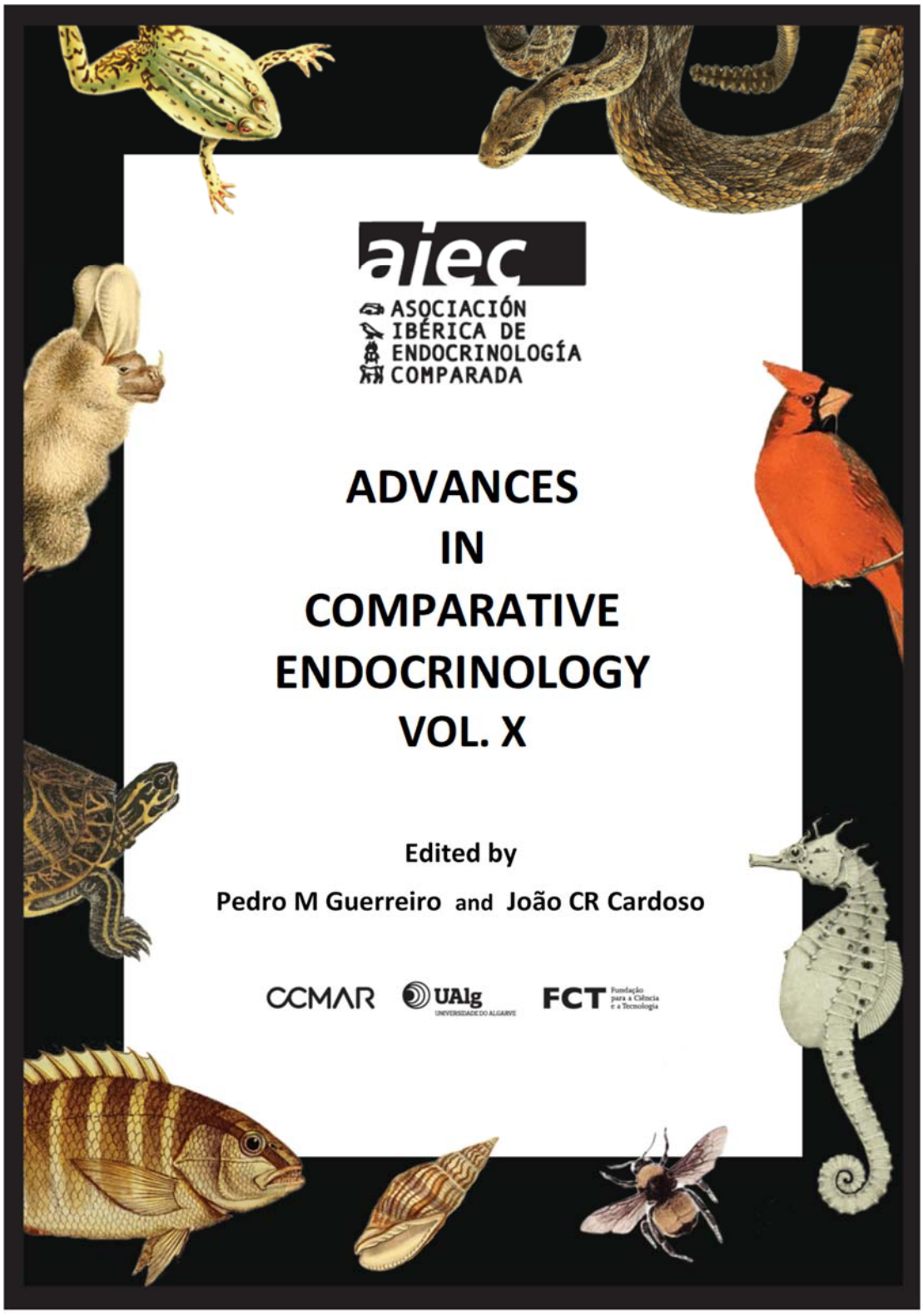


aiec

ASOCIACIÓN IBÉRICA DE ENDOCRINOLOGÍA COMPARADA

ADVANCES IN COMPARATIVE ENDOCRINOLOGY VOL. X

Edited by

Pedro M Guerreiro and João CR Cardoso

CCMAR UAlg UNIVERSIDADE DO ALGARVE FCT Fundação para a Ciência e a Tecnologia

ADVANCES IN COMPARATIVE ENDOCRINOLOGY

VOL. X

ASOCIACIÓN IBÉRICA DE ENDOCRINOLOGÍA COMPARADA
ASSOCIAÇÃO IBÉRICA DE ENDOCRINOLOGIA COMPARATIVA
(AIEC)

CCMAR UAlg UNIVERSIDADE DO ALGARVE FCT Fundação para a Ciência e a Tecnologia

Title: Advances in Comparative Endocrinology: Vol. X

Proceedings from communications presented at the *XII Conference of the Iberian Association for Comparative Endocrinology (AIEC),* held from the 26th to the 28th of September 2019 at the University of Algarve, Faro, Portugal

Editors: Pedro M. Guerreiro e João C. R. Cardoso

Scientific Committee:

Isabel Navarro, *Universidad de Barcelona, Spain*
Jose Luis Soengas, *Universidad de Vigo, Spain*
Francisco Prat Bella, *Instituto de Ciencias Marinas de Andalucía, Spain*
Juan Miguel Mancera, *Universidad de Cadiz, Spain*
Pedro Miguel Guerreiro, *Centre of Marine Sciences, Portugal*
Luz Pérez, *Universidad Politecnica de Valencia, Spain*
Francisco Javier Sánchez-Vásquez, *Universidad de Murcia, Spain*
Jaume Pérez-Sánchez, *Instituto de Acuicultura Torre de la Sal, Spain*
João C. Cardoso, *Centre of Marine Sciences, Portugal*
Adelino V. M. Canário, *Universidade do Algarve, Portugal*
Deborah M. Power, *Universidade do Algarve, Portugal*

Edition: Universidade do Algarve Editora

1st Edition

Place of Edition: Faro

Edition Date: 2021

Graphic Design and Pagination: Barbara Pintar, P.M.Guerreiro

Printing: Secção de Reprodução Documental da Universidade do Algarve

ISBN: 978-989-9023-41-3 (printed version)

Legal Deposit: 479798/21

ISBN: 978-989-9023-31-4 (electronic version)

DOI: http://dx.doi.org/10.34623/q8db-sg56

Available at: http://hdl.handle.net/10400.1/14988


aiec
ASOCIACIÓN IBÉRICA DE ENDOCRINOLOGÍA COMPARADA
CCMAR
Centro de Ciências do Mar
12th Conference of the Iberian Association for Comparative Endocrinology
26th-28th September 2019, Faro, Portugal

IN MEMORIAN

RAFAEL MARTÍNEZ PARDO

24 March 1950 - 08 September 2020

Rafael Martínez Pardo was a founding member of the AIEC and appointed as the first secretary of the Directive Board, a position he held from 1998 to 2011, taking one of the most laborious jobs in moving AIEC forward.

As Secretary of the AIEC (until his retirement), he participated in six directive boards. During this time, he worked to make the Association, and its mission, known in both Spain and Portugal: to promote the research in comparative endocrinology, and to be a forum for established and young scientists.

His goal was to have Iberian conferences where students and young researchers would have not just the opportunity but indeed the primacy to present their studies, in a friendly and supporting environment. Twelve editions of AIEC conferences and this X volume, like the previous 9 volumes of Advances in Comparative Endocrinology, show he was successful.

PTO →

Rafael was a Doctor in Biological Sciences, and his interest in the physiology and particularly in the endocrinology of insects, began during his years at the Entomology Department, New York State Agricultural Experiment Station, Cornell University, USA, first in 1976 as a predoctoral fellow and in later 1983-1984 as an Assistant Professor.

He then returned to a long and fruitful career in Spain. In addition to its merits as a Professor of Physiology for over 28 years, at the University of Valencia, researching and publishing new discoveries on the biology and endocrinology of insects, Rafael had just initiated a path as a fiction novelist.
So, in many ways, his legacy will endure.

Thank You Rafa!

CONTENTS

PRESENTATION

This is the 10th volume of Advances in Comparative Endocrinology, a series of books that collect the communications presented at the congresses of the Iberian Association for Comparative Endocrinology (AIEC). This round number marks roughly 20 years since the foundation of AIEC and the beginning of the biennial meetings organized by AIEC affiliated groups. In 2005, our Comparative Endocrinology and Integrative Biology group CEIB-CCMAR organized the 5th AIEC meeting and it was a pleasure to welcome the 12th AIEC Congress back again to Faro, Portugal, in 2019 (26-28 September).

Volume X includes contributions resulting from plenary, keynote, oral or poster presentations, and authored by almost 150 senior or young scientists and students, which show the latest advances in the fields of reproduction, energy metabolism, genomics, evolution, stress, immune response and growth in both vertebrates and invertebrates. Studies range from fundamental questions on endocrinology to environmental challenges, animal production and welfare, or the mechanisms of disease, and reach beyond the Iberian borders. In addition to Portugal and Spain, the science presented here comes from institutions in Canada, Chile, France, Norway and USA. About 30% of the communications result from international cooperation, and over 55% were direct collaborations between institutions. AIEC aims to promote collaborations and its congresses are a privileged stage for networking.

Many communications were wonderfully presented by PhD and MSc students, and postdoctoral fellows, in line with AIEC efforts to promote the younger generations of Iberian scientists. Our congratulations to all, and specially to the recipients of the best presentation awards. However, we cannot leave unnoticed the excellent plenary talks, which encompassed insects, fish and humans. The prestigious *Prof. Josep Planas Lecture* was presented by Prof. Xavier Bellés Ros (Institut de Biologia Evolutiva, CSIC-Univ. Pompeu Fabra, Barcelona, Spain) on the fascinating endocrine regulation of metamorphosis and physiology of the cockroach. The captivating opening lecture on mammalian reproduction and cancer was delivered by Prof. Silvia Socorro (Health Sciences Research Centre, Univ. of Beira Interior, Portugal). In the closing lecture Prof. José Antonio Muñoz-Cueto (Instituto Universitario de Investigación Marina, Univ. of Cádiz, Spain), and former AIEC secretary, enthusiastically spoke on the contributions of fish models to the knowledge of neuroendocrine control of reproduction.

We would like to acknowledge the help of the AIEC board, the scientific committee and the local committee that made this meeting possible. We are also grateful to Andreia Pinto and Barbara Pintar (CCMAR) and all our colleagues at CEIB for their valuable assistance in the organization, and to University of Algarve for the excellent infrastructures made available for the meeting. We also thank Câmara Municipal de Faro and the Municipal Museum for the welcoming reception.

Finally, we are grateful to all the contributors for their active participation and the interesting discussions during the sessions. We hope that this publication stimulates participation in AIEC, to improve research and promote fruitful collaborations in comparative endocrinology. As we finish this text during the 2020 pandemics, times are uncertain, and more than ever we wish all the best to our colleagues that will organize our next meeting, the 13th AIEC, to be held in the Basque Country, Spain.

The Editors
Pedro Miguel Guerreiro and João Carlos dos Reis Cardoso

THE RELATIONSHIP OF CALCIUM-BINDING PROTEIN REGUCALCIN WITH CANCER AND MAMMALIAN REPRODUCTION

L. Fonseca, S. Correia, C.V. Vaz, S. Socorro

CICS-UBI - Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal.

Regucalcin (RGN) was first identified as a highly conserved calcium (Ca^{2+})-binding protein that is present throughout the evolution line, from prokaryotes to eukaryotes. The RGN protein is an X-chromosome gene product, whose transcription is regulated by a myriad of hormonal and non-hormonal factors. RGN is highly expressed in the liver and kidney of mammalian vertebrates, but its broad expression in a panoply of reproductive and non-reproductive tissues has been reported. Interestingly, RGN expression is downregulated with aging, and for this reason, it is also known as senescence marker protein-30. RGN plays a relevant role in the maintenance of intracellular Ca^{2+} concentration, but its actions in the regulation of several pathways apart from this ion homeostasis were being unveiled over the years. Research work developed by our research group and others has established RGN as a multifunctional protein involved in the regulation of intracellular signaling pathways, oxidative stress, cell proliferation, apoptosis, and energetic metabolism. Overall, these are all basic biological processes recognized as hallmarks of cancer, which are also known as tightly regulated events crucial for the successful production of male gametes. This has propelled research towards clarifying the RGN role in male reproduction and cancer. The experimental evidence indicating RGN as a new tumor suppressor protein and its association with the cancer hallmarks will be presented. The RGN function as a germ cell protective agent supporting spermatogenesis and sperm function also will be discussed.

Introduction

Regucalcin (RGN) was first described by M. Yamaguchi as a calcium (Ca^{2+})-binding protein (1). Despite not having the typical EF-hand Ca^{2+}-binding motif, RGN has been shown to bind this cation and plays a relevant role in the maintenance of intracellular Ca^{2+} levels, and in the regulation of Ca^{2+} signaling. The action of RGN in Ca^{2+} homeostasis occurs through the modulation of Ca^{2+}-pumps activity at the plasma membrane, endoplasmic reticulum and mitochondria (2). It was also reported that RGN could bind other divalent cations, such as Zn^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+}, which in rodents was related with different levels of its activity as a gluconolactonase, catalyzing one of the last steps of ascorbic acid synthesis (3).

More recently, RGN has been implicated in the regulation of basic biological processes, such as cell survival and growth, and for this reason, its role in tissue homeostasis has been proposed (4-6). Moreover, RGN displays a protective role against oxidative stress (OS) and damaging factors (4,5), and its function in the regulation of energy metabolism also has been described (4). All these processes, cell survival, OS and metabolic reprogramming underlie the development of tumour cells, representing some of the well-known cancer hallmarks (7), which raises the curiosity about the protective role of RGN in tumorigenesis. On the other hand, ensuring germ cell survival and their

metabolic support, as well as maintaining appropriate levels of OS are vital requisites for a successful spermatogenesis and male fertility. Indeed, the last years, have witnessed the emergence of RGN as a reproduction-related protein (8), with a likely protective role for male germ cells.

Concerning the tissue expression of RGN, it is highly expressed in the liver, where it was first described, but its expression in several other tissues has been characterized. RGN is detected in a wide variety of reproductive and non-reproductive mammalian tissues (reviewed by (5)). The tissue expression levels of RGN have been shown to be regulated by several factors displaying organ- and species-specific patterns. Moreover, RGN also displays a characteristic down-regulated expression with aging (4,5), and because of that it is also called senescence marker protein-30 (SMP30).

Besides aging, other physiological and pathological conditions have been associated with the altered expression of RGN. Deregulated expression of RGN has been described accompanying the carcinogenic process of several tissues. RGN expression is down-regulated in a set of animal and human cancers, being the loss of RGN expression directly correlated with tumour onset, progression, aggressiveness and degree of cellular differentiation (4,5). On the contrary, *in vivo* overexpression of RGN markedly suppressed the development of carcinogen-induced rat mammary gland tumors (9).

Overall, there is a substantial amount of evidence supporting the protective role of RGN against cancer development. In the same way, the role of RGN in male reproduction and sperm function started to be disclosed. The main facts associated with the RGN role in reproduction and cancer are discussed.

Regucalcin: from gene to protein

The *RGN* gene is localized in the p11.3-q11.2 and q11.1-12 segments of the human and rat X chromosomes, respectively (8). The X chromosome is widely known to encode several crucial genes for male fertility, and this particular location of the *RGN* gene is one of the facts that supports the interest in studying this protein in male reproduction.

The transcription of the *RGN* gene is regulated by several transcription factors, and other molecular players and physiological conditions, such as sex steroid hormones, OS and Ca^{2+} (4,5). The activator protein 1, the nuclear factor1-A1, and the *RGN* gene promoter region-related protein p117 are among the most well-known transcription factors regulating RGN expression (reviewed by (10)).

The mRNA product of the human *RGN* gene encodes a protein with 229 amino acid residues and an estimated molecular weight of 33 kDa. Besides the full-length RGN mRNA, two alternatively spliced mRNA variants have been identified for RGN (8). The RGN $\Delta 4$ transcript is originated by the deletion of exon 4 and gives rise to a protein of 227 amino acid residues with a molecular weight of approximately 25 kDa. Similarly, the RGN $\Delta 4,5$ arises from the deletion of exons 4 and 5 and is predicted to originate a protein with 183 amino acid residues and approximately 20 kDa. These spliced variants have been described in several healthy human tissues, namely, liver, kidney, brain, lung, breast, prostate and testis (8,11), which indicates that alternative splicing is a normal event for the *RGN* gene, occurring constitutively.

It has also been reported that the full-length RGN protein undergoes proteolytic processing originating 28 and 24 kDa proteins, which were identified in the rat liver, kidney and liver human cell lines (12), and in human liver (11). However, in the human kidney, only one protein with 27 kDa arises from the proteolytic processing of the full-length RGN (11). The functional role of the RGN mRNA and protein variants remains largely unknown. However, Yamaguchi *et. al.*

demonstrated that the effect of RGN in regulating proliferation and death of human pancreatic cancer MIA PaCa-2 cells is exclusively due to the full-length RGN protein (13).
Bioinformatics analysis demonstrated that the RGN protein is highly conserved along the evolution line, from prokaryotes to eukaryotes (8). 18 % of the RGN protein residues are conserved in all species and, for example, human and bacteria RGN share between 22-32% of overall homology, which is significantly high (5,8). These findings strongly indicate that RGN should have an important role in cell physiology. Indeed, relevant evidence has been showing that RGN has a cytoprotective role contributing to tissue homeostasis, as discussed below.

Regucalcin and the hallmarks of cancer

Tumor onset and progression are promoted by distinct but inter-related processes, namely, the continuous increase of OS, self-sustained proliferation and resistance to apoptosis, activation of angiogenesis and migration/invasion pathways, as well as by the metabolic reprogramming (7). In the last years, the role of RGN in regulating several of these cancer hallmarks started being disclosed. For this, much has contributed the diverse studies using *in vitro* and *in vivo* models. Cell lines models stably or transiently overexpressing, or knocked-down for RGN, have demonstrated to be good tools to ascertain this protein's role. A transgenic rat model overexpressing RGN (Tg-RGN), developed by M. Yamaguchi through oocyte transgene pronuclear injection (14), was made commercially available, and has been used to address the biological role of RGN, and its association with the cancer hallmarks (4,5).
The protective role of RGN against OS has been proposed. RGN was capable of decreasing the production of reactive oxygen species and lipid peroxidation in HepG2 cells, and in rat heart and liver, through the increase of the antioxidant capacity and enhancement of the activity of antioxidant enzymes, such as superoxide dismutase (4,5). In addition, experiments in rat tissues and cell lines have reported that RGN inhibits the nitric oxide synthase (4,5). A study made in Tg-RGN rats reported that RGN could also counteract the increase of OS characteristic of aging by increasing the antioxidant capacity and glutathione-S-transferase (GST) activity (4). Studies using a knockout model linked the antioxidant role of RGN with its gluconolactonase activity (15), which is somehow predicted as ascorbic acid is a powerful antioxidant molecule.
Besides being involved in the regulation of intracellular Ca^{2+} levels, RGN also controls cell survival and growth. RGN has been described as a protein distributed to distinct cellular compartments, namely cytoplasm, mitochondria, perinuclear space, and nucleus (5). Although the mechanisms that underlie the translocation of RGN to the nucleus still are unknown, RGN' actions controlling cell proliferation and apoptosis have been closely associated with its nuclear localization, as discussed below.
In vitro and *in vivo* studies in different types of tissues have shown that RGN overexpression strongly suppresses cell proliferation (4-6). Related to this fact was the RGN regulation of the cytoplasmic protein turnover by inhibiting protein synthesis and increasing protein degradation. Overexpression of RGN in cell lines also inhibited DNA and RNA synthesis. Moreover, RGN overexpression was concomitant with the diminished expression levels of several oncogenes, for example, c-src, c-myc, c-kit, PI3K, Akt, Ras, MAPKs, or β-catenin; and with the augmented expression of tumour suppressor genes, such as p21, p53 and retinoblastoma protein (4-6).
RGN was also implicated in the suppression of migration, invasion and metastatic potential of cancer cells. Reduced migration was reported in liver

(HepG2), lung (A549) and pancreatic (MIA PaCa-2) cancer cell lines overexpressing RGN (13,16,17). In human cervical adenocarcinoma HeLa cells RGN suppressed migration, invasion and metastization (18). Moreover, overexpression and silencing experiments showed that these RGN effects are mediated through the inhibition of epithelial-mesenchymal transition and Wnt/β-catenin signalling, a signalling pathway that induces the expression of metalloproteases (18).

Several metabolic pathways are known to be altered in cancer cells, and the reprogramming of energy metabolism is recognized as a cancer hallmark since 2011 (7). RGN was also identified as a putative metabolic regulator having a suppressive action in glycolysis by inhibiting pyruvate kinase, an effect that was demonstrated in rat liver (19). *In vivo* overexpression of RGN also diminished the expression of glucose transporter 3 and phosphofructokinase, as well as lactate dehydrogenase and monocarboxylate transporter 4 in rat prostate, with a reduction in lactate production and export (4). Further studies are needed to establish a liaison between the metabolic actions of RGN and cancer. However, all these findings demonstrate the RGN's role suppressing glycolytic metabolism, which can be seen as a protection against the development of the hyper-glycolytic phenotype that characterizes tumour cells (4,20).

Overall, the broad action of RGN counteracting several cancer hallmarks supports the role of this protein as a tumour suppressor protein.

Regucalcin' actions in male reproduction

The localization of *RGN* gene at the X chromosome, as well as the description of the RGN protein in the male reproductive tract, and its identification as a sex steroid-target gene (4,5,8), have encouraged the study of the RGN' role in testicular function and male reproduction.

Relevant information about the RGN actions associated with spermatogenesis and sperm function have resulted from the use of Tg-RGN rats. Although presenting lower sperm counts and reduced sperm motility, Tg-RGN rats are fertile, and the epididymal sperm collected from these animals displays higher viability and diminished incidence of tail defects (21). Therefore, the diminished sperm counts found in Tg-RGN seem to be counterbalanced by the better performance of spermatozoa, do not affecting the fertility potential of these animals.

It is during the transit through the epididymis, mainly in the caput and corpus regions (proximal), that sperm undergo a set of events necessary for acquiring fertilization potential (22). Histological and functional analysis of the epididymis of Tg-RGN rats also provided some clues for the RGN actions in sperm physiology. The morphology of the epithelium in the caput region differs in Tg-RGN and WT rats (21). Tg-RGN animals present a lower height of caput epithelial cells, indicating that the secretory and resorptive activity of this area could be altered. Indeed, a reduced capacity of Ca^{2+} influx was observed in the epididymis of Tg-RGN rats. As a consequence, Ca^{2+} concentration was increased in the epididymal fluid of Tg-RGN, which was suggested to be the cause for the reduced sperm motility observed in these animals (21).

Oxidative stress has been identified as one of the major causes of male infertility since it contributes to germ cell death and increased incidence of defects in the germ line (23). The anti-oxidant role reported for RGN has also been observed in reproductive tissues. Lower levels of OS were found in the testis and epididymis of Tg-RGN rats(21,24) , and overexpression of RGN exerted a protective effect against OS in rat testicular cells, likely by increasing the anti-oxidant defences, namely the expression levels of GST (24). Also, it was

demonstrated that RGN overexpression alleviates the age-related decline in sperm quality, concomitantly with the suppression of OS (25).
Germ cells are also highly sensitive to apoptotic cell death induced by endogenous and exogenous damaging factors (26). The anti-apoptotic role of RGN has been shown in several *in vitro* and *in vivo* models (4,5,27), which prompted the study in testicular cells. Thapsigargin- and actinomycin D-induced apoptosis was suppressed in the seminiferous tubules of Tg-RGN rats, which depended on the modulation of the expression and activity of key regulators of apoptosis (28). Moreover, lower apoptotic rates were found in the testis of Tg-RGN rats compared with WT counterparts when exposed to radiation (29). Based on these findings, the protective role of RGN against apoptosis in the testis has been proposed.
After ejaculation, and following the maturation in the epididymis, mammalian sperm undergo a set of biochemical, structural and physiological changes that allow fertilization. This process, known as capacitation, occurs in the female reproductive tract, after the removal of the decapacitation factors from the seminal plasma, and is determinant for the subsequent acrosomal reaction, and ovum fertilization. An anti-capacitation role for RGN was proposed by Pillai *et. al*., because RGN is degraded in the buffalo's ejaculated seminal plasma (30). In addition, the same study showed that the percentage of capacitated spermatozoa is significantly reduced in the presence of recombinant RGN. The role of RGN as an anti-capacitation factor is also supported by the reduced motility of epididymal spermatozoa found in Tg-RGN rats (21).

Conclusion

RGN was initially identified as a Ca^{2+}-binding protein playing a role in Ca^{2+} homeostasis, but researchers rapidly understood the multifunctional role of this protein. Actually, it is widely accepted that RGN controls several processes essential for tissue homeostasis, namely cell proliferation, apoptosis, OS and metabolism. Also, protecting against the age-related deterioration of cell function.
The loss of RGN expression in human cancer cases, correlated with tumour aggressiveness and degree of differentiation, together with its capacity in suppressing the cancer hallmarks, supports the role of RGN as a tumour suppressor gene. Moreover, considering the panoply of hormonal and non-hormonal factors that regulate RGN expression, the discovery of mechanisms that maintain RGN tissue levels, or the development of methodologies for use RGN as a therapeutic tool, are exciting fields of research.
Similarly, the beneficial role of RGN in male reproduction, counteracting oxidative damage, protecting germ cells from apoptosis, and improving sperm function, suggests that it can be used, for example, in assisted reproductive technologies. This is supported by the recent findings demonstrating that RGN improved the cryopreservation of buffalo's spermatozoa, which opens new avenues of research in the cryopreservation of human spermatozoa.
In sum, the actions disclosed for RGN in pathological and non-pathological conditions, render this protein an exciting and promising molecular tool both in cancer research and in male fertility.

Acknowledgements: This work was supported by FEDER funds through the POCI-COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I - Strengthening research, technological development and innovation (Project No. 007491 and 029114) and National

Funds by FCT-Foundation for Science and Technology, Portugal (Project UID/Multi/00709/2013).

References

1. Yamaguchi M, Yamamoto T. (1978). Chem Pharm Bull (Tokyo). 26,1915-8
2. Yamaguchi M. (2011). Mol Cell Biochem 353,101
3. Chakraborti S, Bahnson BJ. (2010). Biochemistry 49,3436-44
4. V Vaz C, Correia S, J Cardoso H, I Figueira M, Marques R, J Maia C, Socorro S. (2016). Curr Mol Med 16,607-19
5. Marques R, Maia CJ, Vaz C, Correia S, Socorro S. (2014). Cell Mol Life Sci 71,93-111
6. Yamaguchi M. (2015). J Cancer Res Clin Oncol 141,1333-41
7. Hanahan D, Weinberg RA. (2011). Cell 144,646-74
8. Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Sousa Md, Barros A, Socorro S. (2011). Mol Hum Reprod 18,161-70
9. Marques R, Vaz CV, Maia CJ, Gomes M, Gama A, Alves G, Santos CR, Schmitt F, Socorro S. (2015). Exp Cell Res 330,325-35
10. Yamaguchi M. (2011). Mol Cell Biochem 346,147-71
11. Murata T, Yamaguchi M. (2014). Int J Mol Med 34,1141-6
12. Arun P, Aleti V, Parikh K, Manne V, Chilukuri N. (2011). PLoS One 6,e16545
13. Yamaguchi M, Osuka S, Weitzmann MN, El-Rayes BF, Shoji M, Murata T. (2016). Int J Oncol 48,1955-64
14. Yamaguchi M, Morooka Y, Misawa H, Tsurusaki Y, Nakajima R. (2002). J Cell Biochem 86,520-9
15. Koike K, Kondo Y, Sekiya M, Sato Y, Tobino K, Iwakami S-i, Goto S, Takahashi K, Maruyama N, Seyama K. (2010). Am J Physiol Lung Cell Mol Physiol 298,L784-L92
16. Yamaguchi M, Osuka S, Shoji M, Weitzmann MN, Murata T. (2017). Mol Cell Biochem 430,37-46
17. Yamaguchi M, Osuka S, Weitzmann MN, El-Rayes BF, Shoji M, Murata T. (2016). Int J Oncol 49,1686-94
18. Li X, Huang Y, Guo S, Xie M, Bin X, Shi M, Chen A, Chen S, Wu F, Hu Q. (2019). Oncol Lett 18,609-16
19. Yamaguchi M, Shibano H. (1987). Chem Pharm Bull (Tokyo). 35,2025-9
20. Warburg O, Wind F, Negelein E. (1927). J Gen Physiol 8,519
21. Correia S, Oliveira P, Guerreiro P, Lopes G, Alves M, Canário A, Cavaco J, Socorro S. (2013). Mol Hum Reprod 19,581-9
22. Cornwall GA. (2009). Hum Reprod Update 15,213-27
23. Bisht S, Faiq M, Tolahunase M, Dada R. (2017). Nat Rev Urol 14,470-85
24. Correia S, Vaz CV, Silva AM, Cavaco JE, Socorro S. (2017). J Appl Toxicol 37,159-66
25. Silva A, Correia S, Vaz C, Socorro S, Maia C. (2018). J Sex Med 15,S324
26. Gunes S, Al-Sadaan M, Agarwal A. (2015). Reprod Biomed Online 31,309-19
27. Yamaguchi M. (2013). Apoptosis 18,1145-53
28. Correia S, Alves M, Oliveira P, Alves M, Van Pelt A, Cavaco J, Socorro S. (2014). Andrology 2,290-8
29. Silva AM, Correia S, Casalta-Lopes JE, Mamede AC, Cavaco JE, Botelho MF, Socorro S, Maia CJ. (2016). Life Sci 164,31-41
30. Pillai H, Shende AM, Parmar MS, Kumaresan A, Bhure SK. (2017). Mol Reprod Dev 84,212-21

THE ENDOCRINE REGULATION OF INSECT METAMORPHOSIS. MOLECULAR MECHANISMS AND EVOLUTION

X. Belles

Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Barcelona, Spain.

Insect metamorphosis is mainly regulated by two hormones: 20-hydroxyecdysone (20E), which triggers molting, and juvenile hormone (JH), which represses adult morphogenesis. The hormonal signal transducers include the transcription factors Krüppel homolog 1 (Kr-h1) and E93, which are JH- and 20E-dependent, respectively. Kr-h1 is the master transducer of the JH antimetamorphic action, while E93 triggers metamorphosis. The regulation in hemimetabolans is explained by the MEKRE93 pathway, by which Kr-h1 represses *E93* expression in the last nymphal instar; when JH production ceases, *Kr-h1* expression is interrupted, *E93* expression becomes upregulated, and metamorphosis proceeds. The holometabolan metamorphosis includes the pupal stage, a sort of intermediate between the last larval instar and the adult. In holometabolans, Broad-Complex (BR-C) transcription factors determine the pupal stage, and E93 stimulates the expression of *BR-C* in the prepupa. The MEKRE93 pathway is, thus, conserved in holometabolan insects, which have added the E93/BR-C interaction loop to the hemimetabolan pathway during the evolution from hemimetaboly to holometaboly. Modern data on the regulation of metamorphosis have shed new light to the study of its evolution, which is currently explained by two alternative theories: the pronymph theory and the theory of direct homology between stages. The present data support the theory of direct homology between stages, modernly reformulated with information on molecular regulatory mechanisms.

Introduction

Metamorphosis is a crucial innovation in insect evolution, wherein the individual acquires characteristic adult features and stops molting during postembryonic development. The ancestral type of metamorphosis was hemimetaboly, in which embryo development gives rise to a first nymphal instar with the essential adult body structure. The nymphs grow through successive instars, and the final molt to the adult stage completes the formation of wings and genital structures. The type of metamorphosis known as holometaboly originated from hemimetaboly and is characterized because embryo development produces a larva with a body form that is generally very different from that of the adult. The larva grows through successive instars until molting to the pupal stage (which bridges the morphological gap between the larva and the adult), and then to the adult (1). In the hemimetabolan and holometabolan types, metamorphosis is regulated by two hormones: the juvenile hormone (JH) and the ecdysone and its more bioactive derivative, 20-hydroxyecdysone (20E). Ecdysone and 20E are steroids, and its main role is to promote the successive molts, including the metamorphic one, whereas JHs are terpenoids, whose function is to repress metamorphosis (2). The action of these hormones is underpinned by the mechanisms that transduce the endocrine signal through a pathway of gene activation. The 20E signaling pathway was first described in the 1990s (see (3) and references therein), whereas the most important details of the JH pathway

have been unveiled recently. Important components of the JH signaling pathway are the JH receptor, which is the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) protein known as Methoprene tolerant (Met), to which JH binds (4, 5). Another important component is Taiman, also a bHLH-PAS protein that plays the role of co-receptor. JH bound to the receptor complex Met-Taiman induces the expression of the transcription factor Krüppel homolog 1 (Kr-h1), which is the main transducer of the antimetamorphic signal of JH (see (6) and references therein).

Krüppel homolog 1, the doorkeeper of metamorphosis

Kr-h1 was discovered in the fruit fly *Drosophila melanogaster* as a gene structurally similar to the segmentation gene *Krüppel*, with which it shares the zinc-finger motifs and amino acid spacers between them. The first observations that connected Kr-h1 and JH were also made in *D. melanogaster*, in which the adult abdominal epidermis derives from larval histoblasts that start proliferating after puparium formation. In 1970, Ashburner showed that administration of JH before the prepupal stage prevents the differentiation of the adult abdominal epidermis, and the bristles that should be formed in the adult are shorter or absent. In 2008, Minakuchi and coworkers, working also on *D. melanogaster*, revealed that *Kr-h1* expressed ectopically in the abdominal epidermis during metamorphosis resulted in missing or short bristles, which suggested that Kr-h1 mediates the antimetamorphic action of JH. New experiments performed in the beetle *Tribolium castaneum* showed that depletion of Kr-h1 in young larvae triggered a precocious formation of the pupa (see (7) and references therein). This gave a clear proof that Kr-h1 represses metamorphosis, acting downstream of Met in the JH signaling pathway. The antimetamorphic action of Kr-h1 was generalized to hemimetabolan species in two studies carried out, respectively, on the cockroach *Blattella germanica* (8), and the bugs *Pyrrhocoris apterus* and *Rhodnius prolixus* (9). In these species, RNAi experiments showed that Kr-h1 depletion in nymphs in the penultimate or antepenultimate nymphal stage triggers the formation of a precocious adult (see also (1, 6, 10) and references therein).

E93, the key of insect metamorphosis

E93 is an early gene in the ecdysone signaling cascade specifically expressed in late prepupae of *D. melanogaster*. The gene encodes for a protein with RHF domains similar to pipsqueak motifs, which was found to be a key player in salivary glands degeneration during *D. melanogaster* metamorphosis (11–13). Importantly, E93 not only regulates programmed cell death, but it also plays morphogenetic roles. Mou and coworkers (14) reported that *E93* is widely expressed in adult cells of the pupa of *D. melanogaster*, where it is required for patterning processes. These authors investigated the induction of the *Distal-less* (*Dll*) gene within bract cells of the pupal leg through epidermal growth factor (EGF) receptor signaling, and found that E93 causes *Dll* to become responsive to EGF receptor signaling, which indicates that E93 is both necessary and sufficient for determining this switch. These results suggested that E93 controls the responsiveness of many other target genes and that it is required for general patterning during metamorphosis. Further experiments showed that E93-depleted *D. melanogaster* larvae are able to pupate but die at the end of the pupal stage. In *T. castaneum*, E93 depletion by RNAi prevented the pupa-to-adult transition, resulting in the formation of a supernumerary second pupa. Similar results were obtained in the cockroach *B. germanica*, where E93 depletion in nymphs prevented the nymph-to-adult transition, giving rise to

repeated supernumerary nymphal instars (15). Subsequently, it was shown that the expression of *E93* in juvenile nymphs of *B. germanica* is inhibited by the transcription factor Kr-h1, thus uncovering the essential mechanism by which JH represses metamorphosis, which was named MEKRE93 pathway (16).

The MEKRE93 pathway

The observation that Kr-h1 represses *E93* expression led to propose the MEKRE93 pathway as the general axis regulating insect metamorphosis. Accordingly, in nymph-to-nymph transitions, JH, acting through its receptor Met-Taiman, induces the expression of *Kr-h1*, while Kr-h1 represses the expression of *E93*. However, the decline of JH production in the last juvenile stage interrupts *Kr-h1* expression, with which *E93* becomes de-repressed and triggers adult morphogenesis (Fig. 1) (10, 16). The inhibitory action of Kr-h1 on *E93* expression was later corroborated in the holometabolan *T. castaneum* (17), which extended the MEKRE93 pathway framework to holometabolan metamorphosis (Fig. 1).

Regarding the regulatory mechanisms, the main difference between the two types of metamorphosis is the regulation and functions of the Broad-complex (BR-C) zinc-finger transcription factors. In hemimetabolans, *BR-C* expression is

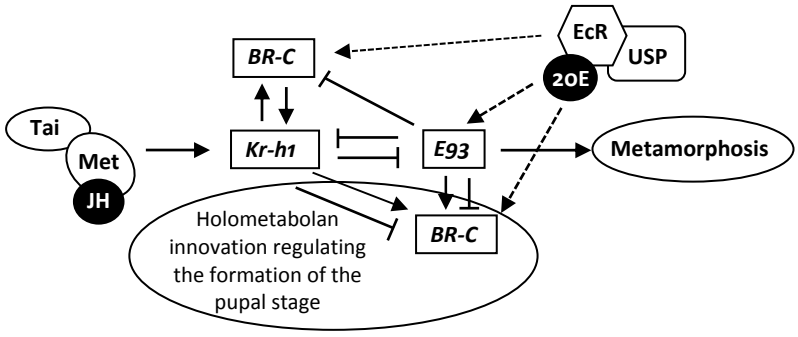


Figure 1. The MEKRE93 pathway in hemimetabolan and holometabolan insects. In the nymph-to-nymph transitions of hemimetabolan metamorphosis, the JH (through Met-Tai) induces the expression of *Kr-h1*, and Kr-h1 represses *E93* expression. However, the drop of JH production in the last nymphal instar interrupts the expression of *Kr-h1* and allows a strong induction of *E93* expression, which triggers metamorphosis. The main difference between the hemimetabolan and holometabolan modes is the regulation (and function) of BR-C. In hemimetabolans, BR-C is mainly involved in promoting the development of wing primordia, whereas in the holometabolans, BR-C triggers the formation of the pupa. References reporting the data that have led to the model are mentioned in the text.

enhanced by JH through Kr-h1 during juvenile stages, and BR-C factors are mainly involved in promoting wing primordia development, as shown in the cockroach *B. germanica*, and the hemipterans *Pyrrhocoris apterus* and *Oncopeltus fasciatus* (see (18) and references therein). Moreover, studies in the cricket *Gryllus bimaculatus* confirmed the mentioned interactions, and

additionally revealed that BR-C and Kr-h1 are reciprocally activated (19). In sharp contrast, JH inhibits the expression of *BR-C* during the larval stages of holometabolan species (20), whereas BR-C factors trigger the formation of the pupal stage (20–22). Work in *T. castaneum* revealed that E93 is involved in triggering the pupal stage, as it promotes *BR-C* expression (23), while E93 represses *BR-C* expression once in the pupal period (15). The whole data indicates that the MEKRE93 pathway is conserved in the holometabolan species, which added the E93/BR-C interaction loop to the ancestral (hemimetabolan) pathway during the evolution from hemimetaboly to holometaboly (Fig.1).

Regulatory mechanisms and evolution

Recent data on regulatory mechanisms have shed new light to the study of metamorphosis evolution. The ancestral metamorphosis mode, hemimetaboly, emerged around middle Devonian. Then, during early Carboniferous, holometaboly evolved from hemimetaboly (1, 24). Two alternative theories have been proposed to explain the evolution of metamorphosis: the pronymph theory and the theory of direct homology between stages. The pronymph theory was formalized by Truman and Riddiford (25), and in its modern version it essentially contends that the holometabolan embryo arrests development in the stage previous to the pronymph, and resumes it at the pupal stage. This implies that the hemimetabolan pronymph would be homologous to the set of holometabolan larval instars, while the set of hemimetabolan nymphal instars would be homologous to the holometabolan pupa (26, 27). The first ideas on the theory of direct homology between stages were proposed by Hinton (28) and received further physiological, paleontological and embryological contributions by Sehnal, Švácha and Zrzavý (29). It contends that embryonic and postembryonic development is equivalent in all insects, so that the pupa would be homologous to the last nymphal instar. Recent embryological studies have shown that all insects follow an equivalent embryonic development (30). Moreover, according to the MEKRE93 pathway, the drop in expression of *Kr-h1* and the increase in *E93* occur in the last nymphal instar (hemimetabolans) or in the pupa (holometabolans), which strongly supports that the last nymphal instar and the pupa are homologous, as argued in a number of recent papers (18, 30, 31). In conclusion, the currently available data does not properly fit with the pronymph theory and rather support the theory of direct homology between stages, as modernly reformulated with information on molecular regulatory mechanisms (1).

Acknowledgements: Supported by the Spanish Ministry of Economy and Competitiveness (Grants CGL2012–36251 and CGL2015–64727-P), the Catalan Government (Grant 2017 SGR 1030), and the European Fund for Economic and Regional Development (FEDER funds).

