was statistically analyzed using one-way analysis of variance (ANOVA) and Tukey-HSD Post-Hoc test. Significant values were considered when $P < 0.01$.

Results

Partial cDNA sequence obtained was 498 bp-long and can be accessed at GeneBank (acc. no.: KP984534). BLAST comparison displayed 91 % nucleotide sequence identity with *Sparus aurata prl* or 89 % with *Acanthopagrus schlegelii*. Aminoacidic sequence translated from partial CDS encoded a 165 aa-long partial protein that also showed a high identity percent.

After primer optimization, oligos used for qPCR analysis showed the best amplification efficiency (E= 0.96) and assay linearity (r^2= 0.996) at 200 nM concentration, setting melting temperature at 60 ºC. Average relative expression of each group is displayed in Figure 1. Expression levels are affected inversely by salinity degree, revealing the highest expression in the hypoosmotic environment and decreasing gradually as the salinity rises.

Discussion

prl expression reaches three-fold gain when fish exposed to 5 ‰ were compared with the control group (38 ‰), which is nearly the salinity of the sea. This result agrees with the reported in other euryhaline teleost acclimated to hypoosmotic environments and support the role of PRL as a fresh-water promoting hormone (3). In 12 ‰ of salinity, the mRNA expression of *prl* also increases comparing to seawater salinity condition. This salinity, corresponding with the isosmotic point, is hypothetically where fishes show greater growing rates because less energy is required for osmoregulatory processes. This *prl* variation at gene expression level could be exploited to determine the optimal salinity of culture where the growth is maximized, since this optimal state could be measured through *prl* level expressed. Once this condition is identified, routine qPCR could be performed to check the osmotic stress of cultured specimens.

In conclusion, in this study *prl* partial CDS has been sequenced in *A. regius*, a popular commercial fish, and a qPCR assay has been described to measure the relative expression of this gene, which can allow future applications on aquaculture industries as a weapon to estimate osmotic stress in cultured specimens.

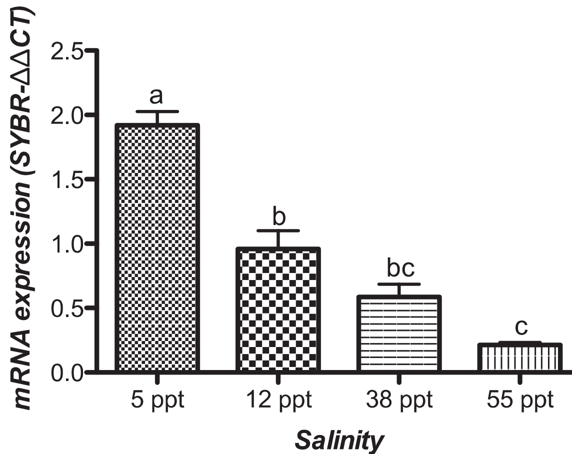

Figure 1. Hypophyseal *prl* mRNA expression levels (relative to *actb*) in specimens of *A. regius* acclimated to four different environmental salinities Values are represented as mean ± S.E.M. (n = 8 fish per group). Significant differences within each group are identified with different letters (P<0.01, one-way ANOVA followed by Tukey's test).

Acknowledgments

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CLONING, SEQUENCING, AND mRNA EXPRESSION PATTERNS OF GH AND IGF-1 GENES IN MEAGRE (*ARGYROSOMUS REGIUS*) JUVENILES ACCLIMATED TO DIFFERENT ENVIRONMENTAL SALINITIES

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The Mediterranean meagre *Argyrosomus regius* is a species of growing interest for the aquaculture industry in the Mediterranean region. However, the roles of growth hormone (Gh)/insulin growth factor-I (Igf-1) axis in mediating euryhalinity of this species are not yet completely unveiled. We cloned partial cDNAs for β-actin (*actb*), pituitary growth hormone (*gh*), and hepatic insulin-like growth factor (*igf-1*), and assessed their expression patterns in juveniles acclimated to 4 different environmental salinities: 5, 12, 38, and 55 ‰: These represent hypo-, iso, hyper- and extremely hyperosmotic salinities, respectively. All investigated transcripts shared high sequence identities with their counterparts in other perciformes. *gh* enhanced significantly in both 12 ‰ and 55 ‰ salinity groups in comparison to control group. However, *igf-1* showed its maximum expression levels under isosmotic condition. The results indicated that enhanced Gh/Igf-1 expression under isosmotic environment could stimulate growth and that the high euryhaline capacity of *A. regius* is supported by the actions of Gh/Igf-1 axis.

Introduction

The meagre, *Argyrosmus regius* (Family Serranidae), exhibits rapid growth rate, easiness in processing, low fat content and somewhat rigid texture, so it gained good popularity for both aquaculture producers and consumers. To our knowledge, there is no information on euryhalinity endocrine control on *A. regius*. The osmoregulatory role of growth hormone (Gh) and insulin-like growth factor type 1 (Igf-1) in teleost fish is depending on the analyzed species (1). The aim of this study was to investigate the patterns of Gh/Igf-1 axis in *A. regius* juvenile specimens acclimated to different environmental salinities (5, 12, 38 and 55 ‰). This is the first study dealing with molecular events on the main elements of the Gh/Igf-1 axis in this species upon exposure to different environmental salinity regimes.

Materials and Methods

Juvenile specimens of *A. regius* (n = 32, 150-180 g body mass) were provided by IFAPA Centro “El Toruño” (El Puerto de Santa María, Cádiz, Spain), transferred to the laboratories in the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz), and acclimated to seawater conditions for 7 days. 8 animals were transferred to four tanks at 5, 12, 38 (control) and 55 ‰ salinity. Fish were fed a daily ration of 1 % of their body mass with commercial pellets. They were fasted for 1 day before sampling. After 14 days of acclimation, fish were sacrificed and sampled for the

pituitary gland and biopsies from the liver. Target tissues were preserved in RNAlater® (Ambion, LifeTechnologies) until processing.

GenBank database sequences for perciform *gh, igf-1* and *actb* were used for cloning primers designing. Total RNA was extracted from pituitary glands and liver biopsies using commercial kits. RNA quantity and quality was checked using Bioanalyzer 2100 (Agilent). cDNA was synthesized using ~3 µg of total RNA from liver and ~100 ng from pituitary. PCRs were run over these cDNAs and their amplicons were directly cloned into the pCR®4-TOPO cloning vector (LifeTechnologies) and sequenced (Bioarray, Alicante, Spain) for designing quantitative reverse transcription PCR (qPCR) primers. Total RNA from experimental livers and pituitaries was extracted and quantified as mentioned before. 250 ng from pituitaries total RNA and 500 ng from liver were separately used for cDNA synthesis using qScript™ cDNA Synthesis Kit and quantified by qPCR using PerfeCTa™ SYBR®Green FastMix™ (Quanta BioSciences). 400 pg cDNA template were used in each qPCR reaction. qPCRs were performed in a Master cycler®ep Realplex^2 (Eppendorf) operated with Realplex 2.2 software (Eppendorf). *actb* was used as the internal reference gene for normalizing mRNA expression data, owing its low C_T variability as we found during the qPCR runs. Relative gene quantification was performed using the $\Delta\Delta C_T$ method. Statistical analyses were performed using one way analysis of variance (ANOVA) and Tukey-HSD Post-Hoc test. Significant values were considered when $P<0.01$.

Results and Discussion

A. regius partial *gh* cDNA consisted of 552 base pairs (bp), sharing 93 %-97 % identities with other sciaenids *gh* transcripts. *gh* mRNA significantly showed 3-fold increase in the group maintained in 55 ‰ salinity in comparison to the control group (Figure 1a). Two cDNA could be identified for the *igf-1*, one with 594 bp long and the other with 679 bp long, sharing 100 % sequence identity with the isoforms Ea2 and Ea4 of other sciaenid species, respectively. mRNA expression patterns of both forms were similar, showing their maximum levels in the nearly isosmotic salinity (12 ‰) group, and the minimum in specimens under 5 or 38 ‰ salinities (Figure 1b,c). *A. regius actb* cDNA was 1,113 bp long sharing 98-99 % sequence identity with other sciaenids *actb*.

The osmoregulatory capability in fish is mainly mediated by hormones like Prl, Gh, Igf-1 and cortisol (2). Gh/Igf-1 axis modifications in different environmental salinities in family Sciaenidae in general are very poorly studied. There is a general tendency in the members of the family Sciaenidae to exhibit low energy expenditure and better growth under isosmotic salinities, as it is the case in another meagre, *Argyrosomus japonicus* (3-4). So, the enhanced Gh/Igf-1 axis expression under isosmotic conditions could explain the better growth observed in *A. regius* at this environmental salinity (5). In addition, *gh* up-regulation under hyperosmotic salinity (55 ‰) agrees with the hypoosmotic role of this hormone, which normally aids increasing opercular chloride cell numbers, gill Na^+,K^+-ATPase activity and mRNA expression of Na^+,K^+-ATPase subunits, and salinity tolerance. In conclusions, *gh* and *igf-1* transcripts showed active differential regulations with changing salinities, which normally mediates metabolic stimulation required for proper osmotic adjustment preceding fish survival under extreme saline environments.

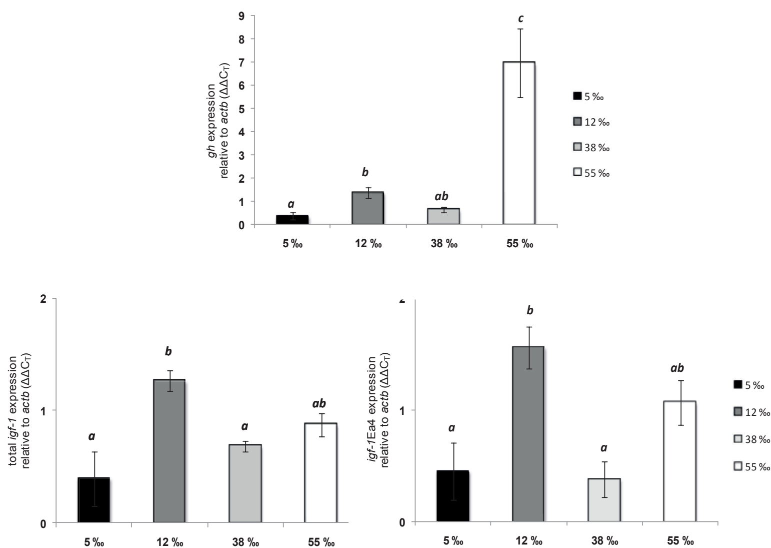

Figure 1. mRNA expression patterns for (a) *gh*, (b) *igf-1* total, (c) *igf-1* isoform Ea4 in *A. regius* juveniles acclimated to different environmental salinities (5; 12; 38; and 55 ‰) during 14 days. Data are represented as mean ± SEM (n = 8). Different letters indicate significant differences among experimental groups (one way ANOVA, $P < 0.01$).

Acknowledgments

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PHYSIOLOGICAL CHANGES IN SILVER CATFISH (*RHAMDIA QUELEN*) TRANSPORTED WITH ESSENTIAL OIL OF *MYRCIA SYLVATICA*

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Aquaculture practices include several procedures as capture, handling and transport, which cause stress in fish. Anesthetics or sedative substances have been used to reduce this stress. The aim of the present study was to evaluate the effects of adding essential oil of *Myrcia sylvatica* (EOMS) in the water on the activation of the stress system in the silver catfish *Rhamdia quelen* submitted to transport. Fish were captured in the production ponds and transferred to a 250-L tanks (density of 54 g/L). After 24 hours, 10 fish were caught and sampled (before transport group). The remaining fish were placed in plastic bags containing 5 L of water (density of 150 g/L) with different concentrations of EOMS (0, 25 or 35 µL/L diluted in 315 µL/L ethanol) in triplicate, transported for 6 h and them sampled (10 animals of each group). Cortisol levels and corticotrophin-releasing hormone (CRH) expression increased after 24 h of handling, decreasing during the transport with addition of EOMS, whereas plasma glucose enhanced their values in both control and ethanol groups. mRNA expression of proopiomelanocortin a (POMCa) was the highest in fish transported with 25 µL/L, while in specimens transported with 35 µL/L POMCb was the lowest. Therefore, it is advised the use of EOMS for transporting *R. quelen* individuals in order to avoid the stress associated with this procedure.

Introduction

The transport of live fish for commercial or biological purposes is considered a traumatic procedure that exposes these animals to a series of physiological responses triggered by various adverse stimuli. To reduce this stress, anesthetics or sedatives have been used in the water during transport. However, synthetic substances used for anesthesia may cause undesirable systemic effects such as increased cortisol levels and adverse reactions. Thus, the demand for natural products such as essential oils, which exhibit anesthetic effect and lower toxicity to animals, consumers and the environment, has increased. In this study, we evaluated the essential oil extracted from *Myrcia sylvatica* (EOMS) as a putative stress suppressor during transport of silver catfish (*Rhamdia quelen*).

Materials and Methods

Juveniles of *R. quelen* (n = 54, 8.87 ± 0.71 g) were captured from local production ponds and transferred to a 250 L tank (density of 54 g/L). After 24 hours, 10 fish were caught, euthanized by section of the spinal cord and sampled (before transport group, BT). The remaining fish were placed in plastic bags containing 5 L of water and 10 L of pure oxygen (density of 150 g/L). Treatments were as follows: control, ethanol (315 µL/L, the highest concentration used to dilute the EOMS), 25 and 35 µL/L EOMS, in triplicate. The EOMS concentrations were determined in previous experiments (data not shown). After transportation for 6 h, 10 animals of each group were captured, euthanized and sampled. Plasma cortisol levels were measured by

indirect enzyme immunoassay (ELISA) as previously described (1) and glucose levels were measured using commercial kits from Spinreact (Barcelona, Spain). Total RNA was extracted from the pituitary gland and the hypothalamus using NucleoSpin®RNA XS and RNA II kits (Macherey-Nagel), respectively. After reverse transcription (qSCRIPT™cDNA Synthesis Kit Quanta BioSciences), qPCR reactions were carried out with the Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep realplex^2 S) for analysis of hypothalamic CRH and hypophyseal POMCa and POMCb mRNA expressions. Results were normalized to *R. quelen* β-actin (GenBank acc. no. KC195970) due to its low variability (less than 0.25 C_T) in our experimental condition. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (2).

Results and Discussion

Both plasma cortisol (Figure 1A) and hypothalamic CRH expression (Figure 2) levels were lower after transport with addition of EOMS compared to BT group. In contrast, glucose levels increased after transport in control and ethanol groups compared to the group not transported (Figure 1B).

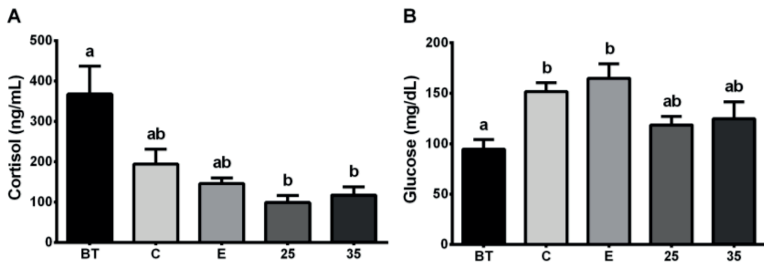

Figure 1. Plasma cortisol (A) and glucose (B) levels in *R. quelen* transported with addition of EOMS. Values are mean ± SEM (n = 10). BT- before transport, C-control, E- ethanol, 25- 25 µL/L EOMS and 35- 35 µL/L EOMS. Different letters indicate significantly difference between treatments based on one-way ANOVA and Tukey's test ($P < 0.05$).

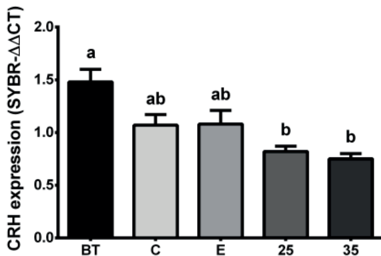

Figure 2. Hypothalamic CRH expression in *R. quelen* transported with addition of EOMS. Further details as described in legend of Figure 1.

CRH plays an important role in stress response, as it is the main mediator for hypothalamus-pituitary-interrenal axis activation. According to previous studies, plasma cortisol levels in *R. quelen* resting or non-stressed are in the range 16-30 ng/mL (3). In this study, cortisol levels and expression of CRH changed after events of handling and high stocking density, decreasing during transport with the addition of EOMS.

POMCa expression was higher in the group transported with 25 µL/L EOMS than in ethanol and 35 µL/L EOMS groups (Figure 3A). POMCb expression was significantly lower after transport with addition of 35 µL/L EOMS than to the BT group (Figure 3B).

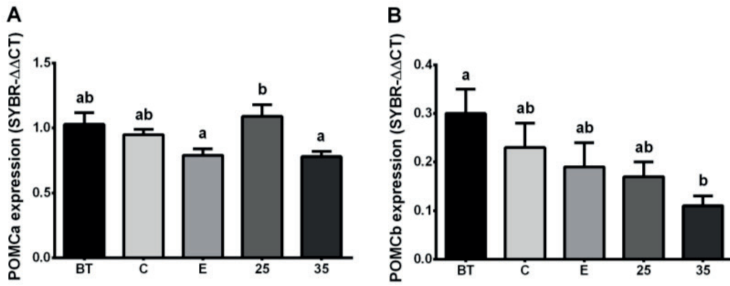

Figure 3. Pituitary POMCa (A) and POMCb (B) expression in *R. quelen* submitted to transport with the addition of EOMS. Further details as described in legend of Figure 1.

POMC is a precursor for several important peptide hormones involved in a variety of functions ranging from stress response to energy homeostasis. According to our results, POMCb is mainly involved in the endocrine response to stress, and the observed changes in its expression is related to the reduction in cortisol release after transport with the addition of EOMS. On the other hand, POMCa has different physiological roles in fish and its higher expression in individuals transported with 25 µL/L EOMS does not seem linked to stress response.

In conclusion, our results suggest that the use of EOMS can minimize stress associated with aquaculture procedures, even after a sequence of stressful events.

Acknowledgments

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CLCK GENES AS TARGET FOR FEEDING IN THE FOOD ENTRAINABLE OSCILLATOR OF LIVER IN GOLDFISH, *CARASSIUS AURATUS*

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Although daily photocycle is the most well-known synchronizer of circadian oscillators, scheduled feeding can also drive circadian rhythms, especially in peripheral tissues, as liver. However, very little is known about how food intake is detected by the hepatic clock in mammals, and nothing in fish. The objective of this study was to identify the clock genes that could be acting as possible targets for feeding signalling in the liver clock of goldfish (*Carassius auratus*). To this aim, the 24 h expression pattern of several clock genes was analyzed in the liver of goldfish. Secondly, we studied the periprandial fluctuations of hepatic clock genes in fed and unfed fish. Finally, clock genes expression in liver was analyzed in three fish groups: daily fed (1% bw), fasted (48 h), and fasted plus refed. Significant daily rhythms were found for all the studied clock genes in the liver. Results from periprandial experiment showed that expression of *per1a*, *per1b* and *rev-erbα* decreases 3 h after food intake in both experimental groups (fed and unfed fish). Thus, observed changes seem to be a consequence of their daily rhythmic profiles, regardless of feeding. However, expression of *bmal1a* increased only in fed fish, suggesting that the daily profile found in this gene is mainly due to food intake. In the last experiment, 48 h fasting decreased *per1a* and *bmal1a* expression, and this effect was reverted by refeeding, in agreement with the *bmal1a* induction by feeding observed in the periprandial experiment. Taken all together, present results suggest that *bmal1a* could be involved in the food entrainment of hepatic oscillator in fish.

Introduction

The circadian system in vertebrates consists of a net of oscillators whose molecular functioning is based on auto-regulatory feedback loops of a series of genes named clock genes (1). These oscillators can be synchronized by photocycle (Light-Entrainable Oscillators, LEO) and also by scheduled feeding (Food-Entrainable Oscillators, FEO) (1, 2, 3). To date, it is unknown the anatomical location of the FEO, but it has been suggested that it may be composed of several central and/or peripheral oscillators (1). Among such peripheral oscillators, the liver, a key organ in metabolism regulation, has been proposed as a principal element of the FEO in fish (2). However, how food intake is detected by the hepatic clock remains unclear. Therefore, the aim of this study was to identify the clock genes that could be acting as possible targets for feeding signalling in the liver clock of goldfish (*Carassius auratus*).

Materials and Methods

Three experiments were performed. In all of them goldfish were kept under a 12 h light:12 h darkness (12L:12D) photoperiod, and daily fed at *zeitgeber time* 2 (ZT2), excepting in the second experiment that fish were fed at ZT4. Fish were sacrificed and liver was sampled and stored at –80 ºC until used.

The experimental designs of the three experiments were as follow:

Daily expression pattern. Goldfish were sacrificed every 4 h through a 24 h cycle and liver was dissected.

Periprandial changes. Goldfish liver samples were obtained at 3 h before feeding and at scheduled feeding time (-3 and 0 groups, respectively). Then, some fish were fed, while others remained unfed. Liver samples were obtained at 1 and 3 h post-feeding time (+1F, +3F, +1U, +3U groups).

Fasting effects. Fish were divided into three groups: daily fed (1% bw), fasted (48 h), and fasted (48 h) plus refed (1% bw) the day of sampling. Fish were sacrificed 1.5 h after last meal (or subjective last meal in the case of fasted group) to collect the liver.

In all liver samples, clock genes (*per1a*, *per1b*, *bmal1a* and *rev-erbα*) expression was analyzed by real-time quantitative PCR (RT-qPCR), using *ef-1α* as a reference gene, and the $2^{-\Delta\Delta Ct}$ method to determine the relative mRNA expression as previously described (4). Statistical differences were determined by one-way ANOVA followed by SNK test. The significance of the rhythms (experiment 1) was also analyzed by fitting data to sinusoidal functions by Cosinor analysis.

Results and Discussion

Daily expression pattern of hepatic clock genes

The four studied clock genes showed significant daily rhythms in the liver of goldfish (Fig. 1). The acrophase of *per1a* and *per1b* rhythms occurred at the end of the darkness (ZT 19.9 ± 0.6 h and 20.2 ± 0.4 h, respectively), while *bmal1a* and *rev-erbα* peak was at the middle of the photophase and scotophase, respectively. These results confirm that *per1a* and *per1b* anticipate both, lights-on and food delivery, as previously reported (2, 4). Results also suggest that the liver is a functional oscillator, because rhythmic *bmal1a* expression is in antiphase with *per* and *rev-erbα* genes (both of which are supposed to decrease *bmal1a* expression).

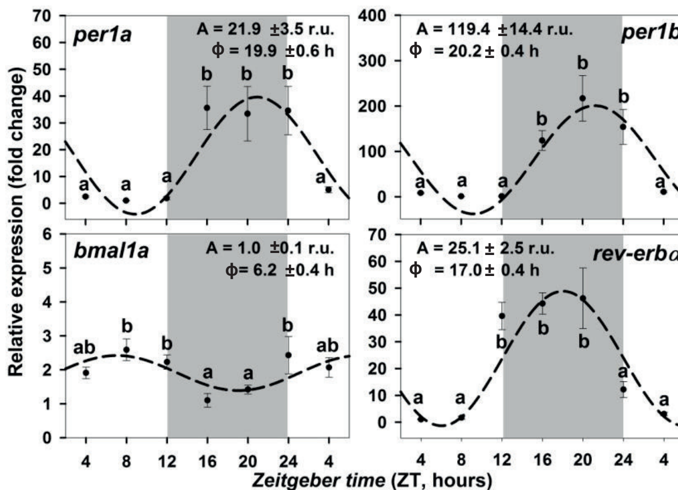

Figure 1. Data are expressed as mean ± SEM (n=6). Shaded area indicates the dark period. Dashed lines represent significant daily rhythms by Cosinor analysis. A: amplitude; ϕ: acrophase. Relative units: r.u. Different letters indicate significant differences by post-hoc analysis (SNK test, p<0.05).