References

1. Belles, X. (2020). *Insect metamorphosis. From natural history to regulation of development and evolution*. London: Academic Press.
2. Nijhout, H.F. (1994). *Insect hormones*. Princeton, New Jersey: Princeton University Press.
3. Ou, Q. and King-Jones, K. (2013). What goes up must come down: transcription factors have their say in making ecdysone pulses. *Curr. Top. Dev. Biol.*103, 35–71.

4. Charles, J.-P., Iwema, T., Epa, V.C., Takaki, K., Rynes, J. and Jindra, M. (2011). Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl. Acad. Sci. U S A* 108, 21128–21133.
5. Jindra, M., Uhlirova, M., Charles, J.-P., Smykal, V. and Hill, R.J. (2015). Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. *PLOS Genet.* 11, e1005394.
6. Jindra, M., Belles, X., Shinoda, T. (2015). Molecular basis of juvenile hormone signaling. *Curr. Opin. Insect Sci.* 11, 39–46.
7. Minakuchi, C., Namiki, T. and Shinoda, T. (2009). Krüppel homolog 1, an early juvenile hormone-response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. *Dev. Biol.* 325, 341–350.
8. Lozano, J. and Belles, X. (2011). Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. *Sci. Rep.* 1, 163.
9. Konopová, B., Smykal, V. and Jindra, M. (2011). Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. *PLoS One* 6, e28728.
10. Belles X. (2019). Krüppel homolog 1 and E93: The doorkeeper and the key to insect metamorphosis. *Arch. Insect Biochem. Physiol.* 103, e21609.
11. Woodard, C.T., Baehrecke, E.H. and Thummel, C.S. (1994). A molecular mechanism for the stage specificity of the *Drosophila* prepupal genetic response to ecdysone. *Cell* 79, 607–615.
12. Baehrecke, E.H. and Thummel, C.S. (1995). The *Drosophila* E93 gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. *Dev. Biol.* 171, 85–97.
13. Lee, C.Y., Wendel, D.P., Reid, P., Lam, G., Thummel, C.S. and Baehrecke, E.H. (2000). E93 directs steroid-triggered programmed cell death in *Drosophila*. *Mol. Cell.* 6, 433–443.
14. Mou, X., Duncan, D.M., Baehrecke, E.H. and Duncan, I. (2012). Control of target gene specificity during metamorphosis by the steroid response gene E93. *Proc. Natl. Acad. Sci. U S A* 109, 2949–2954.
15. Ureña, E., Manjón, C., Franch-Marro, X. and Martín, D. (2014). Transcription factor E93 specifies adult metamorphosis in hemimetabolous and holometabolous insects. *Proc. Natl. Acad. Sci. U S A* 111, 7024–7029.
16. Belles, X. and Santos, C.G. (2014). The MEKRE93 (Methoprene tolerant-Krüppel homolog 1-E93) pathway in the regulation of insect metamorphosis, and the homology of the pupal stage. *Insect Biochem. Mol. Biol.* 52, 60–68.
17. Ureña, E., Chafino, S., Manjón, C., Franch-Marro, X. and Martín, D. (2016). The occurrence of the holometabolous pupal stage requires the interaction between E93, Krüppel-homolog 1 and Broad-Complex. *PLOS Genet.* 12, e1006020.
18. Huang, J.-H., Lozano, J. and Belles, X. (2013). Broad-complex functions in postembryonic development of the cockroach *Blattella germanica* shed new light on the evolution of insect metamorphosis. *Biochim. Biophys. Acta - Gen Subj.* 1830, 2178–2187.
19. Ishimaru, Y., Tomonari, S., Watanabe, T., Noji, S. and Mito, T. (2019). Regulatory mechanisms underlying the specification of the pupal-homologous stage in a hemimetabolous insect. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374, 20190225.
20. Zhou, B., Hiruma, K., Shinoda, T. and Riddiford, L.M. (1998). Juvenile hormone prevents ecdysteroid-induced expression of Broad complex RNAs in the epidermis of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* 203, 233–244.
21. Karim, F.D., Guild, G.M. and Thummel, C.S. (1993). The *Drosophila* Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. *Development* 118, 977–988.
22. Konopová, B. and Jindra, M. (2008). Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis. *Development* 135, 559–568.
23. Chafino, S., Ureña, E., Casanova, J., Casacuberta, E., Franch-Marro, X. and Martín, D. (2019). Upregulation of E93 gene expression acts as the trigger for metamorphosis independently of the threshold size in the beetle *Tribolium castaneum*. *Cell. Rep.* 27, 1039-1049.e2.

24. Belles, X. (2019). The innovation of the final moult and the origin of insect metamorphosis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374, 20180415.
25. Truman, J.W. and Riddiford, L.M. (1999). The origins of insect metamorphosis. *Nature* 401, 447–452.
26. Truman, J.W. (2019). The evolution of insect metamorphosis. *Curr. Biol.* 23, R1252-R1268.
27. Truman, J.W. and Riddiford, L.M. (2019). The evolution of insect metamorphosis: a developmental and endocrine view. *Philos. Trans. R. Soc. B Biol. Sci.* 374, 20190070.
28. Hinton, H.E. (1963). The origin and function of the pupal stage. *Proc. R. Entomol. Soc. London* 38, 77–85.
29. Sehnal, F., Švácha, P. and Zrzavý, J. (1996). Evolution of insect metamorphosis. In: Gilbert, L.I., Tata, J.R. and Atkinson, B.G., editors. *Metamorphosis. Postembryonic reprogramming of gene expression in amphibian and insect cells*. San Diego: Academic Press; pp. 3–58.
30. Konopová, B. and Zrzavý, J. (2005). Ultrastructure, development, and homology of insect embryonic cuticles. *J. Morphol.* 264, 339–362.
31. Jindra, M. (2019). Where did the pupa come from? The timing of juvenile hormone signalling supports homology between stages of hemimetabolous and holometabolous insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 374, 20190064.

THE NEUROENDOCRINE CONTROL OF REPRODUCTION: WHAT WE HAVE LEARNT FROM FISH

José A. Muñoz-Cueto

Department of Biology, Faculty of Marine and Environmental Sciences and INMAR, University of Cádiz, CEIMAR, Puerto Real (Cádiz), Spain.

Fish represent a key group in evolution, exhibiting a great variability and success. With almost 32,000 known extant species, of which around 30,000 are teleosts, fish account for more than half of vertebrate species. A third specific genome duplication that occurred in the stem lineage of ray-finned fish seems to be the reason for the astounding biological diversity and evolutionary success of this group of vertebrates. Fish have been of paramount importance for neuroendocrinology. In fact, the concept of neurosecretion was established by Ernest Albert Scharrer in the 1930s after studying a cyprinid fish, the European minnow, *Phoxinus laevis*, and fish have also proven to be of special interest to understand the mechanisms underlying the evolution of gonadotropin-releasing hormone (GnRH) genes in vertebrates. The neuroendocrine brain message reaches the fish pituitary in a different manner compared to other vertebrates. Fish lack the median eminence and the portal vasculature system characteristic of tetrapods, which connect the hypothalamus with the pituitary, and neurohormones that act on the pituitary are released from the neurosecretory terminals more or less directly into the surroundings of the target cells. Thus, the presence of terminals that secrete gonadotropin-releasing hormone (GnRH), dopamine, neuropeptide Y (NPY), gamma-amino butyric acid (GABA) or gonadotropin-inhibitory hormone (GnIH) in the proximity of the gonadotropic cells has constituted an extremely useful neuroanatomical evidence for determining the actions of these neurohormones on the secretion of gonadotropins and on the reproductive cycle of fish. In this chapter, I review the research developed in our laboratory in the last 25 years, focused on the neuroendocrine control of reproduction in fish, and especially, in the GnRH and GnIH systems of sea bass.

Introduction

As in other vertebrates, fish reproduction is controlled by complex environmental and hormonal interactions that occur along the brain-pituitary-gonadal axis. In this axis, the brain plays an integrative role, transducing both external and internal signals but also an effector role through the secretion of neurohormones that control gonadotropin secretion. From these neurohormones, gonadotropin-releasing hormone (GnRH) represents the main stimulatory factor in the control of reproduction (1).

In turn, dopamine constitutes the main inhibitory neurohormone in the fish reproductive axis. This dopaminergic inhibition was reported for the first time by Richard E. Peter in goldfish and is also functional in fish groups as salmonids, eels and catfishes, between others (2). However, this dopaminergic inhibition does not seem to operate in marine fish species as stripped bass, sea bream or the European sea bass. So, the question is whether there is another brain factor

antagonizing GnRH actions in fish species that do not exhibit a dopaminergic inhibition.

This picture changes in 2000, when Professor Kazuyoshi Tsutsui, from the Waseda University in Japan, discovered in quail a new dodecapeptide belonging to the RFamide family and named as gonadotropin inhibitory hormone or GnIH (3). This neuropeptide was secreted in neurons of the hypothalamic paraventricular nucleus projecting to the median eminence and inhibited the LH release in a dose-dependent manner. These actions are mediated through a GPCR receptor exhibiting the characteristic seven transmembrane domains named as GPR147, and also known as NPFFR1 and OT7T022. The GnIH receptors were identified in gonadotrophic cells but also in GnRH cells and kiss neurons. Following these pioneer studies performed in birds, it was shown that an active peptide inhibiting gonadotropin release was also present in mammals, and now we know that GnIH orthologues are present in all vertebrate groups, from lampreys to humans (4). We also know that GnIH is a pleiotropic neuropeptide acting not only as an inhibitory factor on gonadotropin, GnRH and kisspeptin secretion, but also in the transduction of photoperiod and melatonin effects, in the mediation of stress response, in the modulation of reproductive and social behavior and in the control of food intake through its effects of different orexigenic and anorexigenic peptides.

The main research activity of our laboratory in the last 2 decades has been focused in the study of GnRH and GnIH neuroendocrine systems in the European sea bass, which will be summarized in this chapter.

The fish GnRH system: brain localization, function, ontogeny and targets

The first GnRH peptide was discovered in pig and sheep hypothalamus, and its staple sequence of 10 amino acids was revealed in the laboratories of Andrew V. Schally and Roger Guillemin in 1971. Soon after the discovery of mammalian GnRH, it was demonstrated that GnRH was also able to stimulate the release of gonadotropins in the carp, and ten years later, the first form of GnRH was characterized in a teleost fish, the chum salmon (5). In the mid 90's, it was believed that there were two different GnRH systems in vertebrates, but three different forms of GnRH were characterized in the brain of seabream (6), with the cells producing these three forms of GnRH being apparently segregated in different brain regions. This basic scheme was later elucidated in other teleosts including the European sea bass and many more (1), in which GnRH2 soma were localized exclusively in the dorsal synencephalon-midbrain tegmentum, while GnRH1 and GnRH3 perikarya overlapped in the olfactory bulbs/terminal nerve (OB/TN), ventral telencephalon and preoptic area (POA).

Because GnRH neurons are innervating the pituitary directly, the presence of the GnRH peptides in the pituitary conveys a strong indication of their hypophysiotropic function. In fish with two GnRH forms, one GnRH variant (GnRH3 in most of them), which is expressed both in the OB/TN and POA, is the more abundant form in the pituitary and its levels correlate with gonadal development (1). In fish with three GnRH forms, the situation seems to be more diverse but GnRH1 levels were markedly higher in the pituitary and increased in correlation with the increase in oocyte diameter, indicating that this GnRH form is the most relevant form for the control of Lh release and reproduction. We demonstrated using specific GnRH-associated peptide (GAP) riboprobes and

antibodies that the GnRH1 neurons represented the main source of pituitary GnRH innervation in sea bass, arriving at the proximal pars distalis and the border of the pars intermedia, where gonadotrophic cells and GnRH receptors were also found (1). This result corroborated physiological evidence suggesting a major role for GnRH1 in the stimulation of the secretion of gonadotropins in three-GnRH perciform species. Although GnRH3 axons also reach the pituitary of sea bass, this innervation was strongly reduced as compared to GnRH1 projections. In contrast, no prepro-GnRH2 axons were detected in the pituitary of sea bass, suggesting that the putative role of GnRH2 in the control of reproduction does not involve a direct action of cerebral GnRH2 on gonadotrophic cells, at least in this species (1). In turn, our neuroanatomical results also suggested that GnRH2 and GnRH3 seem to develop a sensory role in the modulation of pineal and retinal functions, respectively (1, 7).

The ontogeny of GnRH system in the European sea bass was also analyzed in our laboratory. In this species, GnRH2 neurons represented the earliest detectable GnRH cell population during development, being detected at day 4 after hatching at a diencephalic/mesencephalic transitional area (8, 9). An early expression of this synencephalic/midbrain GnRH form was also reported in other vertebrate species suggesting an important function of this conserved GnRH form during ontogenesis. Although there was a debate concerning the origin of forebrain GnRH systems and, in particular, of preoptic GnRH neurons, it seems now clear that both GnRH1 and GnRH3 neurons develop from the olfactory placode region in teleost fish but exhibit a differential migration pattern to their final positions, as shown in sea bass using immunohistochemistry and *in situ* hybridization and in medaka using transgenic lines (7, 9, 10).

The action of GnRHs on target cells is mediated by binding to specific membrane GnRH receptors (GnRHRs), which belong to the rhodopsin family of G protein-coupled receptors (1). In fish, multiple GnRHRs have been reported (five GnRHRs in the case of the European sea bass), and *in vitro* studies have revealed that different GnRHs can activate the GnRHRs, with a clear preference for GnRH2, followed by GnRH3 and GnRH1. However, each receptor subtype displays different relative potencies of the different GnRH ligands. In the European sea bass, only sbGnRHR-II-1a subtype shows some sensitivity to GnRH1, the most abundant form of GnRH present in the sea bass pituitary. In turn, GnRHR-II-2b subtype is highly expressed in the pineal and the brain, and only exhibit sensitivity to GnRH2 form, which is the only GnRH form that reach the sea bass pineal (1, 10).

What we know about GnIH in fish

In a recent review, we summarized the information available in the literature on GnIH sequences identified in fish, the distribution of GnIH and GnIH receptors in central and peripheral tissues, the physiological actions of GnIH on the reproductive brain-pituitary-gonadal axis, as well as other reported effects of this neuropeptide, and existing knowledge on the regulatory mechanisms of GnIH in fish (4). Interestingly, most of the GnIH precursors identified in fish exhibited three LPXRFamide peptides whereas those present in modern teleost orders such as Perciformes, Pleuronectiformes or Tetraodontiformes only contained two LPXRFamide peptides.
But this is not only difference found in fish GnIH system because both stimulatory and inhibitory effects of GnIH on the reproductive axis have been

reported depending on the species, dose, sex, reproductive stages, the peptides used, their route of administration and/or the elapsed time after treatment. Moreover, several GnIH cell populations have been reported in fish brain outside the preoptic area in regions such as the terminal nerve of the olfactory bulbs (4).

In this context, we decided in our group to approach the study on the gonadotropin-inhibitory hormone system in the European sea bass, a species that did not exhibit a dopaminergic inhibition of gonadotropin secretion and reproduction. As a first step, we cloned a cDNA containing the complete ORF of the sea bass GnIH precursor. This precursor encoded a 200 aminoacids protein that exhibits two putative RFamide peptides of twelve aminoacids, with C-terminal MPMRF and MPQRF residues (4, 11). GnIH gene was highly expressed in the brain of sea bass, not only in the diencephalon but also in the olfactory bulbs and cerebral hemispheres, in the mesencephalon and rhombencephalon, as well as in peripheral tissues as gonads and intestine, most of them also expressing the GnIH receptor. Furthermore, we developed specific antibodies against sea bass GnIH1 and GnIH2 peptides, which were used to localise immunoreactive GnIH cells and fibers in the brain and pituitary of sea bass (11). This immunofluorescent analysis permitted us to identify GnIH cell bodies in the diencephalic posterior periventricular nucleus, in agreement with results obtained in other teleost species. However, we also detected GnIH cells in the olfactory bulbs, corresponding to the terminal nerve ganglion cells, the ventral telencephalon, the dorsal tegmentun and the rhombencephalic isthmus.

In addition, we analysed the seasonal expression pattern of different GnIH cell populations. Whereas telencephalic GnIH cells did not exhibit significant differences in GnIH mRNA levels, diencephalic GnIH cell population showed lower GnIH expression during the reproductive season and increased GnIH transcript levels at the resting period, exhibiting the GnIH cell populations of the mid and posterior brain an inverse pattern of expression. We also tested the effects of pinealectomy on GnIH expression and observed that only tegmental GnIH cells are affected by pinealectomy in a seasonal-dependent manner. These results suggest that different GnIH cell populations can have distinct functions, with diencephalic GnIH cells being involved in the neuroendocrine control of reproduction, whereas midbrain tegmental GnIH cells could transduce the pineal message (12).

Another aim of our work was to elucidate which were the functions of GnIH in sea bass (12). All the previous functional studies addressing the effects of GnIH in fish used *in vitro* approaches or intraperitoneal injections. Considering that GnIH is a neuropeptide that is mainly synthesized and delivered into the brain, we decided to analyse, for the first time in fish, the effects of intracerebroventricular injections of GnIH1 and 2 peptides in male sea bass. At the brain level, GnIH1 was able to decrease the expression of GnRH1 isoform at the three doses tested (12). These effects were consistent with immunohistochemical studies that revealed an intense GnIH innervation in the ventral telencephalic and preoptic regions where GnRH1 cells were found, and double immunostaining followed by confocal analysis showed that GnIH axons appear in contact with cell bodies and dendrites of preoptic GnRH1 cells (12). In turn, the three doses tested of GnIH2 reduced the transcripts levels of the GnRH 2 form. In addition, GnIH2 also inhibited the kiss system by decreasing the expression of kiss1 and its receptor and kiss2 transcript levels. Again, this was consistent with morphofunctional studies showing the presence of a GnIH

innervation in the habenula of sea bass, where kiss1 cells are placed, and abundant GnIH terminals in the nucleus of the lateral recess, where kiss2 cells are located (12). In the pituitary, GnIH 2 decreased the expression of both FSH beta and LH beta subunits, and both GnIH forms reduced LH plasma levels. These actions can be elicited directly at the pituitary level because GnIH fibers enter into the pituitary and immunolabeled axon terminals were observed in contact with FSH and LH cells. In turn, GnIH actions in the pituitary can also be exerted by modulating the GnRH signaling because the GnRH receptor that is present in most LH cells and some FSH cells, also decreases its transcript levels after GnIH treatment (12). Moreover, we performed a long-term study, from October (pre-spermatogenesis) to February (spermiation), to evaluate the effects of peripheral GnIH implants on gonadal development and steroidogenesis in male sea bass. We showed that GnIHeffects on testicular steroidogenesis were restricted to particular stages of the reproductive cycle (early and mid-spermatogenesis) and steroids (androgens, not progestins) but determined marked effects on the progression of gametogenesis, as revealed in the histological analysis that showed abundant type A spermatogonia and partial spermatogenesis in GnIH treated animals (12).

We have also analysed in our laboratory the ontogenic expression of GnIH system and the effects of rearing temperature during the first year of life in sea bass (12). The expression of GnIH and its receptor increased significantly during the first three week of life, declining thereafter. A dramatic increase (15 to 30 times) in GnIH and GnIH receptor transcript levels was observed between 120 and 150 days post fertilization, coinciding with the beginning of the sex differentiation period, with a subsequent decrease in GnIH expression. In this experiment, animals were maintained under two different thermal conditions, low temperature (15 °C) or high temperature (21 °C), throughout the thermosensitive period (from 5 to 60 dpf). Our results showed significant effects of temperature on GnIH and GnIH receptor expression during the thermosensitive period, with higher transcript levels under low temperature condition, being some differences also evident after the completion of the sex differentiation process (12). From works performed in sea bass in the laboratory of Dr. Piferrer, it is known that low temperature regimes during this thermosensitive period gives rise to a higher proportion of females. In turn, higher temperatures produce more males in the progeny and these masculinizing effects of temperature seem to be mediated by an inhibition of aromatase mRNA expression and activity in genotypic females. The pattern of expression and the effects of temperature on GnIH system suggest that this neuropeptide could have a role in sex differentiation in sea bass, probably by modulating aromatase expression and/or activity (12).

Summarizing, the sea bass brain contained different GnIH cell populations that exhibited distinct seasonal profiles of expression and, probably, distinct functions. GnIH inhibited GnRH-1, GnRH-2 and kisspeptin expression in the sea bass brain. This neuropeptide also reduced FSH and/or LH synthesis and release and GnRHR expression in the sea bass pituitary. GnIH decreased plasma androgen levels in particular stages of the reproductive cycle, affecting the sea bass gametogenesis. The results obtained also suggested that GnIH could transduce the daily and seasonal message of photoperiod and pineal organ in sea bass as well as the effects of temperature on sex differentiation.

Acknowledgements: Funded by the Junta de Andalucía (P10-AGR-05916), UE (FP7/2007-2013, nº 331964) and EMBRC (OOB–EMBRC FR–AAP2018 nº 2200) grants to JAMC.

References.

1. Muñoz-Cueto, J.A., Zmora, N., Paullada-Salmerón, J.A., Marvel, M., Mañanos, E., Zohar, Y. 2020. The gonadotropin-releasing hormones: lessons from fish. Gen. Comp. Endocrinol. 291: 113422.
2. Dufour, S., Quérat, B., Tostivint, H., Pasqualini, C., Vaudry, H., Rousseau, K., 2020. Origin and evolution of the neuroendocrine control of reproduction in vertebrates, with special focus on genome and gene duplications. Physiol. Rev. 100: 869-943.
Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., et al. 2000. A novel avian hypothalamic peptide inhibiting gonadotropin release. Biochem. Biophys. Res. Commun. 275(2):661-667.
4. Muñoz-Cueto, J.A., Paullada-Salmerón, J.A., Aliaga-Guerrero, M., Cowan, M.E., Parhar, I.S., Ubuka, T., 2017. A journey through the gonadotropin-inhibitory hormone system of fish. Front. Endocrinol. 8, 285.
5. Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J., Vale, W., 1983. Characterization of a teleost gonadotropin-releasing hormone. Proc. Natl. Acad. Sci. USA 80: 2794-2798.
6. Powell, J.F., Zohar, Y., Elizur, A., Park, M., Fischer, W.H., Craig, A.G., Rivier, J.E., Lovejoy, D.A., Sherwood, N.M., 1994. Three forms of gonadotropin-releasing hormone from brains of one species. Proc. Natl. Acad. Sci. USA 91: 12081-12085.
7. Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165: 438-455.
8. Gonzalez-Martinez, D., Zmora, N., Zanuy, S., Sarasquete, C., Elizur, A., Kah, O., Munoz-Cueto, J.A., 2002. Developmental expression of three different prepro-GnRH (gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). J. Chem. Neuroanat. 23: 255-267.
9. Gonzalez-Martinez, D., Zmora, N., Saligaut, D., Zanuy, S., Elizur, A., Kah, O., Munoz-Cueto, J.A., 2004. New insights in developmental origins of different GnRH (gonadotrophin-releasing hormone) systems in perciform fish: an immunohistochemical study in the European sea bass (*Dicentrarchus labrax*). J. Chem. Neuroanat. 28: 1-15.
10. Servili A., Lethimonier, C., Lareyre, J.J., Lopez-Olmeda, J.F., Sanchez-Vazquez, F.J., Kah, O., Muñoz-Cueto, J.A., 2010. The highly conserved gonadotropin-releasing hormone-2 form acts as a melatonin-releasing factor in the pineal of a teleost fish, the European sea bass Dicentrarchus labrax. Endocrinology 151: 2265-2275.
11. Paullada-Salmerón, J.A., Cowan, M., Aliaga-Guerrero, M., Gómez, A., Zanuy, S., Mañanós, E., Muñoz-Cueto, J.A., 2016. LPXRFa peptide system in the European sea bass, a molecular and immunohistochemical approach. J. Comp. Neurology. 524; 176-198.
12. Paullada-Salmerón, J.A., Cowan, M.E., Loentgen, G.H., Aliaga-Guerrero, M., Zanuy, S., Mañanós, E.L., Muñoz-Cueto, J.A. 2019. The gonadotropin-inhibitory hormone system of fish: the case of sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinology. 279: 184-195.

INSULIN SIGNALLING IN *Rhodnius prolixus*, THE VECTOR OF CHAGAS DISEASE

A.B. Lange, J. Leyria, I. Orchard

Department of Biology, University of Toronto Mississauga, Mississauga, Ontario, Canada.

Insulins are peptide hormones widely studied for their important regulatory roles in metabolism, growth and development. In insects, insulin-like peptide (ILP) signalling along with the target of rapamycin (ToR) are involved in detecting and interpreting nutrient levels. Using the blood-gorging triatomine, *Rhodnius prolixus* as a model, we have examined two different nutritional conditions (unfed and fed) and monitored key tissues involved in reproduction (the central nervous system, fat body and ovaries) using next-generation sequencing and transcriptome analysis. Although there is an up-regulation of the genes involved in ILP signalling in unfed insects, western blot analysis reveals that ILP signalling is only activated in tissues of fed insects, i.e. phosphorylation of proteins. Immunofluorescence and RNA interference studies suggest that during the unfed condition, FoxO signalling, which regulates the longevity phenomena via ILP signalling, maybe responsible for the up-regulation of transcripts involved in the ILP signalling cascade. Moreover, insulin stimulates protein phosphorylation in the fat body and ovaries, suggesting that unfed females are in a sensitized state and respond to food by rapidly activating ILP signalling. Using dsRNA to silence molecules involved in ILP/ToR signalling we find that the ILP signalling pathway is involved in the coordination of the synthesis of yolk protein precursors and influences the numbers of eggs laid. Finally, immunohistochemistry indicates that the insulin receptor is differentially expressed in oocytes according to their developmental stage. This is a preliminary study on the network of regulatory pathways in these different tissues being implicated in reproductive performance.

Introduction

In insects, oviparous females must drive, with extraordinary effectiveness, the conversion of their nutritional resources into nutrients for developing eggs. Vitellogenesis, a crucial event in egg development, is the process of yolk formation for the growth of the oocytes at the expense of synthesis of large amounts of yolk protein precursors (YPPs), mainly vitellogenin (Vg), along with lipids and carbohydrates from the fat body (1). The process of vitellogenesis is controlled by a variety of hormonal signals including neuropeptides, juvenile hormone (JH), and ecdysteroids. Insulin-like peptides (ILPs) are neuropeptides that, along with the target of rapamycin (ToR), a direct downstream target of the ILP signalling cascade, play vital roles during vitellogenesis by acting as nutritional sensors (2). Binding of ILPs to the insulin receptor (InR) leads to Akt expression. This in turn phosphorylates a series of mediators leading to the target response. One such mediator is FoxO (forkhead box O transcription factor), a critical intermediary of cellular processes under conditions of low levels of insulin signaling (3). In mosquitoes and other insects, ILP/ToR signalling plays a pivotal role in the transduction of nutritional signals to stimulate the synthesis of YPPs, thereby leading to egg growth (4). However, the relative contribution of

ILP signalling in vitellogenesis is not completely understood and may differ between insect species and life strategies.

In triatomines, which are blood-feeding insects and vectors of the etiological agent of Chagas´ disease, the relationship between ILP signalling and reproductive success has not been studied. The triatomine, *Rhodnius prolixus,* presents a perfect model to examine reproduction since the blood meal is the stimulus that activates egg production. Previous studies on *R. prolixus* have identified one ILP (Rhopr-ILP), one insulin-like growth factor (Rhopr-IGF) and one insulin receptor (Rhopr-IR) (5-7). Expression profile analyses show that the Rhopr-ILP transcript is predominantly present in the brain, located in four medial neurosecretory cells, whilst the Rhopr-IGF transcript is distributed in a variety of tissues. A partial transcript for the Rhopr-IR (a tyrosine kinase receptor) has been cloned and is expressed in all tissues tested, with highest expression in the CNS. Previous studies suggest that Rhopr-ILP operates on an IR-like tyrosine kinase receptor (5). Knocking down the Rhopr-ILP or Rhopr-IR transcripts (with dsRNA) increased the levels of lipids and carbohydrates in the hemolymph, whilst altering lipid and carbohydrate storage in the fat body and skeletal muscle (6). Knockdown of the Rhopr-IGF transcript resulted in defective growth and development of fifth instars into adults, with insects displaying abnormal morphological features such as smaller wings and reduced body size (7). Therefore, in *R. prolixus,* Rhopr-IR signaling is involved in hemolymph nutrient homeostasis and fat body storage both in post-feeding and in non-feeding stages. These metabolic effects are likely regulated by the activation of Akt and downstream cascades similar to mammalian insulin signaling pathways.

In this paper, we present preliminary results on events involved in egg production, focusing on ILP signalling.

Materials and Methods

Reproductive success depends on the regulation of a variety of tissues and therefore we evaluated transcriptional and/or protein expression in the central nervous system (CNS), fat body and ovary from adult females with standardized physiological conditions. Each tissue was analyzed before (unfed condition, UFC) and after (fed condition, FC) a blood meal. For transcriptomic exploration, RNAseq and qRT-PCR were used (5-7). Western blot assays, using specific antibodies against proteins involved in the ILP signalling cascade, and injection of insulin were performed to analyze the signal activation (5). The specificity of these antibodies has previously been reported (5). RNA interference (RNAi) assays (5) using different dsRNA to downregulate ILP/ToR signalling was used to evaluate the role of ILP signalling on the synthesis of YPPs. We also performed immunofluorescence assays to evaluate InR location in ovaries.

Results and Discussion

In insects, the availability of nutrients affects multiple signalling pathways. Nutrient depletion activates processes involved with energy production, stress resistance, and survival. On the other hand, in conditions where nutrients are abundant, an increase in ILP signalling, working cooperatively with other pathways, stimulates successful reproduction in some species of insects (4). Specific genes related to ILP/ToR signalling were selected and analysed in this study. In a preliminary transcriptomic analysis, we find that some of the transcripts involved with ILP/ToR signalling are up-regulated in the fat body and ovaries during the UFC (Table 1 and 2).

Tables 1 and 2: Expression profile of some genes involved in ILP/ToR signalling in the fat body (FB, Table 1) and ovaries (OV, Table 2) during the fed (FC) and unfed conditions (UFC) using transcriptomic exploration. Log_2fold change of FC with respect to UFC.

Table 1. Transcriptome analysis on fat body

Gene	Readcount after normalization		log_2Fold Change	*P* val
	FB_FC	FB_UFC		
InR1	19	38	-1.0	0.09
VKR	142	432	-1.6	9.82E-08
IRS1	100	266	-1.4	0.002
PTEN	68	294	-2.1	8.22E-06
TSC2	113	382	-1.8	3.62E-08
Raptor	163	458	-1.5	8.68E-07
S6	3979	9419	-1.2	8.40E-06
eIF4E	401	761	-0.9	0.002
IGF	827	1318	-0.6	0.01

Table 2. Transcriptome analysis on ovaries

Gene	Readcount after normalization		log_2Fold Change	*P* val
	OV_FC	OV_UFC		
InR	481	675	-0.5	0.00973
VKR	124	257	-1.1	7.52E-07
IRS1	233	662	-1.5	1.22E-18
PI3K	185	372	-1.0	1.41E-05
PTEN	48	108	-1.1	0.000196
TSC1	3503	8804	-1.3	9.66E-17
TSC2	3364	6711	-0.9	1.18E-12
LKB1	2063	3415	-0.7	4.12E-08
TOR	1187	2922	-1.3	8.20E-12
Raptor	1212	2151	-0.8	2.15E-09
S6K	142	243	-0.7	0.000236
eIF4B	1738	2201	-0.3	0.01282
AMPK	780	1387	-0.8	7.82E-08
IGF	99	169	-0.7	0.008778

In a similar manner, in the mosquito *Aedes aegypti*, starvation significantly increases the expression of transcripts involved in insulin signalling in the *corpora allata*, a neuroendocrine gland that synthesizes and releases JH (6). Also, in *Bombyx mori*, the expression of transcripts associated with the ILP cascade are elevated in the fat body when animals cease feeding (7). However, it is important to note that an increase in transcript level does not necessarily mean that the protein is translated and/or active. Insulin uses a phosphorylation cascade and the resultant protein–protein interactions to promote cellular responses (2). Western blot studies indicate that the expression of phosphorylated proteins such as *p*-Akt, *p*-glucogen synthase kinase (*p*-GSK), *p*-FoxO and *p*-mToR, only occurs after a blood meal (Fig 1A) in both fat body and ovaries. So far, the findings suggest that tissues of unfed insects accumulate more transcripts (or proteins) involved in the ILP/ToR signalling cascade, thus establishing a sensitized state in order to respond quickly to changes in ILP levels. In this way, when nutrient conditions become favourable, the cells are able to respond rapidly by turning on the mechanisms that stimulate growth. With this in mind, insulin was injected into unfed insects to activate the ILP signalling cascade. Phosphorylated Akt protein is up-regulated 2 h post-injection demonstrating that unfed insects are able to respond quickly to insulin stimulus (Fig 1B).

In *D. melanogaster*, transcriptional activation of genes by FoxO signalling is a critical step in survival during amino acid withdrawal (8). Examples of genes up-regulated during the starvation are those involved in ILP signalling (3). It is widely known that FoxO is phosphorylated in the presence of ILP and remains in the cytoplasm inhibiting its nuclear translocation and its signalling activation (3). Indeed, FoxO signalling activation represents a pathway that indirectly indicates that insulin signalling is absent. To examine if FoxO could be a factor responsible for increasing transcript levels during ILP signalling in the UFC in *R. prolixus*, we interfered with FoxO signalling in unfed insects by dsRNA treatment. The results indicate that transcripts involved with ILP signalling decrease in dsFoxO treatment of unfed insects with respect to the control (dsARG) (unpublished). Also, by western blot we find that in unfed insects, *p*-FoxO expression is undetectable in both fat body and ovaries (Fig 1A), promoting the hypothesis that FoxO is translocated to the nucleus to initiate its signalling response when

the nutritional conditions are not favourable. Indeed, by immunofluorescence we find that FoxO (red signal) co-localizes with a DAPI, a blue fluorescent nucleic acid stain, in numerous cells of the fat body of unfed insects (Fig 2, merge). No cells with FoxO in the nucleus are found in fed females (Fig 2).

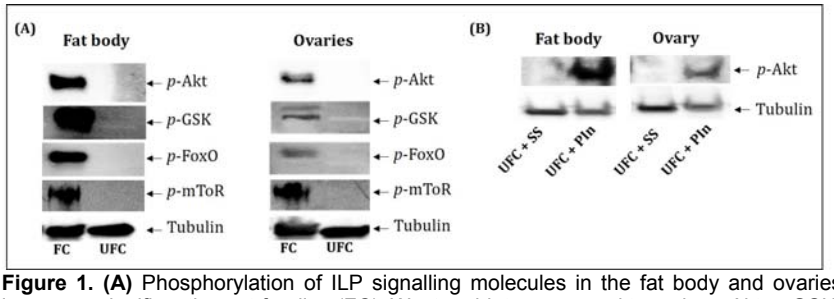


Figure 1. (A) Phosphorylation of ILP signalling molecules in the fat body and ovaries increases significantly post-feeding (FC). Western blots were used to probe *p*-Akt, *p*-GSK, *p*-FoxO and *p*-mToR (visualized by chemiluminescence). **(B)** In unfed females (UFC), ILP signalling (*p*-Akt) was activated by injection of porcine insulin (PIn). SS: Saline solution. Images are representative of 3 independent assays.

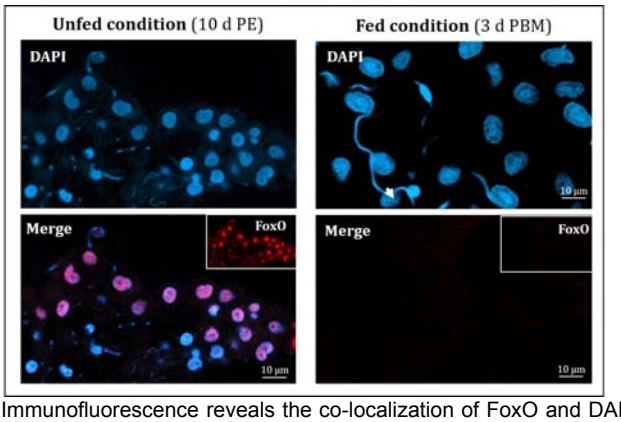


Figure 2. Immunofluorescence reveals the co-localization of FoxO and DAPI (merge) in the nuclei of unfed insects (left panel) but not of fed insects (right panel). PE, post-ecdysis; PBM, post-blood meal.

We also examined the distribution of the InR in the ovaries from fed insects using immunohistochemistry and the results show positive signal in the tropharium and beta oocytes (Fig 3). This result suggests that the tropharium, which contains nurse cells, is incorporating nutrients from the hemolymph, probably to maintain the immature oocytes, and the beta oocytes, that are in actively in vitellogenesis and incorporating yolk proteins for egg growth. These findings are in agreement with the immunolocalization of a downstream protein of InR activation, the *p*-AKT (unpublished).

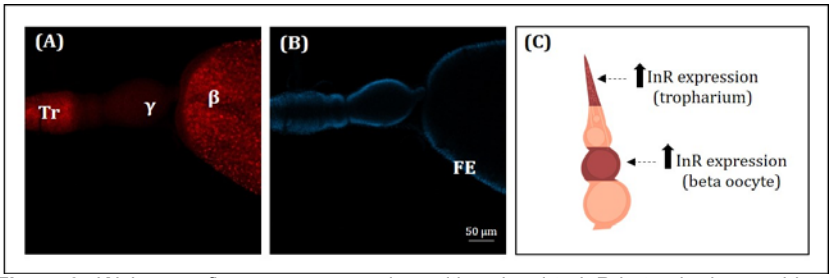


Figure 3. **(A)** Immunofluorescence reveals positive signal to InR in tropharium and beta oocyte. **(B)** DAPI staining of (A). **(C)** Schematic drawing of *R. prolixus* ovariole. Tr, tropharium; Y, gamma oocyte; β, beta oocyte; FE, follicular epithelium.

Overall, the results show that ILP signalling is activated after a blood meal. To examine the functional role of this pathway on reproduction, we performed gene-silencing assays. We injected females with dsRNA to InR or ToR and 2 h post injection insects were given a blood meal (Fig. 4A). Knockdown of ILP signalling reduces the number of eggs laid per day per female compared to controls, thereby reducing the total number of eggs laid per female in one reproductive cycle (unpublished). Could this result be related to a change in the synthesis of YPPs? To answer this question, we analyzed the synthesis of vitellogenin (Vg), the main YPP from the fat body, in ILP/ToR signalling knockdown insects. The results show a reduction in Vg transcript expression levels in females that had a reduced ILP signalling pathway (Fig. 4B). To support this finding we used an analog of JH and specific inhibitors of the ILP/ToR pathway to examine Vg expression in the fat body and find that Vg transcript expression in fat body depends on ILP/ToR signalling (unpublished).

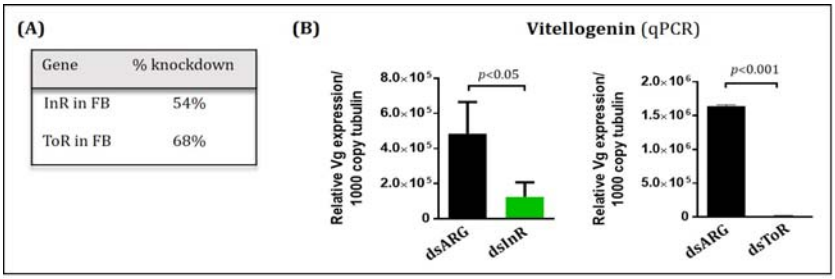


Figure 4 (A-B) Fat body was collected 3 days post-dsRNA injection/feeding and the Vg expression was evaluated by qPCR. In all experiments, each value is the mean ± SEM of n=3. The statistical significance of the data was calculated using Student's t-test.

Overall, we propose a preliminary model for the influence of nutritional status on the ILP/ToR pathway on reproductive performance (Fig 5). In *R. prolixus*, after a blood meal, ILPs are released from the CNS to circulate and affect target tissues by activating a phosphorylation cascade via the ILP signalling pathway. ILP signalling is activated in the fat body to promote Vg expression and in ovaries to promote egg growth. This pathway is under the influence of the nutritional state of the insect and tissues are stimulated to enhance the number of eggs laid in *R. prolixus*. During the UFC, a significant increase in transcript involved

in ILP signalling is observed; however, activated signalling, i.e. phosphorylated proteins, are not detected. We suggest that FoxO is translocated to the nucleus during the UFC and that it is responsible for increasing insulin sensitivity and modulating longevity signalling.

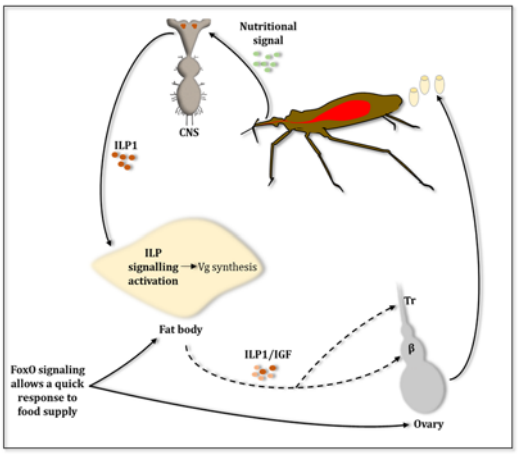


Figure 5. Preliminary model for the role of ILP signalling pathway influenced by nutritional status and its effect on reproductive performance. Details described above in text.

Acknowledgements: This research was supported through NSERC.