Periprandial changes of hepatic clock genes

Expression of *per1a*, *per1b* (data not shown) and *rev-erbα* decreased at 3 h post-feeding in both, fed and unfed fish (Fig. 2), suggesting that such changes could

reflect their daily rhythmic profiles regardless of feeding. An increment in *bmal1a* 3 h after feeding was observed in fed, but not in unfed fish, suggesting that food could be involved in *bmal1*a induction.

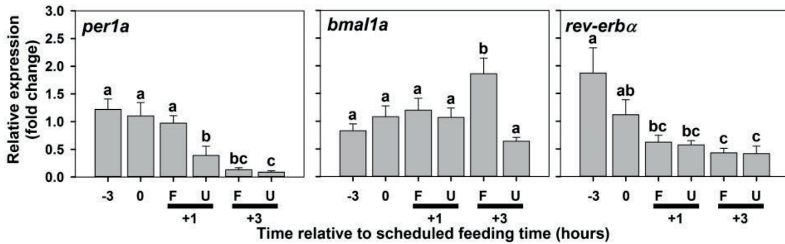

Figure 2. Data are expressed as mean ± SEM (n=6). F: fed fish; U: unfed fish. Different letters indicate significant differences by post-hoc analysis (SNK test, $p<0.05$).

Fasting effects on hepatic clock gene

Fasting (48 h) decreased *per1a* and *bmal1a* expression, and this effect was reverted by refeeding, in agreement with *bmal1a* induction by feeding observed in the periprandial experiment. Meanwhile, *per1b* remained unchanged despite the treatment (data not shown), and *rev-erbα* expression was not modified by fasting, but it was overexpressed with refeeding.

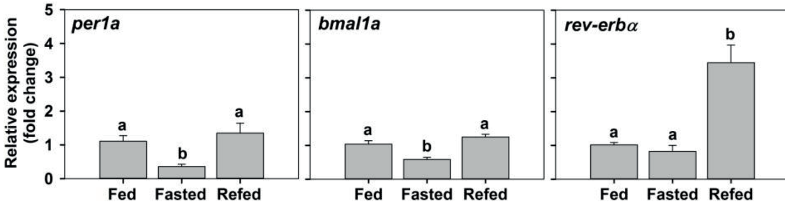

Figure 3. Data are expressed as mean ± SEM (n=6). Different letters indicate significant differences by post-hoc analysis (SNK test, $p<0.05$).

In conclusion, present results indicate that the clock gene *bmal1a* seems to be a target for feeding in the liver of goldfish, suggesting that it may be involved in the entrainment of hepatic oscillator by feeding in this teleost.

Acknowledgments

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INSULIN AND IGF-1 PLAY A RELEVANT ROLE IN THE REGULATION OF NORMAL AND TUMORAL PROSTATE CELL FUNCTION

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Obesity (OB), a multifactorial chronic endocrine-metabolic disease, represents one of the most serious global health threats. OB is associated with an increase in the incidence of certain cancer types, such as prostate cancer (PC), a hormone-related, heterogeneous, and complex cancer with a high incidence. Unfortunately, the precise molecular and cellular and endocrine-metabolic mechanisms underlying the pathophysiological association between OB and the higher incidence/aggressiveness of PC are still not well understood. Indeed, although androgens play a primary, crucial role in PC development and progression, there is growing evidence that other hormones and growth factors that are significantly altered in OB, such as insulin (Ins) and IGF-1, might also play a key role in the development and progression of PC. However, the direct and relative effects of Ins and IGF-1 or the combination of both (I+I) in controlling prostate cell function has not been completely elucidated. Here, we used normal prostate (NP) tissues and primary NP cell cultures from mice, as well as two human PC cell lines (PC3 and LNCaP), as models to evaluate, in parallel, the presence of Ins/IGF-1-axes and the direct role of Ins and IGF-1 alone or in combination. We found that IGF-1R was consistently more expressed than Ins-R in all experimental models analyzed. Remarkably, both Ins and IGF-1 treatment increased IGF-1/IGF-1R, but not Ins-R expression in prostate cells, which could be considered as an indication of PC progression. In fact, IGF-1, but not Ins or I+I, treatment was able to increase cell proliferation in PC3 and LNCap; whereas, Ins, IGF-1 and I+I treatments increased migration capacity in PC3. Interestingly, Ins and IGF-1 alone did not alter PSA secretion in LNCaP cells. Altogether, our data indicate that Ins and IGF-1 could contribute to the regulation of malignancy features in normal and tumoral prostate cells.

Introduction

Obesity (OB) is a multifactorial chronic endocrine-metabolic disease that nowadays represents one of the most serious threats for the global population (1). In addition to the well-known OB-related metabolic and cardiovascular complications, OB is also associated with increased incidence of certain cancer types (2, 3), such as prostate cancer (PC) (3, 4). In particular, PC is a heterogeneous and complex hormone-related cancer with the highest incidence in the male population (5). Unfortunately, the precise molecular/cellular and endocrine-metabolic mechanisms underlying the pathophysiological association between OB and the higher incidence/aggressiveness of PC are still not well understood. Indeed, although androgens play a primary, crucial role in PC development and progression, there is growing evidence that other hormones and growth factors that are significantly altered in OB, such as insulin (Ins) and IGF-1 (6, 7), might also play a key role in the development and progression of PC.

Materials and Methods

Mouse models. C57BL/6J male mice were obtained at weaning from Janvier Labs and housed individually. Starting at 4 weeks and until 23 weeks of age, mice were fed a high-fat (n=10; HF; fat, 60 kcal%; carbohydrate, 20 kcal%; protein, 20 kcal%) or a low-fat (n=10; LF; fat, 10 kcal%; carbohydrate, 70 kcal%; protein, 20 kcal%) diets (Research Diets). Mice were handled daily for one week before blood sampling or

sacrifice in order to acclimate them to the experimental procedures. At 23 weeks of age, mice were injected with vehicle or insulin (10 U/kg) and 8 min later, mice were killed and blood and tissues were collected for hormone and metabolite determinations.

Primary normal prostate (NP) cell cultures from mice. Prostates of 8-12 week-old C57BL/6J male mice (n=2-3 pooled/experiment, four separate experiments) were dispersed into single cells and plated at 100,000/well in DMEM with 4,5 g/L glucose and D-Valine (SeraLab), supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (Sigma), and 2mM L-glutamine at 37ºC and 5% CO_2. After a 24-h incubation, cultures were preincubated in serum-free medium for 2h, and subsequently, the medium was replaced with serum-free medium containing Ins, IGF-1 or their combination (3 wells per treatment; 10nM each). Cultures were incubated for an additional 24h and then, total RNA was extracted and PSA secretion was measured (see below).

PC derived cell lines. Two human PC cell lines were maintained in RPMI 1640 Medium (Lonza) with 1g/L glucose and supplemented with 10% FBS, 1% antibiotic-antimycotic (Sigma), and 2mM L-glutamine at 37ºC and 5% CO2.

RNA isolation, reverse transcription (RT) and qPCR. RNA was isolated [using columns (Qiagen) or TRI-reagent (Sigma)] and treated with DNAse (Qiagen or Promega) for removing genomic DNA. Total RNA (1μg) was RT with the cDNA First-Strand Synthesis Kit (Fermentas). qPCRs were run with 50ng of cDNA using the Brilliant III SYBRgreen QPCR Master Mix (Agilent) in a Stratagene Mx3000P (Agilent).

Proliferation assay. PC cell lines were plated into 96-well plates (5,000/well) and treated with Ins, IGF-1, or the combination of both and proliferation was measured at different times (24, 48 and 72 hours) using the Alamar Blue method. Absorbance was measured by the FlexStation System 3 (Molecular Devices).

Migration assay. Migration was assessed by wound healing technique. Cells were plated into 12-well plates (250,000/well) and allowed to grow for 24h in RPMI medium with 10% FBS to obtain confluence. Then, a 1-mm wide scratch (wound) was made across the cell layer using a sterile pipette tip. Wells were rinsed using PBS and subsequently, cells were incubated with Ins, IGF-1 or their combination for 14h in FBS supplemented medium. Wells were photographed immediately after scratching and after the 14h-incubation period and, wound healing was evaluated. All experiments were performed at least three times in duplicates.

PSA measurement. PSA was determined by specific ELISA (EIA-3719, DRG) following the manufacturer's instructions.

Glucose and insulin Tolerance Test (GTT and ITT). GTT (glucose: 1g/kg) were implemented at 17 week of age under 8h fasting conditions and ITT (insulin: 10 U/kg) was performed one week later under fed conditions.

Results and Discussion

IGF-1 receptor was significantly more expressed than insulin receptor in all the experimental models analyzed (mouse and human prostate cells). In addition, GH-R was also more expressed than insulin receptor in mouse tissue but not in mouse and human PC cell cultures. Interestingly, both IGF-1 and insulin treatment directly regulate the expression of IGF-1/insulin/GH receptors in mouse and human prostate cell cultures. In terms of human PC cells, IGF-1, but not insulin, treatment increased in vitro cell proliferation in PC3 and LNCap. Treatment with IGF-1, insulin and their combination (I+I) increased migration capacity in PC3 however, treatment with IGF-1 and insulin did not alter PSA secretion in LNCaP cells at 4- or 24-h.

Furthermore, to study the impact of obesity on prostate cell function, a mouse model of obesity and its respective control (lean) was generated. Obese mice had higher body weight, showed impaired glucose tolerance and insulin resistance, and had higher fat mass and lower lean mass, compared with their low fat-fed controls. Expression of specific components of the insulin/IGF-1 and GH-axes were altered at the prostate level in obese mice under basal conditions, as well as in obese mice treated with an acute injection of insulin. Specifically, expression of IGF-1 binding protein-3 was elevated while glucose transporter-4 was decreased in obese mice compared with lean-control mice. Interestingly, expression of GH and IGF-1 binding protein-3 was decreased while expression of IGF-1 receptor tends to be elevated in obese mice treated with insulin compared with vehicle-treated controls.

Altogether, the data presented in this study indicated that insulin and IGF-1 could play a relevant role in the regulation of prostate cell function under normal and obesity conditions, as well as on the control of malignancy features in prostate tumor cells.

Acknowledgments

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DIETARY Pi RESTRICTION IN SEA BASS AND MODULATION OF PHOSPHORUS AND CALCIUM INTESTINAL ABSORPTION

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In the present study we evaluated the impact of dietary available inorganic phosphorus (Pi) on growth performance, circulating Pi and Ca levels in plasma, skeletal development, mineral deposition and Pi and Ca intestinal absorption. Fish were fed diets containing low (0.3 e 0.05% diet), optimum (1.1% diets) and excess (3% diet) levels of P, and sampled at 10th and 30th days. No significant effect of different dietary P was observed on growth weight or survival rate. Deficient P diets appear to modify the incidence and type of skeletal malformations. Fish fed with 0.05% Pi diet showed a marked decreased on plasmatic Pi levels, but Ca levels were not affected in any group. Pi and Ca intestinal absorption studies showed a regional differentiation that was affected by the dietary available P. Preliminary data indicates that duodenal absorption of these two ions in different Pi dietary regimes is modified by Parathyroid hormone-related peptide.

Introduction

The endocrine factors regulating inorganic phosphorus (Pi) homeostasis in vertebrates are not completely understood and our knowledge comes from its association with calcium (Ca) where parathyroid hormone (PTH) or 1,25-dihydroxyvitamin D3 (VitD) play relevant roles. A novel factor, fibroblast growth factor-23 (FGF23) was described as a key Pi regulator, with actions on urinary Pi excretion but also on the expression of PTH and VitD. There is no information on how these factors may interplay in fish but our studies show that the Corpuscles of Stannius, the source of the anti-hypercalcemic stanniocalcin also express PTHrP and FGF23. PTHrP was shown to increase intestinal Ca uptake [1,2] but its action on Pi absorption is unclear. Contrary to Ca, Pi is limiting in seawater, and so does Pi availability determine hormonal profiles and the response of absorption/excretion mechanisms?

Materials and Methods

Dicentrarchus labrax (~80g) were allocated in 5 groups in tall 500L open system tanks and fed a commercial control diet (1.1%P) for 1 month prior to the experimental dietary regime. For the feeding trial four experimental diets containing 0.05, 0.3, 1.1 and 3.0 %P, plus the control diet, were used. Fish were fed (2-3% w/w) twice a day and the remaining pellets collected 30 minutes after feeding to establish Pi consumption and growth and feeding parameters. At 10 and 30 days into the trial 5 fishes for each group were sampled, weight and fork length were measured, blood samples were collected and evaluated for electrolytes and energetic substrates and x-ray and mineral contents were analyzed as well. Intestinal sections from fish in different conditions were dissected and mounted in Ussing chambers and uni-direccional fluxes were measured in symmetric and asymmetric conditions with or without hormonal exposure using radioactive tracers.

Results and Discussion

1. Growth performance. At day 0, the total mean wet weight of all treatments was 84±3 g, which increased to 98±4g, 126±8g, 163±9g, at 10, 30 and 70 days, respectively. During the experimental regime, no significant effects were observed on growth or survival rates by the different diets despite the huge difference in Pi intake (Fig. 1). Similar results were also report in other studies in sea bass [3]) and Atlantic salmon [4].

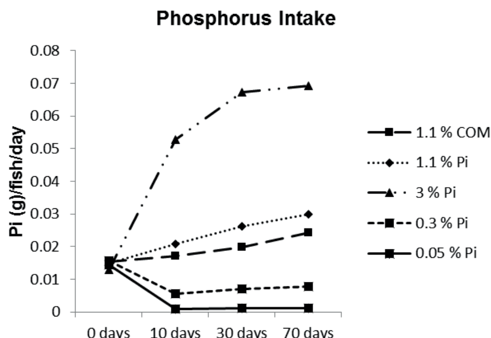

Figure 1. *Daily phosphorus intake - feed utilization of juvenile seabass (Dicentrarchus labrax) fed diets containing graded levels of phosphorus (Pi).*

2. Plasma parameters. At an initial stage of Pi dietary restriction, the most deficient diet showed a marked effect on circulating Pi with a reduction of 20% in comparison to the control diet. Meanwhile, the diet with excess Pi presented a reduction of 10%, and surprisingly, the 0.3% Pi diet presented a 20% increase of circulating Pi, perhaps due to increased absorption rate. Dietary Pi manipulations showed no effect over circulating total calcium.

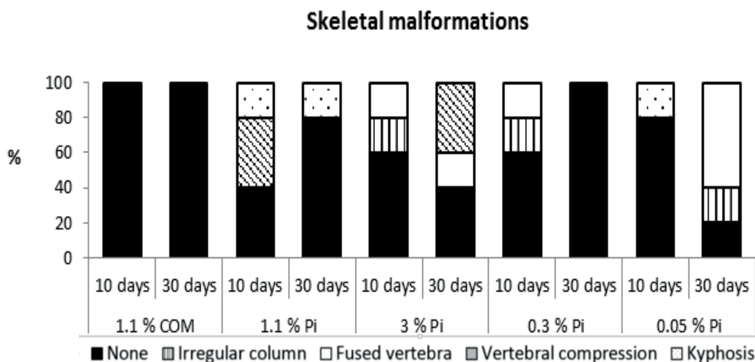

Figure 2. *Percentage of internal malformations detected per experimental diet group after examination by radiography (n = 5 individuals per sampling days).*

3. Mineralization and abnormalities. To detect morphological bone alterations, fish were x-rayed on the 10th and 30th days. Regardless the lack of effects in growth,, the experimental diets influenced the frequency of abnormalities, and prolonged dietary Pi restriction had an impact on the vertebral column morphology (Fig. 2), which is in keeping with the major role of this nutrient in mineralization [3, 4] and may indicate that bone Pi is recruited in an attempt to maintain normal circulating levels.

4. Intestinal uptake. A regional differentiation (duodenum vs middle intestine) was observed for both Pi and Ca [see 2] intestinal absorption rates in control conditions, but such difference was reduced in altered dietary conditions (Fig. 3). Preliminary data shows that PTHrP seems to increase Pi and Ca intestinal uptake as well as transepithelial resistance. Ouabain (a Na^+/K^+-ATPase inhibitor) had a delayed effect on transepithelial potential and an inhibitory effect in Pi and Ca uptake confirming the transport is Sodium-dependent.

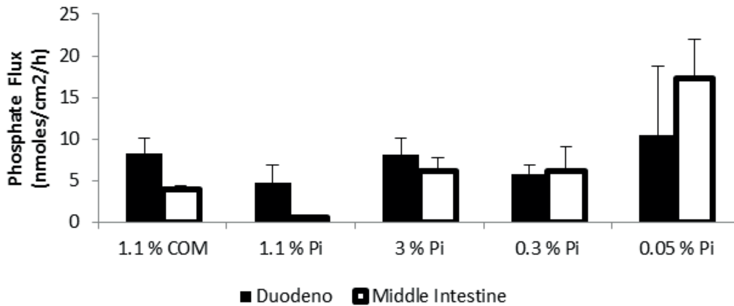

Figure 3. *Effect of dietary Pi concentration on intestinal Pi uptake after 60 minutes of the tracers' addition in two different segments of seabass' intestine - duodenum and middle intestine.*

Our study shows that despite low Pi intake fish sustain growth and quasi-normal circulating Pi levels, probably at the cost of bone mobilization skeletal malformation, and changes in intestinal absorption rates. The hormonal factors regulating Pi homeostasis are under investigation.

Acknowledgments

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EXPRESSION OF FGF23/KLOTHO SYSTEM IN THE SEA BASS: TISSUE DISTRIBUTION AND REGULATION BY DIETARY Pi

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Fibroblast growth factor-23 (FGF23) is a key regulator of phosphorus (Pi) homeostasis, secreted in response to increased serum Pi. FGF23 binds to FGF receptor 1 (FGFR1) when coupled to the transmembrane protein αKLOTHO, decreasing PTH secretion and increasing urinary phosphorus excretion. Dietary Pi imbalance produce significant alterations in expression of genes related to Pi uptake in transporting tissue such as kidney and pyloric ceaca within 10 days of treatment. A longer period seems to be necessary to induce changes in expression of genes coding for the receptors for regulatory factors such are the receptors for PTHrP (PTHR3) and the co-factor αKLOTHO, required for FGF23 activation.

Introduction

Inorganic phosphorus (Pi) is paramount for many physiological functions and an essential element in bone formation in vertebrates. Its regulation is usually associated to that of calcium (Ca), through the actions of factors such as parathyroid hormone/parathyroid hormone related protein (PTH/PTHrP), 1,25-dihydroxycalciferol (VitD) or stanniocalcin (STC) [1]. The gene coding for FGF23 exists in fish and abundant expression as been found in the Corpuscles of Stannius of the developing zebrafish [2]. However there are no functional studies to show its role in fish Pi homesotasis.

Given its crucial physiological role, abundant amounts of Pi are provided in fish feeds, which may have deleterious effects to either fish or the environment [3]. Whether the amount of Pi in feed affects the complex interaction between those endocrine factors is not clear, and such information can be useful for the production of optimized diets. Here we aimed to characterize the presence and regulation of the genes encoding the main players in Pi uptake, internal balance and excretion in the European seabass, *Dicentrarchus labrax*, fed diets differing in the amount of Pi.

Materials and Methods

Tissue distribution was performed in 3 male and 3 female sea bass (~250g). Total RNA was purified using the E.Z.N.A kit (from Omega Biotek) and quantification and quality verified by NanoDrop 1000 Spectrophotometer and it's integrity was checked in 1% agarose gel electrophoresis. cDNA synthesis was carried out by RT-PCR with Total RNA previously treated with DNAse (from Thermo Fisher Scientific). Ribolock/RevertAid from Life Technologies. Fish were pooled by gender and expression evaluated by PCR using primers designed upon the sea bass genome. For the diet experiment, fish (~85g) allocated in 5 groups fed commercial control diet: 1.1%P, and four experimental diets: 0.05, 0.3, 1.1 (control) and 3.0 % P. At 10 and 30 days into the feeding trial tissues were collected in RNA later and processed as above. Gene expression was performed by qPCR using EvaGreen® Supermix and a StepOnePlus thermocycler (Applied Biosystems).

Results and Discussion

1. Tissue distribution of Pi-related genes. FGF23 and FGFR1 (data not shown) are expressed in all the tissues analyzed but αKLOTHO expression occurs only in liver, kidney, CS, pyloric ceca and pituitary indicating these may be targeted for FGF23 since a FGFR1/ αKLOTHO complex is required for transactivation [1,2]. The sodium-phosphate cotransporter NaPi-IIa was expressed in kidney alone (Figure 1) while NaPi-IIb (not shown) presents stronger expression in the gastrointestinal tract. Both PiT-1 and PiT-2 are ubiquitously expressed, as is XPR-1, which may be in keeping with a housekeeping function for cellular Pi balance. Interestingly and quite surprisingly there is a wide distribution of FGF23.

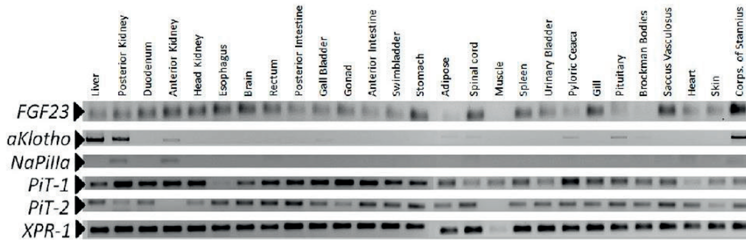

Figure 1. *Tissue distribution of expressed phosphate-regulating genes. While those relating directly to the regulation and facilitation of Pi uptake and excretion are just locally expressed, a few possible housekeeping genes show widespread expression.*

2. Dietary effects on gene expression. Different genes show modifications at different times, indicating that short-term and long-term conservatory mechanisms may be in action. PTHR3 expression is in keeping to its know roles in fish [4]. Surprisingly no evident increases in the expression of the phosphaturic factor FGF23 in CS occurred when fish were fed with an excessive Pi diet. On the contrary a marked rise in expression was seen in the Pi restricted group within 10 days of treatment (Table 1).

Table 1. *Variation in gene expression in relation to the control diet (1.1% Pi) at 10 and 30 days of feeding. (0)=no significant difference; (+)=up-regulation; (–)=down-regulation.*

		Dietary phosphate					
		0.05%Pi		0.3%Pi		3%Pi	
Gene	***Tissue***	***10 d***	***30 d***	***10 d***	***30 d***	***10 d***	***30 d***
NaPiIIa	Kidney	0	0	+	0	+	0
NapiIIb	Pyloric caeca	+	+	0	+	0	0
αKlotho	Kidney	0	0	+	0	0	0
	Pyloric caeca	+	++	0	0	0	0
PTHR3	Kidney	0	0	0	+	-	0
	Pyloric caeca	+	+	0	+	0	0
PiT-1	Kidney	0	0	0	0	0	0
	C. Stannius	0	-	0	-	+	0
FGF23	C. Stannius	+	0	0	0	0	0

Expression of the putative Pi-sensing PiT-1 [1] did not change in the kidney, but it was significant altered in the CS in response to changes in available Pi in the diet, which may indicate a role in the modulation of FGF23 and STC secretion in relation to circulating Pi.