References

1. Swevers L, Raikhel AS, Sappington TW, Shirk P, Latrou, K. 2005. Vitellogenesis and post-vitellogenic maturation of the insect ovarian follicle. Entomology Publications. 482:87–155.
2. Badisco L, Van Wielendaele P, Vanden Broeck J. 2013. Eat to reproduce: a key role for the insulin signalling pathway in adult insects. Front. Physiol., 4: 202.
3. Puig O, Tijan R. 2006. Nutrient availability and growth: regulation of insulin signalling by dFOXO/FOXO1. Cell cycle 5:503–505.
4. Roy S, Saha TT, Zou Z. Raikhel AS. 2018. Regulatory pathways controlling female insect reproduction. Annu. Rev. Entomol. 63:489–511.
5. Defferrari MS, da Silva SR, Orchard I, Lange AB. 2018. *A Rhodnius prolixus* insulin receptor and its conserved intracellular signalling pathway and regulation of metabolism. Front Endocrinol (Lausanne). 9:745.
6. Defferrari MS, Orchard I, Lange AB. 2016a. Identification of the first insulin-like peptide in the disease vector *Rhodnius prolixus*: Involvement in metabolic homeostasis of lipids and carbohydrates. Insect Biochem Mol Biol. 70:148-59.
7. Defferrari MS, Orchard I, Lange AB. 2016b. An insulin-like growth factor in *Rhodnius prolixus* is involved in post-feeding nutrient balance and growth. Front Neurosci 10, 566.
6. Perez-Hedo M, Rivera-Perez C, Noriega FG. 2014. Starvation increases insulin sensitivity and reduces juvenile hormone synthesis in mosquitoes. PLoS One. 9:e86183
7. Liu Y, Zhou S, Ma L, Tian L, Wang S, et al. 2010. Transcriptional regulation of the insulin signalling pathway genes by starvation and 20-hydroxyecdysone in the *Bombyx* fat body. J Insect Physiol. 56:1436-44.
8. Kramer JM, Slade JD, Staveley BE. 2008. Foxo is required for resistance to amino acid starvation in *Drosophila*. Genome. 51:668-72.

CHEMICAL SENSING AT THE RAT CHOROID PLEXUS IS REGULATED BY SEX HORMONES

C.R.A. Santos, A.C. Duarte, T. Quintela, J. Tomás, I. Gonçalves

CICS-UBI - Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.

The choroid plexuses play several critical functions to the central nervous system homeostasis. Interestingly, our previous studies showed that many of these functions are regulated by the sex hormone background. These studies also unveiled the presence of several chemosensing receptors, regulated by sex hormones. Among these receptors, taste, odorant and vomeronasal receptors were highly represented. Here, we analyzed the taste signalling functionality in rat and human choroid plexus. We demonstrated that several taste receptors and taste effector-proteins are expressed and functional in both choroid plexus models by gene/protein expression analysis and calcium imaging. Moreover, we showed that the sex hormone, 17-βestradiol, downregulates various taste-related genes in the rat CP. In conclusion, we present evidence of chemical surveillance mechanisms operating at the choroid plexus which is under sex hormone regulation.

Introduction

The choroid plexuses (CPs) are highly vascularized structures, located in the ventricular system of the brain (1). Many different functions have been attributed to CP including cerebrospinal fluid (CSF) production, nutrient and hormone supply to the CSF, immune surveillance, and brain detoxification. Another important feature of the CP is the establishment of the blood-CSF barrier that confers brain protection against noxious compounds in circulation (2). Interestingly, these CP functions are regulated by the sex hormone background (3). In addition, we found that several chemosensing receptors, such as taste, olfactory and vomeronasal receptors are also regulated by sex hormones in the CP (2, 4). Considering these findings, we propose that chemosensing receptors may be active in the CP and relevant for monitoring the chemical composition of blood and CSF fluids.

In the last years, several studies showed that taste receptors are not restricted to the oral cavity. Instead, they are also expressed in many other organs (5, 6). Taste receptors, that belong to the G-protein coupled receptors family, can be classified in type 1 taste receptors (T1Rs) and type 2 taste receptors (T2Rs). T1Rs subunits display three subunits (T1R1, T1R2 and T1R3) and can dimerize to recognize sweet (T1R2+T1R3) or umami (T1R1+T1R3) compounds (7, 8). On the other hand, bitter compounds are recognized by bitter receptors or T2Rs (9, 10). In the mouth, T1Rs or T2Rs activation are similar and include the activation of the G-protein α-gustducin (GNAT3), that activates phospholipase C Beta 2 (PLCβ2) and induces the production of IP3 which triggers an increase in intracellular calcium levels. In turn, calcium elevation activates the transient receptor potential cation channel subfamily M member 5 (TRPM5) causing cell depolarization (11, 12). Interestingly, many studies have evidenced a role of T2Rs in different biological functions in response to internal and external chemical stimuli, including thyroid and gastrointestinal function, spermatogenesis, and innate immunity (6).

Therefore, we aimed to confirm the expression of these receptors and assess their functionality in the rat and human CP, as well as asses the role of sex hormones in their regulation.

Materials and Methods

The expression of the taste transduction pathway components, taste receptors and effector proteins were analyzed in rat and human CP tissues and cell lines (CPEC, Z310 and HIBCPP) by different techniques: RT-PCR, immunohistochemistry, immunocytochemistry, and Western blot. Functional assays were also carried out to evaluate the taste pathway functionality using single cell calcium imaging in rat Z310 and human HIBCPP cells, in the presence of bitter agonists.

The effect of sex hormones on the expression of taste transduction related genes was also evaluated by incubating rat CP explants with 17-βestradiol (1, 10, and 100 nM) for 6h. Moreover, CP explants were also incubated with ICI/fluvestran (100nM).

Results and Discussion

Our studies demonstrated the mRNA and protein expression of several bitter receptors in rat CP cells (CPEC and Z310), Tas1R1, Tas1R2, Tas2R144, and taste-effector proteins GNAT3, PLCβ2, and TRPM5. In addition, calcium functional assays in CPEC cells showed an increase of intracellular calcium levels in response to a bitter stimulus (D-Salicin). Moreover, using a specific blocker of bitter taste receptors (probenecid), calcium response was significantly reduced. Therefore, these data show that the rat CP present a functional taste transduction pathway (13).
Regarding the effect of 17-βestradiol in taste pathway related genes, we observed a downregulation of Tas1R1, Tas2R109, PLCβ2, and TrpM5 in rat CP explants (Figure 1). Additionally, we also showed that 17-βestradiol effects are estrogen receptor-dependent since the treatment of rat CP explants with ICI or fluvestran, a specific antagonist of estrogen receptor abolished downregulation of taste related genes' expression (14).

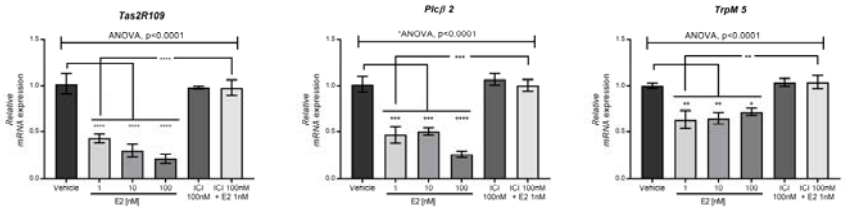


Figure 1. Expression of taste signaling pathway genes in rat CP explants exposed to increasing concentrations of 17-βestradiol (E2) for 6h. Effect of E2 in Tas2R109, Plcβ2 and TrpM5 gene expression, in the absence or in the presence of the respective receptor antagonist (ICI/fluvestran). ANOVA, followed by Tukey´s multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001. T-test, ###p<0.001, ####p<0.0001.

Regarding the bitter taste transduction pathway in the human CP we confirmed the mRNA expression of 13 T2Rs and taste-effector proteins GNAT3, PLCβ2, and TRPM5 in HIBCPP cells, an in vitro model of the blood-CSF barrier (15). Protein expression of T2R4, 5, 14 and 39 as well as taste related proteins was confirmed by immunocytochemistry and Western blot. Additionally, T2R4, 5, 14

and 39, but not 10 were detected in men and women CP tissues by immunohistochemistry. In order to further characterize the expression of bitter taste receptors in the human CP we analysed the localization of various bitter receptors within HIBCPP cells basolateral and apical membranes facing the bloodstream and CSF, respectively. The human T2R5, 14 and 39 localized in the basolateral membrane, while T2R4 showed a sub-apical distribution. These might indicate their role in the assessment of blood or CSF composition due to the interaction with bitter taste compounds found in circulation in each fluid. Bitter taste signalling functionality was assessed by using different bitter agonists (haloperidol, resveratrol, quercetin and epigallocatechin gallate) as stimuli. Intracellular calcium levels increased in HIBCPP cells in response to haloperidol and resveratrol in a dose-dependent manner and to quercetin in a dose-independent manner. Considering the therapeutic potential of resveratrol for CNS diseases we further analyzed its ability to bind and activate T2R14 and 39, previously described as the receptors for resveratrol. Using siRNA knockout experiments, we observed a specific response of HIBCPP cells to resveratrol (50μM) through T2R14 (Figure 2) (16).

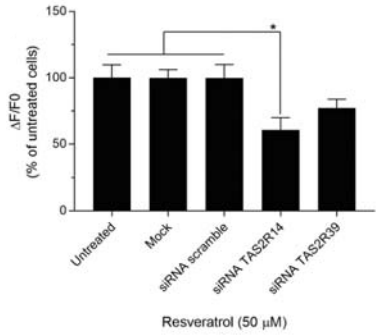


Figure 2. Calcium responses to resveratrol stimulus in HIBCPP cells transfected with bitter receptor 14 or 39 siRNAs. Intracellular calcium levels were measured in HIBCPP cells transfected or mock-transfected for 72 h with bitter receptor 14 or 39 siRNA, or with scramble siRNA, after resveratrol (50 μM) stimulus. Graph bars indicate the mean ± SEM ($N \geq 4$, independent cultures; $^*p < 0.05$; One-way ANOVA followed by Bonferroni's post hoc test).

In summary, we demonstrated that taste signalling pathway is present and functional in rat and human CP suggesting a role for this pathway in the assessment of the chemical surveillance of blood and CSF composition. These findings are relevant to understand the mechanisms operating at the blood-CSF barrier responsible for the surveillance of blood and CSF composition and put in evidence a novel type of interaction between therapeutic drugs and brain barriers. In addition, we showed that sex hormones, specifically, 17-βestradiol regulates genes of the taste transduction pathway. In the future, it would be interesting to evaluate the functional implications of this regulation, namely regarding the ability of bitter agonists to access the CNS trough the blood-CSF barrier. Additionally, taste and olfactory pathways responses to relevant compounds in the frame of the neurological field would also be important to understand its role in the chemical surveillance of blood and CSF.

Acknowledgements: Supported by the Portuguese Foundation for Science and Technology (FCT, Portugal) project grants (PTDC/SAU-NEU/114800/2009, project UID/Multi/04326/2013, project UID/Multi/00709/2013 and UID / Multi / 00709/2019), and FEDER funds: project POCI-01-0145-FEDER-007491 and ICON project (Interdisciplinary Challenges On Neurodegeneration; CENTRO-01- 0145-FEDER-000013).

References

1.Redzic, Z.B., Segal, M.B., 2004. The structure of the choroid plexus and the physiology of the choroid plexus epithelium. *Adv. Drug Deliv. Rev.* 56, 1695– 1716.
2.C.R.A. Santos, A.C. Duarte, T. Quintela, J. Tomás, T. Albuquerque, F. Marques, J.A. Palha, I. Gonçalves, The choroid plexus as a sex hormone target: Functional implications, *Front. Neuroendocrinol.* 44 (2017) 103–121
3.Quintela, T., Gonçalves, I., Carreto, L.C., Santos, M.A.S., Marcelino, H., Patriarca, F. M., Santos, C.R.A., Goncalves, I., Carreto, L.C., Santos, M.A.S., Marcelino, H., Patriarca, F.M., Santos, C.R.A., 2013. Analysis of the effects of sex hormone background on the rat choroid plexus transcriptome by cDNA microarrays. *PLoS ONE* 8, e60199
4.C.R.A. Santos, A.C. Duarte, A.R. Costa, J. Tomás, T. Quintela, I. Gonçalves, The senses of choroid plexus, *Progress in Neurobiology.* 182 (2019)
5.N.M. Dalesio, S.F. Barreto Ortiz, J.L. Pluznick, D.E. Berkowitz, Olfactory, Taste, and Photo Sensory Receptors in Non-sensory Organs: It Just Makes Sense, *Front. Physiol.* 9 (2018) 1673–1691.
6.S.J. Lee, I. Depoortere, H. Hatt, Therapeutic potential of ectopic olfactory and taste receptors, *Nat. Rev. Drug Discov.* 18 (2019) 116–138
7.G. Nelson, M.A. Hoon, J. Chandrashekar, Y. Zhang, N.J.P. Ryba, C.S. Zuker, Mammalian sweet taste receptors, *Cell.* 106 (2001) 381–390
8.G. Nelson, J. Chandrashekar, M.A. Hoon, L. Feng, G. Zhao, N.J.P. Ryba, C.S. Zuker, An amino-acid taste receptor, *Nature.* 416 (2002) 199–202
9.E. Adler, M.A. Hoon, K.L. Mueller, J. Chandrashekar, N.J.P. Ryba, C.S. Zuker, A novel family of mammalian taste receptors, *Cell.* 100 (2000) 693–702
10. J. Chandrashekar, K.L. Mueller, M.A. Hoon, E. Adler, L. Feng, W. Guo, C.S. Zuker, N.J. Ryba, T2Rs function as bitter taste receptors, *Cell.* 100 (2000) 703–711
11. J. Chandrashekar, M.A. Hoon, N.J. Ryba, C.S. Zuker, The receptors and cells for mammalian taste, *Nature*. 444 (2006) 288–294
12. N. Chaudhari, S.D. Roper, The cell biology of taste, *J. Cell Biol.* 190 (2010) 285–296
13. J. Tomás, C.R.A. Santos, T. Quintela, I. Gonçalves, "Tasting" the cerebrospinal fluid: Another function of the choroid plexus? *Neuroscience*. 320 (2016) 160–171
14. J. Tomás, C.R.A. Santos, A.C. Duarte, M. Maltez, T. Quintela, M.C. Lemos, I. Gonçalves, Bitter taste signaling mediated by Tas2r144 is down-regulated by 17β-estradiol and progesterone in the rat choroid plexus, *Mol. Cell. Endocrinol.* 495 (2019)
15. A.C. Duarte, J. Santos, A.R. Costa, C.L. Ferreira, J. Tomás, T. Quintela, H. Ishikawa, C. Schwerk, H. Schroten, I. Ferrer, E. Carro, I. Gonçalves, C.R.A. Santos, Bitter taste receptors profiing in the human blood-cerebrospinal flid-barrier, *Biochem. Pharmacol.* (2020)
16. Ana C. Duarte, Tiago Rosado, Ana R. Costa, José Santos, Eugénia Gallardo, Telma Quintela, Hiroshi Ishikawa, Christian Schwerk, Horst Schroten, Isabel Gonçalves, Cecília R.A. Santos, The bitter taste receptor TAS2R14 regulates resveratrol transport across the human blood-cerebrospinal flid barrier, *Biochem. Pharmacol.* (2020)

THE GONADOTROPHIN-INHIBITORY HORMONE OF SEA BASS: MUCH MORE THAN REPRODUCTION

J.A. Paullada-Salmerón[1], G.H. Loentgen[1], M.E. Cowan[1], L. Aielli[1], M. Fuentès[2], L. Besseu[2], E.L. Mañanós[3], J.A. Muñoz-Cueto[1]

1. Department of Biology, Faculty of Marine and Environmental Sciences and INMAR, University of Cádiz, CEIMAR, Puerto Real (Cádiz), Spain.
2. Sorbonne Université, CNRS, Observatoire Océanologique, Banyuls sur Mer, France.
3. Institute of Aquaculture of Torre de la Sal, CSIC, Castellón, Spain.

Gonadotrophin-inhibitory hormone (GnIH) is a hypothalamic neuropeptide belonging to the RFamide peptide family. Following on from pioneer research in avian species, subsequent studies performed in other vertebrates demonstrated that GnIH could modulate the reproductive process from mammals to fish. In previous studies, we have identified a *gnih* gene encoding two sea bass Gnih peptides, localized Gnih cells and fibres in the sea bass brain and pituitary, and evidenced the inhibitory role of centrally- and peripherally-administered Gnih in the reproductive brain-pituitary-gonadal axis of male sea bass. In addition, GNIH is also involved in the regulation of feeding, growth, stress response and social/agonistic behaviour in birds and mammals. Unfortunately, all these aspects remain almost unexplored in fish. In our laboratory, we have recently addressed the study of non-reproductive actions of Gnih in sea bass, showing that this neuropeptide can modulate the expression of some orexigenic/anorexigenic hormones and stress-related genes. Moreover, we revealed that Gnih affected the neurosteroid production and inhibited the aggressive behaviour of male sea bass. Altogether, our results show that Gnih could be involved in functions other than reproduction and could exert pleiotropic actions in fish.

Introduction

Since professor Tsutsui discovered in 2000 that gonadotrophin-inhibitory hormone (GNIH), a hypothalamic neuropeptide of the RFamide family, inhibited LH release from the pituitary gland of birds, important progress has been made to understand its role in vertebrates' reproduction, but also in other processes (1). However, although fish represents around half of all living vertebrate species, the study of Gnih and its physiological effects is limited and has been approached only in a few fish species, including sea bass (2). To understand the function of Gnih system in the sea bass brain, we previously cloned *gnih* gene, whose precursor encodes two putative Rfamide peptides, termed sbGnih1 and sbGnih2, respectively. In our laboratory, we have also developed specific antibodies against endogenous sea bass Gnih peptides, for the first time in a teleost fish. These antibodies allowed us to localized the presence of Gnih-immunoreactive cells in the olfactory bulbs, ventral telencephalon, diencephalon, dorsal tegmentum and rhombencephalic isthmus (3). In addition, we observed that Gnih-immunoreactive cells not only projected to the pituitary but also to other brain areas such as the ventral and dorsal telencephalon, habenula, pineal organ, ventral thalamus, vascular sac, pretectum, rostral midbrain tegmentum, posterior tuberculum, reticular formation and facial-vagal sensory lobes, suggesting that Gnih could play functions other than

reproduction, e.g., feeding behaviour and social aggressive behaviour (3). Previously, it has been shown that the central administration of GNIH increased food intake in rats and chicks. Interestingly, it has been reported that GNIH activates aromatase activity and increases estrogen levels in the preoptic area of quail, and these effects have been associated with an inhibition of aggressive and sexual behaviour (4). However, although GNIH is involved in the regulation of feeding, social and aggressive behaviours in birds and mammals, these aspects remain unexplored in fish. Accordingly, we investigated the effects of Gnih intracerebroventricular (icv) injections on orexigenic and anorexigenic neuropeptides involved in the control of feeding. Moreover, we also analysed its actions on neurosteroidogenic enzymes in the sea bass brain and performed a behavioural mirror test to determine Gnih effects on agonistic behaviour and aggression levels in this species.

Materials and methods

Animals for the expression analysis of orexigenic/anorexigenic neuropeptides and neurosteroidogenic enzymes were housed in the "Laboratorio de Cultivos Marinos" (University of Cádiz, Puerto Real, Spain), and reared under natural photoperiod, at a temperature and salinity of 19±1ºC and 39 ppt. respectively. Sexually mature males at the beginning of the reproductive season (end of November), were injected intracerebroventricularly (icv) with sbGnih1 and sbGnih2 peptides, at doses of 1, 2 and 4 µg/animals. Subsequently, fish from each group were sampled 6h and 12h post-injection (hpi) and the brain was dissected. Animals for the behaviour analysis were obtained from Aqualande (Salses-le-Château, France), housed in the aquarium of the Biodiversarium (Sorbonne Université) and maintained under natural conditions. Sexually mature males were icv-injected with sbGnih2 peptide at a single dose of 2 µg/animal or saline (controls). Subsequently, fish from the same condition were placed by pairs in the experimental tanks and recovered during 15 min before behavioural tests. The period of behavioural analysis and recording was 30 min. The video recordings were analysed using Noldus EthoVision XT software and some parameters defined by Noldus system (proximity, relative movement, mobility, acceleration state, velocity and distance moved), as well as others such as latency, C-shape bending of the body in front of the mirror were determined. All animals were anesthetised by immersion with MS-222 before sacrifice and all samples dissected were frozen in liquid nitrogen and stored at -80ºC until used.

Results and discussion.

We examined the effects of icv injections of both sbGnih1 and sbGnih2 peptides on the expression of orexigenic (*npy, agrp*) and anorexigenic (*crh, pomc*) neuropeptides. The administration of sbGnih1 induced a significant increase in brain *npy* expression with two doses tested (2µg, 4µg) when compared to the control group at 12 hpi (fig.1A). In contrast, the administration of sbGnih2 significantly decreased the expression of *npy* at 2µg and 4µg doses at 6hpi (fig.1C). Both stimulatory and inhibitory effects of GNIH on NPY neurons have been reported in birds and mammals, respectively (1). Interestingly, NPY is not only involved in feeding but also in the stimulatory control of reproduction of sea bass. According to previous results obtained in sea bass, sbGnih2 appeared to be the most active form in down-regulating the reproductive axis of this species (2). Therefore, decreased *npy* expression after sbGnih2 treatment could be reinforcing the inhibitory role of sbGnih2 on sea bass reproduction. Whether sbGnih1 is modulating Npy effects on feeding and sbGnih2 is controlling Npy

actions on reproduction of sea bass will require further investigation. Regarding anorexigenic neuropeptides, the administration of sbGnih1 resulted in a significant decrease of *pomc* expression at 6 hpi with the dose of 1µg (fig.1B). In turn, injection of sbGnih2 decreased *crh* transcript levels at the dose of 2µg (fig.1D). Altogether, these results reinforce the assumption that Gnih could represent an orexigenic neuropeptide in sea bass.

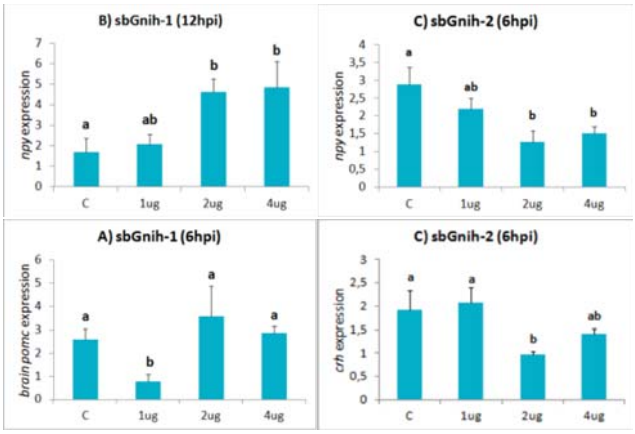


Figure 1. Effect of *in vivo* intracerebroventricular (icv) injection of different doses (1µg, 2µg and 4µg) of sbGnih1 and sbGnih2 peptides on *npy* (A, C), *pomc* (B) and *crh* (D) expression at 6 and 12 h post injection (hpi) in the brain of male sea bass. Data are presented as mean ± SEM (n= 4-5). Different lowercase lettering denotes statistically significant differences as revealed by ANOVA ($p<0.05$). C, control.

GnIH can also modulate behaviour through its action on neurosteroid biosynthesis in the brain of vertebrates (1, 4). To elucidate whether Gnih is also involved in the modulation of brain neurosteroidogenesis in sea bass, we analysed the effect of the central administration of both Gnih peptides in the brain expression of the main neurosteroids-synthesizing enzymes in this species. Injection with sbGnih1 resulted in a significant decrease in *3bhsd* and *17bhsd* expression at the dose of 2µg (fig. 2A, C) and a significant increase of *cyp19b* transcript levels at doses of 1µg and 4µg (fig. 2B). Similarly, central administration of sbGnih2 induced a decrease in the brain *3bhsd* gene expression with the three doses tested when compared to the control group and *17bhsd* mRNA levels at doses of 2µg and 4µg (fig. 2D, F). In addition, sbGnih2 also determined an increase of *cyp19b* expression at the lower dose tested (fig. 2E). Considering that 17 Hsd is a key enzyme for the synthesis of androgens (e.g. testosterone) and aromatase converts testosterone to estradiol, these results suggest that Gnih could decrease neuroandrogens and increase neuroestrogens synthesis in the brain of sea bass.

In addition, we tested the effect of the central administration of sbGnih2 on several parameters related to agonistic and aggressive behaviour using the mirror test. Our results showed that animals treated with Gnih significantly decreased the number of contacts with the mirror and the time that animals spent in the mirror zone, and increased the time needed to establish the first contact with the mirror and the mean distance to the mirror zone.

Several studies have revealed that testosterone and cortisol play an important role on social-aggressive behaviour in vertebrates (5). Moreover, in Japanese quail, GNIH administration stimulated the activity of aromatase and increased estradiol synthesis in the preoptic area both *in vivo* and *in vitro,* suggesting that GNIH inhibits social-sexual behaviour by increasing neuroestrogens synthesis in this species (4). Therefore, the results obtained in our study suggest that Gnih could inhibit male aggressive behaviour in sea bass, likely by decreasing neuroandrogens and increasing neuroestrogens synthesis in the brain of this species.

Acknowledgements: Funded by the Junta de Andalucía (P10-AGR-05916), UE (FP7/2007-2013, nº 331964) and EMBRC (OOB–EMBRC FR–AAP2018 nº 2200) grants to JAMC.

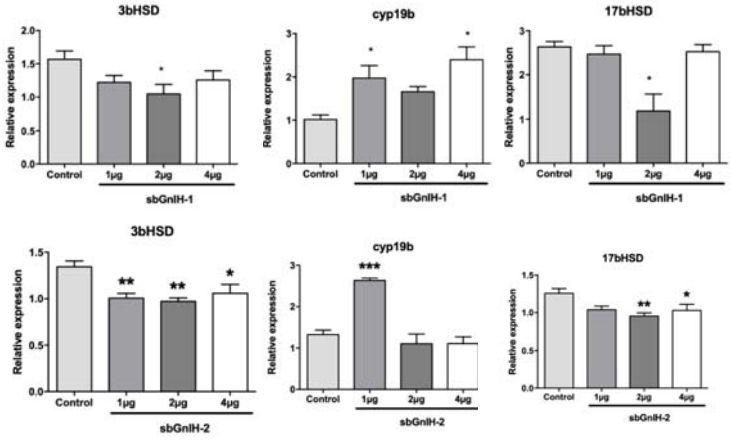


Figure 2. Effect of *in vivo* intracerebroventricular (icv) injection at different doses (1, 2, and 4 µg) of sbGnIH1 (**A**, **B**, **C**) and sbGnIH2 (**D**, **E**, **F**) peptides on *3bhsd, cyp19b* and *17bhsd* relative expression at 6 hpi in the brain of male sea bass. Values are expressed as mean ± SEM (n = 5). Asterisks denote significant differences as revealerd by one-way ANOVA (*p<0.05, **p<0.01, ***p<0.001).

References

1. Ubuka, T., Son, Y.L., Tsutsui, K., 2016. Molecular, cellular, morphological, physiological and behavioural aspects of gonadotropin-inhibitory hormone. Gen. Comp. Endocrinol. 227, 27-50.

2. Muñoz-Cueto, J.A., Paullada-Salmerón, J.A., Aliaga-Guerrero, M., Cowan, M.E., Parhar, I.S., Ubuka, T., 2017. A journey through the gonadotropin-inhibitory hormone system of fish. Front. Endocrinol. 8, 285.

3. Paullada-Salmerón, J.A., Cowan, M., Aliaga-Guerrero, M., Gómez, A., Zanuy, S., Mañanós, E., Muñoz-Cueto, J.A., 2016. LPXRFa peptide system in the European sea bass, a molecular and immunohistochemical approach. J. Comp. Neurology. 524; 176-198.

4. Ubuka T, Haraguchi S, Tobari Y, Narihiro M, Ishikawa K, Hayashi T, et al. Hypothalamic inhibition of socio-sexual behaviour by increasing neuroestrogen synthesis. Nat. Commun. (2014) 5:3061.

5. Chang C, Li CY, Earley RL, Hsu Y. Aggression and related behavioral traits: the impact of winning and losing and the role of hormones. Integr Comp Biol. 2012;52(6):801-813.

FUNCTIONAL ACTIVITY OF RECOMBINANT FORMS OF ANTIMÜLLERIAN HORMONE FROM EUROPEAN SEA BASS (*Dicentrarchus labrax*)

C. Zapater, A. Rocha[1], G. Molés, S. Ibáñez, S. Zanuy, A. Gómez

Department of Fish Physiology and Biotechnology, Institute of Aquaculture Torre la Sal, CSIC, Castellon, Spain.
[1]Present address: CIIMAR, MARE IPLeiria, Portugal.

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β superfamily that classically has been correlated to the regression of Müllerian ducts in higher vertebrates. Despite the absence of Müllerian ducts in teleosts, orthologues of mammalian AMH have been described in some fish species, and a role of this hormone in sex determination and gonad differentiation has been demonstrated. In adult teleost gonads, Amh acts at early stages of germ cell development in both males and females, however, the exact mechanism of Amh signaling and its implication in gonad development are poorly investigated. We have recently demonstrated that Amh signals through a transmembrane AMH type II receptor (Amhr2) with serine-threonine kinase activity, and that Amh is localized in Sertoli cells surrounding early germ-cell generations, but still lacking knowledge about the function of this growth factor. As tool for studying the mechanisms of Amh action, we have produced specific recombinant mature forms of this hormone, and have used them in *in vitro* transactivation experiments to study the intracellular signaling pathways of sea bass Amhr2. In addition, we have performed *in vitro* tissue cultures of ovaries and testis treated with recombinant Amh to study the impact of this hormone in steroidogenesis, and its interaction with Fsh, both by analyzing steroid production and gene expression.
We finally concluded that Amh promotes steroid production in immature testis and early vitellogenic ovaries, showing a clear involvement of sea bass Amh in gametogenesis.

Introduction

The Anti-Müllerian hormone (AMH) belongs to the transforming growth factor β (TGF-β) superfamily and it is an inhibiting factor that promotes the regression of the Müllerian duct during normal male sexual differentiation in amniotes [1]. AMH signals through a heterodimeric receptor complex consisting of two related serine/threonine kinase receptors, type 2 and type 1 receptors. In teleosts, the existence of an *amh* orthologue gene was described in different species [2]. Most teleosts present a male-biased *amh* expression during sex differentiation or at least in differentiated juvenile gonads [3-5], corroborating the involvement of Amh in male sex differentiation as observed in mammals. In addition, expression of *amh* and localization of Amh polypeptide are found in Sertoli cells in adult testis and granulosa cells of previtellogenic and vitellogenic follicles in adult ovaries [6, 7], suggesting an involvement of Amh in gonadal steroidogenesis and follicular development. The role of Amh in some teleost species is similar to that observed in mammals, namely inhibition of androgen- or Fsh-stimulated spermatogenesis in males, and involvement in vitellogenin uptake and transition from primary to secondary growth in females [8, 9]. However, most studies have been conducted on model species such as zebrafish or medaka and still knowledge is lacking about the function of this

growth factor in other fish species. In the European sea bass (*Dicentrarchus labrax*), an *amh* gene had been previously isolated and its expression and that of alternatively spliced isoforms analyzed [10]. We have demonstrated that sea bass Amh signals through a transmembrane AMH type II receptor (Amhr2) with serine-threonine kinase activity [11]. As tool for studying the mechanisms of Amh action, we report the production of specific recombinant mature forms of this hormone, and their use to study the intracellular signaling pathways of the sea bass Amhr2. In addition, we have performed *in vitro* tissue cultures of ovaries and testis treated with recombinant Amh to study the impact of this hormone in steroidogenesis.

Materials and Methods

The expression plasmids pPICK9-His6Amh and pPICK9-AmhHis6, to be used with the *Pichia pastoris* expression system, and pcDNA3-His6Amh, to be used in the Chinese hamster ovary (CHO) expression system, were generated in several steps by PCR amplification. Each plasmid contains a modified sea bass *amh* cDNA. The specific sequence modifications have been introduced to optimize, for each expression system, the putative cleavage site that generates the mature peptide, and to facilitate its purification by addition of a his-tag. After verifying by Western blot the production of recombinant mature Amh, its bioactivity has been tested using transactivation assays. The sea bass *amhr2* cDNA was expressed in African green monkey kidney fibroblast-like (COS-7) cells seeded in 24-well plates (~1.5 x 10^5 cells per well). Cells were grown in Dulbecco modified Eagle medium (DMEM) with GlutaMAX (Life Technologies, Inc.) supplemented with 10% v/v heat inactivated fetal bovine serum (FBS), and 100 U/ml of penicillin and streptomycin, at 37ºC in a 5% CO2 incubator. When cells reached 75-80% confluence they were cotransfected using FuGENE® HD Transfection Reagent (Promega) with 100 ng of the reporter plasmid pBRE-Luc, 415 ng of pcDNA3-Amhr2 plasmid [11], and 20 ng of pRL-TK reporter plasmid (Promega Corp.) to normalize transfection. After 24 h, the medium was replaced with DMEM supplemented with 1% FBS containing different concentrations of sea bass Amh (0.25, 0.5 and 1 µg/ml of Amh from *Pichia pastoris*, and 0.2, 2, 5.4 µg/ml from CHO). Media obtained from *Pichia pastoris* expressing the empty pPIC9K vector or native CHO cells served as control. After incubation at 37ºC for 24 h, cells were washed twice with PBS, pH 7.4 and harvested in 20 µl of Passive Reporter Lysis buffer (Promega). Determination of luciferase activity was done using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

For *in vitro* tissue culture, ovaries and testis of adult sea bass, sacrificed in October, were dissected and rapidly immersed in ice-cold Sea Bass Ringer (SBR). The explants were preincubated for 60 min at 21ºC under shaking conditions (100 rpm). Then, the medium was replaced with 0.1 ml of fresh SBR containing different quantities of sea bass Amh. After 24 h of incubation, 300 ng/ml of seabass Fsh or DMEM control medium were added, and samples were further incubated during 24 h. Finally, the medium was collected and gonadal explants deep-frozen in liquid nitrogen and stored at −80 °C until posterior analyses. Medium samples were used to quantify steroid production by specific immunoassays, and tissue explants were used to quantify expression of steroidogenic enzyme genes by quantitative real-time PCR (qPCR).

Results and Discussion

With the aim of producing a bioactive recombinant sea bass Amh propeptide, a combination of strategies was adopted (Fig. 1). For each construct, expression levels of recombinant protein were screened by Western blot on up-concentrated media, and the highest expressing clone used in all subsequent experiments. High levels of recombinant sea bass Amh were produced. To test the bioactivity of recombinant sea bass Amh proteins, we used a cell-based reporter assay that depends on sea bass Amhr2 activity. All recombinant sea bass Amh proteins were able to increase the activity of the BRE-Luc reporter gene in a dose-dependent manner. Recombinant Amh produced in the *Pichia pastoris* expression system (Fig. 1B) activated the receptor with more efficiency than Amh produced in CHO cells [11]. The presence of a his-tag did not have a major impact on binding. Nevertheless, the recombinant sea bass Amh-His significantly activated the receptor with all the tested doses (0.25-1 µg/ml), while recombinant sea bass His-Amh was less efficient and significant activation above control levels was only observed for the highest doses (0.5 and 1 µg/ml). Considering the higher bioactivity and production scale of Amh-His, we only used this recombinant hormone in the explant culture experiments.

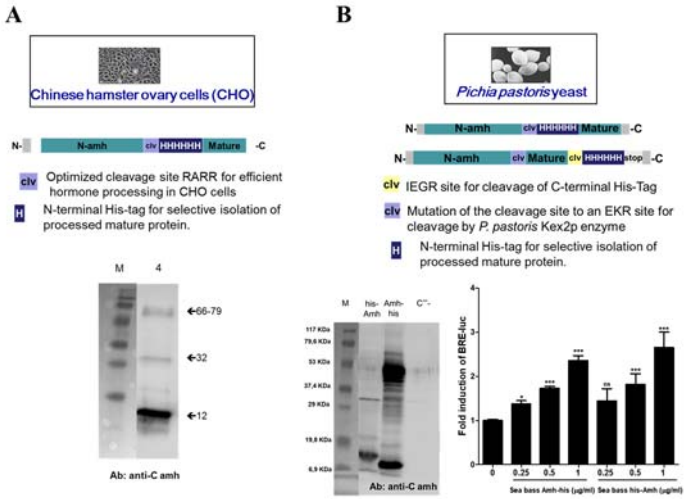


Figure 1. A). Production of European sea bass Amh in CHO cells. B) Production and bioactivity of European sea bass His-Amh and Amh-His in *Pichia pastoris* expression system.

Next, we assessed the role(s) of Amh in adult sea bass ovaries and testis. The results show that in ovarian explant cultures, estradiol (E2) levels increased in response to Fsh treatment although not significantly. Addition of different doses of sea bass Amh to Fsh treatments resulted in a significant increase in E2 production, respect to that produced by Fsh alone, but only when using the highest dose of Amh, therefore showing a synergistic effect of Amh on Fsh-induced E2 synthesis in vitellogenic ovaries. Results for *cyp19a1a* expression exhibited a similar profile to that observed for E2 production. A non-significant increase in *cyp19a1a* transcript levels was observed in Fsh treatments. When

these were combined with sea bass Amh treatments, the increase became significantly different from the control, and more prominent when the highest dose of Amh was used. In testis explants, levels of steroids and expression of steroidogenic enzyme genes increased only with the addition of Amh, while Fsh had no effect on steroid production.

In conclusion, by using a recombinant sea bass Amh we found that sea bass Amh is processed in a similar way to mammalian Amh, becoming a biologically active protein able to bind and activate sea bass Amhr2. The results obtained with the *in vitro* tissue culture suggest that Amh is involved in sea bass gametogenesis promoting steroid production both in males and females.

Acknowledgements: Supported by funds from MICINN (AGL2015-67477-C2-1-R, RTI2018-094667-B-C22) and EU (LIFECYCLE FP7-22719-1). C. Z. was recipient of a postdoctoral fellowship from the Ministerio de Ciencia e Innovación (Programa Juan de la Cierva).

References

1. Adolfi MC, Nakajima RT, Nóbrega RH, Schartl M. (2019) Annu Rev Anim Biosci. 7:149-72..
2. Pfennig F, Standke A, Gutzeit HO. (2015). Gen Comp Endocrinol. 223:87-107.
3. Yoshinaga N, Shiraishi E, Yamamoto T, Iguchi T, Abe S, Kitano T. (2004). Biochem Biophys Res Commun. 322:508-513.
4. Wang XG, Orban L. (2007). Dev Dyn. 236:1329-1338.
5. Maugars G, Schmitz M. (2008). Gen Comp Endocrinol. 159:178-187.
6. Rodriguez-Mari A, Yan YL, Bremiller RA, Wilson C, Canestro C, Postlethwait JH. (2005). Gene Expr Patterns. 5:655-667.
7. Kluver N, Pfennig F, Pala I, Storch K, Schlieder M, Froschauer A, Gutzeit HO, Schartl M. (2007). Dev Dyn. 236:271-281.
8. Skaar KS, Nobrega RH, Magaraki A, Olsen LC, Schulz RW, Male R. (2011). Endocrinology. 152:3527-3540.
9. Morinaga C, Saito D, Nakamura S, Sasaki T, Asakawa S, Shimizu N, Mitani H, Furutani-Seiki M, Tanaka M, Kondoh H. (2007). Proc Natl Acad Sci U S A. 104:9691-9696.
10. Halm S, Rocha A, Miura T, Prat F, Zanuy S. (2007). Gene. 388:148-158.
11. Rocha A, Zanuy S, Gomez A. (2016). Biol Reprod. 94:141.

FISH EMBRYONIC STEM CELLS AS TOOLS FOR CHRONOBIOLOGICAL AND ENDOCRINOLOGICAL STUDIES

A. Vergès-Castillo[1,2], C. Pendón[1], J.A. Muñoz-Cueto[2], Á.J. Martín-Robles[1]

[1]Department of Biomedicine, Biotechnology and Public Health, Biochemistry and Molecular Biology Area. INBIO, Faculty of Sciences, University of Cádiz, Spain.
[2]Department of Biology, Faculty of Marine and Environmental Sciences, INMAR, University of Cádiz, Spain.

Circadian rhythms exist in many biochemical, physiological and cellular processes, including hormonal synthesis and/or secretion. These rhythms are driven by endogenous clocks that are ultimately entrained by environmental factors, being the light-dark cycle (LD) the most important synchronizer. Fish circadian rhythms appear sustained by cell-autonomous mechanisms, but unfortunately, fish cellular clocks have been poorly studied in marine species. An important bottleneck in fish research is the supply and maintenance of embryos, larvae, juvenile and adult specimens. In this context, cell lines constitute powerful and alternative tools with important applications in fish chronobiology and endocrinology. In this study, we have used a recently developed monoclonal embryonic cell line derived from the blastula stage of gilthead seabream embryos (SAEC-H7) to analyse the oscillation of central clock components and the response to different photoregimes. Our results showed rhythmic expression of key clock genes in embryonic cells, which were able to re-entrain to a light cycle inversion. Rhythmic expression of some core clock genes persisted under constant light and dark conditions after several days, but with decreased mesors and amplitudes, reinforcing the circadian and endogenous nature of the cellular rhythms. These results indicate that SAEC-H7 contain a functional molecular clock entrained by light, representing an important tool for chronobiological and endocrinological studies in fish.