3. **Phylogeny of fish FGF23 genes.** Annotation of piscine FGF23 is still uncertain and its homology to the mammalian protein is relatively low. Several fish show both a FGF23 and a FGF23-like gene, which may derive from the fish specific genome duplication round. No information exists on its distribution and whether all these genes express circulating proteins is still unknown.

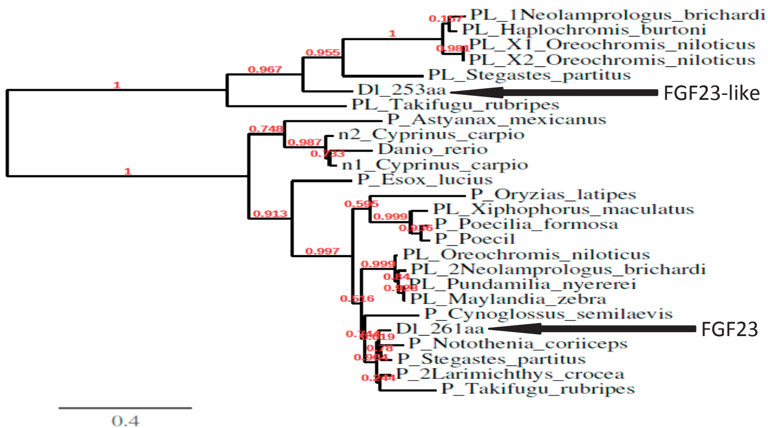

Figure 1. *Phylogenetic tree showing the relations between FGF23 protein in different fish species, showing a FGF23 and a FGF23-like in the sea bass.*

The elevated expression of FGF23 in the CS, which secretes both STC and expresses PTHrP suggests it may be a central player in Pi regulation. However its unexpected expression profiles in relation to dietary Pi are puzzling. The role of a FGF23-like gene is also unclear and is also under investigation.

Acknowledgments

This work received national funds from FCT- Foundation for Science and Technology through project grant PTDC/BIA-ANM/4225/2012. Additional support through institutional grant CCMAR/Multi/04326/2013. Thanks to Mrs Elsa Couto for laboratorial assistance, and Mr Joao Reis and Mr Cristovão Nunes for animal husbandry.

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GONADOTROPIN-INHIBITORY HORMONE IN THE FLATFISH *SOLEA SENEGALENSIS:* MOLECULAR CLONING, BRAIN LOCALIZATION AND PHYSIOLOGICAL EFFECTS

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Gonadotropin-inhibitory hormone (GnIH) is a recently discovered neuropeptide that plays a very important role in the regulation of reproduction in birds and mammals. This neuropeptide from the RFamide family has neuromodulatory functions and controls the synthesis and/or release of brain gonadotropin-releasing hormone (GnRH) and pituitary gonadotropins. Unfortunately, scientific and technological advances on fish GnIH system are, up to date, scarce, contradictory and inconclusive. For this reason, research on GnIH system appears necessary to better clarify its role in the neuroendocrine and environmental control of reproduction in this important group of vertebrates. In this context, we have cloned a full-length sequence for the GnIH precursor of Senegal sole (ssGnIH), which codifies for three putative GnIH peptides. We have also generated specific antisera against ssGnIH peptides, and used them to elucidate the localization of GnIH cells and their projections in the brain and pituitary of sole. ssGnIH-immunoreactive cell bodies were present in the olfactory bulbs, ventral telencephalon, caudal preoptic area, dorsal tegmentum and rostral rhombencephalon. These GnIH cells innervated profusely the brain and pituitary of sole. Intramuscular injection of ssGnIH-3 provokes a significant reduction in GnRH-3 (dose of 1.0 µg/g) and LH (doses of 0.1 µg/g and 1.0 µg/g) expression at 4 h post-injection. In contrast, no effect of ssGnIH-2 on the transcript levels of the main reproductive genes was evident. Our immunohistochemical and physiological results reveal that GnIH could represent an important actor in the neuroendocrine control of reproduction of sole. Further studies are being directed to elucidate if this inhibitory neurohormone is involved in the reproductive dysfunctions that occur in the F1 generation of aquacultured sole.

Introduction

Fifteen years ago, Tsutsui and co-workers discovered an avian hypothalamic dodecapeptide that suppressed the synthesis and release of gonadotropins (1). Successively, GnIH orthologs have also been identified in other vertebrate groups including fish. Previous studies in mammals (2), birds (3) and fish (4) showed that GnIH decreases GnRH and/or gonadotropin expression and protein levels. However, the reproductive effects of GnIH peptides remain controversial in fish and both stimulatory and inhibitory actions have been reported (4). Aquaculture of sole is seen as an excellent choice for diversification and economic expansion of European aquaculture. Reproduction of wild sole broodstock in captivity is successful, but sole specimens from F1 generation still present reproductive dysfunctions. In the present work, we tried to elucidate whether GnIH is present and has a role in the neurohormonal inhibition of reproduction in the flatfish *Solea senegalensis*.

Materials and Methods

Cloning strategy: PCR products obtained with degenerated primers were ligated in pSpark-TA vectors and transformed in competent *E. Coli* DHα 5 cells. Ten positive

colonies were checked. Analysis of sequences was done with Chromas Lite 2.01, Blast, Expasy Proteomics server and PhyloWin 2.0.

Generation of specific antibodies and immunohistochemistry (IHC): We have designed and synthesised two putative peptides (ssGnIH-2 and ssGnIH-3), which were used to immunize rabbits and goats and generate specific anti-GnIH antibodies. For IHC, male sole were anaesthetized in MS-222 (Sigma) and perfused via aortic bulb with 0.65% NaCl and paraformaldehyde fixative solution (4% PAF) in 0.1 M phosphate buffer pH 7.4 (PBS). Brains with pituitaries were dissected and post-fixed in 4% PAF overnight at 4ºC. Tissues were cryoprotected in 15% sucrose solution in 0.1 M PBS overnight, embedded in Tissue-Tek, frozen in cold isopentane, sectioned with a cryostat, and immunostained according to Paullada-Salmerón et al. (5).

Quantitative PCR (qPCR): Sole were injected with saline, ssGnIH-2 and ssGnIH-3 (doses of 0.1 µg/g and 1.0 µg/g body weight) and brains and pituitaries were analysed at 4 and 8 h post-injections by qPCR. qPCR was performed in a CFX Touch™ Real-Time PCR Detection System (Biorad). PCR reactions were developed in a 20µl volume using cDNA generated from 1µg of RNA. SensiFAST™ SYBR No-Rox kit (Bioline) and specific primers from sole *gnrh-1, gnrh-2, gnrh-3, kiss2, fsh, lh and gh* sequences were used. For normalization, sole *βactin-2 and elongation factor* were used as housekeeping gene. The relative mRNA expression was determined by the ΔΔCt method.

Results and Discussion

Cloning and expression of GnIH precursor: In the present study, we have cloned a full-length sequence for the GnIH precursor of Senegal sole. As in other teleosts (4), the sole GnIH precursor contains three putative GnIH peptides exhibiting MPMRF-a, MPQRF-a and LPXRF-a motifs, respectively. GnIH precursor from sole is positioned in a GnIH branch close to teleost fish LPXRFa sequences. The results obtained by RT-PCR in the brain of sole specimens evidenced a conspicuous expression of GnIH precursor in the diencephalon, and a much lower expression in other central areas analysed.

Brain localization of GnIH cells and fibers: Specific antisera generated against ssGnIH peptides have permitted us to reveal the precise localization of immunoreactive (ir) GnIH cells and fibers in the brain of sole. GnIH-ir cells were found in the terminal nerve, ventral telencephalon and preoptic area, three regions that have been related with reproductive process (Fig. 1A). Moreover, we localized GnIH-ir cells in the mesencephalic tegmentum and the isthmic region of the rhombencephalon. These GnIH-ir cells innervated profusely the preoptic area, hypothalamus, optic tectum, torus semicircularis and caudal tegmentum, but conspicuous projections were also evident in the olfactory bulbs, ventral and dorsal telencephalon, habenula, ventral thalamus, pretectal area, rostral tegmentum, posterior tuberculum, reticular formation and vagal lobe. The retina, pineal organ, vascular sac and pituitary (Fig. 1B) were also targets of GnIH-ir fibers. A similar pattern of GnIH distribution was reported in the brain and pituitary of sea bass (5). Distribution of sole GnIH-ir cells and fibers suggest that this neurohormone might also be involved in the neuroendocrine and photoperiodic control of other functions such as food intake, growth and behaviour, as it has been reported in other vertebrates (4).

Physiological effects of ssGnIH: In the present study we have evidenced the inhibitory role of GnIH in the reproductive axis of sole. Thus, ssGnIH-3, but not ssGnIH-2, decreased *gnrh-3* (Fig. 1C) and *lh* (Fig 1D) mRNA expression at 4 h post-

injection. Previous studies performed in fish have shown that the GnIH system plays a significant role in the regulation of gonadotrophin secretion (3-5). However, both stimulatory and inhibitory effects have been reported, and the nature of these effects seems to vary considerably depending on the species, the physiological status, and the route of administration of the GnIH peptide (3, 4). The presence of GnIH cells in the same regions where GnRH-3 cells are placed and the fact that GnIH fibers innervate profusely the preoptic area and the hypothalamus, also reaching the adenohypophysis of sole, allow us to suggest that GnIH could be controlling the reproductive process in this species by acting at both brain and pituitary level.

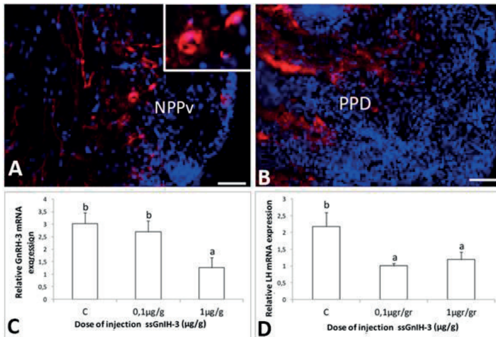

Figure 1. A. GnIH- immunoreactive cells from the posterior periventricular nucleus (NPPv). **B.** GnIH fibers entering the proximal pars distalis of the adeno-hypophysis, PPD. Scale bars = 100 µm. **C, D.** ssGnIH-3 effects on GnRH-3 (C) and LH (D) mRNA levels determined at 4 h post-injection in male sole. (C: control group, NaCl 0.7%). Different letters indicate statistically significant differences (ANOVA, $p<0.05$).

Acknowledgments

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THE ONTOGENY OF SOLE (*SOLEA SENEGALENSIS*) NEUROENDOCRINE SYSTEMS IS AFFECTED BY ENVIRONMENTAL AND CULTURE CONDITIONS.

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Information on how environmental and culture conditions affect the establishment of fish reproductive systems is rather scarce. In this study, sole larvae were exposed to: 1) daily thermocycle and blue light (18-21ºC; TCblue) or, 2) constant temperature and white light (19.5ºC; cteW; routine aquaculture conditions). The TCblue conditions were applied in different time frames: 1) until metamorphosis, 2) post-metamorphosis or, 3) gonad differentiation. In addition, larval development was compared between two culture systems, intensive (routine aquaculture) *vs* mesocosm. Effects were determined by analyzing gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH) and kisspeptin gene expression by qPCR. Results showed that under routine aquaculture conditions, developing sole exhibited higher GnIH transcript levels in critical periods (sex differentiation) compared to specimens reared under daily thermocycles and blue light. Mesocosm conditions also affected GnIH and GnRH-3 ontogenetic expression patterns.