Introduction

Embryonic stem (ES) cells have been revealed as powerful tools to improve our knowledge in many research fields, including chronobiology and endocrinology. Abundant studies performed in ES cells have reinforced the importance of daily light cycles as strong synchronizers of the molecular clock and many cellular processes, providing useful information for understanding the functioning of living organisms (1). ES cells have also been used for improving our knowledge of cell development and differentiation, cellular metabolism, cytotoxicity, intracellular mechanisms of hormonal action, gene expression and its regulation. Therefore, ES cell lines can be considered as helpful experimental models for *in vitro* studies that complements *in vivo* analyses, permitting easier experimental design and sampling and avoiding the sacrifice of animals. The gilthead seabream (*Sparus aurata*) is one of the most important commercial fish species for European aquaculture. Recently, several studies have focused on the circadian system of developing seabream, characterising daily rhythms of clock genes expression and performing a functional analysis of circadian transcriptome in larvae (2, 3). In the present work, we have used a recently developed monoclonal ES cell line derived from seabream embryos to

characterise cellular circadian rhythms in the molecular clock and their response to light photoperiod.

Materials and Methods

Development of a Sparus aurata cell culture and routine maintenance: Seabream fertilized oocytes at blastula stage were properly disinfected and washed with sterile sea water and PBS. Embryonic cells were obtained by mechanical disruption and cultured at 22°C in Leibovitz's media (L-15) supplemented with 10% fetal bovine serum and NaCl. At passage 20, a limited dilution was performed to develop monoclonal embryonic stem cell lines.

Circadian rhythms analysis: $2x10^5$ cells were plated in 6-well plates and maintained under light-dark (LD) cycles (12h:12h) of white light and constant temperature during three days. Thereafter, cells were maintained under LD, LD-DL, constant light (LL) or dark (DD) photoregimes during three days and sampled. RNA was extracted every 4 or 6 hours during four complete cycles and aliquots of 500 ng were retrotranscribed to cDNA. Relative expression of clock genes (*clock*, *bmal1*, *per2*, *cry*) was analysed by real-time qPCR, using *β-actin* as reference gene. El Temps software was used to analyse clock rhythms by the cosinor method.

Results and Discussion

Sparus aurata ES cell line (SAEC-H7) has been maintained during more than 150 passages displaying embryonic shape, continuous and unmodified growing, indicating the establishment of long-term immortalized cell cultures. All clock genes analysed in SAEC-H7 cells exposed to 12 h light and 12 h darkness (LD:LD) cycles during four consecutive days showed light-entrained daily rhythms (Fig. 1), as it was reported in zebrafish cells (1).

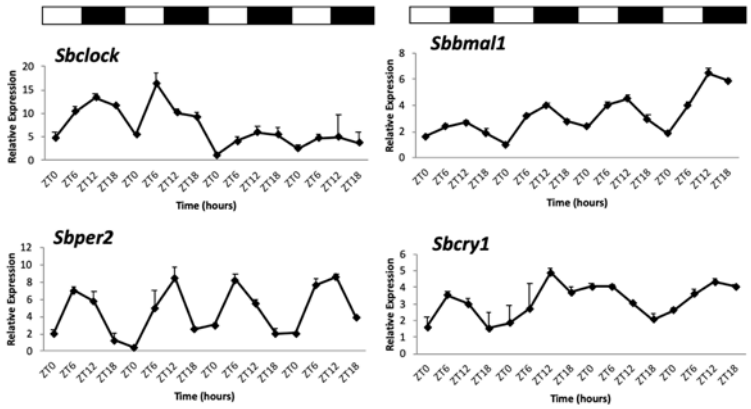


Figure 1. Relative mRNA expression of clock components (*clock*, *bmal1*, *per2* and *cry1*) in SAEC-H7 cell line. Cells were collected every 6 hours during 4 complete days under 12L:12D cycles. Bars above graphs represent photoperiod conditions (white bars indicate light period and black bars indicate darkness period). ZT, zeitgeber time.

When seabream cells were maintained under LD cycle (Fig. 2A) and the photoperiod inverted into DL cycle (Fig. 2B), clock genes profiles also inverted. In LD conditions, *clock* has its expression peak in the transition from light to darkness phase (Fig. 2A *Sbclock*).

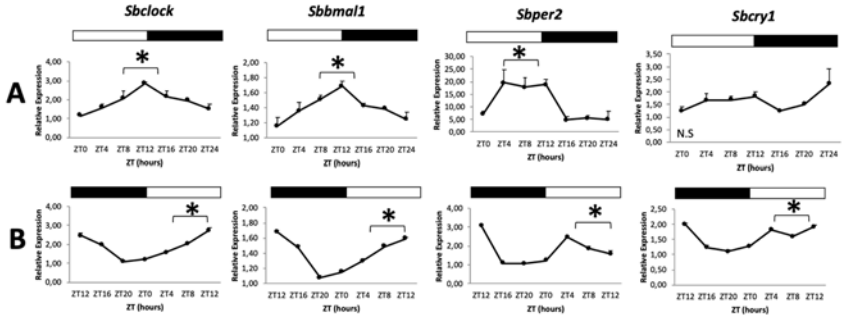


Figure 2. Relative mRNA expression of *clock*, *bmal1*, *per2* and *cry1* in SAEC-H7 cell line under 12L:12D (A) and 12D:12L (B) photoregimes. Cells were collected every four hours from ZT0 to ZT24 in LD cycle and from ZT24 to ZT12 in DL cycle. The bars above graphs represent the photoperiod conditions. White bars indicate light period (ZT0 to ZT12) and black bars indicate darkness period (ZT12 to ZT24). Asterisks show acrophases as revealed by cosinor analysis. ZT, zeitgeber time.

When photoperiod was inverted, its profile also inverted, exhibiting a minimum in the transition from dark to light phase (Fig. 2B *Sbclock*). The same expression rhythm profile and response to light inversion was observed in *bmal1* (Fig. 2B *Sbbmal1*). This result suggests that *clock* and *bmal1* have the capability to be entrained by light, as previously described in seabream larvae (2). *Per2* displays its higher expression during the light phase and a decrease in the transition from light to darkness (Fig. 2A *Sbper2*). When photoperiod was inverted, *per2* profile peaked at the beginning of light and dark phases declining thereafter, showing the lowest transcript levels during dark phase (Fig. 2B *Sbper2*). This result indicates that SAEC-H7 cell line is light-sensitive and exhibits a robust oscillation in clock gene expression, being able to be entrained by the LD cycle and re-entrained effectively after three days of inverted DL cycles. A similar fact was previously reported in several developing and adult fish species (1,4). SAEC-H7 cells maintained under constant light conditions (LL) maintained the oscillation of *clock* and *bmal* expression.

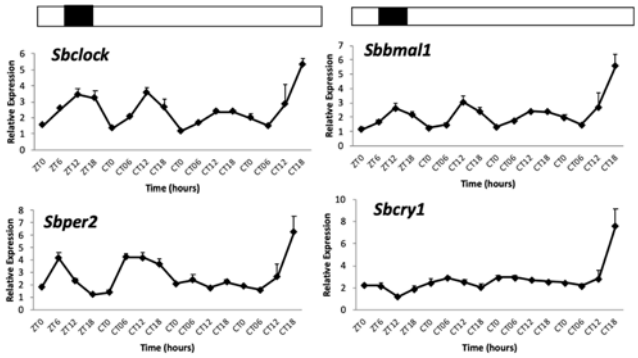


Figure 3. Relative mRNA expression of *clock*, *bmal1*, *per2* and *cry1* in SAEC-H7 cells under constant light conditions (LL) after three days under LD cycles. Cells were collected every six hours. Bars above graphs represented the photoperiod (white bars indicate light period and black bars indicate darkness period). ZT, zeitgeber time. CT, circadian time.

These fluctuating patterns were evident during two LL cycles but rhythms were affected because acrophases were shifted. In turn, oscillation of *per2* continued during one full day (Fig. 3). In constant darkness conditions (DD), *clock* and *bmal1* rhythmic patterns persisted for 2 days and *cry* for 1 day, but with a gradual decline in amplitudes. In contrast, *per2* expression rhythms appear to be light-driven because non-oscillating transcript levels were observed from the first day under constant darkness (Fig. 4). The existence of robust daily rhythms in clock gene expression following exposure to LD cycles, their response to inverted light cycles and the maintaining of rhythmicity under constant conditions support that seabream ES cell line SAEC-H7 contain a light-entrained circadian clock (1, 4).

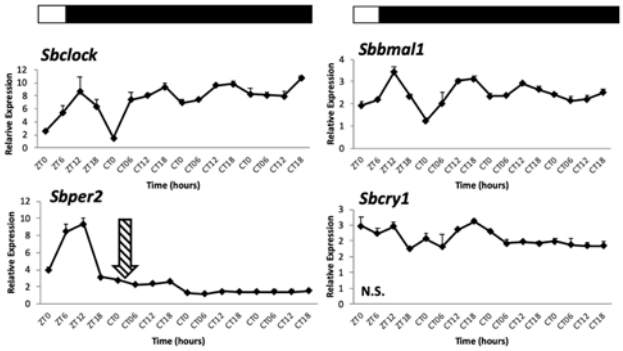


Figure 4. Relative mRNA expression of *clock*, *bmal1*, *per2* and *cry1* in SAEC-H7 cells under constant dark conditions (DD) after three days of LD cycles. Cells were collected every six hours. Bars above graphs represented the photoperiod (white bars indicate light period and black bars indicate darkness period). Arrow indicates *per2* loss in oscillation after the first 12 h in darkness. ZT, zeitgeber time. CT, circadian time.

Acknowledgements: Funded by MINECO Grants to JAMC and CP (AGL2013-49027-C3-2-R) and to JAMC (AGL2017-82582-C3-1-R). AVC has a predoctoral fellowship from the Spanish Ministry of Science, Innovation and Universities (FPI Program PRE2018-085642).

References

1. Whitmore, D., Foulkes N.S., Sassone-Corsi P. (2000). Light acts directly on organs and cells in culture to set the vertebrate circadian clock. Nature. 404(6773):87-91.
2. Mata-Sotres, J.A., Martínez-Rodríguez, G., Pérez-Sánchez, J., Sánchez-Vázquez F.J., Yúfera M. (2015). Daily rhythms of clock gene expression and feeding behaviour during the larval development in gilthead seabream, Sparus aurata. Chronobiology International. 32(8):1061-1074.
3. Yúfera, M., Perera, E., Mata-Sotres, J.A., Calduch-Giner, J., Martínez-Rodríguez, G., Pérez-Sánchez, J. (2017). The circadian transcriptome of marine fish (Sparus aurata) larvae reveals highly synchronized biological processes at the whole organism level. Sci Rep. 7(1):12943.
4. Martín-Robles, A.J., Whitmore, D., Pendón, C., Muñoz-Cueto, J.A. (2013). Differential effects of transient constant light-dark conditions on daily rhythms of period and clock transcripts during Senegalese sole metamorphosis. Chronobiology International. 30(5):699-710.

REV-ERBα CIRCADIAN RHYTHMS: PUTATIVE ROLE ON FOOD INTAKE AND METABOLISM IN GOLDFISH

A. Cebrián[1], N. Saiz[1], M. Gómez-Boronat[1], C. Velasco[2], J.L. Soengas[2], M.J. Delgado[1], E. Isorna[1], N. De Pedro[1]

[1]*Neuroendocrinología de peces, Departamento de Genética, Fisiología Animal y Microbiología, Universidad Complutense de Madrid, Spain.*
[2]*Laboratorio de Fisioloxía Animal, Facultade de Bioloxía, Universidade de Vigo, Spain.*

In this work we explore the role of the nuclear receptor REV-ERBα as a link in the crosstalking between the circadian system and the energy homeostasis in goldfish (*Carassius auratus*). First, we performed experiments using a REV-ERBα agonist (SR9009), showing that this compound significantly decreased food intake at 2 and 8 h post-injection. In addition, the agonist reduced the gluconeogenic capacity and showed a lipolytic effect. No modifications were observed in the expression of *bmal1a*, a core clock gene known to be a direct REV-ERBα target in mammals. We also studied the daily expression profile of *rev-erbα* in central and peripheral tissues of goldfish maintained under different feeding and light conditions. Results reveal that the feeding schedule is the main input for *rev-erbα* rhythmic expression in the goldfish liver, while it seems to be dependent on the light-dark cycle in the hypothalamus. In summary, our results suggest that REV-ERBα could be involved in the regulation of feeding and metabolism in fish, while it would also be an output of the core circadian clock.

Introduction

REV-ERBα is a nuclear receptor that acts as a negative transcription factor for those target genes that have a RORE site in their promoter. In mammals it is known that REV-ERBα takes part in glucidic and lipidic metabolism, by repressing *g6pase* (glucose 6-phosporilase) and *pepck* (phosphoenolpyruvate carboxykinase) transcription in liver, leading to a gluconeogenesis reduction (1). Also, REV-ERBα promotes fatty acids β-oxidation by acting on liver *cpt-1* (carnitine palmotoyltransferase I) (2). Some studies in mammals also suggest that REV-ERBα may exert an anorectic response through repression of orexigenic genes such as orexin (3). Not only acting as a metabolism trigger, REV-ERBα is involved in the circadian clock forming an auxiliary loop that helps stabilize the core clock oscillator (5). Since REV-ERBα is a clock-controlled gene (CCG) and it targets genes implied in metabolism (1), it could play a role in coordinating metabolism and circadian rhythms. So, the objective of this work was to explore the role of this nuclear receptor as a possible link between the circadian system and energy homeostasis in fish.

Materials and Methods

Otherwise noted experiments were performed with goldfish (10–15 g of body weight, b.w.) kept under a 12L:12D photoperiod (lights on at 8 a.m.), water temperature of 21±2ºC and daily fed with 1% b.w. at 10 a.m. Fish were anesthetized (MS-222, 160 mg/l) before handling, and when sacrificed a dose of anaesthetic 320 mg/ml was used. All procedures comply with Spain and European Union current legislation (RD53/2013, EU63/2010).

Role of REV-ERBα in feeding and metabolism. Goldfish (n=9/group) were intraperitoneally (IP) injected with either SR9009 (REV-ERBα agonist, 100 µg/g b.w.) or the vehicle (15% Kolliphor + 85% teleost saline), at 10:00 h (24 h of fasting). Food intake was individually quantified at 2 and 8 h post-injection. In a second experiment, 24-h fasted goldfish were IP injected with SR9009 as above described and blood samples were extracted at 3 h post-injection to determine plasma levels of glucose, triglycerides and fatty acids. Liver samples were used to quantify enzymatic activity (CPT-1 and PEPCK) by real time colorimetry technics previously adapted for goldfish tissues (6). The expression of *ghrelin* in the intestine, *leptin* in the liver, and *orexin*, *npy,* and *pomc* in the hypothalamus was quantified by qPCR as previously described (6).

Periprandrial variations of hepatic expression of *rev-erbα*. Goldfish were sampled 3 h before the scheduled feeding time (10:00 a.m.). At the scheduled feeding time (10:00 a.m.), some fish were fed while others were not. Then, fed and unfed fish were sampled at 1 h and 3 h after feeding time. Expression of *rev-erbα* in goldfish liver (n=6/sampling point) was determined by qPCR as previously described (6).

Daily rhythms of *rev-erbα* expression in liver and hypothalamus. Goldfish (n=42/group) were divided into the following groups: 1st group (12L:12D photoperiod + feeding at 10:00 a.m.); 2nd (12L:12D photoperiod + random feeding time); 3rd (24D + feeding at 10:00 a.m.); 4th (12L:12D + feeding at 15:00 p.m.); and 5th (12L:12D + feeding at 3:00 a.m.). After 1 month under these conditions, goldfish were sacrificed every 4 h (n=7/sampling point) through a 24 h cycle, and the hypothalamus and liver were obtained to analyse rhythmic expression of *rev-erbα*.

Statistical analysis was performed by SigmaPlot® software (t-Student and ANOVA). Cosinor® software was used to analyse daily sinusoidal rhythms of gene expression.

Results and Discussion

Results from the first trial show that SR9009 had a short-term anorectic effect, as intake was 28% reduced in the 0-2 h interval, and 34% in the 0-8 h interval. The relative expression of feeding regulators genes like *leptin, pomc, orexin* and *npy* was not modified by treatment with the REV-ERBα agonist, but modifications of the tisular concentrations of these peptides cannot be discarded. Moreover, REV-ERBα might also exert this anorectic effect by interfering in the hedonic component of food intake, as in rodents REV-ERBα activation represses tyrosine hydroxylase activity (7). Regarding metabolism, hepatic PEPCK activity was slightly reduced (from 5.5±0.8 to 3.7±1 mU/mg protein) in fish injected with the REV-ERBα agonist. Plasma glucose levels also decreased in this group (vehicle 76±9 *vs* SR9009 57±6 mg/dL), suggesting that REV-ERBα activation reduces gluconeogenesis, and therefore glycaemia in fish. Treatment with SR9009 also increased the hepatic activity of CPT-1 (from 16±3 to 23±3 mU/mg protein), which takes part in fatty acid beta oxidation. A concurrent decrease in triglyceride plasma levels (156±7 *vs* 118±10 mg/dL) could indicate that REV-ERBα activation reduces triglyceridemia by increasing lipolysis.

Regarding periprandrial *rev-erbα* expression in the liver, there were no significant differences between fed and unfed fish at 1 and 3 h after feeding time. In both groups (fed and unfed fish), *rev-erbα* hepatic expression was significantly

lower in fish at 1 h (around 30%) and 3 h (around 20%) postfeeding compared to fish at 3 h before feeding time, suggesting that such changes could be a consequence of their daily rhythmic profiles, regardless of feeding.

Finally, we investigated how feeding time and photoperiod affect *rev-erbα* daily rhythms in liver and hypothalamus of goldfish. Amplitudes and acrophases of these daily rhythms are shown in Table 1. In both, hypothalamus and liver, 24-h rhythms are conserved under all the studied different conditions. However, the amplitude of the rhythm in hypothalamus significantly decreases under constant darkness, while this downturn occurs in liver when feeding time is random compared to when it was fixed at 10:00 a.m. In addition, a 12-h shift in the daily rhythms of *rev-erbα* was observed in the liver, but not in the hypothalamus, when scheduled feeding was shifted from midday (15:00 p.m.) to middark (3:00 a.m.). These results indicate that *rev-erbα* has a daily rhythm that could be influenced by both *Zeitgebers* (photoperiod and feeding schedule) differently in each tissue. In hypothalamus, the *rev-erbα* rhythms are entrained by photoperiod, categorizing this tissue as a LEO (light entrainable oscillator). In contrast, the liver seems to behave like a FEO (food entrainable oscillator), since *rev-erbα* rhythms are conditioned by feeding schedule in this tissue (5).

Table 1. Amplitudes and acrophases of gene expression daily rhythms of *rev-erbα* in hypothalamus and liver under different feeding and lighting conditions.

Lighting and feeding conditions	HYPOTHALAMUS		LIVER	
	Amplitude	Acrophase	Amplitude	Acrophase
12L:12D Feeding 10:00	10.8 ± 1	18.6 ± 0.3	22.6 ± 3	17.5 ± 0,5
12L:12D Random Feeding	2.5 ± 0.2	17.6 ± 0.3	9.8 ± 0,9	16.9 ± 0,4
24D Feeding 10:00	0.9 ± 0.2	16.2 ± 1	24.5 ± 4	16.9 ± 0,6
12L:12D Feeding 15:00	5.48 ± 0.5	18.2 ± 0.4	7.69 ± 1	19.0 ± 0,5
12L:12D Feeding 03:00	1.61 ± 0.5	15.4 ± 1.07	10.13 ± 2.8	7.4 ± 1

Parameters from the sinusoidal functions obtained by Cosinor analysis when the adjustment was significant. Data are expressed as mean ± standard error. The amplitude (difference between the maximum and mean value) of the rhythm is expressed in relative units (fold change). The acrophases (time of maximum expression) are expressed in ZT (*Zeitgeber* time*,* hours) or CT (Circadian time, hours) under 24h-darkness.

In summary (Figure 1), REV-ERB agonist raises fatty acid oxidation and decreases gluconeogenesis, acting as a short-term anorectic signal in goldfish. Also, the rhythmic expression of REV-ERBα in liver and hypothalamus (influenced by feeding time and lighting conditions) suggests that this nuclear receptor can be acting as an output of the circadian clock. In this sense, REV-ERBα is proposed as a link between energy homeostasis and circadian system in fish, as previously demonstrated in mammals.

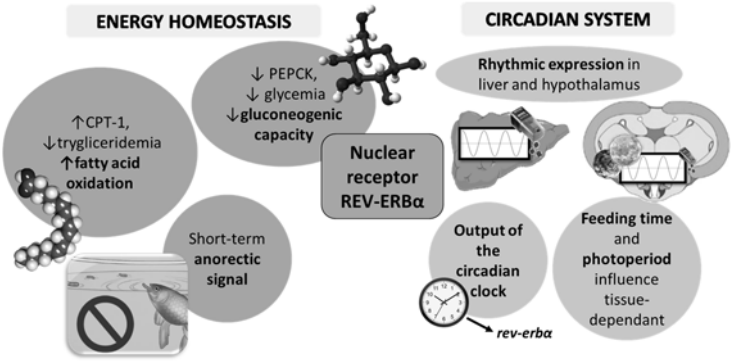


Figure 1. Summary of the role of REV-ERBα as a link between energy homeostasis and circadian system. REV-ERBα reduces food intake and gluconeogenic capacity, and raises fatty acid oxidation. This receptor has a rhythmic expression (influenced by feeding time or lighting conditions) in liver and hypothalamus, and it could be an output of the circadian clock.

Acknowledgements: Supported by funds from Ministry of Science, Innovation and Universities (AGL2016-74857-C3-2R to M.J.D. and AGL2016-74857-C3-1R to J.L.S.). A.C. is a collaborative scholar from UCM. M.G-B. and N.S. are predoctoral fellowships from the FPI program (BES-2014-0681038) and the UCM (CT42/18-CT43/18), respectively.

References

1. Yin L., Wu N., Curtin J.C. (2007). REV-ERBα, a heme sensor that coordinates metabolic and circadian pathways. Science 318, 1786-1789.
2. Solt, L.A., Wang, Y., Banerjee S., Hughes, T., Kojetin D.J., Shin, Y., Liu, J., Cameron, M.D., Noel R, Yoo S., Takahashi J.S., Butler, A.A., Kameneka, T.M., Burris, T.P. (2012). Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. Nature 485, 62-68.
3. Amador A., Wang Y., Banerjee S., Kameneka T.M., Solt L.A., Burris T.P. (2016). Pharmacological and genetic modulation of REV-ERB activity and expression affects orexigenic gene expression. PLoS ONE 11(5), e0156367.
4. Duez H., Staels B. (2009). REV-ERBα: an integrator of circadian rhythms and metabolism. J. Applied Physiol. 107, 1972-1980.
5. Isorna E., De Pedro N., Valenciano A.I., Alonso-Gómez A.L., Delgado M.J. (2017). Interplay between the endocrine and circadian systems in fishes. J. Endocrinol. 232, 141-159.
6. Gómez-Boronat, M., Isorna, E., Conde-Sieira, M., Delgado, M.J., Soengas, J.L., De Pedro, N. (2019). First evidences on the role of palmitoylethanolamide in energy homeostasis in fish. Horm. Behav. 117, 104609.
7. Chung S., Lee E.J., Yun S., Choe H.K., Seong-Beom, P., Son H.J., Kim K.S., Dluzen D.E, Lee I., Hwang O., Son G.H., Kim K. (2014). Impact of circadian nuclear receptor REV-ERBα on midbrain dopamine production and mood regulation. Cell 157, 858-868.

CHARACTERIZATION OF NOCTURNIN PARALOGS OF GOLDFISH: GENE STRUCTURE, LIVER RHYTMICITY AND THE EFFECT OF FASTING

D. Madera, A. Alonso-Gómez, A.I. Valenciano, M.J. Delgado, A.L. Alonso-Gómez

Department of Genetics, Physiology and Microbiology, Faculty of Biological Sciences, Complutense University of Madrid, Spain.

Nocturnin (NOC) is a protein related with the circadian system and the endocrine control of lipid metabolism. From its discovery, and based on mammalian molecular structure, it has been proposed as a putative deadenylase involved in post-transcriptional regulation of mRNAs. A recent hypothesis suggests that NOC is involved in dephosphorylation of NADPH to NADH. The aim of this study is to characterize gene structure and rhythmicity of the expressed paralogs of *noc* in goldfish. An *in silico* analysis let us to propose the existence of 4 *noc* paralogs as a result of a double genomic duplication 3R (teleost specific) and 4Rc (*Cyprininae* specific). By RT-PCR, whole coding sequences have been obtained for three paralogs that we named *noc-a1, noc-a2* and *noc-b1* according to the phylogenetic sequence analysis. Expected *noc-b2* seems to be a pseudogene not expressed in goldfish. Besides, we confirm the existence and expression of two splicing variants for both *noc-a1* and *noc-a2* in goldfish which expression is modify by fasting. By RT-qPCR, we quantified the tissue relative abundance of all *noc* coding transcripts, showing *noc-a1* and *noc-a2* higher expression than *noc-b1* in the nervous system, and vice-versa for the non-neural tissues, with the exception of adipose tissue where all paralogs are highly expressed. Finally, we determine the daily variation of these paralogs in liver, where *noc-a2* and *noc-b1* expression peaks around five hours post-feeding. In conclusion, this study presents the first description of full-length sequences and splicing variants of *nocturnin* in fish, proving to be an important tool for the study of relationships of this enzyme with the metabolism and circadian system.

Introduction

Nocturnin (NOC) is a molecule discovered in the retinal photoreceptors of *Xenopus laevis*, with a robust rhythmic expression pattern (1). Different studies have identified the relationship between NOC and metabolism in mammals, suggesting the implication of this molecule in adipogenesis, lipid absorption and energy homeostasis (2). Currently, there are two hypothesis to explain NOC activity. The first one supports that NOC is a putative deadenylase, involved in posttranscriptional control of half-life of some mRNAs (3). Recently, a new putative function of NOC being involved in dephosphorylation of NADPH to NADH has been proposed (4). In fish, it has been identified the presence of *noc* and its relationship with feeding in a teleost model, goldfish (*Carassius auratus*) (5). The aim of this study is to characterize all paralogs of NOC in goldfish. First, we obtained the full gene sequence of the three *noc* transcripts expressed in goldfish. Using bioinformatics tools, we determined the protein structure of NOC paralogs and performed a phylogenetic analysis. Second, we analyzed the tissue distribution of *noc* expression in goldfish. Finally, we investigated the possible influence of light/dark cycle and fasting on the daily variations of *noc* expression in liver.

Materials and Methods

Goldfish (15-25 g) were maintained at 21±2ºC on a 12L:12D photocycle, with feeding (1% body weight) at 10:00 h (*Zeitgeber time,* ZT2). Full sequences of *noc* paralogs and tissue expression pattern were obtained as previously described (5). To characterize the *noc* daily expression, liver samples were collected at ZT3, 7, 11, 15, 19, 23 and 27 throughout a 24-h cycle (n=7/sampling time). To determine the possible effect of fasting on *noc* expression, we studied the effect of short- and long-term food deprivation. Liver was sampled after 7-days and 30-days of fasting (n=9/group). RNA extraction and cDNA synthesis were performed as previously described (5). The relative expression of *noc-a1*, *noc-a2*, *noc-b1*, and the splicing variants *noc-a2 (I)* and *noc-a2 (II)* was quantified by RT-qPCR, using *ef-1α* as a housekeeping gene for the $2^{-\Delta\Delta Ct}$ analysis. Statistical differences were determined by ANOVA and a post-*hoc* test (Student-Newman-Keuls). Data from daily variations were checked by COSINOR analysis to determine sinusoidal functions.

Results and Discussion

1) Phylogenetic analysis of *noc* sequences.

Using splicing variant II of all *noc* paralogs, we generated a phylogenetic tree (**Fig. 1**). Different Sarcopterygii models were included as outgroup. By an *in silico* approach, we determined the lack of *noc-b2* expression in goldfish, because many accumulated mutations in its gene sequence were observed (data not shown). Thus, goldfish *noc-b2* was not included in the analysis. We can conclude that all *noc* paralogs are clustered into orthologous groups of all analysed teleost models.

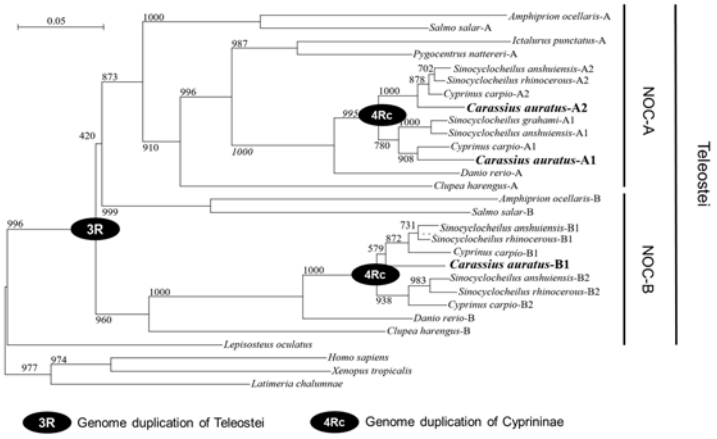


Figure 1. Phylogenetic tree showing the relationships 4Rc among NOC-A1, NOC-A2 and NOC-B1 sequences. The tree was inferred by the *Neighbor-joining* method. The numbers at tree nodes refer to bootstrap values of a total of 1000 replicates. The scale bar indicates the average number of substitutions per position (a relative measure of evolutionary distance). The binomial name of the species used for the alignment is given on the right side of the tree. Letters A and B indicate NOC isoforms in teleost. 3R and 4Rc indicate the proposed whole genome duplication events in Teleostei and Cyprininae, respectively. Species names and GenBank accession numbers of the sequences are as follows: *L. chalumnae*, XP_005996687.1; *X. tropicalis*, XP_005996687.1; *H. sapiens*, NP_036250.2; *L. oculatus*, XP_006629686.2; *C. harengus*-B, XP_012692428.1; *D. rerio*-B, XP_009305064.1; *C. carpio*-B2, XP_018949899.1; *S. rhinocerous*-B2, XP_016375510.1;

S. anshuiensis-B2, XP_016375510.1; *C. carpio,* XP_018919123.1; *S. rhinocerous*-B1, XP_016410990.1; *S. anshuiensis*-B1, XP_016347208.1; *S. salar*-B, XP_014043159.1; *A. ocellaris*-B, XP_023150318.1; *C. harengus*-A, XP_012689840.1; *D. rerio*-A, XP_700794.1; *C. carpio*-A1, KTG06093.1; *S. anshuiensis*-A1, XP_016326574.1; *S. grahami*-A1, XP_016149199.1; *C. carpio*-A2, KTG46929.1; *S. rhinocerous*-A2, XP_016429113.1; *S. anshuiensis*-A2, XP_016332140.1; *P. nattereri*-A, XP_017547495.1; *I. punctatus*-A, XP_017329139.1; *S. salar*-A, XP_014055069.1; *A. ocellaris*-A, XP_019723093.1.

2) Tissue expression pattern of *noc* in goldfish.

A different pattern in tissue expression of *noc-a* and *noc-b1* paralogs was found. In neural tissues (**Fig. 2A**), the *noc-a1* and *noc-a2* transcripts levels were higher than *noc-b1* mRNAs, especially in pituitary and retina. In non-neural locations (**Fig. 2B**), the abundance of *noc-a* paralogs was lower than *noc-b1* expression, which shows the highest levels in adipose tissue, skin and muscle.

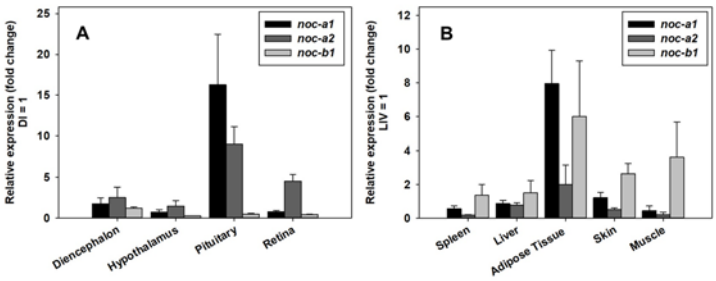


Figure 2. Tissue distribution of *noc* expression in goldfish**.** Data are expressed as mean + SEM (n=6).

3) Daily rhythms of *noc* expression in goldfish liver.

The analysis of daily variations in liver expression of *noc* shows a rhytmic profile of *noc-a2* and *noc-b1* mRNAs (**Table 1**), with the acrophases around ZT7: five hours after feeding (**Fig. 3**). These rhythms could suggest a link between NOC activity and the circadian system in fish, as it was suggested in mammals (3).

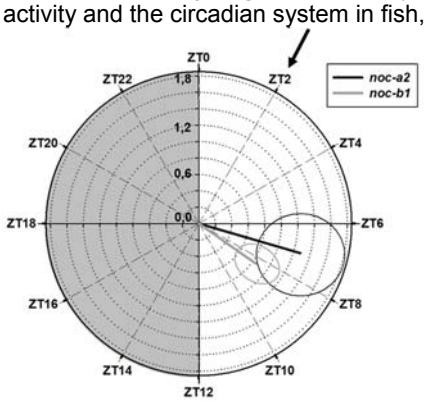


Figure 3. Polar representation of daily rhythmic parameters of *noc-a2* and *noc-b1* expression in goldfish liver**.** The grey semicircle shows the dark phase of photocycle. The bars angle and length indicate the acrophase and sinusoidal amplitude, respectively. The ellipses represent the SE of amplitude and acrophase. The arrow indicates the scheduled feeding time.

Table 1. Parameters defining *noc* expression in goldfish liver.

Parameters	***noc-a1***	***noc-a2***	***noc-b1***
Amplitude (fold change)	n.s.	1.28 ± 0.54	0.85 ± 0.31
Acrophase (*ZT*, hours)	n.s.	7.09 ± 1.55	8.35 ± 1.16

Data were obtained by Cosinor analysis and are expressed as estimation + SE. Not significant (n.s.)

4) Regulation of *noc* expression by fasting.

The *noc* paralogs respond differently to a short- and long-term fasting (**Table 2**). After 7-days fasting, *noc-a2* expression decreases, while *noc-b1* is increased. The *noc-a1* paralog is not affected by a short-term food deprivation, but after 30-days fasting, its mRNA levels show a severe increase. Then, using specific primers for each splicing variants, we focused on the analysis of the two variants (I and II) of *noc-a2* (**Table 2**). Despite of the decline of general *noc-a2* levels, 7-days fasting increase splicing II / splicing I ratio. Then, the low expression of *noc-a2* is related with splicing I, and not with splicing II variant. Thus, splicing I levels are lower on fasting conditions than on regular feeding situation. We can suggest that each *noc* paralog is induced differently depending on the fasting duration.

Table 2. Effect of fasting on *noc* expression in liver of goldfish**.**

Short / Long-term fasting							
noc-a1 (fold change)		***noc-a2*** (fold change)		***noc-b1*** (fold change)		***noc-a2 (II)*** (%)	
7d	**30d**	**7d**	**30d**	**7d**	**30d**	**7d**	**30d**
=	+10	-10	=	+2	=	+500	=

(+) Upregulation, (-) downregulation, (=) unaltered *noc* mRNA levels at 7-days (7d) and 30-days (30d) of fasting.

In summary, this study presents the first characterization of full code sequences of *noc* paralogs in fish, and shows its evolutionary relationships in the teleost group. The tissue distribution and the different responses of *noc-a1*, *noc-a2* and *noc-b1* to fasting, suggest a possible implication in specific physiological processes, such as neo- and sub-functionalization of each *noc* paralogue in teleost. In addition, considering the daily rhythms of *noc-a2* and *noc-b1*, we must emphasize the relevance of investigate the possible role of NOC as a link between the circadian system and metabolism.

Acknowledgements: Supported by the Spanish MICIU (AGL2016-74857-C3-2-R). D.M and A.A-G are predoctoral fellows from the UCM (CT42/18- CT43/18) and MICIU (FPI, BES-2017-081398).

References

1. Green CB, Besharse JC. (1996). Use of a high stringency differential display screen for identification of retinal mRNAs that are regulated by a circadian clock. Mol. Brain Res. 37, 157-165.
2. Green CB, Douris N, Kojima S, Strayer CA, Fogerty J, Lourim D, Keller SR, Besharse JC. (2007). Loss of nocturnin, a circadian deadenylase, confers resistance to hepatic steatosis and diet-induced obesity. Proc. Natl. Acad. Sci. 104, 9888-9893.
3. Hussain MM, Pan X. (2015). Circadian regulators of intestinal lipid absorption. J. Lipid Res. 56, 761-770.
4. Estrella MA, Du J, Chen L, Rath S, Prangley E, Chitrakar A, Aoki T, Schedl P, Rabinowitz J, Korennykh A. (2019). The metabolites $NADP^+$ and NADPH are the targets of the circadian protein Nocturnin (Curled). Nat. Commun. 10, 2367.
5. Blanco AM, Gómez-Boronat M, Madera D, Valenciano AI, Alonso-Gómez AL, Delgado MJ. (2018). First evidence of nocturnin in fish: two isoforms in goldfish differentially regulated by feeding. Am J. Physiol. Regul. Integr. Comp. Physiol. 314, R304-R312.

SEROTONERGIC SIGNALING IN THE REGULATION OF FOOD INTAKE IN FISH

Mauro Chivite; N. Fatemeh, C. Velasco, M.A. Lopez-Patiño, J.M. Míguez

Laboratorio de Fisioloxía Animal. Dept. Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía, Universidade de Vigo; and ECIMAT 36310, España.

Introduction

A bi-directional functional axis between periphery and central tissues has been postulated to control food intake. Brain receive signals from the periphery about digestive and nutritional status via neuronal inputs, peptides and hormones, thus modulating a response that stimulates or inhibits food intake (1). The goal of this mechanism is achieve a balance between food intake, fuel stored and energy expenditure; In other words, reach energy homeostasis in the processes facing the animal.

Serotonin, 5-hydroxytryptamine (5HT) is a neurotransmitter widely distributed in trout brain (2). 5HT has been implicate in a large number of physiological and behavioral processes, including food intake regulation. In teleost, the central integration networks related to food intake are mainly located in the hypothalamus. The serotonergic system integrates neuronal circuits in this brain area and was suggested as an actor in the modulation of feeding related signals, promoting the expression of hypothalamic anorexigenic neuropeptides (3).

The complex process of achieve energy homeostasis predetermine the fish feeding behavior by stimulating or inhibiting appetite and satiety. In teleost, the hypothalamus contains population cells that express the orexigenic AgRP and NPY and the anorexigenic CART and POMC neuropeptides(4). Previous studies suggest that serotonin evokes an anorexigenic response, which is mediated by the activation of 5-HT receptors of the subtype 5-htr2c. In mammals, this receptor is associated with α-MSH producing neurons, whereas in fish, its precursor pomca1 responds with increases to central serotonin treatments, suggesting a similar functional regulation than mammals (3). Additionally, other serotonin receptors such as 5htr1b and 5htr1a have been involved in feeding regulation in mammals. However, the role of these receptors in teleost is poorly known.

Previous studies of our group in rainbow trout evidence an activation of the serotonergic system after food intake in teleost, as well as the upregulation of pomca1 mRNA abundance (Chivite, et al. Unpublished data). These data are in line with previous pharmacological studies, suggesting that an interaction between both systems exists. Moreover, IP treatments with peripheral hormones and peptides related to satiety provoke an upregulation of pomca1 and cartpt among other anorexigenic neuropeptides in fish brain (5). In mammals, an interaction between peripheral peptides and central 5HT has been observed, which might explain a functional relationship between peripheral feeding signals and brain serotonergic activity (6).

With this in mind, we design experiments to evaluate the modulation of central serotonergic system by peripheral peptides related to satiety (Cholecystokinin and Glucagon-like peptide) in trout. In addition, we evaluate the role of 5-htr2c in the presence of this peripheral signals and the implication on the hypothalamic neuropeptidergic system. In other experimental set, we evaluate the role of 5htr1a and 5-htr1b receptor subtypes in the modulation of serotonergic and neuropeptidergic systems in normal-fed and fasted trout, trying to get new knowledge about feeding regulation in teleost fish.

Materials and Methods

Immature *Onchorynkiss mykiss* were acclimated for one month under controlled conditions of 12:12 h light/dark photoperiod (lights on at 08:00) and 13±1°C water temperature in running, aerated, and dechlorinated freshwater. Trout were fed once a day ad libitum with commercial dry pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed).

In a first experiment, fish groups were anaesthetized with 2-phenoxyethanol 0,02% v/v directly in their tanks, weighed, and intraperitoneally (IP) injected with saline 1ul/g BW or the 5htr2c antagonist (SB 242084, Tocris) at a dose of 1 ug/g BW. A second IP treatment consisted in saline (control), CCK 100 ng/g BW or GLP-1 100ng/g BW. 90 minutes after second injection, fish were deeply anesthetized directly in their tanks and sacrifice. Hypothalamus were quickly remove and placed in dry ice, then stored at -80˚C until analysis. In a second experiment fish were intra-cerebroventricular (ICV) injected (1 µL) with 5htr1a agonist (10mM 8-Hydroxy-DPAT), 5htr1b agonist (, 30mM CP 93129) or Saline (control). In this experiment, treated trout were from groups submitted to two different feeding conditions: On the one side fish that were fed 1h before ICV, and on the other side, fish were not fed since 24h before ICV injection. 30 min after treatment fish were sacrificed and sampled as above.

Hypothalamic samples were assessed to quantify mRNA levels of *npy pomca1, tph1* and *tph2* transcripts by real time qPCR and tissue concentration of 5HT and 5HIAA (5-hydroyindolactic acid) by HPLC. All the experiments carried out in this studied has been approved by the Ethics Committee of the University of Vigo, in concordance with the Directives of the European Union Council (2010/63/UE), and of the Spanish Government (RD 53/2013) for the use of animals in research.

Results and Discussion

Our results demonstrated that intraperitoneal injection of the gastrointestinal peptides CCK and GLP-1 activates hypothalamic serotonergic system, as demonstrated by increased metabolite/amine contents ratio (Fig.1). In addition, data provided in Table 1 corroborate the typical anorexigenic profile previously demonstrated for these peptides at the level of the hypothalamic neuropeptides (5, 7). This effect was partially reverted by pretreatment with the selective 5htr2c antagonist, SB 242084, suggesting that serotonergic system plays a key role in the anorexigenic response promoted by peripheral CCK and GLP1.

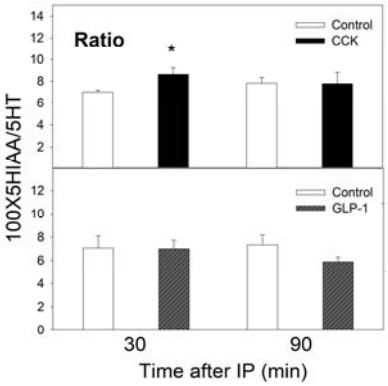


Figure 1. Ratio between 5HIAA and 5HT contents in hypothalamus of rainbow trout 30 or 90 min after IP treatment with 100ng/g BW of CCK (top) or 100ng/g BW of GLP1 (bottom). Data represent the average + S.E.M (n=10) Asterisk indicates significant differences respect to control (saline). P<0,05.

The existence of a gut-brain axis explains the interaction between peripheral inputs and the central satiety centers at short term during the post-prandial period. For instance, in rodents activation of CCK receptors located in nodose ganglion, the inferior ganglion of the vagus nerve, has been reported to increase the activity of brainstem serotonergic fibers (6). In addition, studies carried out in trout demonstrate the implication of the gut brain axis in the anorexigenic response to GLP1 (7). Comparing with our results, the potential existence of a similar circuit might explain the activation of serotonergic system after peripheral treatment with CCK and GLP1 in the rainbow trout, which agrees with a role of central serotonin in the integration of peripheral signals involved in feeding regulation in teleost

Table 1. Data are expressed as fold-change with respect to the control group and represent the average ± SEM of n=6 samples. **Bold letters** indicate significant differences (P<0.05) as compared to 30 and 90 min control (data not shown), respectively.

Time	**30 min**			
	CCK	**ant2c+CCK**	**GLP-1**	**ant2c+GLP1**
pomca1	**2,03±0,41**	0,80±0,16	1,04±0,21	0,69±0,15
npy	**0,15±0,04**	**0,52±0,10**	**1,58±0,20**	**1,86±0,32**
tph1	1,18±0,37	1,75±0,40	0,79±0,09	0,77±0,06
tph2	**1,47±0,22**	**1,88±0,26**	**1,64±0,30**	**1,58±0,04**
Time	**90 min**			
	CCK	**ant2c+CCK**	**GLP-1**	**ant2c+GLP1**
pomca1	**1,85±0,25**	1,25±0,25	**1,75±0,24**	0,86±0,26
npy	0,70±0,17	0,91±0,22	1,14±0,27	1,25±0,22
tph1	1,07±0,10	0,90±0,11	1,06±0,07	1,09±0,12
tph2	**1,77±0,23**	**1,58±0,07**	**0,55±0,10**	**0,60±0,11**

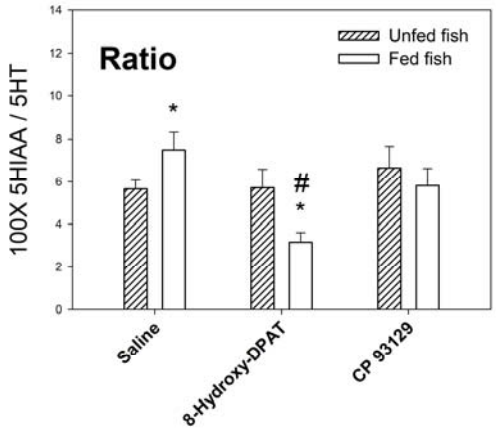


Figure 2. Ratio between 5HIAA and 5HT contents in hypothalamus of rainbow trout 30 min after ICV treatment with 1µl saline (274mOsm), 10mM 8-Hydroxy-DPAT or 30mM CP93129. Data represent the average + S.E.M (n=10). Asterisks represent significant differences between fed and unfed fish. Double barred symbol indicates significant differences with control (saline). P≤0,05.

Results obtained after 5-htr agonists treatment demonstrate that 5HTergic activity ratio decreased after treatment with the 5htr1a agonist 8-OH-DPAT,

varying this effect with feeding condition (Fig. 2). Moreover, mRNA abundance of *tph1* and *tph2* displays lower values after ICV treatment of 5htr1a (8-OH-DPAT) and 5htr1b (CP931229) agonists, independently to feeding condition. It suggests that, as in mammals, 5htr1a and 5htr1b receptors downregulate 5HTergic turnover, probably by acting as somatodendritic autoreceptors. Regarding the hypothalamic neuropeptides, both receptor agonists resulted in decreased *npy* (orexigenic) and *pomca1* (anorexigenic) mRNA abundance in unfed fish (Table 2) but not in fed fish, which is a controversial response. A possibility exists that feeding promotes the inhibition of *npy* mRNA abundance and this effect involves a role of 5-htr1A or 5htr1B hetero-receptors located on postsynaptic NPY cells. Following this reasoning, the higher serotonergic activity displayed in fed fish, as compared to unfed ones, could be responsible for inhibition of *npy* mRNA abundance by acting serotonin on post-synaptic receptors. In this condition, agonistic treatment did not display additional negative effect. Meanwhile, the opposite takes place in unfed fish in which serotonergic activity in controls is lower than in fed fish (Fig.2), and treatments with 5htr1a and 5htr1b agonists inhibit neuropeptides (*npy*, *pomc*) mRNA expression due to increased activation of post-synaptic 5HT receptors.

Table.2 Data are shown as a relative fold change in comparison to unfed control group. Means ± SEM, n=6. **Bold** indicates significantly different (P<0.05) from control group.

	Unfed fish			**fed fish**		
	Control (saline)	**8-OH-DPAT**	**CP93129**	**Control (saline)**	**8-OH-DPAT**	**CP93129**
pomca1	1±0,06	**0,72±0,04**	**0,69±0,08**	1,25±0,32	0,70±0,22	0,99±0,07
npy	1±0,20	**0,36±0,06**	0,63±0,10	0,47±0,06	0,63±0,17	0,73±0,13
tph1	1±0,12	**0,58±0,06**	**0,58±0,05**	1,05±0,16	**0,52±0,12**	**0,70±0,08**
tph2	1±0,27	1,81±0,29	1,37±0,26	1,45±0,26	2,22±0,59	1,35±0,62

Altogether, the results suggest that serotonergic system play a tonic role on hypothalamic neuropeptides, in such a way that it limits appetite and triggers satiety signals. We here report an interaction between serotonin receptors and hypothalamic neuropeptides in trout, although more studies are needed to in deep in the complex role of serotonin in feeding regulation.

Acknowledgements: The study was supported by Spanish Agencia Estatal de Investigación and European Fund of Regional Development (AGL2016-74857-C3-1-R and FEDER).

References

1. Soengas JL, Cerdá-Reverter JM, & Delgado MJ. Central regulation of food intake in fish: An evolutionary perspective. Journal of Molecular Endocrinology 2018 **60** R171–R199. (doi:10.1530/JME-17-0320)

2. Frankenhuis-van den Heuvel THM & Nieuwenhuys R. Distribution of serotonin-immunoreactivity in the diencephalon and mesencephalon of the trout, Salmo gairdneri - Cellbodies, fibres and terminals. Anatomy and Embryology 1984 . (doi:10.1007/BF00303149)

3. Pérez-Maceira JJ, Otero-Rodiño C, Mancebo MJ, Soengas JL, & Aldegunde M. Food intake inhibition in rainbow trout induced by activation of serotonin 5-HT2C receptors is associated with increases in POMC, CART and CRF mRNA abundance in hypothalamus. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental

Physiology 2016 **186** 313–321. (doi:10.1007/s00360-016-0961-9)
4. Otero-Rodiño C, Rocha A, Sánchez E, Álvarez-Otero R, Soengas JL, & Cerdá-Reverter JM. Sensing glucose in the central melanocortin circuits of rainbow trout: A morphological study. Frontiers in Endocrinology 2019 . (doi:10.3389/fendo.2019.00254)
5. Zhang X, Tang N, Qi J, Wang S, Hao J, Wu Y, Chen H, Tian Z, Wang B, Chen D, & Li Z. CCK reduces the food intake mainly through CCK1R in Siberian sturgeon (Acipenser baerii Brandt). Scientific Reports 2017 **7** 1–13. (doi:10.1038/s41598-017-12646-3)
6. Engster KM, Frommelt L, Hofmann T, Nolte S, Fischer F, Rose M, Stengel A, & Kobelt P. Peripheral injected cholecystokinin-8S modulates the concentration of serotonin in nerve fibers of the rat brainstem. Peptides 2014 . (doi:10.1016/j.peptides.2014.07.003)
7. Polakof S, Míguez JM, & Soengas JL. Evidence for a gut-brain axis used by glucagon-like peptide-1 to elicit hyperglycaemia in fish. Journal of Neuroendocrinology 2011 . (doi:10.1111/j.1365-2826.2011.02137.x)

DISRUPTION OF THE SEA BASS (*Dicentrarchus labrax)* SKIN-SCALE PROTEOME BY THE EMERGING POLLUTANT FLUOXETINE

P. I. Pinto[1,*], L. Anjos[1,*], S. Santos[1], M. D. Estêvão[1,2], C. Moreira[3], C. Zapater[4], C. Santa[5,6], B. Manadas[5], A. Gomez[4], T. Monsinjon[3], D.M. Power[1]

*[1]Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Faro, Portugal. [2]Escola Superior de Saúde da Universidade do Algarve, Faro, Portugal. [3]Environmental Stress and Aquatic Biomonitoring (SEBIO), Université Le Havre Normandie, Le Havre, France. [4]Instituto de Acuicultura Torre la Sal (IATS), CSIC, Castellón, Spain. [5]Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Coimbra, Portugal. [6]Institute for Interdisciplinary Research (IIIUC), University of Coimbra. * Equal contribution*

Accumulation of chemicals in aquatic ecosystems remains a concern, despite extensive legislation to control discharges. In this study, the impacts of *in vivo* exposures of juvenile sea bass (*Dicentrarchus labrax*) to the antidepressant fluoxetine (FLX), and to the natural estrogen 17β-estradiol (E_2) were investigated. A significant increase in E_2 and FLX plasma levels confirmed the effectiveness of exposure by injection. Plasma estrogen-responsive parameters including calcium, phosphorus and vitellogenin were significantly altered in response to E_2 but not FLX, while enzyme activities related to mineral turnover in scales were not affected. The scale proteome of fish exposed to E_2 and FLX for 5 days revealed that 213 proteins had significantly altered levels compared to the control group, 31 of which were altered by both E_2 and FLX. *In vitro* transactivation assays revealed antiestrogenic activities of FLX via nuclear estrogen receptors when combined with E_2 and indicated one of the potential mechanisms through which FLX may interfere with scales.

Introduction

Contamination of aquatic environments with anthropogenic pollutants, including pharmaceutical drugs, is a major concern worldwide. Many of these toxicants are endocrine disrupting chemicals (EDCs) that interfere with the endocrine system of aquatic organisms, which are at particular risk from aquatic toxicants due to lifelong, multi-route exposures or bioaccumulation (1,2).

Many studies have characterized the effects of EDCs and pollutants on the reproductive system, brain and liver of aquatic species, but their impacts on fish mineralized tissues such as the scales, that form a barrier between the fish and its environment, have been poorly investigated (2). We have previously shown that fish scales express multiple forms of membrane and nuclear estrogen receptors (3-6) and are responsive to estrogens and also potentially disrupted by estrogenic EDCs. Exogenous exposure to 17β-estradiol (E_2) may influence fish scale regeneration, mineral turnover and estrogen receptor expression (4,5,7). Recently, the global impacts of E_2 and genistein (a phytoestrogen EDC) were analyzed for the first time on the scale transcriptome and revealed common and specific effects (8), although the effect on scale proteins was not performed.

Fluoxetine is a widely prescribed SSRI (selective serotonin-reuptake inhibitor) antidepressant and is accumulating in aquatic ecosystems. Studies have reported disruptive effects of FLX on developmental, behavior, reproduction and stress in fish (9), but its impacts on mineralized tissues is unknown.

The objectives of this study were to evaluate E_2 or FLX effects on plasma parameters and scale proteome profiles in juvenile sea bass, exposed for 1 and 5 days to these compounds via intra-peritoneal injections.

Materials and Methods

In vitro transactivation assays used FLX hydrochloride (Cayman) at different concentrations (0.08, 0.8 or 1.6 nM), alone or in combination with 1 nM of E_2 (Sigma-Aldrich) to expose Human Embryonic Kidney 293 (HEK293) cells co-transfected with one of the three sea bass nuclear estrogen receptors (Esr1, Esr2a or Esr2b) and the estrogen-responsive pERE-TK-Luc reporter plasmid. This was followed by measurements of luciferase activity, as previously described (8).

Immature sea bass with approximately 1 year (130g) were maintained at the Ramalhete Marine Station (Faro, Portugal) in 1000 L tanks with flow-through natural seawater and fed daily with dry pellets (1% wet weight). Fish were randomly subdivided (n=5 per tank, with replicate tanks for each treatment and sampling time) and acclimatized for 7 weeks. Fish were anesthetized with MS-222 and received intra-peritoneal injections of coconut oil, in the control group, or coconut oil containing 5 mg/kg of FLX or of E_2. After 1 and 5 days of treatment, 10 fish per group were anesthetized with MS-222 and blood and scales were collected. Plasma levels of E_2 were measured by radioimmunoassay and FLX / nor-FLX levels were measured by LC-MS/MS; total plasma calcium (Ca) and phosphorus (P) were measured using colorimetric assays and vitellogenin (Vtg) was analyzed by SDS-PAGE, as previously described (5,10). Tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) activities were determined in scales using a colorimetric assay (5).

Global proteome profiles were analyzed by SWATH-MS (Sequential Windowed data independent Acquisition of the Total High-resolution-Mass Spectra) on a Sciex Triple TOF 5600 System, as detailed in (11). Total protein extracts were prepared in extraction buffer from 30-60 mg of frozen scales from individual sea bass sampled at day 5 post-injection (n=7 / group), homogenized on ice with an Ultra Turrax homogenizer (IKA). Protein extracts were validated by SDS-PAGE (10%) and total protein content assessed with the 2-D Quant Kit (GE Healthcare). The short GeLC-SWATH-MS strategy was used for each of 7 biological replicates per group. LC-MS data was acquired using Information-Dependent Acquisition (IDA) of the pooled samples for each group (Control, E_2 or FLX) for protein library construction (using searches against the sea bass genome database). SWATH acquisition was performed in each sample for protein relative quantification of the proteins identified with FDR ≥ 5%. Differences between E_2 or FLX-exposed scales compared to the control group were evaluated in SPSS using a Mann-Whitney (MW) pairwise test (significant p-value ≤ 0.05).

Results and Discussion

Significant increases in the circulating levels of E_2 were detected in the E_2-injected group after 1 and 5 days (Table 1-A), confirming the effectiveness of the treatments. FLX and its metabolite nor-fluoxetine (nor-FLX) could be detected in the plasma of FLX-injected fish but not in E_2 and control groups, confirming that the FLX administered through coconut oil implants could pass to the blood and was metabolized in the fish.

Table 1. Plasma parameters in sea bass from control (Ct), E_2 and FLX treated groups, 1 or 5 days after intraperitoneal injection. Panel A presents the levels of E_2, FLX and nor-FLX used to confirm the treatment effectiveness; Panel B shows calcium (Ca), phosphorus (P) and vitellogenin (Vtg) levels measured to assess the treatment effects.

A	**E_2 (ng/ml)**		**FLX (pg/mg)**		**nor-FLX (pg/mg)**	
	1 d	**5 d**	**1 d**	**5 d**	**1 d**	**5 d**
Ct	0.06±0.012	0.19±0.05	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
E_2	55.63±7.52*	14.93±1.75*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLX	0.17±0.04	0.20±0.09	992.38±79.29*	745.27±52.77*	476.67±40.47*	755.06±28.29*
B	**Ca (mM)**		**P (mM)**		**Vtg (relative intensity)**	
	1 d	**5 d**	**1 d**	**5 d**	**1 d**	**5 d**
Ct	2.30±0.15	2.66±0.11	2.88±0.13	2.77±0.19	0.99±0.13	1.00±0.17
E_2	2.18±0.26	4.23±0.13*	2.70±0.18	6.91±0.42*	1.11±0.048	4.09±0.44*
FLX	2.34±0.18	2.49±0.11	3.52±0.25	2.55±0.14	1.15±0.12	1.66±0.62

Data is expressed as mean ± standard error. * significantly different ($p<0.01$) levels relatively to the respective control for each time of exposure (1 or 5 days)

SWATH proteomic analysis identified 1254 proteins in the scale proteome, 57% of which (715) could be confidently quantified. Modified levels of a total of 213 proteins were observed after E_2 and/or FLX treatment, 31 of which were altered by both compounds (Fig. 1).

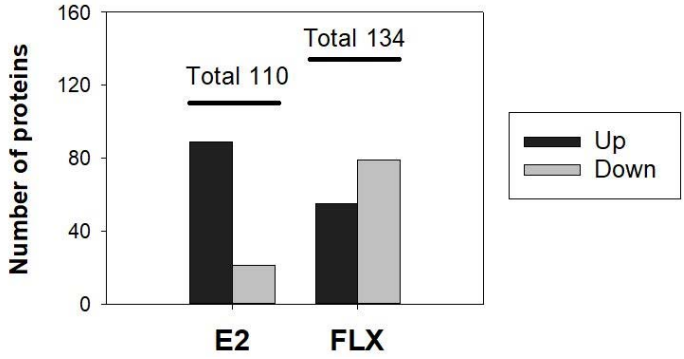


Figure 1. Number of proteins with significantly modified levels in sea bass scales, 5 days after injection with E_2 or FLX.

The proteins identified in the scale proteome in response to E_2 and FLX may provide insight into the biological processes and pathways that are physiologically regulated by E_2 in fish scales and those that can be disrupted in cases of environmental exposure to E_2 disruptors or to FLX.

Finally, in the *in vitro* transactivation assays, FLX (0.08 to 1.6 nM) could not activate any of the sea bass nuclear estrogen receptors but, when combined with 1 nM of E_2, caused an inhibition of the E_2-induced transactivation through Esr-1 and Esr-2b receptors (data not shown). This suggests that some of the actions detected for FLX in the sea bass scales may result from its effects (mainly antiestrogenic) at the estrogen receptors expressed in the scales.

Acknowledgements: Supported by FCT through projects PTDC/AAG-GLO/4003/2012, UID/Multi/04326/2019 and UID/NEU/04539/2019, researcher contracts under "Norma Transitória"-DL57/2016/CP1361/CT0015 to PP and DL57/2016/CP1361/CT0011 to LA and grant SFRH/BD/88419/2012 to CS, and by the Spanish Ministry of Science through grant AGL2015-67477-C2-1-R to AG.

References

1. Scholz S., Mayer I. (2008). Molecular biomarkers of endocrine disruption in small model fish. *Mol. Cell. Endocrinol.* 293, 57-70.
2. Pinto P.I., Estevao M.D., Power D.M. (2014). Effects of estrogens and estrogenic disrupting compounds on fish mineralized tissues. *Mar. Drugs* 12, 4474-4494.
3. Pinto P.I., Estevao M.D., Redruello B., Socorro S.M., Canario A.V., Power D.M. (2009). Immunohistochemical detection of estrogen receptors in fish scales. *Gen. Comp. Endocrinol.* 160, 19-29.
4. Ibarz A., Pinto P.I., Power D.M. (2013). Proteomic approach to skin regeneration in a marine teleost: modulation by oestradiol-17beta. *Mar. Biotechnol* 15, 629-646.
5. Pinto P.I., Estevao M.D., Andrade A., Santos S., Power D.M. (2016). Tissue responsiveness to estradiol and genistein in the sea bass liver and scale. *J. Steroid Biochem. Mol. Biol.* 158, 127-137.
6. Pinto P.I.S., Estevao M.D., Santos S., Andrade A., Power D.M. (2017). *In vitro* screening for estrogenic endocrine disrupting compounds using Mozambique tilapia and sea bass scales. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 199, 106-113.
7. Persson P., Takagi T., Björnsson B.T. (1995). Tartrate resistant acid phosphatase as a marker for scale resorption in rainbow trout, *Oncorhynchus mykiss*: effects of estradiol-17β treatment and refeeding. *Fish Physiol. Biochem.* 14, 329 - 339.
8. Pinto P.I.S., Andrade A.R., Moreira C., Zapater C., Thorne M.A.S., Santos S., Estevao M.D., Gomez A., Canario A.V.M., Power D.M. (2019). Genistein and estradiol have common and specific impacts on the sea bass (*Dicentrarchus labrax*) skin-scale barrier. *J. Steroid Biochem. Mol. Biol.* 195, 105448.
9. Brooks B.W. (2014). Fish on Prozac (and Zoloft): ten years later. *Aquat. Toxicol.* 151, 61-67.
10. Robert A., Schultz I.R., Hucher N., Monsinjon T., Knigge T. (2017). Toxicokinetics, disposition and metabolism of fluoxetine in crabs. *Chemosphere* 186, 958-967.
11. Anjos L., Pinto P.I.S., Tsironi T., Dimopoulos G., Santos S., Santa C., Manadas B., Canario A., Taoukis P., Power D.M. (2019). Experimental data from flesh quality assessment and shelf life monitoring of high pressure processed European sea bass (*Dicentrarchus labrax*) fillets. *Data Brief* 26, 104451.

CHRONIC DIETARY AFLATOXIN B1 (AFB1) EXPOSITION ALTERS GROWTH AND STRESS AXIS IN JUVENILE GILTHEAD SEA BREAM (*Sparus aurata*)

A. Barany[1,2], M. Guilloto[1], G. Martínez-Rodríguez[3], J. Fuentes[2], J.M. Mancera[1]

[1]Department of Biology, Faculty of Marine and Environmental Sciences, Instituto Universitario de Investigación Marina (INMAR), University of Cádiz, Campus de Excelencia Internacional del Mar (CEI-MAR), Puerto Real, Cadiz, Spain. [2]Centre of Marine Sciences (CCMar), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. [3]Department of Marine Biology and Aquaculture, Institute of Marine Sciences of Andalusia (ICMAN-CSIC), Puerto Real, Cadiz, Spain.

The impacts of a chronic toxicity with aflatoxin B1 (AFB1) in gilthead sea bream (*Sparus aurata*) through the diet (1 and 2 mg AFB1 kg^{-1} fish feed) were assessed. This AFB1 chronic treatment during 85 days resulted in 80% inhibition of the total weight gain, among other significant decreases in biometric indexes. Carbohydrate and lipid metabolisms, both in plasma and liver, were also significantly altered. Relative expression levels of gene transcripts for growth control were affected, with a significantly enhancement in hypophyseal *gh* mRNA and decrease in hepatic *igf1* mRNA. Plasma cortisol levels were no affected by AFB1, however relative expression levels of gene transcripts for stress regulation were enhanced (hypothalamic *trh*, *crh* and *crhbp*). In a second experiment, we evaluated influence of high stocking density (HSD) versus low stocking density (LSD) in individuals from control group and group receiving 2 mg AFB1 kg^{-1} fish feed. HSD alone increased plasma cortisol levels but dietary AFB1 prevented the increase in plasma cortisol.

Introduction

In recent years, the aquaculture industry is switching to a more sustainable and productive industry model. To reach these challenges alternative protein sources are needed for aquafeeds, as plant-based ingredients and fish feed additives to prevent or repair negative impacts due to extreme diet formulations in carnivorous fishes. Most carnivorous farmed fish, including the gilthead sea bream, have been successfully reared with plant-based diets containing <10% marine feedstuff (1). The new aquafeeds are plant-based, due to the increase in the incorporation of vegetal ingredients. This generates new issues associated with the agricultural sector, increasing the risk for contamination and proliferation of mycotoxins (2), linked to ongoing climate change (3) and their potential impacts in the aquaculture industry and the final consumers (4). Aflatoxin B1 (AFB1) is a secondary metabolite produced by the group of molds *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (5), which are well adapted to warm and dry weather conditions. AFB1 is the most potent carcinogen among aflatoxins, classified as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1993) and highly hepatocarcinogenic. These harmful effects of AFB1 are believed to be directly linked to its bioactivation. After ingestion, AFB1 is bioactivated and responsible for binding to cellular macromolecules such as DNA, RNA, cell membranes and constituent proteins (6). The objectives of this study were to investigate the putative physiological negative effects of dietary AFB1 contamination combined with confinement, in the sea bream to evaluate

the real implications for aquaculture using a wide spread farmed fish as biological model.

Materials and Methods

Juvenile individuals of *S. aurata* about 55 g body mass were purchased from a local fish farm, CUPIBAR S.L. (Cádiz, Spain). Fish were maintained under natural photoperiod (February-March 2017) for our latitude (36º 31' 44'' N) and constant temperature (18-19 ºC).

Experiment I: Growth performance and physiological parameters
Fish were distributed in nine 500 L-fiber glass tanks (triplicate tanks per treatment) continuously aerated in a flow through system. In each tank, the number of individuals was adjusted in order to get the same experimental density (4 g L^{-1}) and number (n = 30). Fish were fed for 85 days with: i) CT (fish feed not containing AFB1); ii) D_1 (1 mg AFB1 kg^{-1} fish feed); and iii) D_2 (2 mg AFB1 kg^{-1} fish feed). Sampling points for biometric data (body mas and length) were used to adjust food ration to maintain the initial daily ration of 1.5% of their body mass.
Experiment II: Effects of AFB1 on physiological parameters of S. aurata subjected to different stocking density
After the feeding trial, fish from groups CT and D_2 were distributed into eight tanks (n = 10 fish/tank) as previously described. Two experimental conditions were stablished, in duplicated tank, for an additional period of 10 days for each of the two different diets: i) low stocking density (LSD, 4 g L^{-1}), and ii) high stocking density (HSD, 40 g L^{-1}).

At end point of both Experiments, fish were netted and anesthetized with a lethal dose of 2-phenoxyethanol (1 mL L^{-1} in seawater), weighed, measured and sampled. Blood was collected from the caudal peduncle into 1-mL syringes rinsed with a solution containing 25,000 U heparin ammonium salt (Sigma H6279) in 3 mL 0.9% NaCl. Plasma was obtained by centrifugation, frozen in liquid nitrogen and stored at −80 °C. In addition, representative biopsies of liver and head kidney tissues, as well as whole pituitaries and brains were placed in Eppendorf tubes containing 500% (v/w) of RNAlater (Ambion®, Applied BioSystems) until total RNA isolation.

Results and Discussion

Dietary treatment of *S. aurata* with different AFB1 doses induced several alterations in productive parameters but did not affect survival (Table 1). These results are correlated in a dose-response manner, being the D_2 group the most affected. Growth performance was significantly affected by AFB1 by a 20% and 30% reduction of the total biomass production, for D_1 and D_2 groups respectively (Figure 1).
No significant differences were detected in cortisol levels for Experiment I (Figure 2A). However, in Experiment II cortisol levels increased significantly in CT-HSD when compared to CT-LSD groups, but not in the groups fed with AFB1 (D_2-LSD and D_2-HSD, Figure 2B). Changes in mRNA levels assessed for Experiment I are shown in Table 2. AFB1 fed groups compared to CT group, significantly up-regulated mRNA expression levels of *gh* (D_2), *crh* (D_2), *crhbp* (D_1 and D_2) and *trh* (D_2). In contrast, a significant down-regulation of hepatic *igf1* mRNA expression was observed in D_1 and D_2. Expression of head kidney *star* was lower in D_1 when compared to CT group. Surprisingly, *star* in D_2 remained statistically the same as in the CT group.

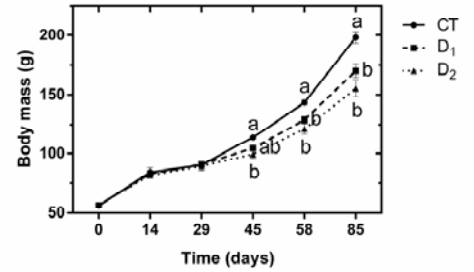


Figure 1. Evolution of body mass in *S. aurata* individuals fed with different experimental diets (CT, D_1 and D_2) during 85 days (Experiment I). Data are presented as mean ± SEM (n = 3 experimental units). Different letters indicate significant differences ($P < 0.05$, two-way ANOVA followed by a Tukey's *post hoc* analysis).

Table 1. Survival, growth and nutritional indexes in *S. aurata* individuals fed with different experimental diets (CT, D_1 and D_2) during 85 days (Experiment I). Data are presented as mean ± SEM (n= 3 experimental units) Different letters indicate significant differences ($P < 0.05$, one-way ANOVA followed by a Tukey's *post hoc* analysis).

Parameter	CT	D_1	D_2
Survival (%)	98.89 ± 1.11	97.78 ± 1.11	97.78 ± 1.11
WG (g)	142.40 ± 4.60^a	113.40 ± 6.50^b	98.57 ± 5.92^b
WG (%)	253.80 ± 6.61^a	201.10 ± 14.89^b	173.90 ± 7.39^b
K (%)	2.53 ± 0.06	2.45 ± 0.06	2.47 ± 0.01

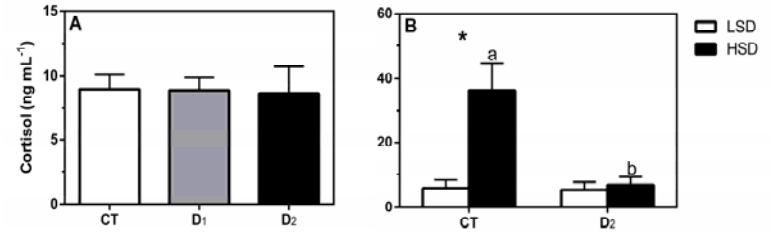


Figure 2. (A) Changes in plasma cortisol levels in *S. aurata* individuals fed with different experimental diets (CT, D_1 and D_2) during 85 days (Experiment I) and (B) at different stocking densities (low stocking density, LSD, 4 g L^{-1}; and high stocking density, HSD, 40 g L^{-1}) for 10 extra days (Experiment II). Data are presented as mean ± SEM (n = 7-8). Different letters indicate significant differences between groups fed with different experimental diets within the same stocking density. Asterisk* represents differences between stocking densities within the same experimental diet ($P < 0.05$, one-way or two-way ANOVA followed by a Tukey's *post hoc* analysis).

In summary, our results indicated that chronic exposure to contaminated aquafeeds with AFB1 impairs growth, altering metabolism (data not shown), tissue integrity (data not shown) as well as both the endocrine growth and the stress axes (Experiment I). These observations reinforced the negative effects of AFB1 on biometric parameters reported in other species. In addition, AFB1 also seemed to compromise the expected physiological stress response of *S. aurata* individuals to a subsequent stress situation by confinement (Experiment

II). Since no survival differences were observed this problem can persist in the industry for the production cycle, even reaching the final consumers. Hereby, special attention should be paid to the legislative aspect. Maximal concentration of aflatoxins in agricultural food and feed products and their commodities (*e.g.* terrestrial livestock) is regulated worldwide, with specific restrictions in Europe (Commission Regulation EU/574/2011, 2006/1881/EC and amendments). Unfortunately, a specific aquaculture mycotoxin maximum concentration for both aquaculture feeds and foods (7) remains unlegislated.

Table 2. Changes in mRNA expression levels (relative to *ef1a* and *actb*) in hypophyseal *gh*, hepatic *igf1*, in *crh*, *crhbp*, *trh* at brain and *star* at head kidney from *S. aurata* individuals fed with different experimental diets (CT, D_1 and D_2) during 85 days (Experiment I). Data are presented as mean ± SEM (n = 6-9). Different letters indicate significant differences among different experimental diets ($P < 0.05$, one-way ANOVA followed by a Tukey's *post hoc* analysis).

Gene	CT	D_1	D_2
gh	0.68 ± 0.06^{a}	1.00 ± 0.16^{ab}	1.25 ± 0.15^{b}
igf1	2.05 ± 0.27^{a}	0.75 ± 0.11^{b}	0.39 ± 0.13^{b}
crh	0.82 ± 0.04^{a}	0.91 ± 0.05^{ab}	1.03 ± 0.08^{b}
crhbp	0.77 ± 0.05^{a}	0.99 ± 0.04^{b}	1.10 ± 0.06^{b}
trh	0.85 ± 0.04^{a}	0.99 ± 0.06^{a}	1.22 ± 0.07^{b}
star	1.22 ± 0.54^{ab}	0.14 ± 0.05^{a}	2.77 ± 0.78^{b}

Acknowledgements: This work was funded by Spanish Ministry of Economy and Competitiveness-MINECO (AGL2016-76069-C2-1-R) awarded to JMM. AB and JMM belong to the Fish Welfare and Stress Network (AGL2016-81808-REDT), supported by the Agencia Estatal de Investigación (MINECO, Spanish Government). A. Barany is currently supported by University of Cadiz Ph.D. scholarship (PIF UCA/REC02VIT/2014).

References

1. Pérez-Sánchez, J., Simó-Mirabet, P., Naya-Català, F., Martos-Sitcha J. A., Perera, E., Bermejo-Nogales, A., Benedito-Palos, L., Calduch-Giner, J. A. (2018). Somatotropic Axis Regulation Unravels the Differential Effects of Nutritional and Environmental Factors in Growth Performance of Marine Farmed Fishes. Frontiers in Endocrinology, 9 (November).
2. CAST (2003). Mycotoxins: risks in plant, animal, and human systems. Report No. 13. Council for Agricultural Science and Technology, Ames, Iowa, USA.
3. Battilani, P., Toscano, P., Van Der Fels-Klerx, H. J., Moretti, A., Camardo Leggieri, M., Brera, C., Robinson, T. (2016). Aflatoxin B1 contamination in maize in Europe increases due to climate change. Scientific Reports, 6 (December 2015), 1.
4. Michelin, E. C., Massocco, M. M., Godoy, S. H. S., Baldin, J. C., Yasui, G. S., Lima, C. G., Fernandes, A. M. (2017). Carryover of aflatoxins from feed to lambari fish (Astyanax altiparanae) tissues. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 34(2): 265–272.
5. Miliţă, N.M., Mihăescu, G., Chifiriuc, C. (2010). Aflatoxins—Health risk factors. Bacteriol Virusol Parazitol Epidemiol., 55: 19–24.
6. Wang, X. (2016). Response of yellow catfish (Pelteobagrus fulvidraco) to different dietary concentrations of aflatoxin B1 and evaluation of an aflatoxin binder in offsetting its negative effects. Ciencias Marinas, 42(1): 15–29.
7. Gonçalves, R. A., Schatzmayr, D., Albalat, A., Mackenzie, S. (2018). Mycotoxins in aquaculture: feed and food. Reviews in Aquaculture, 1–31.

SCREENING OF PHYTOESTROGENS' EFFECTS ON RAINBOW TROUT AND GILTHEAD SEA BREAM PREADIPOCYTES AND BONE-DERIVED CELLS

S. Balbuena-Pecino, E. Gasch-Navalón, E. Lutfi, N. Riera-Heredia, J. Gutiérrez, I. Navarro, E. Capilla

Departament de Biologia Cellular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Spain.

Seeking to accomplish sustainable aquaculture, a high demand of plant components for inclusion in fish feeds exists nowadays. Efforts to investigate how these ingredients might affect cultured species are critical to optimize diets and produce healthy animals with good growth rates and an adequate level of fat. This study aims to evaluate the effects on viability and lipid accumulation of several phytoestrogens in two different cell culture models of gilthead sea bream and rainbow trout. The phytoestrogens genistein (GEN), daidzein (DZN), glycitein (GLY) and coumestrol (COU) were analyzed in comparison to estradiol (E2) at two doses each, either 1, 10 or 100 μM, in confluent preadipocytes (days 7-8 of culture) and bone-derived cells (day 4). Concerning viability, a reduction was observed in response to GEN100, DZN100 and COU100 in rainbow trout preadipocytes. In gilthead sea bream preadipocytes, GEN100 and DZN100 also caused a significant decrease, while DZN10, COU10 and COU100 increased viability. Differences between species were found as well regarding the effects on adipocyte fat content, with GEN100 and DZN100 showing a decrease in rainbow trout, but all the phytoestrogens inducing an increase in gilthead sea bream cells. Moreover, experiments in bone-derived cells of this species appeared to support the negative effects of GEN100 and DZN100 in cell viability and the suppressing effects of GEN100 on lipid accumulation. Otherwise, E2 did not significantly affect the cells in any case. In summary, these results indicate that high levels of the phytoestrogens GEN and DZN, compounds present in the most commonly used vegetable ingredients for aquafeeds (i.e. soybeans), seem to have at a cellular level a deleterious effect, and to modulate lipid deposition in fish. Nevertheless, the physiological significance of these effects needs to be further explored both, *in vitro* and *in vivo*.

Introduction

The increasing need of fish for human consumption has led to a parallel growth of aquaculture, which has been faster than in other food production sectors. Nevertheless, a serious bottleneck in keeping the current trend is the future of the supplies, fishmeal and fish oil, used in diet formulations. For this reason, it is expected that plant sources will progressively replace in a major degree the scarcity of these marine ingredients in fish feeds, to achieve a more sustainable production (1). However, numerous plants contain phytoestrogens, which are biologically active molecules that structurally mimic animal estrogens. These phytoestrogens can be mainly divided into three classes: isoflavones, lignans and coumestans, and the consequences of exposure to these different compounds when present in the diet are still unclear in fish, since both, estrogenic and antiestrogenic effects have been reported in vertebrates (2).

Materials and Methods

Preadipocytes were isolated from both rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) adipose tissue, according to the previously established protocols by Bouraoui *et al*. (3) and Salmerón *et al*. (4), respectively. Cells were seeded at a final density of 2-2.5×10^4 or 4.3×10^4 cells/cm^2, respectively, in 1% gelatin pretreated 12-well plates. Preadipocytes coming from rainbow trout were cultured in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotic-antimycotic (A/A) solution, whereas DMEM with 10% FBS, 1% A/A and 60 mM NaCl was used for the gilthead sea bream preadipocyte cells. After confluence was reached (day 7-8 of culture), cells were induced to differentiate with media supplemented with 10 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 µM dexamethasone and 5 µl/ml lipid mixture. At this point, the phytoestrogens genistein (GE), coumestrol (COU) daidzein (DZN), glycitein (GLY) and 17β-estradiol (E2) (as a positive control of estrogenic effect), at two doses each, either 1, 10 or 100 µM, were applied for 72 h to determine cell viability (MTT assay) and lipid accumulation (Oil Red O staining).

On the other hand, primary cultures of gilthead sea bream bone-derived cells were performed as previously described in Capilla *et al*. (5). Cells were seeded at a density of 1×10^4 cells/cm^2 into 6 or 12-well plates, for the gene expression and viability and lipid accumulation analyses, respectively. Briefly, at day 3 the different treatments were added for 72h with or without lipid. With regards to gene expression, total RNA was extracted from two duplicate wells of the 6 well-plates and cDNA synthesis was obtained. The mRNA levels of the genes of interest were examined in a CFX384™ real-time system (Bio-Rad). The expression level of each target gene analyzed was calculated using the Pfaffl method relative to the corresponding reference gene.

Results and Discussion

To determine the appropriate concentrations to work, cell viability was first evaluated. As observed in Fig. 1A, C, in rainbow trout preadipocytes, high doses of GE, COU and DZN (at 100 µM each) resulted toxic, and the same was observed with GE100 and DZN100 in gilthead sea bream preadipocytes. Contrarily, in those same cells, a significant increase in viability was observed with the two doses of COU, as well as DZN at 10 µM (Fig. 1C). In the gilthead sea bream bone-derived cells, only GE100 significantly decreased viability, while COU at the dose of 10 µM showed the same stimulatory pattern as that observed in preadipocytes from the same species (Fig. 1E). These data indicate that gilthead sea bream cells appear to be more resistant to the potential deleterious effects of phytoestrogens, especially the bone-derived cells, as well as pointed to COU as an interesting inducer of cell proliferation in this species.

Furthermore, changes in adipocyte differentiation and lipid accumulation induced by the phytoestrogens were assessed by ORO staining. In rainbow trout preadipocytes, the high dose of GE and the low doses of GLY and E2 increased the formation of lipid droplets respect to the control cells (Fig. 1B). Similarly, in gilthead sea bream, the two doses of GE and the high dose of COU, DZN and GLY also induced a significant rise in the fat content of the adipose cells (Fig. 1D). Contrarily, DZN100 caused lower values of apparent adipocyte differentiation compared to the control cells in rainbow trout. Finally, in the bone-derived cells model, GLY 1 µM and E2 10 µM decreased lipid accumulation whereas the remaining phytoestrogens did not cause any effect (Fig. 1F).