Introduction

Aquaculture of sole is seen as an excellent choice for diversification and expansion of the European aquaculture. Reproduction of wild sole breeders in captivity is successful whilst F1 specimens present reproductive dysfunctions. Reproduction is under the control of environmental (light and temperature) and endocrine factors produced along the brain-pituitary-gonad axis. But information of how environmental and culture conditions affect the ontogenetic establishment of fish reproductive systems is rather scarce. Light information is a crucial environmental factor that controls the onset of neuroendocrine machinery during fish development. In marine environments blue lights reach deep waters, where flatfish species as sole inhabits. Previous studies highlight the important role of blue light as a factor improving early fish development [1,2]. Nowadays, aquaculture practices are conducted under established protocols that, in most cases, differ notably from those found in the nature. As a result, these unnatural conditions could alter the optimal development of the cultivated species. Therefore, to mimic natural conditions for each species should represent one of the main objectives of sustainable aquaculture.

Materials and Methods

Experiment on thermophotocycles: Sole eggs were collected after spawning during the night and subdivided in different groups that were acclimated to routine aquaculture conditions of light-dark (LD) 12L:12D cycles of white light (LDW) and constant temperature (19.5ºC), or different time frames (AZ15: 15 days post hatching dph, AZ30: 30 dph and AZ100: 100 dph) of 12L:12D cycles of blue light (LDB, λ peak=463nm) and daily thermocycles (18ºC-21ºC), and later transferred to light-dark cycles of white light until 170 dpf. Sampling points (0, 2, 6, 12, M1, M2, M3, 26, 51, 79, 107, 117, 127, 138, 149 and 170 dph) were carried out at ZT4 (n=6; larvae pools=2-20).

Experiment on mesocosm: Sole eggs were collected after spawning during the night and acclimated to two culture conditions. First group was cultured (duplicate) under routine conditions, in 450 L tanks, density of 40 larvae L^{-1} and natural illumination supplemented with white fluorescent tubes. Second group was cultured (duplicate) under mesocosm conditions, in 2500 L tanks with "green water" supplemented with a mixture of natural zooplankton, a density of 7 larvae L^{-1} and natural illumination enhanced with blue LEDs. Mesocosm conditions were maintained until weaning (64 dph) and then, routine aquaculture conditions were used till the end of the experiment (147 dph). Sampling points were at 0, 4, 12, 15, 18, 22, 29, 56, 84, 98, 119, 126, 140 and 147 dph.

Quantitative PCR: PCR reactions were developed in a CFX Touch™ Real-Time PCR Detection System (Biorad) in 20µl volume using cDNA generated from 1µg of RNA. SensiFAST™ SYBR No-Rox kit (Bioline) and specific primers from sole *GnIH, GnRH-2, GnRH-3 and Kiss2* sequences were used. For normalization, sole *βactin-2 and Elongation factor* were used as housekeeping gene. The relative mRNA expression was determined by the ΔΔCt method.

Results and Discussion

Effects of thermophotocycles: GnIH, GnRH-2 and GnRH-3 presented similar ontogenetic expression patterns in all conditions. We found higher GnIH levels under routine conditions (LDW), whilst animals reared under blue lights (AZ15, AZ30, AZ100) and daily thermocycles exhibited lower GnIH expression in critical ontogenetic periods such as sex differentiation (Fig. 1). GnRH-2 and GnRH-3 expression was unaffected by the culture conditions tested. Previous studies in zebrafish and sole have shown the impact of photoperiod, light spectra and daily thermocycles on hatching rhythms, larval performance and sex differentiation [1-4]. In addition, GnIH has been involved in the regulation of reproductive development and maintenance in birds and mammals [5]. Taken together, these results highlight the important role of natural light and environmental cycles during fish development, which shaped the daily rhythms in embryo and larvae and, ultimately, can influence sex differentiation and reproductive performance, at least in part by the actions of GnIH.

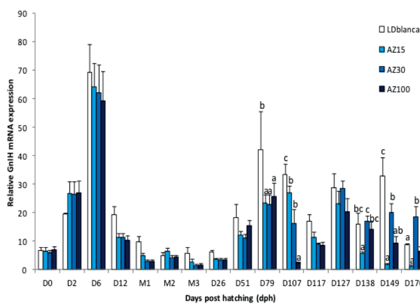

Figure 1. Relative mRNA expression of GnIH under different culture conditions. Different letters indicate significant statistical differences between conditions in the same day (ANOVA $p<0.05$). M1, M2 and M3 refer to early, mid and final metamorphosis, respectively.

Effects of culture conditions: Under mesocosm conditions, the rise of GnIH and GnRH-3 expression was advanced compared to routine intensive conditions (Fig. 2). Mesocosm determined lower GnIH mRNA levels during metamorphosis and sexual differentiation periods (Fig. 2). A role of GnIH in metamorphosis has not yet been reported, but it was previously shown that the thyroid and GnIH systems interact to control seasonal reproduction in photoperiodic animals [5,6]. Whether both hormonal systems are also cooperating in the modulation of sole metamorphosis remains to be deciphered. In addition, increasing evidences suggest a role of GnIH in the regulation

of reproductive development and maintenance in birds, mammals and fish [5]. In fish, it has been proposed that GnIH and its receptor are involved in sex reversal from female to male in protogynous hermaphroditic orange-spotted grouper [7]. The potential effects of the observed reduced GnIH levels during sex differentiation on reproductive development and sex ratio should be elucidated.

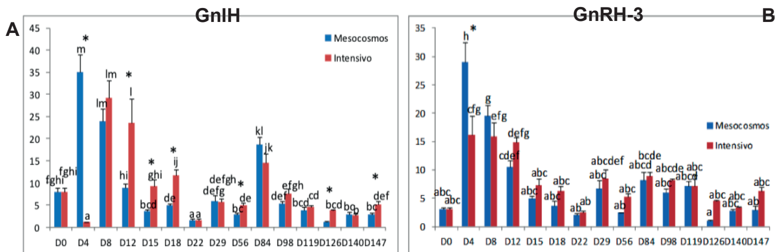

Figure 2. Relative mRNA expression of GnIH (A) and GnRH-3 (B) under mesocosm (blue bars) and routine intensive conditions (red bars). Different letters indicate significant statistical ANOVA differences ($p<0.05$). Asterisks (*) denote significant statistical differences between groups.

In conclusion, our results show that light and temperature have remarkable effects in the development of GnIH systems of sole. Fifteen days of light-dark 12L:12D cycle of blue wavelengths and daily thermocycles (AZ15) are able to modify GnIH gene expression during sole development. Our results evidence that routine conditions used in aquaculture practice can disturb the natural organization of sole neuroendocrine systems, reinforcing the importance of mimicking natural environmental conditions during fish rearing. These evidences could underlie the reproductive dysfunctions of F1 soles in aquaculture.

Acknowledgments

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ONTOGENY OF LIGHT-SENSOR SYSTEMS OF SENEGAL SOLE EXPOSED TO DIFFERENT LIGHT PHOTOPERIOD AND SPECTRA

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The light that penetrates into the seawater column changes in intensity and wavelength due to selective absorption. As a result, shorter and longer wavelengths are absorbed in the upper waters, while the green and blue lights reach greater depths. The Senegal sole undergoes a real metamorphosis between days 12 to 19 after hatching and during this process it changes its life habits from pelagic to benthonic and from diurnal to nocturnal. In the present study we investigated the expression of four classes of opsins (green sensitive cone opsin, red sensitive cone opsin, UV opsin and teleost multiple tissue opsin) during the development of this flatfish using qPCR. The eggs were collected immediately after spawning during the night and exposed to light-dark (LD)12L:12D cycles of white (LDW), blue (LDB, λ peak = 463 nm), red (LDR, λ peak = 685 nm), continuous white light (LL) or continuous darkness (DD). Our results show a peak of expression of green sensitive cone opsin between day 6 and 12 in all the conditions, decreasing thereafter. Red opsin expression shows a peak at day 12 in all the conditions and decreases during metamorphosis, but in LL, LDW and LDB it increases again in post-metamorphosis (day 25). UV opsin expression presents a first peak at day 1 in all the conditions, increasing during metamorphosis in LDB, LDW and LL and decreasing in post-metamorphic animals (day 25). Finally, TMT opsin transcript levels peaked at day 12 (onset of metamorphosis) in all conditions.

Introduction

Light is one of the most important environmental signals in nature. It has a strong influence on the life cycle of the living organisms and actually it regulates some really important biological processes, including circadian clock functions and vision. In the underwater environment, the light spectrum changes radically depending on the depth. In fact, some wavelengths of light, which include UV, red and yellow/orange colors, are absorbed in the surface water, whereas green and, in particular, blue wavelengths of light reach deeper depths [1]. During their life cycle, fishes can change their habitats and go through different developmental stages exposed to different light environments [2]. In *Solea senegalensis* these changes are related with a metamorphic process that occurs between day 12 and 19 after hatching, in which animals change their life habits from pelagic to benthonic and their behaviour from diurnal to nocturnal [3]. The first step in light perception is light detection, which is promoted by photopigments. A photopigment is composed of a protein called opsin and a chromophore derived from vitamin A named retinal. When retinal absorbs a photon of light, it undergoes a change in his molecular shape and it allows the activation of a G protein, which mediates an enzymatic cascade that permits to transform the light signal in an electrical signal. In the present study we have evaluated the expression of four classes of opsins: green sensitive cone opsin, red sensitive cone opsin, UV opsin (Opn5 or neuropsin), and teleost multiple tissue (TMT) opsin during the development of the flatfish *Solea senegalensis*.

Materials and Methods

The experiment was carried out at the IFAPA Centro El Toruño (Puerto de Santa María, Spain). Fertilized eggs were collected in complete darkness and divided into 5

experimental groups: 12L:12D cycle with red (LDR, λ peak = 685 nm), blue (LDB, λ peak = 463 nm), or white (LDW) lights, constant white light (LL) and constant dark (DD) conditions. The larvae of each group (n=4) were sampled at two different zeitgeber time points, ZT4 (12 h) and ZT16 (24 h), at 1, 6, 12, 15, 19 and 25 days post fertilization (dpf). Primers for green sensitive cone opsin, red sensitive cone opsin, TMT and UV opsins were designed from the sequence available in Solea database, using Primer3 software. Total RNA was extracted using TRIsure Reagent® (Bioline, London, UK), cDNA synthesis was performed using TAKARA PrimeScript™ RT Master Mix and relative expression was carried out using Bio-Rad CFX96 TOUCH detection system.

Results and Discussion

In this study we have shown significant developmental differences in the four opsin classes analysed in *Solea senegalensis* (Fig. 1). Although at low levels, all opsins were expressed from the first day of embryonic development in all tested conditions, confirming that sole embryo is light sensitive before the differentiation of any photoreceptor structure such as the pineal organ and retina [4,5]. Green, red and TMT opsins showed high expression levels during 12 dpf, coinciding with the beginning of metamorphosis (Fig. 1), suggesting a key role of these photopigments in the onset of this crucial process. In contrast, UV opsin displayed a high expression levels during 1 dpf, significantly decreasing until the beginning of metamorphosis and reaching the highest levels at metamorphic end (Fig. 1). The high expression levels of UV found at 19 dpf could be related with the increase of thyroid hormones and their receptors, which also reach maximum levels at this stage in sole [6].

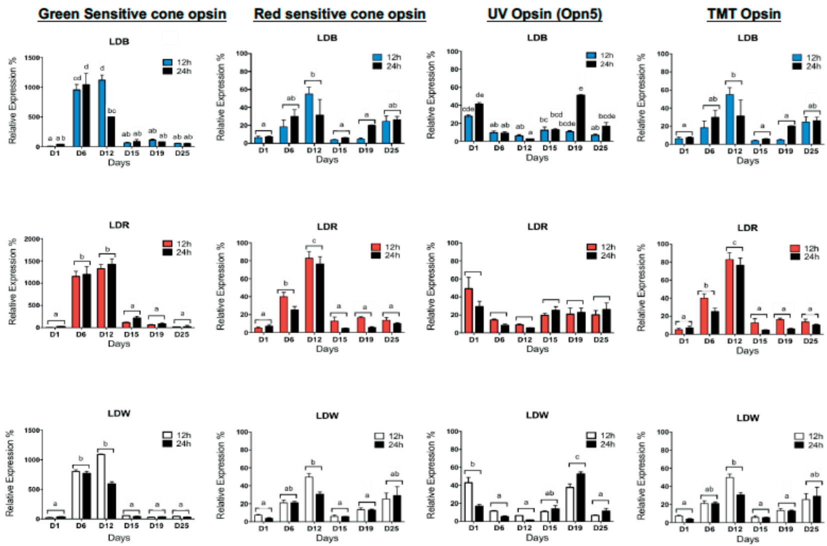

Figure 1. *Relative expression of green cone, red cone, UV and TMT opsins during Senegalese sole development. Statistical analysis was performed by two-way ANOVA. The black lines above the bars indicate a non-significant correlation between the two factors. Different letters indicate statistically significant differences (ANOVA, p*<0.05).