Overall, an obesogenic effect for most phytoestrogens was observed in gilthead sea bream and to a lesser extent in rainbow trout preadipocytes, while in bone-derived cells a change of lineage into adipocyte-like cells was not induced.

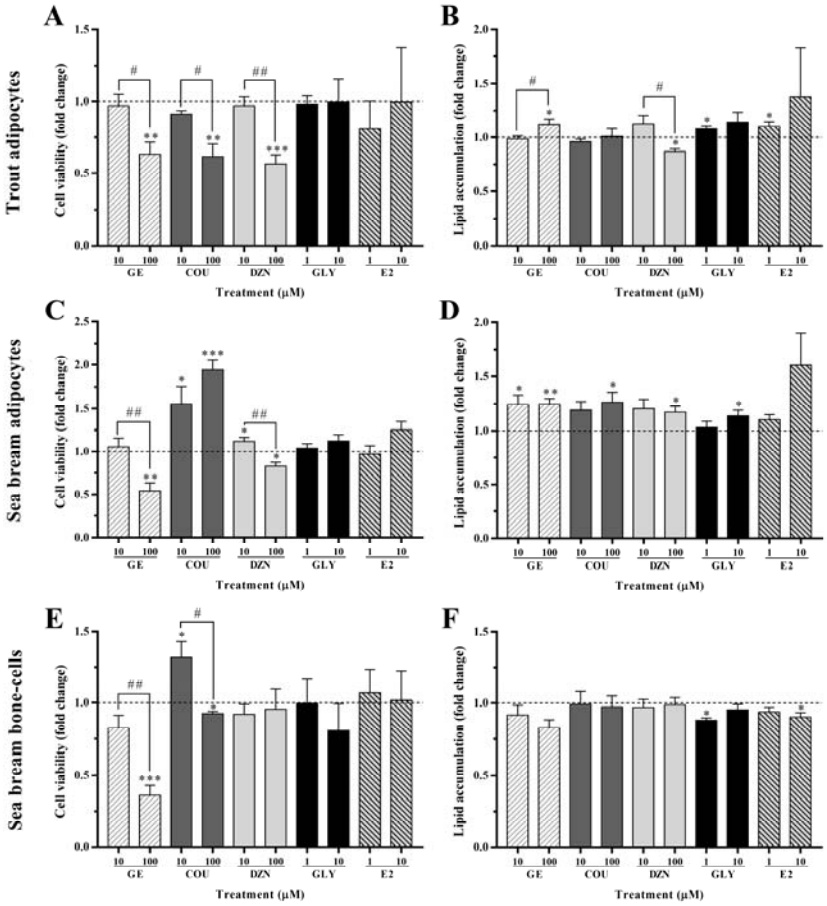


Figure 1. Effects of phytoestrogens on the viability and adipocyte differentiation of rainbow trout and gilthead sea bream cultured preadipocytes or bone-derived cells Quantification of (**A, C, E**) cell viability using an MTT assay, and (**B, D, F**) lipid accumulation determined by Oil Red O staining. Data are shown as Mean + SEM (n = 6-8) and presented as fold change respect to the control condition (dotted line). Asterisks indicate significant differences compared to the control group [p<0.05 (*), p<0.01 (**), p<0.001 (***)], and hash indicate significant differences between doses within each phytoestrogen [p<0.05 (#), p<0.01 (##)], determined by Student's *t* test. Genistein (GE); Coumestrol (COU); Daidzein (DZN); Glycitein (GLY); 17β-estradiol (E2).

Regarding gene expression results in gilthead sea bream bone-derived cells, the mRNA levels of *runx2* (the key transcription factor inducer of osteogenesis) were significantly up-regulated upon incubation with GE 10 μM, COU 10 μM and GLY 1 μM. In addition, differences between doses were only found after GLY incubation (Fig. 2A). In parallel to this, the levels of expression of osteonectin (*on*), a molecule involved in the regulation of mineral deposition, were significantly lower after treatment with both doses of COU and E2 compared to the control (Fig. 2B), whereas the expression of another osteogenic

differentiation marker, osteopontin, revealed no differences among groups (data not shown). Therefore, in this context, the results suggest that the main regulators of bone development can be modulated at a transcriptional level in gilthead sea bream by the presence of phytoestrogens in the diet.

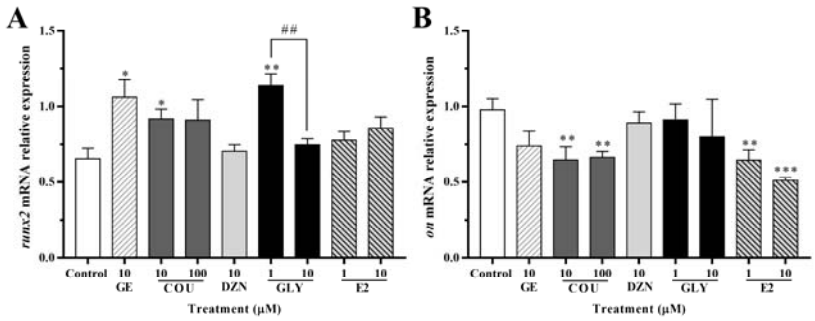


Figure 2. Effects of phytoestrogens on the expression of osteogenic-related genes in gilthead sea bream bone-derived cells. Relative mRNA expression normalized to *rps18* and *rpl27a* of (**A**) *runx2* and (**B**) *on*. Data are shown as Mean + SEM (n = 5-8). Asterisks indicate significant differences compared to the control group [p<0.05 (*), p<0.01 (**), p<0.001 (***)], and hash indicate significant differences between doses within each phytoestrogen [p<0.05 (#), p<0.01 (##)], determined by Student's *t* test. Genistein (GE); Coumestrol (COU); Daidzein (DZN); Glycitein (GLY); 17β-estradiol (E2); Osteonectin (on).

To conclude, high levels of the phytoestrogens GE and DZN, the two major isoflavones present in plants, appear to have negative effects at a cellular level; thus, their content in diets for these species should be properly controlled. On the other hand, vegetable ingredients containing the coumestan COU seem to be promising sources with healthy cell effects to be included in feed formulations for gilthead sea bream. In this sense, the data also demonstrate that some species-specific effects exist; therefore supporting the need to test and fine-tune the amount of phytoestrogens that can be added to aquafeeds, specifically for each species of interest.

Acknowledgements: Supported by the Ministerio de Ciencia, Innovación y Universidades (MICIUN) AGL2014-57974-R, AGL2017-89436-R and the Generalitat de Catalunya 2017SGR-1574. S.B-P and N.R-H were predoctoral fellows from the MICIUN (PRE2018-085580 and BES-2015-074654).

References

1. FAO. The State of World Fisheries and Aquaculture – (SOFIA) (2018).
2. Cederroth CR, Nef S. (2009). Soy, phytoestrogens and metabolism: A review. *Mol Cell Endocrinol*. 304, 30-42.
3. Bouraoui L, Gutiérrez J, Navarro I. (2008). Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*). *J Endocrinol*. 198, 459-469.
4. Salmerón C, Acerete L, Gutiérrez J, Navarro I, Capilla E. (2013). Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*). *Dom Animal Endocrinol*. 45, 1-10.
5. Capilla E, Teles-García A, Acerete L, Navarro I, Gutiérrez J. (2011). Insulin and IGF-I effects on the proliferation of an osteoblast primary culture from sea bream (*Sparus aurata*). *Gen Comp Endocrinol*. 172, 107–114.

FISHING A PUTATIVE MEMBRANE RECEPTOR IN THE GILTHEAD SEA BREAM (*Sparus aurata*): ROLE IN ACUTE STRESS

Jorge E. Aedo[1,2], I. Ruiz-Jarabo[3], G. Martínez-Rodríguez[4], S. Boltana[2], A. Molina[1,2], J.A. Valdés[1,2] and J.M. Mancera[3]

[1]Facultad de Ciencias de la Vida, Universidad Andrés Bello, Santiago, Chile. [2]Interdisciplinary Center for Aquaculture Research (INCAR), Universidad de Concepción, Concepción, Chile. [3]Department of Biology, Faculty of Marine and Environmental Sciences, Instituto Universitario de Investigación Marina (INMAR), University of Cádiz, Campus de Excelencia Internacional del Mar (CEI-MAR), Puerto Real, Cadiz, Spain. [4]Department of Marine Biology and Aquaculture, Institute of Marine Sciences of Andalusia (ICMAN-CSIC), Puerto Real, Cadiz, Spain.

Cortisol is the most important hormone involved in the regulation of neuroendocrine stress response in teleosts. Effects of this hormone are mediated through its intracellular receptors, although membrane components are also involved, with unclear roles during the stress response. In this work, the contribution of membrane-initiated cortisol actions on stress-related parameters and glucose metabolism-related genes in gilthead sea bream (*Sparus aurata*) were evaluated. Two *in vivo* experiments were performed. In the first, fish were administered with vehicle, BSA, cortisol and cortisol-BSA (membrane impermeable analogue) dissolved in saline. In the second, the same treatments were replicated but using coconut oil as a vehicle. Fish were sampled after 1 and 6 h (first experiment) and after 3 days (second experiment). Plasma cortisol and glucose levels as well as hepatic transcript levels of the gluconeogenesis, glucose-6-phosphatase (*g6pc*) and phosphoenolpyruvate carboxykinase (*pepck*), were analyzed. Fish implanted with each version of cortisol in both *in vivo* experiments reached plasma levels typically observed in *S. aurata* under an acute stress. Cortisol and cortisol-BSA increased glucose plasma levels after 6 h of treatment. However, cortisol, but not cortisol-BSA, maintained elevated plasma glucose levels after 3 days. Analysis by qPCR showed that expression of *g6pc* increased after 1 hour of cortisol and cortisol-BSA administration. However, cortisol, but not cortisol-BSA, enhanced *g6pc* and *pepck* expression after 3 days. Our results suggested that membrane-initiated cortisol actions contributed to the regulation of early metabolic adaptations in *S. aurata* submitted to an emulated acute stress situation.

Introduction

Teleost fish are exposed to diverse stressors in farming and wildlife conditions during their lifespan (1, 2). Cortisol is the main glucocorticoid hormone involved in the regulation of their metabolic acclimation under physiological stressful conditions (1). The mechanism of cortisol action is associated with its genomic/classic signaling pathway involving the interaction with intracellular receptors and the modulation of stress responsive genes (3). Owing to the lipophilic nature of cortisol, this hormone is capable of crossing inside the cell and interact with its intracellular glucocorticoid receptor (GR) (3). In addition to classical cortisol mechanism action, there is evidence that cortisol and other steroid hormones can also interact with membrane components activating rapid signaling pathways (4, 5). This novel mechanism for cortisol is known as a non-

genomic/ non-canonical or membrane-initiated cortisol action (4) This mechanism has been mainly characterized using steroids analogues coupled to hydrophilic molecules such as bovine serum albumin (BSA) (6, 7). Even though the mechanisms involved in the non-classical cortisol pathways are complex and diverse, until know, its impact on the adaptive response of fish has not been established. In the present study, the effects of the membrane-initiated cortisol actions on the regulation of glucose and transcription of key glucose metabolism-related genes were studied in *S. aurata*.

Materials and Methods

S. aurata were obtained from Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, University of Cadiz, Puerto Real, Cádiz, Spain). For the saline assay, **i**mmature fish (16.1 ± 0.2 g body mass, mean ± SEM, n = 48) were randomly distributed in four 80-L tanks in a flow-through system, under natural photoperiod and constant temperature (18 – 19 °C). After ten days of acclimation, fish of the first and second tanks were intraperitoneally administered with 0.01028 µM of cortisol or cortisol-BSA dissolved in DMSO 1X, PBS 1X, and NP 40 0.05%, respectively. Fish of the third and fourth tanks were administered with vehicle solution (DMSO, PBS 1X, NP 40 0.05%) and BSA (0.00892 mg/kg), respectively. For the coconut oil assay, immature fish (24.74 ± 0.29 g body mass, mean ± SEM, n = 32) were randomly distributed in four 80-L tanks in a flow-through system and maintaining in the same basal condition of the saline assay. Fish of the first and second tanks were intraperitoneally administered with 50 µg/g per fish of cortisol and cortisol-BSA dissolved in coconut oil as a vehicle, respectively. Fish of the third and fourth groups were administered with vehicle solution and BSA (0.3 µg/g per fish), respectively. Both experiments were performed using duplicate tanks for each group. After one and six hours (saline assay) and three days (coconut oil assay), all fish were euthanized by an overdose of 2-phenoxyethanol (1 mL/L) and the plasma and liver were collected and immediately frozen in liquid nitrogen. Plasma cortisol levels were measured by EIA kit. Plasma glucose levels were quantified with the HK Spin react kit. RNA liver was extracted using the Nucleo Spin RNA kit and retro transcription was carried out with the qScript^{TM} cDNA Synthesis Kit. Real time PCR were performed to evaluate the mRNA levels of *pepck* and *g6pc* genes. Results were normalized against beta actin (*actb*) and elongation factor 1a (*ef1a*) as an internal references gene. Relative gene quantification was performed using the ΔΔCT method

Results and Discussion

S. aurata intraperitoneally injected with cortisol and cortisol-BSA dissolved in saline reached plasma cortisol levels of 103.0 ± 0.9 and 107.2 ± 38.1 ng/mL, respectively. However, these values returned to basal levels after 6 h of treatment (Figure 1A). In addition, plasma glucose levels enhanced after 6 h of cortisol and cortisol-BSA treatments (Figure 1B). On the other hand, fish intraperitoneally injected with cortisol and cortisol-BSA dissolved in coconut oil reached plasma cortisol levels of 94.1 ± 13.0 and 270.0 ± 17.3 ng/mL, respectively (Figure 2A). However, cortisol but not cortisol-BSA enhanced plasma glucose levels after 3 days of treatment (Figure 2B). The increase of plasma cortisol levels in the saline assay was correlated with the hepatic enhancement in the expression of the gluconeogenesis-related gene *g6pc* (Figure 3A). Moreover, the expression of *pepck* and *g6pc* were elevated after 3 days of cortisol but no cortisol-BSA treatment (Figure 4A, B).

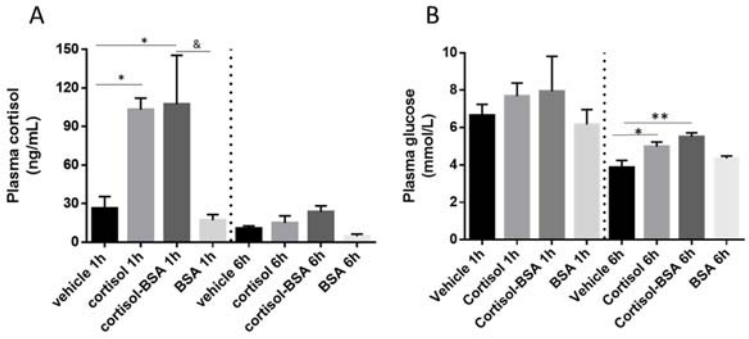


Figure 1. Plasma cortisol (A) and glucose (B) levels in vehicle (sham-control), cortisol, cortisol-BSA and BSA administrated fish at 1- and 6-hours post-treatment. Results are expressed as mean ± SEM (n = 7). Asterisks and & represent significant differences ($p < 0.05$) against vehicle and BSA group at each sampling time, respectively.

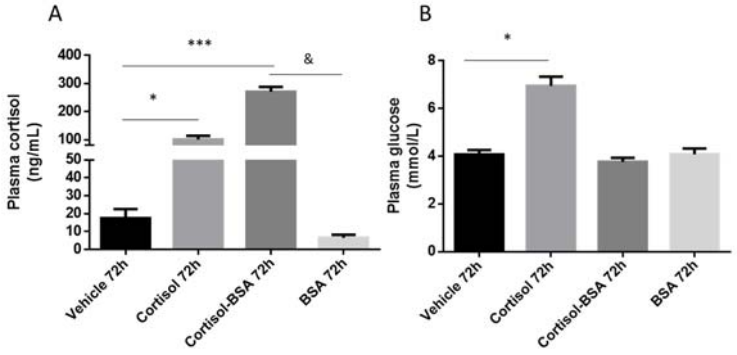


Figure 2. Plasma cortisol (A) and glucose (B) levels in vehicle (sham-control), cortisol, cortisol-BSA and BSA administrated fish at 72 hours post-treatment. Results are expressed as mean ± SEM (n = 8). Asterisks and & represent significant differences ($p < 0.05$) against vehicle and BSA group at each sampling time, respectively.

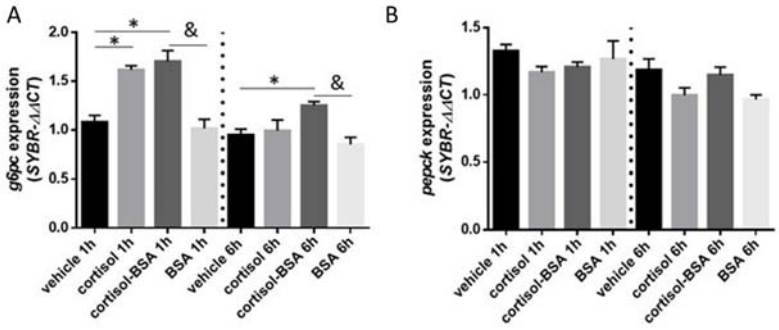


Figure 3. mRNA levels of hepatic gluconeogenesis enzymes glucose 6 phosphatase (*g6pc*) (A) and phosphoenol pyruvate carboxykinase (*pepck*) (B). Expression values were normalized against elongation factor 1 alpha (*ef1α*) and beta actin (*actb*). Results are expressed as mean ± SEM (n = 6). Asterisks and & represent significant differences ($p < 0.05$) against vehicle and BSA group at each sampling time, respectively.

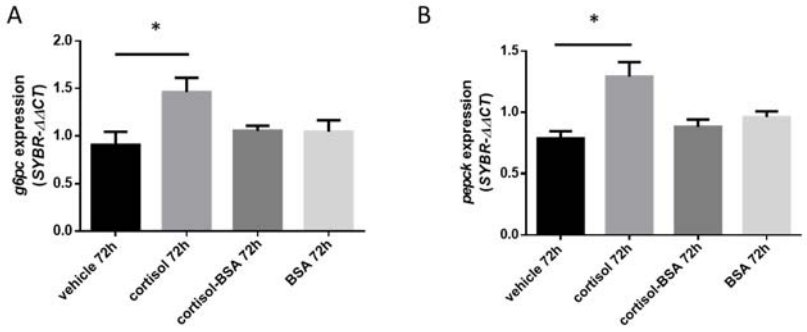


Figure 4. mRNA levels of hepatic gluconeogenesis enzymes glucose 6 phosphatase (*g6pc*) (A) and phosphoenol pyruvate carboxykinase (*pepck*) (B). Expression values were normalized against elongation factor 1 alpha (*ef1α*) and beta actin (*actb*). Results are expressed as mean ± SEM (n = 8). Asterisks represents significant differences ($p < 0.05$) against vehicle.

Our results suggest that the regulation of glucose metabolism (mobilization of glucose and the enhancement of gluconeogenesis-related genes expression) is mediated first by the membrane-initiated cortisol actions, follow by genomics/classic cortisol action in *Sparus aurata.*

Acknowledgements This work was funded by Spanish Ministry of Science, Innovation and Universities - MICINN (AGL2016-76069-C2-1-R) awarded to JMM. The authors belong to the Fish Welfare and Stress Network (AGL2016-81808-REDT), supported by the Agencia Estatal de Investigación (MICINN, Spanish Government).

References

1. Wendelaar Bonga SE. The stress response in fish. Physiological Reviews (1997) 77(3): 591-625. doi: 10.1152/physrev.1997.77.3.591
2. Sneddon LU, Wolfenden DC, Thomson JS. “Stress management and welfare”. In: Fish Physiology. Academic Press (2016). p. 463-539. doi: 10.1016/B978-0-12-802728-8.00012-6
3. Aluru N, Vijayan MM. Stress transcriptomics in fish: a role for genomic cortisol signaling. General and Comparative Endocrinology (2009) 164(2-3): 142-150. doi: 10.1016/j.ygcen.2009.03.020
4. Borski RJ. Nongenomic membrane actions of glucocorticoids in vertebrates. Trends in Endocrinology & Metabolism (2000) 11(10): 427-436. doi: 10.1016/S1043-2760(00)00325-8
5. Lösel R, Wehling M. Nongenomic actions of steroid hormones. Nature Reviews Molecular Cell Biology (2003) 4(1): 46. doi: 10.1038/nrm1009
6. Levin ER, Hammes SR. Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors. Nature Reviews Molecular Cell Biology (2016) 17(12): 783. doi: 10.1038/nrm.2016.122
7. Jozic I, Vukelic S, Stojadinovic O, Liang L, Ramirez HA, Pastar I, et al. Stress signals, mediated by membranous glucocorticoid receptor, activate PLC/PKC/GSK-3β/β-catenin pathway to inhibit wound closure. Journal of Investigative Dermatology (2017) 137(5): 1144-1154. doi: 10.1016/j.jid.2016.11.036

FOOD DEPRIVATION AND REFEEDING AFFECT THE LIVER OF TEMPERATURE IMPRINTED JUVENILE EUROPEAN SEA BASS (*Dicentrarchus labrax*)

R.A. Costa[1], A.P. Mateus[1,2], J. Jimenez, R. Piñera, B. Sadoul[3], D.M. Power[1]

[1]Comparative Endocrinology and Integrative Biology, Centre of Marine Sciences, University of Algarve; [2]Escola Superior de Saúde, University of Algarve; [3]MARBEC, Ifremer, University of Montpellier, CNRS, Palavas Les-Flots, France.

The effect of temperature imprinting (11 ºC, 13 ºC and 16 ºC) during the egg stage on the response to food deprivation was investigated in juvenile European sea bass (*Dicentrarchus labrax*). Temperature imprinted juvenile fish (~9 months) were either maintained on a normal diet (control) or deprived of food for one week and then re-fed for two days. Samples were collected from control fish and fish fasted for one week (0h) and 2 days after refeeding. Plasma cortisol levels were similar in the three temperature imprinted control groups. Food deprivation for one-week increased cortisol levels in all the temperature imprinted fish and it remained significantly higher 2 days after refeeding in the 13ºC and 16ºC imprinted fish when compared to the matched control, fed fish. Plasma glucose and lactate decreased significantly ($p < 0.05$) after one week of fasting in sea bass imprinted at 16ºC compared to the temperature matched control. Histomorphology of the liver from temperature imprinted fish one week after fasting revealed an increase in the number of nuclei / μm^2, which returned to control levels 2 days after refeeding in all groups. The area occupied by the lipids in the liver of all imprinted fish was significantly reduced after one week of fasting. Overall, the results indicate that temperature imprinting during the egg stage caused subtle changes in the response to short term fasting in European sea bass and suggested the physiological response in temperature imprinted fish was modified.

Introduction

Fish are ectotherms and this means that changes in ambient water temperature directly affect fundamental cellular processes and can have a determinant effect on fish physiology (Mozes *et al*. 2011, Somero 2005, 2010). Early life stages of fish are particularly vulnerable to changes in ambient temperature as behavioural thermoregulation (Davies 1988) is very limited and this is a stage when key organs and tissues are developing. Thermal imprinting, the temperature experienced during embryonic life, is likely to influence the adult physiology of fish and may have persistent effects on phenotypic traits (Burgerhout *et al*. 2017). For these reasons there is growing concern about the potential impact of global warming on embryonic and larval stages of fish and if this will affect the fitness of juveniles and adults (Somero 2005, Wood and McDonald 1997). A study in sea bream (*Sparus auratus*), an important Mediterranean aquaculture species, revealed that the thermal regime during early development modified the response of bone to the changes in water temperature that occur during winter (Mateus *et al*. 2017). In the present study we wanted to assess if thermal imprinting during the egg stage in the aquaculture species, European sea bass, modified its metabolism. The liver and

the intestine are the most important organs for the digestion and absorption of nutrients from the food and can reveal the metabolic status of animals (Raskovic *et al*. 2011). Therefore, monitoring biochemical parameters in blood plasma and the structure of the liver is a good way to assess metabolic state. In the present study we used biochemistry and quantitative histological methods to evaluate the effects of temperature imprinting during the egg stage on the response to food deprivation and refeeding in the European sea bass.

Materials and Methods

The animal experiments were carried out in compliance with European legislation in licensed experimental facilities (Ifremer`s experimental station at Palavas-les-Flots, France). Juvenile European sea bass (*Dicentrarchus labrax*) were imprinted with different temperatures during the egg stage (11ºC, 13ºC and 16ºC) and then reared in a common garden (CG) regime until ~9 month of age. Two experimental groups were then established: C – the control groups for 11ºC, 13ºC and 16ºC imprinted eggs, kept under standard rearing conditions, and FR – the fasted/re-fed group for 11ºC, 13ºC and 16ºC imprinted eggs, that were deprived of food for one week and then re-fed for two days. Samples were collected from the FR group after one week of fasting (0h) and 2 days after refeeding. For sampling, the fish were lightly anaesthetised in the experimental tanks with benzocaine (to minimise capture stress), netted and then fully anaesthetised and their weight and length determined and a sample of blood collected and the plasma snap frozen in liquid nitrogen. The fish were sacrificed by decapitation and the liver was fixed in 4% paraformaldehyde for histological analysis. The levels of glucose and lactate in plasma samples were determined using commercial kits Glucose-TR kit GOD-POD (Spinreact, Ref. 1001192) and Lactate LO-POD enzymatic kit (Spinreact, Ref. 1001330) and the levels of cortisol were determined with a fully characterised and optimised radioimmunoassay (Rotllant *et al.* 2005). Histological pictures of serial 5 µm paraffin sections of the liver (stained with haematoxylin and eosin, H&E) from both control and fasted/re-fed fish were assessed with the software ImageJ (Abramoff *et al*. 2004). The following parameters were calculated for the liver: number and area of the nuclei and total area (μm^2 and %) of the lipid in cells.

Results and Discussion

The average weight (32,07±1,302 g) and length (132,2±1,618 mm) of the experimental fish was identical between the different thermally imprinted fish (11ºC, 13ºC and 16ºC) ($p > 0.05$) and did not vary significantly between groups during the experimental trial. The thermal imprinting in the egg stage affected the basal levels of glucose and lactate in the plasma of the fed control (Figure 1A and 1B) and they were significantly higher in the control fish from the 16ºC imprinted group. One week of fasting in the 16ºC imprinted group caused a significant drop in plasma glucose and lactate compared to the matched control. In contrast, in the 11ºC imprinted group glucose increased significantly ($p < 0.05$) compared to the matched control. Basal plasma cortisol levels (Figure 1C) in the fed control was not significantly different between the differently imprinted fish. However, after a week of fasting the plasma cortisol response differed between the imprinted fish and it was significantly increased ($p < 0.05$) in fish from the 11 ºC and 13ºC imprinted groups compared to the matched fed control. Two days after refeeding the fish imprinted at 13ºC and 16ºC had significantly higher levels

of plasma cortisol than the matched, fed control group. Furthermore, the concentration of plasma cortisol was significantly different between the 11ºC and the 16ºC imprinted fish 2 days after refeeding. Overall, the fasting period induced variable changes in plasma glucose, lactate and cortisol in all temperature imprinted groups (Figure 1).

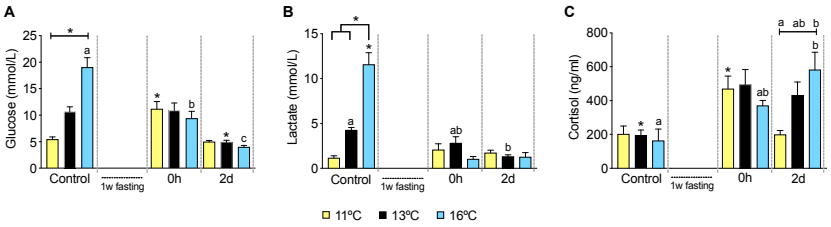
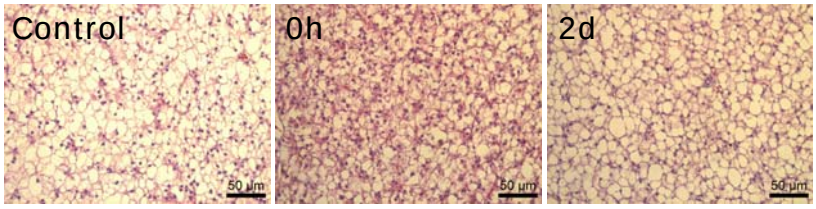


Figure 1. Plasma levels of A) Glucose, B) Lactate and C) Cortisol in Control and fasted/re-fed juvenile European sea bass imprinted during the egg stage at different temperatures (11°C, 13°C and 16°C). Significant differences ($p < 0.05$) between temperature groups at the same timepoint are indicated with horizontal bars; significant differences across time for each of the thermal imprinted groups are indicated with asterisk or different letters.

The morphology of the hepatic tissue (Figure 2A) was used to evaluate the nutritional status of the experimental fish prior to and after one week of food deprivation. The hepatic parenchyma was organized into characteristic polygonal-shaped hepatocytes and basophilic nuclei, displaced to the periphery of the cells due to the accumulation of lipids. The liver from the fed fish from all the thermally imprinted groups had a similar appearance: the hepatocytes had an abundant content of lipid and all cells had a similar regular shape.

A



B

	Control			Fasting-refeeding					
				11°C		13°C		16°C	
	11°C	13°C	16°C	0h	2d	0h	2d	0h	2d
Number of nucleus	239 ± 18	223 ± 20	229 ± 12	390 ± 38 *	259 ± 23	448 ± 32 *	249 ± 18	434 ± 78 *	227 ± 29
Nucleus area (µm²)	7.0 ± 0.6	7.5 ± 0.4	7.6 ± 0.4	6.5 ± 0.6	6.1 ± 0.3	6.9 ± 0.3	7.4 ± 0.4	6.8 ± 0.5	6.2 ± 0.5
Lipids total area (µm²)	65046 ± 3922	66986 ± 1476 [a]	63608 ± 3463	40929 ± 3535 *	63289 ± 1441	36329 ± 3797 [b]	48373 ± 1725 †[c]	28085 ± 2576 *	65387 ± 2308
% lipids area	73 ± 4	75 ± 1 [a]	72 ± 4	46 ± 4 *	71 ± 2	41 ± 4 [b]	54 ± 2 †[c]	32 ± 3 *	74 ± 3

Figure 2. A) Representative pictures of the histological sections of the liver of control and fasted/re-fed juvenile sea bass at 0h and 2 days post-refeeding stained with H&E. B) A table of the results obtained for the histomorphometry of the liver using ImageJ (Abramoff *et al.* 2004). Significant differences ($p < 0.05$) across time for the same thermally imprinted group is indicated with asterisk or different letters; the symbol "†" represents significant differences between thermally imprinted fish 2 days after refeeding.

No significant differences ($p > 0.05$) were observed in the number of nuclei in a given area or in the nuclei area or lipid total area in the liver of fed fish from each of the thermal regimes. The number of hepatocyte nuclei in a given area (μm^2) increased and the area occupied by lipids decreased with one-week fasting (Figure 2A and 2B) in all the thermally imprinted groups. The total area occupied by the lipids increased with refeeding in all thermal regimes however in fish imprinted at 13ºC this increase was significantly different than in the other temperature treatments 2 days after refeeding.

The histomorphometric changes in the liver of juvenile European sea bass represents an adaptive metabolic response to the fasting period. Temperature imprinting during embryonic stage modified the basal resting levels of glucose and lactate and the response to fasting and refeeding. The results suggest that even small changes in temperature of relatively short duration during the egg stage can modify the metabolic response to fasting and refeeding of the European sea bass.

Acknowledgements: This study received Portuguese National funds from FCT - Foundation for Science and Technology through the COFASP ERA-NET project "SUStainable production of High-quality aquaculture FISH using innovative tools and production strategies and integrating novel processing methods and cold chain management (Acronym: SUSHIFISH, COFASP/0002/2015) and the project UIDB/04326/2020 and from the operational programmes CRESC Algarve 2020 and COMPETE 2020 through project EMBRC.PT ALG-01-0145-FEDER-022121. RAC was funded by a research assistant grant CCMAR/BI/0008/2016.

References

1. Mozes N., Papandroulakis N., Vergara J. M., Biswas A., Takii K., Ntatsopoulos A. (2011). *In* M. Pavlidis & C. Mylonas (Eds.*) Sparidae: Biology and aquaculture of gilthead sea bream and other species*, 169-198.
2. Somero G. N. (2005). *Frontiers in Zool.* 2(1), 1-1.
3. Somero GN. (2010) *J. Exp. Biol.* 213(6), 912-920.
4. Davis P. (1988). *J. Fish. Biol.* 33(6), 951-951.
5. Burgerhout E., Mommens M., Johnsen H., Aunsmo A., Santi N., Andersen Ø. (2017) *PLoS ONE* 12(6): e0179918.
6. Wood C. M., McDonald D. G. (1997) Global warming: implications for freshwater and marine fish. Cambridge: Cambridge University Press.
7. Mateus A.P., Costa R.A., Gisbert E. *et al*. (2017) *J. Exp. Biol*. 220, 3442-3454
8. Raskovic B., Stankovic M., Markovic Z., Poleksic V. (2011) *J. Agric. Sci.* Belgrade 56, 87-100.
9. Abramoff M., Magalhães P., Ram S. (2004) *Biophotonics Int.* 11(7):36-42.
10. Rotllant J. Guerreiro P.M., Anjos L., Redruello B., Canario A.V.M., Power D.M. (2005) *Endocrinology* 146(1):71-76.

INVOLVEMENT OF SULFAKININ SIGNALING IN REPRODUCTION IN THE BLOOD GORGING BUG, *Rhodnius prolixus*

I. Orchard, M. Bloom, A.B. Lange

Department of Biology, University of Toronto Mississauga, Ontario, Canada.

The role of sulfakinin in the regulation of spontaneous and stimulated contractions of male and female reproductive tissues was examined in adult *Rhodnius prolixus*. Rhopr-SK-1 decreased the frequency of spontaneous contractions of the oviducts in a dose-dependent manner and reduced the myostimulatory effects of an extended Rhopr-FMRFamide. Rhopr-SK-1 had no effect on the frequency or amplitude of bursal contractions. In male adult *R. prolixus*, Rhopr-SK-1 decreased the frequency of ejaculatory duct contractions in a dose-dependent manner and reduced the myostimulatory effects of *R. prolixus* corazonin (Rhopr-CRZ). These findings highlight the potential roles of Rhopr-SKs in reproduction in *R. prolixus*, implicating Rhopr-SKs in the control of egg and sperm movement.

Introduction

We have previously shown that the sulfakinin signaling pathway is involved in blood feeding behaviour in *Rhodnius prolixus*, acting as a satiation factor (1). Interestingly though, we recently found that the transcripts coding for the two sulfakinin (SK) receptors in *R. prolixus* (Rhopr-SKRs) are expressed in the *R. prolixus* reproductive system (2). This observation, along with the requirement of blood-feeding for reproduction (3) prompted us to investigate the possible physiological roles of the Rhopr-SKs in reproduction. The reproductive system of the female *R. prolixus* contains two ovaries, two lateral oviducts, a common oviduct, a spermatheca, a bursa, as well as a cement gland. A variety of neurochemicals stimulate and relax ovary and oviduct contractions in insects and ovarian contractions are often synchronized with the relaxation of the lateral oviducts during ovulation and oviposition (4). Accordingly, during ovulation, mature eggs are deposited from the ovary into the lateral oviducts. Through peristaltic contractions of the lateral oviducts, the mature eggs are then moved into the common oviduct, where the sperm that are stored in the spermatheca are released to fertilize the egg.. The fertilized egg then moves into the bursa for oviposition. In the bursa, the eggs are coated with secretions from the cement gland and then deposited via strong phasic contractions of the bursa (4,5). Prior to fertilization, spermatozoa are transferred to the female during mating to be stored in the spermatheca. In the male *R. prolixus*, sperm that are stored in the seminal vesicles are transferred to the ejaculatory duct, where they are mixed with glandular secretions. A small duct at the base of the ejaculatory duct then injects the sperm into the female genital canal during mating.

Materials and Methods

Adult male and female *R. prolixus* were obtained from a colony raised at the University of Toronto Mississauga. Insects were reared at 50% humidity, 25°C in incubators and fed defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada).

Rhopr-SK-1 (pQFNEY(SO_3H)GHMRFamide) was custom synthesized by SynPeptide (Shanghai, China) at > 95% purity. Rhopr-FMRFamide (GNDNFMRFamide) and Rhopr-CRZ (pQTFQYSRGWTNamide) were custom synthesized by GenScript (Piscataway, NJ, USA) at > 95% purity. Stock

solutions were stored at -20°C as 10 µl aliquots of 10^{-3}M in double distilled water until working solutions were made using physiological saline (KCl 8.6 mM, NaCl 150 mM, $CaCl_2$ 2 mM, NaHCO3 4 mM, glucose 34 mM, $MgCl_2$ 8.5 mM, HEPES 5 mM [pH 7.0]).

Unfed female adult *R. prolixus*, which were fed as 5th instars four weeks prior to performing the study, were utilized for the oviduct bioassays. The oviducts were dissected under physiological saline. The lateral oviducts were pinned via minuten pins onto a Sylgard-coated dissecting dish (Dow Corning Corporation, Midland, MI, USA). A silk thread was tied to the posterior portion of the common oviduct and the other end of the silk thread was tied onto a Grass FT03 force displacement transducer (Grass Instruments, Warwick, RI, USA) and data was analyzed using PicoScope 2200 (Pico Technology, St. Neots, UK) software. The preparation was rinsed with saline, then left to stabilize in a bath containing 200 µl of saline for 15 minutes at room temperature.

To examine the effects of Rhopr-SK-1 on oviduct contractions, 100 µl of peptide was pipetted onto the *R. prolixus* oviduct preparation, while simultaneously removing 100 µl of saline from the preparation bath. The frequency of spontaneous contractions was compared between saline and Rhopr-SK-1 over 200 second intervals. The bursa contraction bioassay used the same technique as the one described above for the measurement of oviduct contractions.

Ejaculatory duct contractions were monitored in unfed adult male *R. prolixus* using an impedance converter (UFI model 2991, Morro Bay, CA, USA), as previously described for salivary gland contractions (6). Following the addition of Rhopr-SK-1 the tissue was monitored for approximately 200 seconds. The frequency of ejaculatory duct contractions in Rhopr-SK-1 was compared with the frequency in saline over 100 second intervals. Graphical representations and statistical analyses were generated using Graph Pad Prism 5.03 (www.graphpad.com). One-way or two-way ANOVA followed by a Bonferroni's test were utilized to determine statistical significance.

Results and Discussion

Rhopr-SK-1 led to a dose-dependent, reversible inhibition of the frequency of spontaneous oviduct contractions, with threshold effect at 10^{-11}M and maximal effect observed at 10^{-7} M with a 48.7 ± 5.3% reduction in frequency (Fig. 1A). Rhopr-FMRFamide is known to stimulate oviduct contractions (4,7) and at 10^{-7} M doubled the frequency of spontaneous oviduct contractions (Fig. 1A). Rhopr-SK-1 led to a dose-dependent decrease in the stimulatory effect of Rhopr-FMRFamide on the frequency of oviduct contractions (Fig. 1A).

No effects were observed on the frequency of bursal contractions following the addition of Rhopr-SK-1 (data not shown).

Rhopr-SK-1 led to a dose-dependent reversible inhibition of the frequency of spontaneous ejaculatory duct contractions with threshold at 10^{-11}M and maximal effect at 10^{-7} M resulting in a 40.7 ± 4.1% reduction in frequency (Fig. 1B). Rhopr-CRZ (10^{-9}M) stimulates an increase in contraction frequency of the ejaculatory duct of 30% (Fig. 1B). Rhopr-SK-1 led to a dose-dependent decrease in the stimulatory effect of Rhopr-CRZ on the frequency of ejaculatory duct contractions (Fig. 1B).

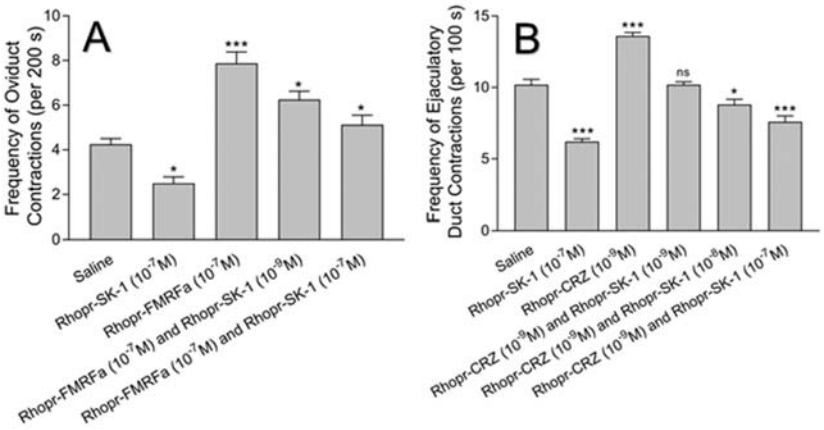


Figure 1. A) Spontaneous oviduct contractions in adult female *R. prolixus*. Frequency of contractions in Rhopr-SK were significantly lower than those observed in saline. Rhopr-SK was able to inhibit the Rhopr-FMRFa-induced increase in frequency in a dose-dependent manner. B) Spontaneous ejaculatory duct contractions in adult male *R. prolixus*. Rhopr-SK significantly lowered the frequency of contractions. Rhopr-SK dose-dependently inhibited the frequency of Rhopr-CRZ-induced contractions. (*p=0.05; **p=0.01; ***p=0.001).

The transcripts of the Rhopr-SKRs are expressed in *R. prolixus* reproductive tissues, suggesting that the reproductive tissues are targets for Rhopr-SKs, and indeed Rhopr-SK-1 inhibits contractions of the oviduct and ejaculatory duct. The inhibitory effects of Rhopr-SK-1 on both male and female reproductive tissue aligns with previous data where SK-1 was found to reduce the frequency of oviduct and ejaculatory duct contractions in the beetle *Z. atratus* (8). Sulfakinins are therefore not limited to the regulation of feeding but might also be involved in influencing reproductive processes in both males and females. Rhopr-SKs action on oviduct muscle contractions may regulate the movement of eggs, and similarly, effects on ejaculatory duct contractions could influence the transfer of sperm from the male to the female during mating. Rhopr-SKs may play a role in coordinating blood-feeding with egg production (oogenesis) in *R. prolixus*. Blood meals are essential to obtain the nutrients (lipids and proteins) required for oogenesis. Oogenesis involves nutrient rich metabolic events such as the production of vitellogenin (a lipoglycoprotein) by the fatbody and the accumulation of lipids into the oocytes (3). The nutrients obtained during blood-feeding are initially concentrated during short term diuresis via the removal of excess water and ions, with the larger elements (such as the red blood cells) processed around 3 days after feeding. This timing coincides with the onset of oogenesis, which starts at approximately 3 days after blood-feeding (9). Rhopr-SKs released during feeding may serve not only as a satiation signal but also as a signal to female reproductive tissues in advance of oogenesis. Oocyte maturation is likely delayed until nutrient absorption is complete and nutrients are available.

Acknowledgements: Supported by the Natural Sciences and Engineering Research Council of Canada.

References

1. Al-Alkawi H., Lange A.B., Orchard I. (2017). Cloning, localization, and physiological effects of sulfakinin in the kissing bug, *Rhodnius prolixus*. *Peptides* 98, 15-22.
2. Bloom M., Lange A.B., Orchard I. (2019). Identification, functional characterization, and pharmacological analysis of two sulfakinin receptors in the medically-important insect *Rhodnius prolixus*. Sci. Rep. 9, 13437
3. Davey K.G. (2007). The interaction of feeding and mating in the hormonal control of egg production in *Rhodnius prolixus*. *J. Insect Physiol.* 53, 208-215.
4. Sedra L., Lange A.B. (2014). The female reproductive system of the kissing bug, *Rhodnius prolixus*: Arrangements of muscles, distribution and myoactivity of two endogenous FMRFamide-like peptides. *Peptides* 53, 140-147.
5. Lococo D., Huebner E. (1980). The ultrastructure of the female accessory-gland, the cement gland, in the insect *Rhodnius prolixus*. *Tissue & Cell* 12, 557-580.
6. Orchard I., TeBrugge V. (2002). Contractions associated with the salivary glands of the blood-feeding bug, *Rhodnius prolixus*: evidence for both a neural and neurohormonal coordination. *Peptides* 23, 693–700.
7. Sedra L., Lange A.B. (2016). Cloning and expression of long neuropeptide F and the role of FMRFamide-like peptides in regulating egg production in the Chagas vector, *Rhodnius prolixus*. *Peptides* 82, 1-11.
8. Marciniak P., Kuczer M., Rosinski, G. (2011) New physiological activities of myosuppressin, sulfakinin and NVP-like peptide in *Zophobas atratus* beetle. *Journal of Comparative Physiology B.* 181, 721-730.
9. Coelho H.S., Atella G.C., Moreira M.F., Gondim K.C., Masuda H. (1997) Lipophorin density variation during oogenesis in *Rhodnius prolixus*. *Arch. Insect Biochem. Physiol.* 35, 301-313.

THE GENOME SIZE OF GILTHEAD SEA BREAM (*Sparus aurata*) REVEALS NOVEL FISH INSIGHTS IN GENE DUPLICATION

J. Pérez-Sánchez[1], F. Naya-Català[1], B. Soriano[2], M. C. Piazzon[3], A. Hafez[2], T. Gabaldón[4], C. Llorens[2], A. Sitjà-Bobadilla[3], J. À. Calduch-Giner[1]

[1]Nutrigenomics and Fish Growth Endocrinology Group, Institute of Aquaculture Torre de la Sal (IATS-CSIC), Castellón, Spain. [2]Biotechvana, Parc Cientific, Universitat de Valencia, Valencia, Spain. [3]Fish Pathology Group, Institute of Aquaculture Torre de la Sal (IATS-CSIC), Castellón, Spain. [4]Bioinformatics and Genomics Unit, Centre for Genomic Regulation (CRG), Barcelona Institute of Science and Technology, Barcelona, Spain.

We report a draft genome assembly for the teleost gilthead sea bream (*Sparus aurata*), reconstructed by combination of short- and long-read high-throughput sequencing, and genetic linkage maps. The assembly comprised 5,039 scaffolds that span 1.24 Gb of the expected 1.59 Gb complete genome size, with 932 scaffolds (~732 Mb) anchored to 24 chromosomes. This sequencing strategy resulted in a high quality fish genome assembly, as supported by the high N50 (1.07 Mb) and lower L50 (227) values. These long and continuous reads allowed the annotation of a large number of coding genes (55,423), non-coding RNA genes (2,991) and 345 Mb of mobile genetic elements (MGEs). Synteny analysis revealed a high level of homology between gilthead sea bream genes. Phylogenomics relationships with other fish species were established to locate temporal speciation and to assign duplication events. A large degree of recent and lineage-specific gene duplications was found, that were mainly enriched in functions related to genome transposition, immune response and response to stimulus. These duplications and gene family expansions could be related in part to the activity of MGEs. This fact makes gilthead sea bream an interesting model for the study of gene repertoire expansion strategies complementary to fish whole genome duplication events. Transcriptional analyses across six different tissues (anterior and posterior intestine, skeletal muscle, liver, gills, spleen) indicated that gene duplication preferentially affected genes expressed in two or more tissues. These findings highlight the genomic complexity of this species and the importance of selective gene duplications in the adaptation of fish to a challenging environment.

Introduction

Gilthead sea bream (*Sparus aurata*) is an economically important fish species highly farmed throughout the Mediterranean area with a yearly production of 218,000 metric tons. This species occurs naturally in the Mediterranean and the Eastern Atlantic Seas with an important gene flow across natural populations in absence of physical or ecological barriers.

This fish is a protandrous hermaphrodite species with a high adaptive plasticity, evidenced in response to changes in water salinity, temperature, dissolved oxygen concentration, social hierarchy or diet composition, which makes this marine species a rather unique fish for physiologists and geneticists.

Here we produced a high quality draft sequence of the gilthead sea bream genome by combining high-throughput sequencing/ with genetic linkage maps.

Homology-based functional annotation was supported by RNA-seq transcripts, and synteny and phylogenomic analyses revealed a high frequency of species-specific duplications, advancing our understating of teleost genome expansion.

Materials and Methods

Fish sampling and DNA/RNA sequencing. Fish were reared from early life stages under natural conditions of photoperiod and temperature at the experimental facilities of IATS-CSIC. Blood genomic DNA was extracted and used for the preparation of two Illumina paired-end (PE) format libraries, two mate pairs (MP) libraries and 12 PacBio RS II single molecule real time cell libraries. Extracted RNA from six white skeletal muscle samples and two pooled samples of anterior and posterior intestine of juvenile fish was used for the construction of six 1x75 nt single reads and two 2x150 PE libraries, respectively.

***De novo* genome assembly and chromosome anchoring**. Reads from sequencing samples were quality-filtered and pre-processed. Genome size was estimated calculating the count distribution of k-mers in PE data. Illumina PE and MP data were introduced in SOAP de Novo. To improve the consensus sequence, we performed two rounds of: 1) elimination of duplicates, 2) gap filling with PacBio reads and PE/MP data, and 3) hybrid re-scaffolding with all reads and sea bream transcriptome. Highly conserved non-coding elements from a previous gilthead sea bream genome draft (2) were aligned against our assembly for increasing super-scaffolding size. A genome browser was built for the navigation and blast-query of the genome, accessible at www.nutrigroup-iats.org/seabreamdb.

Genome annotation. Prediction of coding genes was carried out using a training set of 13 representative fish species and sequences from the IATS-CSIC gilthead sea bream transcriptome. Redundancy analysis were performed in order to detect segmental duplications. To annotate MGEs, RepeatMasker was used to identify repeats using Repbase and GyDB databases as queries. All the annotations corresponding to coding genes associated to MGEs (chimeric/composite genes) were extracted.

Gene synteny and phylogenomics. Synteny and homology detection were performed by aligning gilthead sea bream genes against 9 fish species. Gilthead sea bream phylome was reconstructed using PhylomeDB (3) pipeline against 19 representative fish species. Homologs were selected according to the standard parameters of MetaPhORs (E-value threshold < 10^{-5} and a continuous overlap of 50% over the query sequence). A maximum likelihood tree (aLRT = 1) with one-to-one orthologous in each of the selected species was created.

Functional gene enrichment analysis. Fisher-based enrichment test was used in the analysis of chimeric/composite fraction of genes. Phylogenomics-derived duplicated genes fraction was analyzed using FatiGO.

Gene duplication landscape and tissue gene expression. RNA-seq reads were processed and mapped against *ab initio* gene predictions to generate a gene expression atlas across tissues. To retrieve and annotate duplication events, we considered both the species-specific set of homologous genes from the phylogenomics analysis as well as *ab initio* predictions supported by RNA-seq transcripts. Paralogs were considered as tissue-exclusive when showing expression in only one of the analyzed tissues. An Atlas of expression in humans and other higher vertebrates (www.ebi.ac.uk/gxa) was exploited to retrieve and compare the enrichment of tissue-exclusive paralogs.

Results and Discussion

Our sequencing strategy (~105 Gb, 67.5x assembly coverage) resulted in one of the best fish genome assemblies in terms of metrics (5,039 scaffolds in a 1.24 Gb assembly; N50 = 1.07 Mb; L50 = 227). Previous attempts in closely related fish resulted in highly fragmented genomes due to the use of assembly protocols based solely on short read-approaches. Likewise, the first reported draft of gilthead sea bream genome comprised 55,202 scaffolds in a 760 Mb assembly (2). In concurrence with the present study, a second draft was submitted to NCBI (PRJEB31901) comprising ~833 Mb, which is still below our assembly. In consequence, our unmasked assembled genome rendered a higher number of unique gene annotated descriptions than the two previous releases (21,275 *vs.* 13,835- 19,631) with a full expected genome size of 1.6 Gb by k-mer counting analysis.

A second peak from k-mer count analysis was indicative of a high amount of repeated sequences. This genome expansion was confirmed by synteny analysis, which makes difficult to establish inter-species synteny blocks probably due to the over-representation of gene expansions during the recent evolution of gilthead sea bream. This was verified by the phylome analysis which showed an average of 2.024 copies for 55,423 actively transcribed genes across tissues (**Fig. 1**). In previous studies, the highest percentage of duplicated genes are reported for eel (36.6%) and zebrafish (31.9%) (4), but intriguingly the values reported by us in gilthead sea bream (56.5%) are even higher.

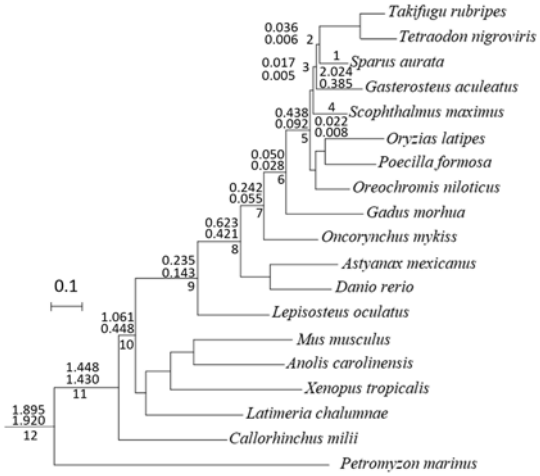


Figure 1. Phylogenomics tree obtained from concatenation of 148 single-copy proteins, with maximally supported nodes. Number on the branches mark the duplication densities (average number of duplication per gene and per lineage) for gilthead sea bream genes in the lineages leading to this species with (up) or without (down) expansions.

Gene functional enrichment in lineage-specific duplicated genes of gilthead sea bream evidenced an increased presence of genes related to DNA integration, transposition, immunoglobulin production and response to stimulus. This finding supports a large-scale gene duplication rather than a whole genome

polyploidization that occurred in the ancestor of teleost fish (3R), and more recently in the common ancestor of cyprinids and salmonids (4R).

The characterized gilthead sea bream mobilome accounts for the 75% of the full genome size (944 Mb), with more than 60% (599 Mb) constituted by introns. A total of 12 Mb are spanned by long- and small-ncRNA. As reported for phylogenomics analysis, enrichment among 3,648 chimeric/composite genes rendered GO terms mainly related to immune system, cell cycle and translational initiation. This provides additional evidence for the involvement of recent genome expansion in the well-recognized plasticity of gilthead sea bream to challenging and pathogen-rich environment.

Analysis of RNA-seq active transcripts across tissues also pointed out the association of gene duplication with different tissue expression patterns. This procedure showed higher duplication levels in genes expressed in two or more tissues as compared to those with a tissue-exclusive expression, being in accordance the annotation and functions of the tissue-exclusive paralogs with the reference Atlas of tissue gene expression of higher vertebrates. Likewise, we observed that gene copies expressed in two or more tissues showed increased duplication rates and percentages of retained paralogs in comparison to tissue-exclusive genes.

In summary, assembly analysis suggests that transposable elements are probably the major cause of the enlarged genome size with a high number of functionally specialized paralogs under tissue-exclusive regulation. These findings highlight the genome plasticity of a protandric, euryhalin and eurytherm fish species, offering the possibility to further orientate domestication and selective breeding towards more robust and efficient fish, making gilthead sea bream an excellent model to investigate the processes driving genome expansion in higher vertebrates.

Acknowledgements: This work was financed by Spanish (Intramural-CSIC, 1201530E025; MICINN, BreamAquaINTECH, RTI2018-094128-B-I00) and European Union (AQUAEXCEL^{2020}, 652831) projects. BS was supported by a predoctoral research fellowship (Doctorados industriales, DI-17-09134) from Spanish MINECO.

References

1. Langham, R. J., Walsh, J., Dunn, M., Ko, C., Goff, S. A., and Freeling, M. (2004). Genomic duplication, fractionation and the origin of regulatory novelty. Genetics 166, 935–945.
2. Pauletto, M., Manousaki, T., Ferraresso, S., Babbucci, M., Tsakogiannis, A., Louro, B., et al. (2018). Genomic analysis of *Sparus aurata* reveals the evolutionary dynamics of sex-biased genes in a sequential hermaphrodite fish. Commun. Biol. 1, 119.
3. Huerta-Cepas, J., Capella-Gutiérrez, S., Pryszcz, L. P., Marcet-Houben, M., and Gabaldón, T. (2014). PhylomeDB v4: zooming into the plurality of evolutionary histories of a genome. Nucleic Acids Res. 42, D897-D902.
4. Rozenfeld, C., Blanca, J., Gallego, V., García-Carpintero, V., Herranz-Jusdado, J. G., Pérez, L., et al. (2019). De novo European eel transcriptome provides insights into the evolutionary history of duplicated genes in teleost lineages. PLoS One 14, e0218085.

ACTIONS OF ESTRADIOL-17β ON THE GONADOTROPIC AXIS AND SPERMATOGENESIS IN MALE EUROPEAN SEA BASS (*Dicentrarchus labrax*)

Gregorio Molés[1], C. Zapater[1], P.I.S. Pinto[2], S. Ibáñez[1], A.V.M. Canario[2], A. Gómez[1]

[1]*Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre la Sal (CSIC), Castellón, Spain.*
[2]*Centre of Marine Sciences (CCMAR), University of Algarve. Faro. Portugal.*

The follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) are central endocrine regulators of gametogenesis in vertebrates, and gonadotropin-releasing hormones (Gnrh) have been postulated as the main regulators of their synthesis and secretion. Gonadal sex steroids have a feedback effect modulating the availability of gonadotropins. All these effects at the level of pituitary have a direct impact in gametogenesis progression. Previous *in vivo* studies in sea bass, during the sexual resting period, showed that Gnrh injections stimulated Lh synthesis and release, but had no effect on the expression of the Fsh beta-subunit gene. At the same time, different steroid implants repressed *fsh-b* expression, but activated the expression of *lh-b* in the pituitary. To elucidate how this system is organized in the pituitary of male sea bass, we analysed the annual expression profile of the three nuclear estrogen receptors (Esrs) in male pituitary and the circulating levels of estradiol in relation with the different stages of spermatogenesis. Also, immunohistochemistry studies have been performed to identify the pituitary cells containing steroid receptors and their relationship with gonadotrophs and GnRh1 fibers. Moreover, we have used an *in vitro* pituitary primary cell cultures stimulated with estradiol-17β (E_2) to study its direct action on gonadotrophs, and E_2 implants to study its effect *in vivo* on the pituitary and the gonad. We have concluded that E_2 has an inhibitory effect on the gonadotropins, although with different specific actions, which is also reflected in the localization of the Esrs in the gonadotrophs, while Gnrh differentially regulates Lh and Fsh cells in male seabass.

Introduction

The follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) are central endocrine regulators of gametogenesis in vertebrates (1). In the pituitary of male sea bass both hormones have overlapping profiles suggesting a similar dynamic in the synthesis and accumulation but different secretion control to the bloodstream. Gonadotropin-releasing hormones (Gnrh) have been postulated as the main regulators of the synthesis and secretion of Fsh and Lh since long. In addition, sex steroids produced by the gonads in response to the action of Fsh and Lh have a feedback effect in the pituitary modulating the availability of these gonadotropins (Gths) and impacting on gametogenesis progression. Our aim was to elucidate how this system is organized in sea bass. In this study we have focused on males, and on the action of E_2 and the three nuclear Esrs (*esr1*, *esr2a* and *esr2b*) during spermatogenesis.

Materials and Methods

Adult male European sea bass were obtained from a stock raised at the facilities of the Aquaculture Institute Torre de la Sal (IATS, 40º NL). Blood and pituitaries from males were sampled monthly during one year (n = 6 / month). For *in vitro* tissue cultures, pituitaries (n=7-10) were extracted, pooled, placed in ice-cold medium for dispersion and cultured in multi-well plates, following established methodology (2). Pituitary cells were stimulated with 100 nM of the steroids (E_2), testosterone (T) or dihydroxyprogesterone (DHP) and/or different doses of Gnrh (10, 50 or 100 nM). For the *in vivo* experiment, E_2 implants were prepared with silastic medical grade elastomer (Dow Corning Corporation) and placed intraperitoneally in male sea bass to give a concentration of 25 μg of E_2/ g of fish. Control animals were treated with empty implants. Treatments lasted for 9 days. In the last 24 hours, half of the animals were injected with the GnRH agonist [des-Gly^{10}, D-Ala^{6}] LHRHa. At the end of the experiment, the animals were sacrificed, blood was taken for hormone assessment, pituitaries were removed and stored at -80ºC for gene expression and hormone analysis. Gonads were taken also for histological processing.
Levels of Lh and Fsh were measured by homologous competitive ELISA according to Mateos et al. (3) and Molés et al. (4), respectively. Plasma levels of E_2 were measured by EIA according to Molés et al. (5). The pituitary expression patterns of *esr1*, *esr2a* and *esr2b* during spermatogenesis as well as *fshb* and *lhb* in E_2-implanted fish were determined by quantitative real-time PCR (qPCR). A double fluorescent immunohistochemical (IHC) detection was performed to study the co-localization of Esrs/Gths and Gths/Gnrh1 in the pituitary following previously described methodology (2).

Results and Discussion

The pituitary expression levels of the three nuclear Esr coding genes, *esr1*, *esr2a* and *esr2b,* were high during summer in animals with immature gonads (stage (st.) I) and low in Autumn, at the beginning of spermatogenesis (st. II). The expression of *esr1* and *esr2a* increased significantly during full spermatogenesis (st. IV) at the end of November, just before of spermiation (st. V) that occurs in winter. The plasma levels of E_2 were high in July (immature stage) and low at the beginning of spermatogenesis (st. II) coinciding with the gene expression profiles of the Esrs. However, E_2 levels were very low in November (st. IV) and towards the end of the cycle (st. V-VI).
The analysis of the primary pituitary cultures confirmed that GnRH was able to stimulate the secretion of Fsh and Lh during spermatogenesis (st. IV). At this stage, the capacity of GnRH to release Lh was stronger. The E_2, T and DHP treatments caused a reduction of the stimulatory effect of GnRH on Fsh and Lh release when they were combined.
The double immunohistochemistry analysis of Esrs and Gths in pituitary sections revealed co-localization of Esrs with Fsh and Lh producing cells. The three Esrs are present in the Fsh and Lh cells with different intensities depending on the region of the pituitary. The signal of Esr2b was more abundant in Fsh cells than in Lh cells. Finally, the results of the double immunohistochemistry GnRH1-Fsh and GnRH-Lh revealed that the GnRH1 fibers just touch Fsh cells but not Lh cells (Fig. 1).
The analyses of pituitaries from st. IV males treated with E_2 indicated E_2 inhibited the expression of *fshb* as well as of secretion of both Gths, especially Fsh. Treatment of half of the animals with LHRHa during the last 24 hours of the experiment showed no effect of this GnRH agonist on the synthesis and

secretion of Fsh. Nevertheless, LHRHa inhibited *lhb* expression and strongly stimulated the release of Lh. The combination of E_2-LHRHa treatment did not modify the inhibitory effect of E_2 on Fsh nor the stimulatory effect of LHRHa on Lh release. The histology revealed that E_2 inhibited the progression of spermatogenesis.

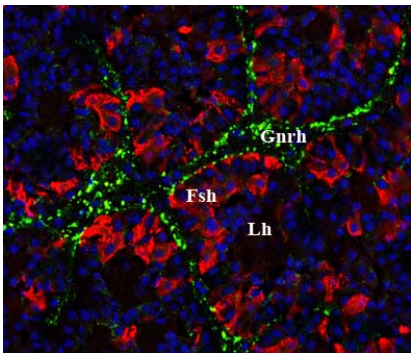


Figure 1. Double immunohistochemistry Gnrh1-Fsh in a pituitary of adult sea bass male in full spermatogenesis (stage IV). The pictures were taken in the central part of the PPD (*proximal pars distalis*).

In conclusion, GnRH differentially regulates the secretion of Lh and Fsh in male sea bass, according to the spermatogenesis stage. The absence of contact between the GnRH1 fibers with the Lh cells suggests different mechanisms of regulation of Fsh and Lh secretion.
Combined treatments with E_2, T and DHP and GnRH cause a reduction of the secretion of both Gths from *in vitro* cultured pituitary cells. E_2 strongly inhibits *in vivo* secretion of both Gths and stops spermatogonia differentiation into spermatocytes. The different expression and distribution of the Esrs in the gonadotropin-producing cells suggest that they could affect differentially the secretion of Fsh or Lh according to reproductive stage.

Acknowledgements: Funded by Spanish MICINN (AGL2015-67477-C2-1-R, RTI2018-094667-B-C22) and CSIC (201640E073). PP was supported by the Portuguese FCT through project UID/Multi/04326/2019 and a researcher contract under “Norma Transitória”-DL57/2016/CP1361/CT0015.

References

1. Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J. (2010). Perspectives on fish gonadotropins and their receptors. *Gen. Comp. Endocrinol.* 165, 412-437
2. Espigares F, Zanuy S, Gómez A. (2015). Kiss2 as a Regulator of Lh and Fsh Secretion via Paracrine/Autocrine Signaling in the Teleost Fish European Sea Bass (*Dicentrarchus labrax*). *Biol Reprod.* 93(5), 114.
3. Mateos, J., Mañanós, E., Swanson, P., Carrillo, M., Zanuy, S. (2006). Purificación de la hormona luteinizante (LH) en la lubina (Dicentrarchus labrax) y desarrollo de un inmunoensayo específico. *Ciencias Mar.* 32, 271–283.
4. Molés, G., Gómez, A., Carrillo, M., Zanuy, S. (2012). Development of a homologous enzyme-linked immunosorbent assay for European sea bass FSH. Reproductive cycle plasma levels in both sexes and in yearling precocious and non-precocious males. *Gen. Comp. Endocrinol.* 176, 70-78.
5. Molés G, Zanuy S, Muñoz I, Crespo B, Martínez I, Mañanós E, Gómez A. (2011). Receptor specificity and functional comparison of recombinant sea bass (*Dicentrarchus labrax*) gonadotropins (FSH and LH) produced in different host systems. *Biol. Reprod.* 84(6), 1171-1181.

MOLECULAR CHARACTERIZATION AND EXPRESSION OF *nanos2* AND *gfra1a* DURING TESTICULAR DEVELOPMENT OF THE EUROPEAN SEABASS (*Dicentrarchus labrax* L.)

F. Prat[1], M. Simón-Díaz[1], J. A. Martos-Sitcha[2], A. Gómez[3]

[1]*Departamento de Biología Marina y Acuicultura, Instituto de Ciencias Marinas de Andalucía, CSIC. 11519 Puerto Real, Cádiz, Spain.*
[2]*Department of Biology, Faculty of Marine and Enviromental Sciences, University of Cádiz, Campus de Excelencia Internacional del Mar (CEI-Mar), 11519 Puerto Real, Cádiz, Spain.*
[3]*Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre la Sal, CSIC, 12595 Torre de la Sal, Castellón, Spain.*

As a first step to determine the involvement of *nanos2* and *gfra1* in the regulation of the self-renewal and maintenance of spermatogonial stem cells (SSC) and preventing spermatogonial differentiation in the European seabass, the aim of this work was to characterize and study their expression at different stages of testicular development. One sequence of *nanos2* and two sequences of *gfra1* (*gfra1a* and *gfra1b*) were found in the genome of seabass by blast analysis against the NCBI whole-genome shotgun contigs (wgs) database. The sequences were confirmed by PCR using testis cDNA and specific primers designed to the respective genomic sequences. Tissue specificity analysis showed expression in all tissues studied, but *nanos2* expression was higher in testis, while *gfra1a* was highly expressed in gonads, brain and pituitary. The expression profiles of *nanos2* and *gfra1a* obtained by qrtPCR in the testis during the reproductive cycle, suggest that both genes can have key roles during the early stages of testicular development.

Introduction

Spermatogenesis starts when undifferentiated type A spermatogonia (und-SpgA), also known as spermatogonial stem cells (SSC), become differentiated SpgA (diff-SpgA). Expression of the RNA binding protein *nanos2* in und-SpgA has been observed in several species and it is recognized as a spermatogonial stem cell marker (1). In the mammalian testis, it is well established that Sertoli cells produce GDNF (glial cell line-derived neurotrophic factor) that binds the GFRA1 (GDNF family receptor alpha-1), which is expressed by und-SpgA promoting its self-renewal and maintenance (2). A few recent studies have addressed the potential involvement of the Gdnf-Gfra1 signaling pathway in the regulation of gametogenesis in cartilaginous (3) and bony fishes (4). As a first step to determine *nanos2* and *gfra1a* involvement in the self-renewal and maintenance of the und-SpgA, and preventing spermatogonial differentiation in the European seabass, the aim of this work was to characterize and study their expression at different stages of testicular development.

Materials and Methods

Blast analysis against the NCBI whole-genome shotgun contigs (wgs) database for *D. labrax* (sequences CBXY010000001 to CBXY010037781), was performed to find the sequences of *nanos2* and *gfra1*. EMBOSS est2genome program was used to identify exons and introns. The deduced mRNA sequences were cloned by PCR using ovarian and testis cDNA and specific primers designed to exons of the respective genomic sequences. Testis samples from immature to fully

mature stages were collected at different times of the reproductive cycle. Levels of expression of *nanos2* and *gfra1a* were analyzed in all testis samples by quantitative real time PCR by the standard curve method using specific primers, SYBR Green dye and cDNA retro-transcribed from 500 ng of total RNA as template. Geometric mean of *rna18s* and *ef1a* reference genes, were used to

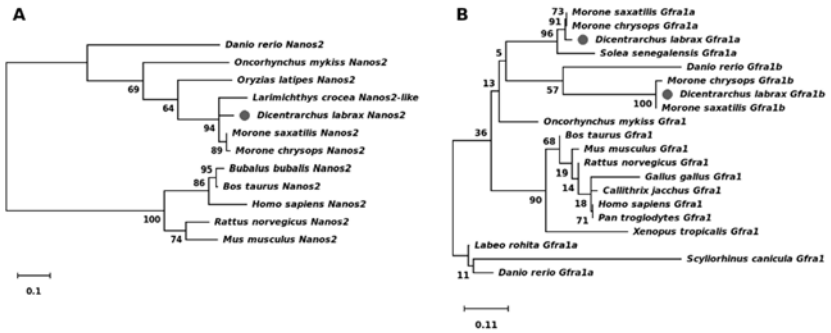


Figure 1. Maximum Likelihood phylogenetic trees for Nanos2 (A) and Gfra1 (B) based on the Jones-Taylor-Thornton (JTT) model and bootstrap method with 1000 replicates. Amino acid sequences from different species of fish, amphibian, birds and mammals were aligned by Clustal Omega. Trees were performed by MEGA-X software. Nanos2: *D.rerio* (DAA64468), *O.mykiss* (AHN91987), *O.latipes* (NP_001153919), *L.crocea* (AHN52225), *M.saxatilis* (GBAA01143918), *M.chrysops* (GAZY01124056), *B.bubalus* (AQY62587), *B.taurus* (NP_001268833), *H.sapiens* (NP_001025032), *R.norvegicus* (NP_001102378), *M.musculus* (NP_918953). Gfra1: *S.canicula* (AGY14507), *O.mykiss* (BAM84261), X.tropicalis (AAI36108), *G.gallus* (AAB61570), *C.jacchus* (XP_009008582), *B.taurus* (NP_001098881), *M.musculus* (AAH54378), *R.norvegicus* (AAC53300), *H.sapiens* (AAH14962), *P.troglodytes* (XP_001150803). Gfra1a: *M.saxatilis* (GBAA01193911), M.chrysops (GAZY01175506*), S.senegalensis* (solea_v4.1_unigene105038), *L.rohita* (ADJ94947), *D.rerio* (AAK11260). Gfra1b: *D.rerio* (AAK11261), *M.saxatilis* (GBAA01114933/GBAA01159730), *M.chrysops* (GAZY01083525/GAZY01061271).

normalize data. Significant differences were tested by Kruskal-Wallis non-parametric test followed by two-sample Mann-Whitney U test.

Results and Discussion

One *nanos2* and two *gfra1* sequences (*gfra1a* and *gfra1b*) were found in the genome of the European seabass. The cloned *nanos2*, *gfra1a* and *gfra1b* sequences showed open reading frames of 531 bp, 1419 bp and 1425 pb coding for 177, 473 and 475 amino acid residues, respectively. Amino acid alignments and phylogenetic trees demonstrate that the cloned cDNA sequences from testis encode for Nanos2, Gfra1a and Gfra1b, and are related to the Nanos2 and Gfra1 of other vertebrate species (Fig.1). Tissue specificity analysis by RT-PCR showed expression in all tissues studied, but *nanos2* expression was high in testis and low in ovary, while *gfra1a* was highly expressed in gonads, brain and pituitary. Expression analysis during the reproductive cycle showed the highest levels of *nanos2* expression in testis containing only SpgA, decreasing as development was progressing. Expression levels of *gfra1a* were also high during early stages of development until spermatocytes were observed in the testis, decreasing later and reaching the lowest levels in the testis of post-spawning fish. These results suggest that *nanos2* is mainly involved in the very early s tages of testicular development, while *gfra1a* could be also involved in later stages of development in the testis of European seabass.

Figure 2. Tissue specificity expression of *nanos2* and *gfra1a*. Non-quantitative RT-PCR product sizes are provided. Ov: ovary, Te: testis, Br: brain, Pi: pituitary, Li: liver, ki: kidney, Mu: muscle, He: heart, Sp: spleen, AT: adipose tissue, (–): negative control.

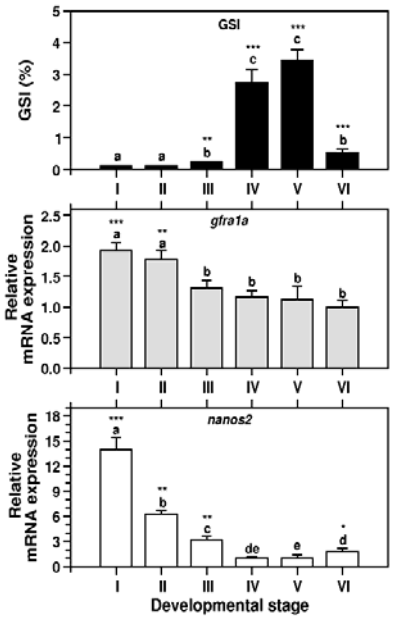


Figure 3. Changes in the GSI and *gfra1a* and *nanos2* mRNA expression at different stages of testicular development determined by histology (data not shown) according to (5). **I**: immature stage with testis containing mainly SpgA (July-September), **II**: early recrudescence with testis containing SpgA and cysts of SpgB (October), **III**: mid-recrudescence with spermatocytes being the dominant germ cell type but SpgA and SpgB were also visible (November), **IV**: late-recrudescence: spermatocytes and spermatids were the dominant cell type and spermatozoa were also observed (December-January), **V**: full spermiating testes containing also spermatocytes and spermatids (February-March), **VI**: post-spawning with testis with no spermatogenic activity and containing residual spermatozoa (April-May). Different letters indicate significant differences among values at $p < 0.05$ level. Asterisks indicate differences compared to the lowest levels of GSI or expression for each gene (*, $p < 0.05$; **, $p < 0.01$: ***, $p< 0.001$.)

Acknowledgements: The authors thank to Mr Juan José Blanco for his technical assistance. Grant RTI2018-094667-B-C21 (MCIU/AEI/FEDER,UE).

References

1. Suzuki H., Sada A., Yoshida S., Saga, Y. (2009). The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. Dev. Biol. 336, 222-231.
2. Meng XJ., Lindahl M., Hyvonen ME., Parvinen M., de Rooij DG., Hess MW., Raatikainen-Ahokas A., Sainio K., Rauvala H., Lakso M., Pichel JG., Westphal H., Saarma M., Sariola H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287, 1489-1493.
3. Gautier A., Bosseboeuf A., Auvray P., Sourdaine P. (2014). Maintenance of potential spermatogonial stem cells in vitro by GDNF treatment in a chondrichthyan model (Scyliorhinus canicula L.). Biol. Reprod. 91, 91, 1-15.
4. Maouche A., Curran E., Goupil A.S., Sambroni E., Bellaiche J., Le Gac F., Lareyre J.J. (2018). New insights into the evolution, hormonal regulation, and spatiotemporal expression profiles of genes involved in the Gfra1/Gdnf and Kit/Kitlg regulatory pathways in rainbow trout testis. Fish Physiol. Biochem. 44, 1599-1616.
5. *Begtashi I., Rodríguez L., Moles G., Zanuy S., Carrillo M. (2004). Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (Dicentrarchus labrax, L.). I. Morphological aspects. Aquaculture 241, 539-559.*

REGULATION OF GH EXPRESSION IN GILTHEAD SEA BREAM PITUITARY

M. Perelló-Amorós[1], A. Sánchez-Moya[1], M. Moosavi[1], M. Hoseinzade[1], M. Hedayatirad[1], E.J. Vélez[1], S. Balbuena-Pecino[1], J.A. Calduch-Giner[2], J. Pérez-Sánchez[2], E. Capilla[1], I. Navarro[1], J. Fernández-Borràs[1], J. Blasco[1], J. Gutiérrez[1]

[1]Department of Cell Biology, Physiology and Immunology, University of Barcelona, Barcelona, Spain. [2]Nutrition and Fish Growth Endocrinology, Institute of Aquaculture Torre de la Sal (CSIC), Castellón, Spain.

In this study, we analyzed the changes on pituitary *gh* and related molecules' expression upon different experimental conditions *in vivo* and *in vitro* in gilthead sea bream (*Sparus aurata*). Gilthead sea bream juveniles were treated with recombinant bovine GH (rbGH, Posilac®), were fasted during 21 days and refed for 7 days, or were exposed to sustained swimming while feeding a diet supplemented with an amino acid (AA) derivative. Moreover, adult fish hemi-pituitaries were treated *in vitro* with different hormones and AA. Pituitary *gh* expression in rbGH-treated fish was down-regulated reaching the lowest values after 6 weeks of injection and maintaining low levels during the rest of the experiment. On the contrary, 21 days of fasting triggered an increase of pituitary *gh* expression parallel to plasma levels, while *gh* and *igf-i* receptors expression strongly decreased; and refeeding partially recovered the basal levels. Sustained swimming and dietary supplementation for 8 weeks increased growth in parallel with a tendency to rise pituitary *gh* expression. Finally, hemi-pituitaries treated with different doses of Lysine presented increased *gh* expression thus demonstrating that this *in vitro* model is suitable to study the GH regulation.

Introduction

The GH/IGF-I system is the main endocrine regulator of growth in vertebrates and abundant bibliography demonstrates that this hormonal axis can be modulated by multiple factors, such as through interaction with other hormone systems, nutritional state, energy balance, physical activity, among many others. This fact has made this GH/IGF system an attractive target for the research directed to establish solid biomarkers associated to the growth condition in vertebrates' species with high economical value, such as aquaculture species.

However, there is still limited knowledge on the regulation of pituitary *gh* expression and its correlation with the plasma protein levels. This information can be very interesting to understand the synthesis and regulation of this hormone and which are the best stimulators of its secretion. Thus, the present study aims to summarize different experiments that were done in our group investigating pituitary *gh* expression under different *in vivo* and *in vitro* models in gilthead sea bream.

Materials and Methods

Gilthead sea bream juveniles were treated with recombinant bovine GH (rbGH, Posilac®) (1), were fasted during 21 days and refed for 7 days (2), or were exposed to moderate sustained swimming activity while fed a diet containing an amino acid (AA) derivative during 8 weeks (3). Moreover, adult fish hemi-

pituitaries were treated *in vitro* with different AA, pituitary RNA was extracted and GH/IGF-I system-related members expression analyzed by qPCR (1), and the results were then compared with circulating GH and IGF-I levels obtained in an *in vivo* intraperitoneal administration of the same AA. Plasma GH and IGF-I were determined by RIA in IATS-CSIC.

Results and Discussion

The GH long-term treatment by means of Posilac® administration induced a significant decrease in pituitary *gh* expression, reaching the lowest values at 6 weeks of injection and only a tendency to increase after 12 weeks, although still not recovering the control *gh* expression level that remained stable (Fig. 1A). The effects of Posilac® treatment in fish and specifically in gilthead sea bream are well characterized resulting in a somatic growth increase due to the activation of the GH/IGF system (1,4). In present conditions, pituitary *gh* expression resulted down-regulated probably as a consequence of the IGF-I increased levels and their GH inhibitory effects as it would be expected considering the high exogenous GH dose. Fasting of 21 days determined a clear increase in the GH plasma levels that were maintained high during early refeeding (24 h) and returned to basal values after 7 days (2). Pituitary *gh* expression followed a similar tendency as plasma GH levels, with a progressive increase, although during early refeeding *gh* expression was still high, recovering control values only after 7 days refeeding (Fig. 1B). Both *gh* pituitary receptors' expression decreased during fasting, and remained low during early refeeding to partially recover at 7 days refeeding (data not shown), which probably represents a counterpart adaptation to food deprivation determined by a simultaneous increase of GH gene and protein expression to facilitate its energy mobilization function.

Fish exposed to sustained swimming and fed with an AA derivative supplemented diet grew more than those fed with the control diet (data not shown) and presented a tendency to increase pituitary *gh* expression (Fig. 1C). It is noticeable that the pituitary *gh* expression in *in vivo* experiments presented a regulation parallel to circulating GH levels. Thus, while in exercised and dietary supplemented fish only a tendency was observed, in the fasting and refeeding condition, pituitary *gh* expression and circulating levels were very coincident. Even the delay in pituitary *gh* expression respect to plasma GH during early refeeding, reflected the regulative differences among protein and gene expression.

When hemi-pituitaries were incubated with Lysine at different doses (2-4 mM) and at different times (1.5-18 h; Fig. 1D), the *gh* expression resulted clearly increased in comparison with the control or other AA conditions that only presented weak responses of *gh* expression (data not shown). This is in agreement with the intraperitoneal Lysine administration *in vivo* that provoked a significant increase of GH plasma levels (Control: 20.29±0.77 ng/ml; Lysine: 24.75±0.50 ng/ml).

These results demonstrated the parallelism between gene expression and circulating hormone regulation, indicating the interest of hemi-pituitary preparations to investigate specific stimulators of GH secretion. The method can be very useful to study GH function taking into account the difficulties to analyze GH plasma levels.

In summary, the present data indicate that pituitary *gh* expression can be a very suitable model to understand the effects of different physiological conditions on GH/IGF-I system in fish.