According to this hypothesis, it was demonstrated that, in salmonids, the degeneration and regeneration of UV-photoreceptors is controlled by thyroid hormones [7]. In addition, in Pacific salmon all single cones express a UV sensitive opsin at hatching, and these cones switch to a blue light sensitive opsin during the juvenile period [8]. UV photoreception can also contribute and improve prey search behaviour [9], and could have an important role in the activation of DNA repair enzymes during early life stages of of sole, as it has been suggested previously [10]. In conclusion, our results show a differential pattern of expression of different opsins before, during and after the completion of metamorphosis in *Solea senegalensis* and suggest that its bottom settlement is accompanied by changes in its photopigments.

Acknowledgments

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PHOTOPERIOD MODULATES THE REGULATION OF *KISS1* AND *GNRH2* NEURONAL EXPRESSION

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In this study, precocious 1 year-old male European sea bass (*Dicentrarchus labrax*) were exposed to three different photoperiod conditions, natural photoperiod (NP), advanced photoperiod (AP) and continuous light (LL). The obtained results indicated that AP and LL were able to modify the rhythms of both *kiss1* and *gnrh2* mRNA levels in the forebrain-midbrain (FB-MB), suggesting that the photoperiodic signalling may modulate the activity of the habenular Kiss1 and the synencephalic Gnrh2 neurons to drive the reproductive axis. Therefore we hypothesize that very likely, as a result of the modulatory role of the photoperiod on the Kiss1 and Gnrh2 activity, the hormone profiles may be shifted/modified and hence advance the onset and progression of gametogenesis under AP, whereas under LL, the disrupted production of reproductive hormones could be limiting the stimulation of germ cell proliferation at the testicular level and thus prevent the normal progression of the spermatogenesis.

Introduction

There are a wide variety of environmental factors that change seasonally and that may modulate the activity of the reproductive axis. Among them, photoperiod is the main environmental signal that most temperate fish species use to predict the changing seasons and therefore to anticipate spawning time through activation of neuroendocrine pathways which, in turn, stimulate gonadotropic axis and gonadal growth. However, many molecular and endocrine mechanism involved in the onset of puberty and the influence of environmental conditions, such as photoperiod signalling, are not well understood in fish. This study aims to contribute to this understanding by investigating the expression profiles of kisspeptin and Gnrh systems in the brain in correlation with sexual hormones in reproductively advanced (exposed to AP) or suppressed (exposed to LL) male European sea bass populations.

European sea bass precocious males are significantly larger than non precocious ones, during their first year of life. In this way, it has been demonstrated that to be precocious male fish must exceed a critical size threshold, while individuals below this limit remain immature until the next reproductive season [1]. Taking advantage of this particularity we hypothesize the that the kisspeptin system acts as a link mediating the response between the light signal and the reproductive axis and thus, differential expression patterns shall be evident between AP and LL treatments during the onset of gametogenesis in large 1 year-old male European sea bass (presumably precocious).

Materials and Methods

Fish were maintained under natural temperature (40º LN) and three different photoperiod treatments in triplicate: a) Natural photoperiod (NP), b) Advanced photoperiod (AP) consisting of a combination of constant long days (15 hours light: 9 hours dark; 15L:9D) in April in otherwise constant short days (9D:15D) and c) Continuous light (24h light day^{-1}; LL) all year round. Group NP represents a positive

control indicating the dynamic of the neuroendocrine cascade under natural conditions. AP provides a sharp photoperiod signal triggering and synchronizing the activity of the reproductive system, allowing the study of the photoperiod effects on early molecular and later developmental events in preparation for puberty. LL provides a negative control since constant light will inhibit testicular recrudescence during the first year. Fish were sampled regularly except the LL group in early October, when some animals exhibited bacterial infection. At each sampling point a sub-sample of large fish (presumable precocious) was sacrificed and samples from forebrain-midbrain (FB-MB) were collected for gene expression. Tissue was frozen in liquid nitrogen and stored at -80ºC until use for total RNA extraction.

Results and Discussion

The histological data show that, in early September, an important number of precocious fish under AP had testicles in stage II of development. Only testicles in stage I were observed in either NP or LL regimes at this time. Continuous light prevented spermiation and thus no further progression beyond stage III was observed in testicles of LL treated fish (**data no shown**). In agreement with these results, AP treatment advanced spermatogenesis and spermiogenesis, while LL prevented early puberty in larger males.

On the other hand, the present study reveals a strong relation between environmental factors (photoperiod) that control the onset of puberty and the Kiss1 and Gnrh2 systems in agreement with previous findings in European sea bass [2, 3]. In this way, AP and LL were able to modify the yearly expression rhythms of both *kiss1* (**Fig. 1**) and *gnrh2* (**Fig. 2**), such that, in large fish these were advanced in AP and completely abolished in LL, compared to their NP counterparts. *Kiss1* expressing cells are located in the habenula [2] a region of the FB-MB linked to the pineal organ by the pineal stalk [4]. Given that sea bass is a highly seasonal species, it seems reasonable to suggest that a Kiss1 neuronal population from the habenular region could likely be involved in the photoperiodic modulation of reproductive function. Moreover, previous studies done by Servili et al., [3] clearly demonstrated the role of Gnrh2 in the modulation of the pineal function in European sea bass. Accordingly, our results provide functional evidence that the photoperiodic signalling may modulate the activity of the habenular Kiss1 and the synencephalic Gnrh2 neurons to drive the reproductive axis.

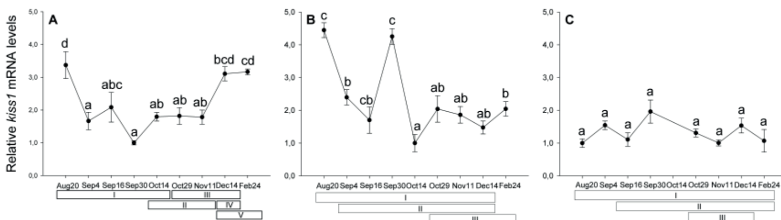

Figure 1. Profile of *kiss1* expression in the FB-MB of large male sea bass kept under NP (**A**), AP (**B**) and LL (**C**). Data are expressed as the mean ± SEM. Distinct lowercase letters show differences between sampling points.

Finally, AP advanced the phase of the Fsh and 11-KT rhythms by one month compared to NP, while the profiles of these hormones in the LL group were similar to those of the two other groups but with lower levels (**data no shown**). This is a relevant aspect since Fsh plays an important role in the regulation of the early

phases of gametogenesis [5] and 11-KT is the most important androgen for stimulating spermatogenesis in several fish species [6], including European sea bass.

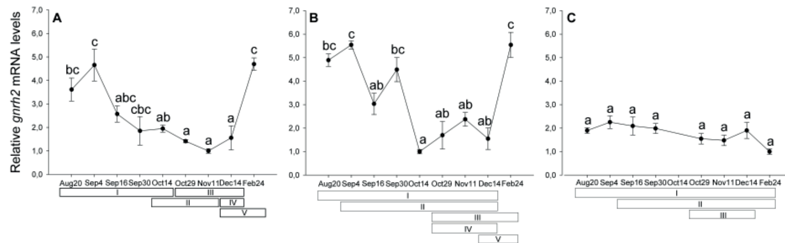

Figure 2. Profile of *gnrh2* expression in the FB-MB of large male sea bass kept under NP (**A**), AP (**B**) and LL (**C**). Data are expressed as the mean ± SEM. Distinct lowercase letters show differences between sampling points.

Altogether, these data indicate that very likely, and as a result of the modulatory role of the photoperiod on Kiss1 and Gnrh2 activity, the hormone profiles may be shifted/modified and hence advance the onset and progression of gametogenesis under AP, whereas under LL, the disrupted production of the reproductive hormones, could be limiting the stimulation of germ cell proliferation at the testicular level and thus prevent the normal progression of the spermatogenesis.

Acknowledgments

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ANOPHELES MOSQUITO SPECIATION MODIFIED ALLATOSTATIN-TYPE A RECEPTOR (AST-AR) GENE STRUCTURE

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Anopheles mosquitoes are known for their role as vectors for malaria transmission. Many Anopheles mosquito species have been identified and their widespread distribution in the globe is associated with their rapid rate of speciation and adaptation to different environments. In this study we report how Anopheles mosquito speciation has impacted on the evolution, structural organization and possibly function of the Allatostatin type-A (AST-A) G-protein coupled receptor (GPCRs) genes. In Diptera duplicate AST-ARs (AST-AR1 and AST-AR2) with divergent gene structures and functions co-exist and in *Anopheles coluzzii* they were recently suggested to play a role in reproduction. AST-AR genes were retrieved from 15 Anopheles genomes and receptor gene structures characterized and compared. Analysis revealed that paralogue receptors have a different organization and in *Anopheles gambiae* the structure of the AST-AR1 and AST-AR2 genes diverges from that of other mosquitoes. In Anopheles, the AST-AR2 gene has been highly modified as a result of exon tandem duplication and exon inversion events.

<u>Introduction</u>

Anopheles mosquitoes have a worldwide distribution as a consequence of their ability to rapidly adapt to different environments (1,2). More than 450 different Anopheles species exist in the world and speciation has generated several complexes of morphologically identical species with rapidly evolving genomes, modified reproductive behavior and distinct vectorial capacities as Malaria parasite transmitters. Anopheles mosquitoes emerged approximately 100 million years ago and speciation was fueled by distinct ecological adaptations (1). The process of speciation has had an impact on mosquito genome architecture and gene content and they have been used as models to understand how the environment and geographic isolation favours speciation at the genome level (1,2).

Allatostatin type A receptors (AST-ARs) are a group of invertebrate G-protein coupled receptors that are activated by FGL-amide (AST-A) peptides to inhibit food intake and development in arthropods (3). In most arthropods a single AST-AR gene exists but in fruit flies and mosquitoes two AST- ARs (AST-AR1 and AST-AR2) are present and arose by a gene duplication event (4). The insect AST-AR genes share a common ancestral origin and potentially overlapping functions with the Kiss and Galanin receptor family that play a key role in the regulation of metabolism and reproduction in vertebrates (4). The two AST-AR receptors in *A. gambiae* (PEST strain) have a different gene organization and both map to chromosome 2R that has suffered strong natural selection (2). In the present study we report how Anopheles speciation has affected the AST-ARs gene structure by isolating and characterizing the genes and comparing them across the anopheles species with a sequenced genome.

Materials and Methods

The mature protein sequences of the duplicate *Anopheles coluzzii* AST-ARs were used to search for homologues in the 15 different Anopheles mosquito species genomes in VectorBase (https://www.vectorbase.org/, accessed in March 2015). Homologue receptor exons were identified by sequence similarity and the predicted mature protein was retrieved and the mosquito AST-AR gene structures were deduced. Multiple nucleotide and amino acid sequence alignment were performed using ClustalW (v2) (http://www.genome.jp/tools/ clustalw/) software and the percent of amino acid sequence identity of the receptors core (the 7 transmembrane domains including intra- and extracellular regions) was calculated using GeneDoc (5).

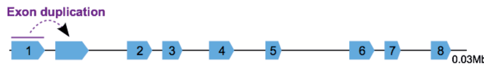
Results and Discussion

Homologues of the *A. coluzzii* AST-ARs were identified in all Anopheles mosquito genomes analyzed and they are highly conserved. In all the mosquito genomes, a putative AST-AR1 gene was identified and the predicted mature protein sequences shared 91-100% amino acid sequence identity. A homologue of the *A. coluzzii* AST-AR2 gene was only retrieved from 9 mosquito genomes and their deduced mature protein sequence shared 82-100% amino acid sequence identity. The failure to identify an AST-AR2 gene in some mosquito genomes is most likely due to their incomplete genome assemblies.

A) **AST-AR1**

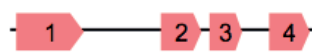
Consensus in most *Anopheles* species

Modified in ***A. gambiae***



B) **AST-AR2**

Consensus in most *Anopheles* species



Modified in ***A. gambiae***, ***A. arabiensis*** and ***A. quadriannulatus***

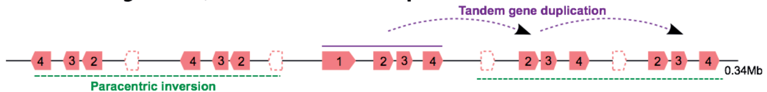


Figure 1. Comparison of the Anopheles AST-AR gene structures. The genome events that may have contributed to modify the structure of AST-AR1 and AST-AR2 gene locus are indicated. In AST-AR2 the duplicate exons are of identical size but degeneration of duplicate exon 1 seems to have occurred (dashed boxes). Exons are represented by colored boxes and are numbered and introns and intergenic regions are represented by lines. The genome region where the AST-AR genes are localized is indicated (Mb). Exon arrowheads indicate the putative orientation for transcription. The figure was not drawn to scale.

Comparisons of AST-AR gene structure between anopheles species confirmed that, in common with *A. gambiae* (PEST strain), the duplicate receptors for a given species had a divergent gene structure but orthologue gene organization was maintained across the Anopheles mosquito genomes. This suggests that both genes evolved under distinct evolutionary pressures after they emerged (Fig 1). The exceptions were AST-AR1 and AST-AR2 in the genome of *A. gambiae* and AST-AR 2 in the genomes of *Anopheles arabiensis* and *Anopheles quadriannulatus*, where extra exon copies were found (Fig 1). The consensus sequence of AST-AR1 in Anopheles mosquitoes consists of 8 exons although tandem duplication of exon 1 occurred in *A. gambiae*. The consensus gene structure of AST-AR2 in Anopheles consists of 4 exons but in the genomes of *A. gambiae*, *A. arabiensis* and *A. quadriannulatus* multiple tandem exon copies and gene paracentric exon inversions were found suggesting that in these species AST-AR2 was under a high pressure to modify. Duplication and translocation events generated 4 incomplete degenerate copies of exon 1 but 4 identical copies of exon 2, 3 and 4 (Fig 1). This suggests that if multiple AST-AR2 transcripts are produced in these species they will all share exon 1. Our study provides genomic evidence that Anopheles AST-ARs genes evolved under distinct selective pressure and that in some mosquito species receptor gene structure was highly modified. This potentially has an impact on the physiological role of the AST-A system in the regulation of mosquito reproduction and suggests the AST-A system may have species-specific roles.

Acknowledgments

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DIFFERENTIAL IMMUNOLOCALIZATION OF SOMATOLACTIN ALPHA IN PITUITARY OF *CYPRINUS CARPIO*

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Somatolactin (SL), a fish specific pituitary hormone belonging to growth hormone (GH) and prolactin (PRL) superfamily, is involved in background adaptation, osmoregulation, reproduction and fatty acid metabolism where effects of potential endocrine disruptors such as estrogenic compounds, are poorly understood. In carp, transcripts of two *sl* genes, α and β, were detected in pituitary gland and only *sl*β increased in adult male in response to 17β-estrogen treatment *in vivo* as shown by RT-qPCR. With the aim to assess anatomically specific expression, a carp SLα specific antibody was raised against an oligopeptide based on the diverging derived amino acid sequence from *sl*α and *sl*β *in silico* cloned gene sequences. Immunohistochemical analyses in adjacent sagittal sections of carp pituitary revealed that the anti-cSLα serum clearly immunodetected a subset of cells in *pars intermedia* (PI) close to *proximal pars distalis* (PPD), compared to the extensive area of immunoreactive cells in PI when applying a coho salmon SL antiserum, that does not distinguish SL variants. The spatially distinct immunodetection in PI of pituitary gland suggests differential expression of SLα thus contributing a morphological clue regarding subfunctionalization of SLα and SLβ in carp.

Introduction

Biologically relevant effects of endocrine disruptor compounds in the aquatic environment imply changes of gene expression that we address in pituitary gland, a central regulatory organ at the interface between the central nervous system and endocrine system (1). SL, a piscine hypophyseal hormone closely related to GH/PRL, is expressed in *pars intermedia* (PI) of pituitary gland (2). As well as in several bony fish species, two active genes *sl*α and *sl*β were identified in carp that responded differentially at the transcriptional level to *in vivo* treatment with 17β-estrogen in adult male carp (1).

Materials and Methods

An antibody specific for carp SLα was raised against the synthetic oligopeptide NH_2-LIYLQTTLNRYDDAPK-COOH comprising amino acids 126-140 from the derived amino acid sequence of *in silico* cloned carp SLα gene (GenBank Acc.No. ADE60529.2) (1) and a lysine added at the carboxyl terminus to facilitate coupling of the 16-mer to carrier protein hemocyanin (*Concholepa concholepa*, Biosonda) thus increasing immunogenicity (3). Total protein extraction, Dot Blot, Western Blot (12% SDS-PAGE), immunohistochemical analyses were performed as described (3). Immunoreaction was visualized with Universal Dako labeled Streptavidin-Biotin2-System (LSAB-HRP Kit, DAKO). Anti coho salmon SL serum was kindly provided by Dr. Mariann Rand-Weaver.

Results and Discussion

Although SLα and β showed only 46.2% identity of the derived amino acid sequences (Fig. 1A), number and position of cysteine residues was conserved, except third cysteine outside the predicted signal peptide which does not participate in intramolecular bisulfite bonds (2). Protein modeling predicted the four antiparallel helical core structure, up(H1)-up(H2)-down(H3)-down(H4), typical for members of class I cytokines, including GH and PRL (4).

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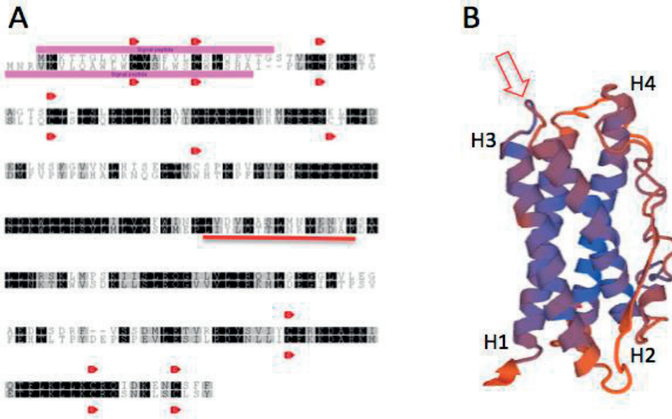
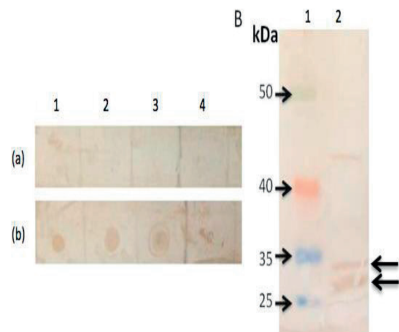


Figure 1. Design of cSLα specific oligopeptide. (A) Alignment of derived amino acid (aa) sequences cSLβ (230 aa, acc. no: AAY45791.2, first line) and cSLα (234 aa, acc. no: ADE60529.2, second line), background color highlights identical (dark) and homologous (grey) aa; at amino terminus signal peptide underlined pink; red arrowheads indicate cysteine residues. (B) SLα predicted 3D structure (SWISS-MODEL). Location of oligopeptide sequence employed for antibody production is underlined in red (A) and depicted with red arrow (B).

Immunoblot analyses confirmed specificity of anti-cSLα (Fig. 2A). The two bands immunodetected in pituitary protein extract (Fig. 2B, arrows) correspond to the expected size of cSLα and an isoform possibly glycosylated at the consensus N-glycosylation site (${}_{145}$N-K-T$_{147}$) as described in other teleost (4).

Figure 2. (A) Characterization of anti-cSLα antibody. (a) rabbit pre-serum, (b) anti-cSLα serum (1:500) on dot blot of (1) cSLα oligopeptide, (2) cSLα oligopeptide linked to hemocyanin carrier protein; (3) carp pituitary total protein; (4) bovine serum albumin (BSA, negative control). (B) Western blot analysis with anti-cSLα (1:500), (1) prestained protein molecular weight marker (Invitrogen), (2) pituitary total protein of *Cyprinus carpio.*



In fish pituitary gland cells are seggretated according to the specific hormone produced (2, 3, 4). Indeed, with anti-coho salmon SL and anti-cSLα immunorection occured exclusively in PI (Fig. 3). However, cSLα reactivity was found to be present in a cell population located in a sub-region of PI (Fig. 3B). In goldfish, on the other hand, anti-SLβ exhibited a restricted immunolocalization in PI bordering PPD in pituitary sections (4), which might be due to difference in genetic, age or other ambient influences (5), since our experiment was performed in adult male *C. carpio* compared to juvenile sexually undifferentiated *Carassius auratus* (4).

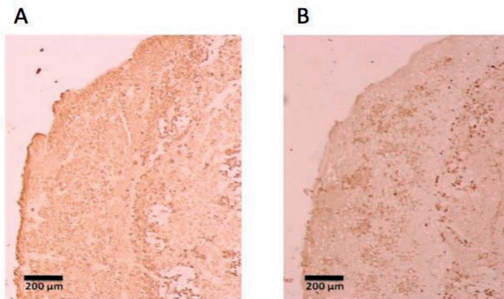


Figure 3. Differential spatial immunodetection of SLα in pituitary of *C. carpio.* SL was immunodetected in *pars intermedia* (PI) in adjacent sagittal sections of adult carp pituitary with antibody (A) anti-coho salmon SL, (B) anti-cSLα in a subset of cells in PI.

The differential expression might reflect subfunctionalization of duplicated *sl* genes playing a role in regulation of various physiological processes, which could be related to particular cell populations in the pituitary gland of *C. carpio*.

Acknowledgments

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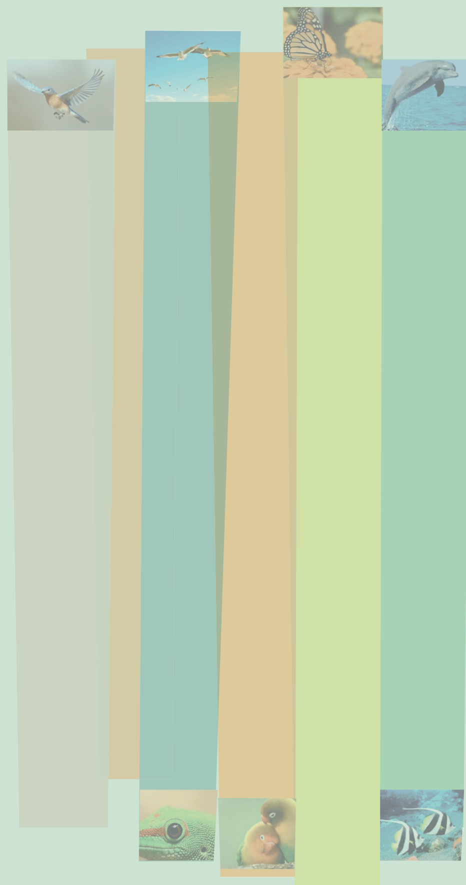

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