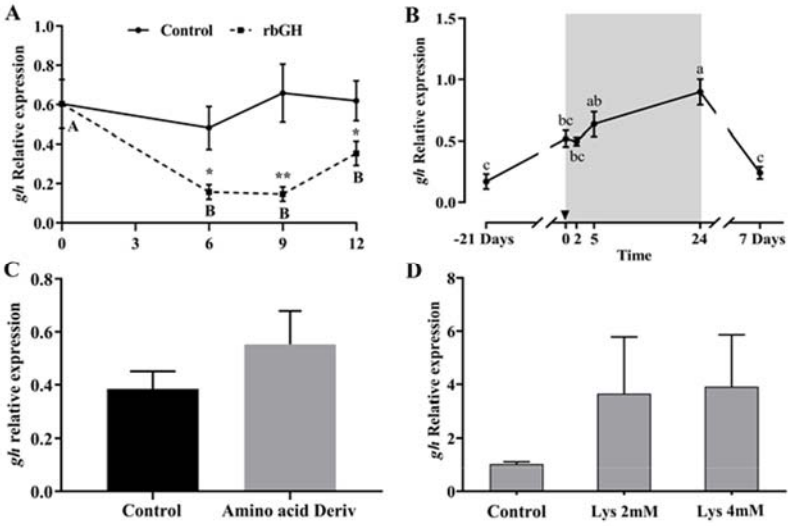


Figure 1. Pituitary *gh* expression in gilthead sea bream A) along the rbGH injection experiment, B) along the fasting and refeeding experiment, C) in the AA derivative supplementation experiment with exercised fishes, and D) in the incubated hemi-pituitaries. The results are shown as mean + S.E.M. Different letters indicate significant differences (P<0.05) among groups.

Acknowledgements: Supported by funds from the Ministerio de Ciencia, Innovación y Universidades (MICIUN) (AGL2017-89436-R and RTI2018-100757-B-I00), 2017SGR-1574 and XRAq. M.P-A and S.B-P are recipients of a FPI fellowship from the MICIUN (BES-2016-078697 and PRE2018-085580 respectively).

References

1. Vélez, E.J., Perelló-amorós, M., Lut, E., Azizi, S., Capilla, E., Navarro, I., Pérez-sánchez, J., Calduch-giner, J.A., Blasco J, Fernández-Borràs J, Gutiérrez J. (2019). A long-term growth hormone treatment stimulates growth and lipolysis in gilthead sea bream juveniles. Comp Biochem Physiol A Mol Integr Physiol. 232, 67–78.
2. Perelló-Amorós, M., Vélez, E.J., Vela-Albesa, J., Sánchez-Moya, A., Riera-Heredia, N., Hedén, I., Fernández-Borràs, J., Blasco, J., Calduch-Giner, J.A., Navarro, I., Capilla, E., Jönsson, E., Pérez-Sánchez, J., Gutiérrez, J., (2018). Ghrelin and Its Receptors in Gilthead Sea Bream: Nutritional Regulation. *Front. Endocrinol. (Lausanne)*. 9, 1–14.
3. Blasco, J., Moya, A., Millán-Cubillo, A., Vélez, E.J., Capilla, E., Pérez-Sánchez, J., Gutiérrez, J., Fernández- Borrás, J., (2015). Growth□promoting effects of sustained swimming in fingerlings of gilthead sea bream (*Sparus aurata* L.). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 185, 859–868.
4. McLean, E., Devlin, R.H., Byatt, J.C., Clarke, W.C., Donaldson, E.M., (1997). Impact of a controlled release formulation of recombinant bovine growth hormone upon growth and seawater adaptation in coho (*Oncorhynchus kisutch*) and chinook (*Oncorhynchus tshawytscha*) salmon. *Aquaculture* 156, 113–128.

STOCKING DENSITY AFFECTS THE GROWTH PERFORMANCE, INTERMEDIARY METABOLISM, OSMOREGULATION, AND RESPONSE TO STRESS IN PATAGONIAN BLENNIE (*Eleginops maclovinus*)

R. Oyarzun[1,2,3], R. Paredes[1], J. Saravia[1,2,3], F.J. Morera[7], J.L.P. Muñoz[4], I. Ruiz-Jarabo[5], J.M. Mancera[5], L. Vargas-Chacoff[1,3]

[1]Instituto de Ciencias Marinas y Limnológicas, Universidad Austral de Chile, Valdivia. [2]Escuela de Graduados, Programa de Doctorado en Ciencias de la Acuicultura, Universidad Austral de Chile, Puerto Montt. [3]Centro Fondap de Investigación de Altas Latitudes (IDEAL), Universidad Austral de Chile, Valdivia. [4]Centro I~mar, Universidad de los Lagos, Puerto Montt, Chile. [5]Department of Biology, Faculty of Marine and Environmental Sciences, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI-MAR), University of Cádiz, 11510 Puerto Real, Spain. [6]Laboratorio de Bioquímica Aplicada, Instituto de Farmacología y Morfofisiología, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile.

Eleginops maclovinus is a native species with potential for Chilean aquaculture, but the influence of stocking density on the physiology of this species is unknown. The objective of this study was to determine the optimal range of stocking density assessing changes in growth parameters (weight and gene expression of the GH/IGF-1 axis), and stress response (plasma cortisol levels, gene expression of glucocorticoid receptor and HSP70). For this, 48 fish (201.60 ± 32.00 g) were randomly assigned to four stocking densities and maintained for 60 days. No mortality was recorded, but growth decreased significantly at highest stocking density. Plasma cortisol levels showed a significant reduction at highest stocking density of 24 kg/m^3, while expression of glucocorticoid receptor enhanced significantly at this density respect to density of 6 kg/m^3. The GH/IGF-1 axis and HSP70 revealed levels of mRNA transcript significantly lower at the highest density. In conclusion, we suggest that the highest stocking density employed in our experiment activated stress system of *Eleginops maclovinus* and decreased growth probably through a reduction in the transcription of the different endocrine players of GH/IGF-1 axis. Our results also suggest to keep *E. maclovinus* in the range of 3-12 kg/m^3, avoiding the high density of 24 kg/m^3.

Introduction

For the aquaculture of any species, stocking density is a crucial factor for the optimization of its culture. Inadequate stocking density can activate the stress system, generating physiological changes, characterized by an increase in the release of cortisol by the hypothalamus-pituitary-interrenal (HPI) axis (1). Cortisol crosses the plasma membrane and binds to glucocorticoid receptors (GR) in the cytoplasm, which dimerize and translocate to the nucleus, modulating the transcription of genes (2). The growth in teleosts is regulated by growth hormone (GH) and insulin-like growth factor (IGF-1) (3,4,5). GH is stored and secreted to the blood by the pituitary gland, and circulating GH stimulates the synthesis and secretion of IGF-1 at the liver (6). Both circulating hormones (GH and IGF-1 in the blood) and their mRNA transcript levels have been considered as growth indicators in teleost, although specific species and context results have been described (7). *E. maclovinus* is a potential candidate for

diversification in Chilean aquaculture. However, to date, the reported growth rate for *E. maclovinus* under captive conditions is low. Therefore, it is important to determine its optimal stocking density since this would provide relevant information for the potential aquaculture of this species. The aim of this study was to evaluate the effect of four stocking densities (3, 6, 12, and 24 kg/m3) on growth performance and stress response.

Materials and Methods

Immature males of *E. maclovinus* (201.60 ± 4.67 g) were obtained from the Valdivia river estuary and transported at the Faculty of Science (Universidad Austral de Chile, Valdivia). Fish were acclimatized for 30 days in seawater (SW 32 psμ, 1085 mOsm/kg) in 500 L tanks using a flow–through system, natural photoperiod, and ambient temperature (12 ± 1 °C). Fish were fed to satiety with commercial dry pellet (BioMar, Golden Optima 5222). After the acclimation period, 48 fish were randomly assigned to eight 500 L tanks (N = 6 fish per tank), with four stocking densities of 3, 6, 12 and 24 kg/m^3. The dissolved oxygen range was 9.2–10.4 and the oxygen saturation was over 80 %. At the end of the experiment (day 60), fish were weighed and sampled. Blood was collected from the caudal peduncle and plasma (for quantification of cortisol) was obtained by centrifugation of whole blood (5 min, 2000 g). Fish were subsequently euthanized by spinal cord sectioning. The liver and pituitary gland was extracted and processing for RNA extraction, synthesis of complementary DNA, and Real-time quantitative PCR (qPCR) analysis, with commercial kits, following the manufacturer recommendations.

Results and Discussion

No mortality was recorded during the experimental time. Fish under higher stocking density (24 kg/m^3) presented a significant reduction in growth (Fig. 1A). Cortisol levels (Fig. 1D) showed the highest values at the density of 3 kg/m^3, with a significant decrease at the higher stocking density. Pituitary *gh* (Fig. 1B) as well as hepatic *igf-1* (Fig. 1C) and *hsp70* (Fig. 1F) mRNA levels were significantly affected by stocking density, being the lowest values observed at 24 kg/m^3. However, the highest *gr* mRNA transcript was detected at this density (Fig. 1E).

Our results suggest that stoking density affects the physiology of *E. maclovinus* through a decrease in growth generated by activation of the stress system and an inhibition in the transcription of different endocrine players of GH/IGF-1 axis. Based on the results, we suggest that *E. maclovinus* should be cultivated at a stocking density range of 3–12 kg/m^3, avoiding the high density of 24 kg/m^3.

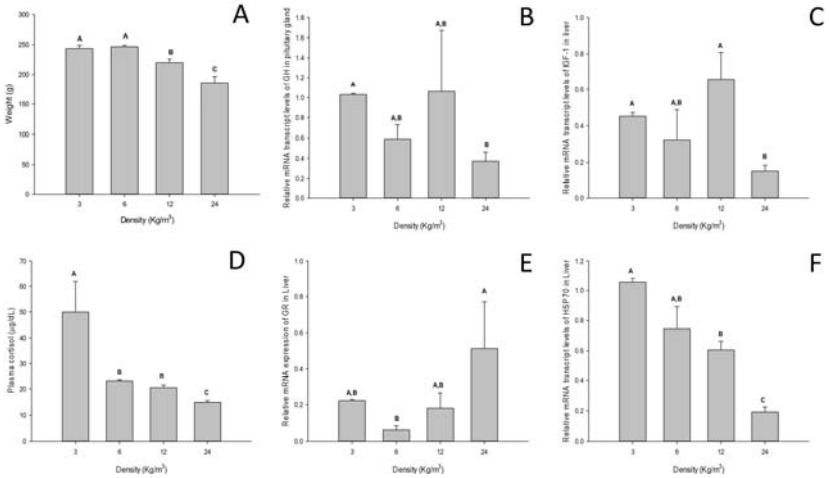


Figure 1. Body weight (A), *gh* (B), *igf-1* (C), Cortisol (D), *gr* (E), and *hsp70* (F) of *E. maclovinus* maintained in four different stocking densities over a 60 day period. Values represent the mean ± S.E.M. of 12 fish (6 fish/tank, 2 tank). Different letters indicate statistically significant differences ($P < 0.05$ one-way ANOVA and Tukey test).

Acknowledgements: Fondecyt project 1160877, Fondap Ideal Center 15150003, Conicyt-PCHA/National Doctorate/2016-21160204 and the PhD program in Sciences of Aquaculture and VIDCA, both belonging to the UACH, for allowing the financing of this study. The authors belong to the Fish Welfare and Stress Network (AGL2016-81808-REDT), supported by the Agencia Estatal de Investigación (MICINN, Spanish Government).

References

1. Barton B.A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* 42, 517–525.

2. Faught E., Aluru N., Vijayan M.M. (2016). The molecular stress response. In: Fish Physiology. Academic Press, pp. 113–166.

3. Sadoul B., Vijayan M.M. (2016). Stress and growth. In: Fish Physiology. Academic Press, pp. 167–205.

4. Saera-Vila A., Calduch-Giner J.A., Prunet P., Pérez-Sánchez J. (2009). Dynamics of liver GH/IGF axis and selected stress markers in juvenile gilthead sea bream (*Sparus aurata*) exposed to acute confinement: differential stress response of growth hormone receptors. *Comp. Biochem. Physiol.* Part A 154, 197–203.

5. Sakamoto T. (2006). Prolactin and growth hormone in fish osmoregulation. *Gen. Comp. Endocrinol.* 147, 24–30.

6. Moriyama S., Ayson F.G., Kawauchi H. (2000). Growth regulation by insulin-like growth factor-I in fish. *Biosci. Biotechnol. Biochem.* 64, 1553–1562.

7. Beckman B.R. (2011). Perspectives on concordant and discordant relations between insulin-like growth factor 1 (IGF1) and growth in fishes. Gen. Comp. Endocrinol. 170, 233–252.

CHARACTERIZATION OF GH/IGF AXIS GENE EXPRESSION DURING ATLANTIC BLUEFIN TUNA (*Thunnus thynnus*) LARVAL DEVELOPMENT IN NW MEDITERRANEAN WATERS

Gonzalo Martínez-Rodríguez[1], N. Gilannejad[1], C. Johnstone[2], J.M. Quintanilla[2], E. Malca[3,4], A. García[2], J.M. Mancera[5], R. Laiz-Carrión[2]

[1] Institute of Marine Sciences of Andalusia (ICMAN), Spanish National Research Council (CSIC), Puerto Real, Cadiz, Spain. [2] Instituto Español de Oceanografía. C. O. de Málaga. 29640 Fuengirola, Málaga. Spain. [3] NOAA National Marine Fisheries Service, Southeast Fisheries Science Center, Miami, FL, USA. [4] Cooperative Institute for Marine and Atmospheric Studies, University of Miami, Miami, FL, USA. [5] Department of Biology, Faculty of Marine and Environmental Sciences, Universidad de Cádiz, Campus de Excelencia Internacional del Mar (CEI-MAR), Puerto Real, Cadiz, Spain.

Atlantic bluefin tuna (ABFT), *Thunnus thynnus* (Linnaeus, 1758) is a large migratory oceanic top predator, considered an important fishery resource worldwide and a key species in pelagic ecosystems. Early life stages represent a critical period due to maximum mortality rates. Survival during this period is crucial for further recruitment success, for which larval growth has a determinant role. Fish growth and development is mainly controlled by the GH/IGF axis involved in skeletal and soft tissue growth, as well as in aspects of immune function and behavior (including appetite, foraging, aggression, and predator avoidance), all having ecological consequences. To characterize the ontogenetic development of the GH/IGF axis, gene expression in wild ABFT larvae, a total of 106 larvae from yolk-sac to post-flexion stage (average standard length SL = 4.73 ± 1.59 mm SD) were collected from the NW Mediterranean spawning area during June 2017. Expression levels of *gh*, *igf1* and *igf2* were evaluated using real-time PCR, normalized to *actb* and *ef1a*, as internal reference genes. No relationship between *gh* gene expression and SL was observed, whereas *igf1* and *igf2* showed an increasing relationship with SL, with significantly higher values in post-flexion compared to pre-flexion larvae.

Introduction

Atlantic bluefin tuna (ABFT), *Thunnus thynnus* (Linnaeus, 1758) is a large migratory fish and an oceanic top predator. It is considered an important fishery resource worldwide and a key species in pelagic ecosystems. Growth hormone (*gh*) is an essential regulator of growth, and also has complex metabolic functions that are widely studied (Björnsson 1997). In fish, *gh* participates in almost all major physiological processes, including the regulation of somatic growth and maintenance of protein, lipid, carbohydrate and mineral metabolisms, skeletal and soft tissue growth, reproduction and immune function (Reinecke et al., 2005). Several aspects of behavior are also affected by *gh* , including appetite, foraging behavior, aggression, and predator avoidance, which in turn has ecological consequences (see e.g. Björnsson, 1997; Pérez-Sanchez, 2000). The *gh* cDNAs of fish have been cloned and sequenced, and their recombinant *gh*s (r*gh*s) have been shown to be potent accelerator of growth rate in fishes (Sato et al., 1988; Tsai et al., 1994). The *gh* immuno-reactive cells in pituitary of laboratory reared Pacific bluefin tuna larvae have been examined

by histological and immuno-histochemical procedures (Kaji et al., 2003). In this study, *gh* cells has been first detected 3 days after hatching (DAH), maintained high levels during few days after first feeding, followed by a rapid decrease, remaining at lowest levels throughout the flexion phases, and increased again in the post-flexion period. Such a V-shaped ontogenetic pattern of relative *gh* expression was also observed in yellow tuna *Thunnus albacares* larvae (Kaji et al., 1999). As in many other species, survival at early life stages is crucial for future recruitment success and viability of the exploited populations. Precise age estimations are required to obtain reliable parameters of growth with a determinant role in larval survival. The aim of this study was to characterize the ontogenetic development of the GH/IGF axis gene expression in wild ABFT larvae from Mediterranean spawning area (Balearic Islands).

Materials and Methods

A total of 106 ABFT larvae from yolk-sac to post-flexion (SL = 4.73 ± 1.59 mm; average ± SD) were collected from the NW Mediterranean spawning area in June 2017 (Fig. 1) on board of SOCIB R/V. Total RNA was extracted from tuna larvae preserved in RNAlater® (Ambion, Applied Biosystems), using the NucleoSpin® XS kit (Macherey-Nagel). Reverse transcription was performed using the qScript™ cDNA synthesis kit (Quanta BioSciences), with 50 ng of total cDNA assumed from total RNA input, including only samples that had a RNA integrity number (RIN) greater than 8.0. Expression levels of *gh*, *igf1* and *igf2* were evaluated using real-time PCR. Primers were designed using Primer3 software (v.0.4.0) and real time PCR was performed in a BioRad CFX Connect™ with BioRad CFX Manager Software v3.1 (BIORAD Laboratories). Expression of target genes were normalized to *actb* and *ef1a*, as internal reference genes (Vandesompele et al., 2002).

Results and Discussion

No significant relationship between *gh* expression and standard length (SL) was observed (Fig. 1A), and no clear conclusion can be achieved due to the high variability in the *gh* expression levels, suggesting a pulsed expression mechanism. The variability of daily *gh* expression should be examined further. However, within the studied size range, and according with the V-shaped ontogenetic pattern of *gh* expression previously described in *Thunnus albacares* larvae (Kaji et al., 1999), a trend can be observed with higher values in smaller and bigger larvae, together with lower values in the middle size larvae.

Expression of *igf1* and *igf2* showed an increasing relationship with body length (Fig. 1B and 1C), with significantly higher values in post-flexion than pre-flexion phases (ANOVA, $p < 0.01$). This suggests a key growth-promoting role of *igf*s, making them good candidates for growth indexes during the ontogenetic development in ABFT.

Experiments using controlled conditions would be necessary to explain the ontogenetic development of the GH/IGF axis, and to understand better its implications in growth regulation of wild animals and therefore, its ecological consequences.

Acknowledgements: This study has been financed by ECOLATUN grant CTM2015-68473-R (MINECO/FEDER) funded by the Spanish Ministry of Economy and Competitiveness and Bluefin tuna project funded by Instituto Español de Oceanografía (IEO) and Balearic Island Observing and Forecasting System (SOCIB).

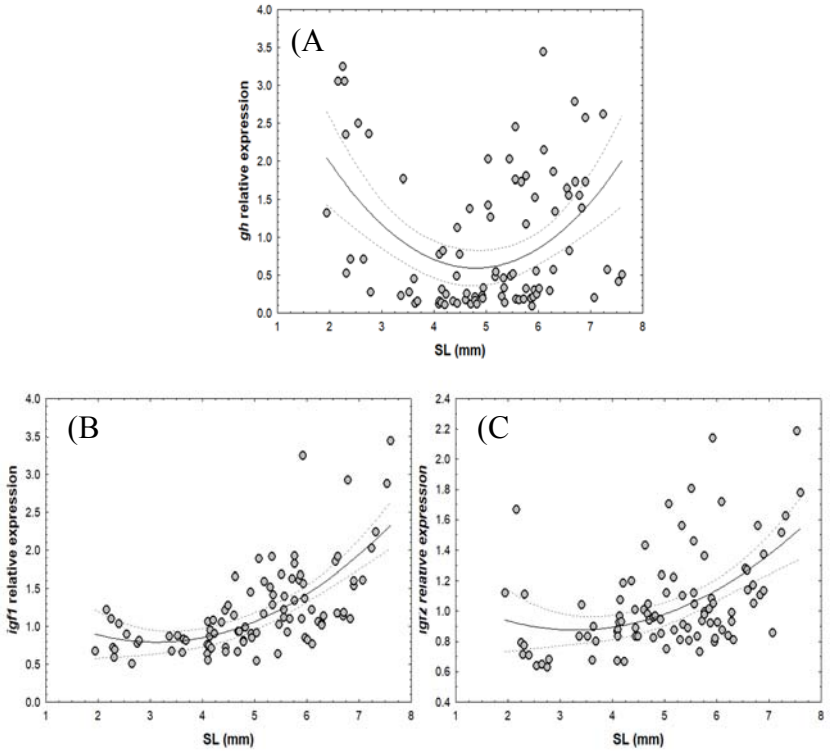


Figure 1. Relative expression levels of A) *gh*, B) *igf1* and C) *igf2* vs SL (mm) in wild ABFT larvae collected in the Mediterranean spawning area (Balearic Islands).

References

1. Donaldson, E.M. (1986) Fish Physiol Biochem 2: 9-24.
2. Björnsson B.Th. (1997). Fish Physiol. Biochem. 17: 9–24.
3. Kaji T. (2003). Cah. Options Méditerr. 60, 85–89.
4. Kaji T., Tanaka M., Oka M., Takeuchi H., Ohsumi S., Teruya K., Hirokawa, J. (1999). Fish. Sci. 65: 700–707.
5. Pérez-Sanchez J. (2000). Fish Physiol. Biochem. 22, 135–144.
6. Reinecke M., Björnsson B.T., Walton W., Dickhoff W.W., McCormick S.D., Navarro I., Power D.M., Gutiérrez J. (2005). Minireview. Gen. Comp. Endo. 142: 20–24.
7. Sato N., Murata K., Nonaka M., Sakaguchi M., Kariya Y., Hayami T., Kimura S., Kimura A., and Watanabe K. (1988). Biotechnol. Appl. Biochem. 10: 385–393.
8. Tsai H.J., Lin K.L. and Chen T.T. (1994). Comp. Biochem. Physiol. 104B: 803–810.
9. Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002). Genome Biology, 3: research0034.

ENVIRONMENTAL pH MODIFIED THE EXPRESSION OF DIFFERENT ADENOHYPOPHYSEAL HORMONES IN GREATER AMBERJACK, *Seriola dumerili* (Risso, 1810)

Liliana Rodríguez-Velásquez[1], N. Gilannejad[2], G. Gónzalez[1], I. Ruiz-Jarabo[1], J. Roo[3], J.L. Muñoz[4], J.M. Mancera[1], G. Martínez-Rodríguez[2]

[1] Department of Biology, Faculty of Marine and Environmental Sciences, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI-MAR), University of Cádiz, 11510 Puerto Real, Cádiz, Spain. [2] Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas (ICMAN-CSIC), E11510 Puerto Real, Cádiz, Spain. [3] Universidad de Las Palmas de Gran Canarias (ULPGC). [4] Centro IFAPA "El Toruño", Junta de Andalucía), Spain.

Greater amberjack (*Seriola dumerili*) is a candidate for diversification in aquaculture production. This work aimed to assess the effect of three environmental pH values on metabolism and expression of some adenohypophysis hormone precursors in greater amberjack individuals, reared in a Recirculating Aquiculture system (RAS). According to our findings, higher pH levels led higher *somatolactin* expression and to a tendency to lower *prolactin* expression levels. Plasmatic metabolites, except lactate, were not influenced by environmental pH levels.

Introduction

S. dumerili is considered as a potential alternative for diversification in the aquaculture sector, due to its fast growth rate and the high quality and commercial value of its meat, among others (1). In RAS, water parameters could be controlled in a highly effective manner and, therefore, this system could lead to an optimized production process. The aim of present work was to evaluate the influence of three environmental pH values (7.0, 7.4, and 7.8) metabolism and expression of some adenohypophyseal hormone precursors in greater amberjack juveniles reared in RAS. Information obtained in this study is useful to determine the optimized pH conditions for the production of this species.

Materials and Methods

Grater amberjack individuals were transported from ULPGC to IFAPA "El Toruño". After an acclimation period of three weeks, fish were randomly distributed into three independent RAS experimental groups, with four tanks per system and at a starting stock density of 3 kg m^{-3} (in each tank N = 11, initial body mass ± SEM of 273.96 ± 15.30 g). During the experimental period, water temperature ranged from 21 to 23 ºC and salinity was 36 ppt. Fish were fed three times a day *ad libitum* (approximately 2-3 % of their total body mass) with a commercial diet containing 52 % of proteins and 16 % of lipids (Skretting®, Stavanger, Norway). At the end of the experimental period (73 days), all fish were anaesthetized with a lethal dose of 2-phenoxyethanol. Blood was collected and plasma was stored in aliquots at -80 ºC until further analysis of some metabolites. Pituitary was removed and conserved in appropriate volume (1/10 w/v) of RNAlater™ (Invitrogen Life Technologies). Pituitary total RNA was extracted using the NucleoSpin® XS kit (Macherey-Nagel). Reverse transcription was performed using the qScript™ cDNA synthesis kit (Quanta BioSciences), with 50 ng of total cDNA assumed from total RNA input. Expression levels of

prolactin (*prl*), *somatolactin* (*sl*), *growth hormone* (*gh*), *proopiomelanocortin a* and *b* (*pomca* and *pomcb*) were evaluated using real-time PCR in a BioRad CFX Connect™. Expression of target genes were normalized to *β-actin* (*actb*) and *elongation factor 1-alpha* (*ef1a*), as internal reference genes (Vandesompele et al., 2002).

Results and Discussion

Regarding the metabolite levels, lactate was the only significantly influenced by the water pH, being significantly higher in fish reared at 7.4 pH (Fig. 1).

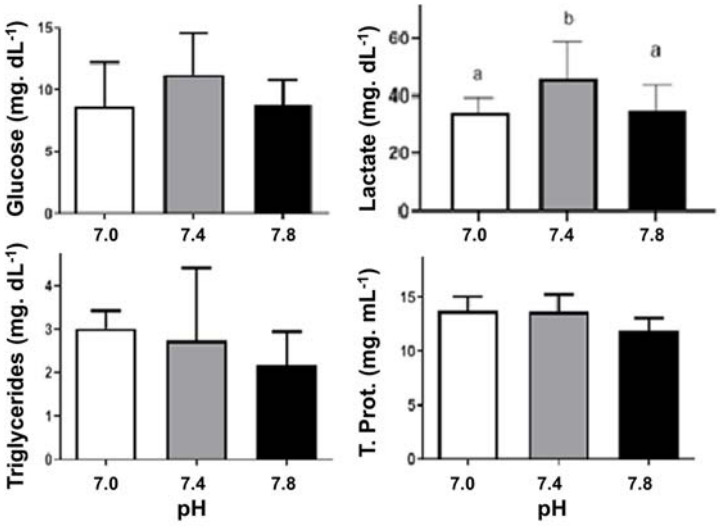


Figure 1. Plasma glucose, triglycerides, lactate, and total protein level in greater amberjack individuals reread under different water pH (7.0, 7.4, and 7.8), over the experimental period (73 days). Data are presented as mean ± SEM (n = 12). Different letters indicate significant differences among pH ($P < 0.05$, one-way ANOVA followed by a Tukey's *post hoc* analysis).

Regarding expression of different adenohypophyseal hormones, *sl* presented a direct lineal relationship with environmental pH. No significant differences were observed for *gh*, *pomca* and *pomcb* expression, while *prl* showed a decreasing tendency with increasing pH levels (Fig. 2). We suggest that the optimal pH for the culture of *S. dumerili* is 7.8.

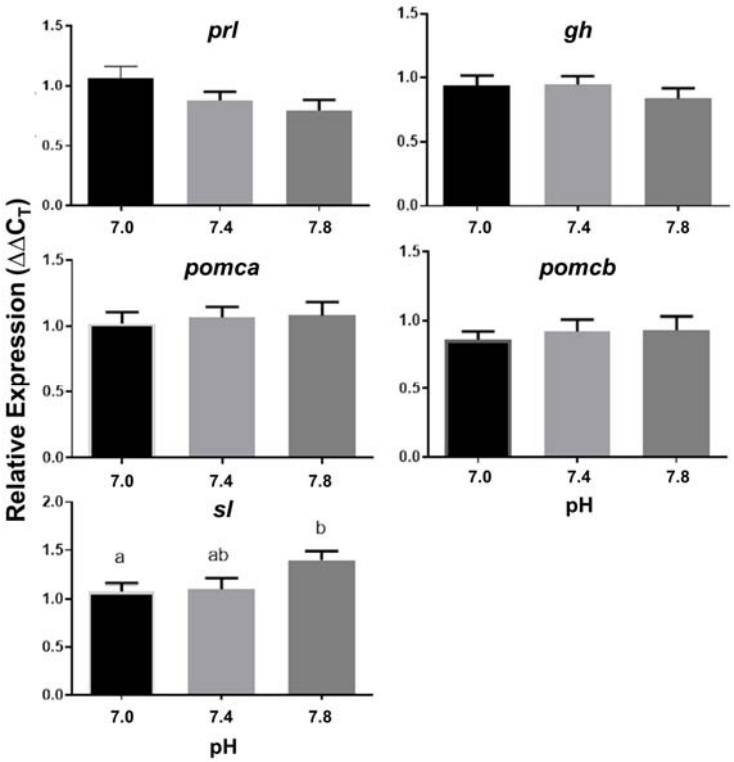


Figure 2. Relative expression of *prl*, *gh*, *pomca*, *pomcb*, and *sl* in greater amberjack individuals reread under different water pH (7.0, 7.4, and 7.8), over the experimental period (73 days). Data are presented as mean ± SEM (n = 12). Different letters indicate significant differences among salinities ($P < 0.05$, one-way ANOVA followed by a Tukey's *post hoc* analysis).

Acknowledgements: This work was funded by Project "Diversificación de la Acuicultura Española mediante la optimización del cultivo de *Seriola dumerili*" JACUMAR 2016 (MAPAMA) and Fondo Europeo Marítimo y de Pesca (FEMP). N. Gilannejad was supported by a grant from MINECO (BES-2015-071662).

References

1. FAO 2016-2020. Cultured Aquatic Species Information Programme. Seriola dumerili. Text by Jerez Herrera S., and Vassallo Agius R. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 1 January 2016.
2. Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: research0034.

PERIPRANDIAL VARIATION OF DIFFERENT PARAMETERS INVOLVED IN CENTRAL HEDONIC REGULATION OF APPETITE IN RAINBOW TROUT *Oncorhynchus mykiss*

A. Díaz-Rúa, S. Comesaña, M. Chivite, C. Velasco, JL. Soengas, M.Conde-Sieira

Laboratorio de Fisioloxía Animal, Facultade de Bioloxía and Centro de Investigación Mariña, Universidade de Vigo.

The mechanisms that regulate homeostatic control of food intake in fish have been broadly studied in the last years. However, information available regarding the mechanisms underlying the hedonic regulation of appetite in fish is still scarce. Previous studies in mammals indicate that cannabinoid system are implicated in homeostatic and hedonic regulation of food intake with forebrain and hypothalamus being the main regions involved. In fish, several studies describe the components of these systems, but only little information is available regarding their role in food intake and energy balance regulation. Therefore, the main objective of this study was to evaluate the main components of these putative hedonic mechanisms related to appetite regulation in different brain areas (telencephalon and hypothalamus) of rainbow trout under different nutritional status (fasted, fed and refed).Thus, blood and brain samples were taken at different post-prandial times (-30, 0, +30 and 180 minutes). Changes in AEA and 2-AG levels were observed in plasma related to the nutritional status and sampling time assessed. At central levels, changes in endocannabinoids levels were observed in hypothalamus and in mRNA abundance of *gpr55* in both brain areas. The results obtained in the present study suggest a role of endocannabinoid system in both homeostatic and hedonic regulation of food intake in fish at central level.

Introduction

Under conditions of food availability, the physiological mechanisms that lead an organism to eat or not are controlled by two types of motivations related to energetic or reward demands: homeostatic and hedonic mechanisms (Lau et al., 2017). In mammals homeostatic mechanisms can be overlapped by sensory or cognitive signals that activate limbic neuronal circuits related to hedonic systems (Demin et al., 2018). The endocannabinoid system (ECs) has been reported to be involved mainly in hedonic aspects but also in homeostatic regulation of food intake and energy balance in mammals with forebrain and hypothalamus being the main regions involved (Di Marzo et al., 2009). In the present study, we focussed on the impact of feeding conditions, i.e food deprivation, feeding or refeeding after food deprivation at different periprandial times (-30, 0, 30, and 180 min) on different parameters related to ECs in hypothalamus and telencephalon of rainbow trout *Oncorhynchus mykiss*.

Materials and Methods

Rainbow trout of about 80g body mass were obtained from a local fish farm (A Estrada, Spain) and were maintained for 1 month in 100 litre tanks under laboratory conditions and a natural photoperiod in dechlorinated tap water at 15 °C. Rainbow trout were 24 or 72 hours fasted before experiment. Samples were

taken at different pre- and postprandial times (-30 min, 0 min, +30 min, +180 min). For that, fish were lightly anaesthetized with 2 phenoxyethanol (Sigma, 0.02% v/v) directly in the tank. Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, deproteinized immediately (using 0.6 M perchloric acid) and neutralized (using 1 M potassium bicarbonate) before freezing on dry ice and storage at –80°C until further assay. Fish were sacrificed by decapitation, and hypothalamus and telencephalon were dissected, snap-frozen, and stored at –80°C. Ten fish per group were used to assess metabolite levels whereas six fish per group were used for the assessment of mRNA levels by qRT-PCR.

Results and Discussion

Endocannabinoids levels in brain areas are shown in Fig.1. Significant differences were found in the parameters assessed regarding the periprandial time and the feeding condition. In hypothalamus, levels of AEA (Fig.1A) increased at all postprandial times under the three feeding treatments with respect to their levels before the feeding time (-30 minutes).
Also in hypothalamus, 2-AG levels (Fig.1B) increased in fed group at all postprandial times with respect to the time before feeding, whereas in refed and fasted group this increase was observed only at 0 and 30 minutes, respectively. On the other hand, an elevation in the mRNA levels of *gpr55* (Fig.1C) was observed in the refed group respect to the other experimental groups 30 minutes before food intake and an increase in the fasted group was observed 180 minutes after food administration time.
In telencephalon, AEA and 2-AG levels did not display significant changes (Fig.1- E, F). An elevation in the mRNA levels of *gpr55* (Fig.1G) was observed in the refed group with respect to the other groups at food administration time and 30 minutes after intake. However, 180 minutes after feeding these levels dropped markedly. In the case of fasted group, decreased levels were observed at the time of food administration with respect to the other sampling times and to the other experimental groups. On the other hand, mRNA levels of *cnr* (Fig.1H) increased 30 minutes after feeding in the fed group respect to the fasted and refed groups, but these values decreased significantly after 180 minutes. Also, significant differences were found at 0 minutes in the fasted group compared to that observed 30 minutes before and after intake.

Endocannabinoids can bind to receptors GPR55 (Brown, 2007) whose presence was also reported in fish brain. In the present study the levels of mRNA gpr55 seems to be more related to nutritional status since refed group showed the highest values before feeding and also fasted fish showed the highest levels at the last sampling time in hypothalamus. Moreover, this gpr55 activation at preprandial time correlates to increased levels of 2-AG and AEA in plasma of the long term fasted fish (refed group) at the same time. In telencephalon, the changes in the mRNA abundance of receptors allow us to suggest the existence of a hedonic activation of the ECs in the telencephalon although the levels of ligands at the sampling times considered remained unchanged.

In summary, in the present study we observed that fish under different nutritional status displayed responses in the ECs that seem to be more related to the periprandial time than to the feeding condition in hypothalamus, suggesting a role of this system in the regulation of appetite and energy balance at central level in fish.

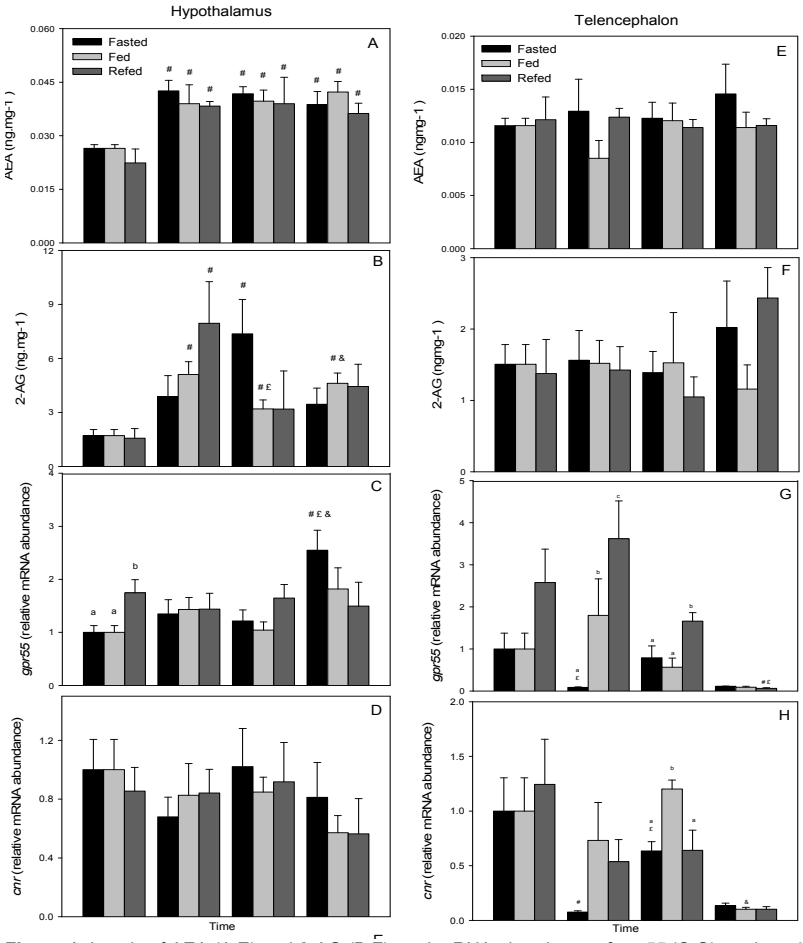


Figure 1. Levels of AEA (A,E) and 2-AG (B,F), and mRNA abundance of *gpr55* (C,G), and *cnr* (D,H), in hypothalamus (A,B,C,D) and telencephalon (E,F,G,H) of fasted, fed an refed rainbow trout sampled at different periprandial times (-30, 0, 30, 180 minutes). Each value is the mean + S.E.M. of 10 fish for metabolites and 6 fish for mRNA abundance. Different letters indicate significant differences (P<0.05) among feeding condition within each sampling time. #, significantly different from group at T = -30; £, significantly different from group at T = 0; & significantly different from group at T = 30.

References

1. Brown, A.J. (2007). Novel cannabinoid receptors. Br. J. Pharm. 152, 567–575.
2. Demin, K. A., Meshalkina, D. A., Kysil, E. V., Antonova, K. A., Volgin, A. D., Yakovlev, O. A., and Barcellos, L. J. (2018). Zebrafish models relevant to studying central opioid and endocannabinoid systems. Prog. Neuro-Psychoph., 86, 301-312.
3. Di Marzo, V., Ligresti, A., and Cristino, L. (2009) The endocannabinoid system as a link between homeostatic and hedonic pathways involved in energy balance regulation. Int. J. Obes. 33, 18–24.
4. Lau, B.K., Cota, D., Cristino, L., and Borgland, S.L. (2017). Endocannabinoid modulation of homeostatic and non-homeostatic feeding circuits. Neuropharmacology 124, 38–51.

VAGAL CONNECTIONS ARE INVOLVED IN THE EFFECT THAT CENTRAL AMPKα2 EXERTS ON THE HEPATIC METABOLISM AND FOOD INTAKE REGULATION IN RAINBOW TROUT

M. Conde-Sieira, S. Comesaña, A. Díaz-Rúa, R.M. Ceinos, R. Álvarez-Otero and J.L. Soengas

Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía and Centro de Investigación Mariña, Universidade de Vigo, Spain.

In fish, hypothalamic inhibition of AMPKα2 resulted in decreased levels of food intake and depressed hepatic metabolism of glucose, lipids and amino acids as has been demonstrated in a previous study in rainbow trout. Here, we aimed to evaluate if the effect of central AMPKα2 on liver metabolism might be mediated by the nervous connection between the brain and liver. Therefore, we administered by i.c.v. injections adenoviral vectors containing green fluorescence protein (GFP) alone (CONTROL) or linked to a constitutive inactive isoform of AMPKα2 (DN-AMPKα2) inducing the inhibition of AMPK expression in rainbow trout hypothalamus. Simultaneously, we sectioned the vagal nerve by bilateral incision (VGX) or made the same incision without sectioning the nerve (SHAM). The presence of the injected adenovirus in the hypothalamic areas was demonstrated by immunohistochemical analyses. The results obtained demonstrate that the central inhibition of AMPKα2 leads to a decrease of food intake levels and to an overall decrease of metabolic parameters in plasma and liver, which is in agreement with the results obtained previously. Vagotomy *per se* also decreased food intake levels and affected some hepatic parameters. When DN-AMPKα2 and vagotomy treatments were simultaneous, some of the effects of central AMPKα2 on hepatic parameters were reverted. Overall, these results indicate that central AMPKα2 effects seem to be mediated by the vagal communication between liver and brain.

Introduction

AMP-activated protein kinase (AMPK) acts as a cellular energy sensor that is mainly activated by the cell energy deficiency and is involved in cellular mechanisms responsible for the restoration of energy balance by inhibiting anabolic pathways and stimulating catabolic pathways (1). In a previous study with rainbow trout *Oncorhynchus mykiss* we demonstrated that in fish, as in mammals, hypothalamic inhibition of AMPKα2 resulted in decreased levels of food intake and depressed hepatic metabolism of glucose, lipids and amino acids (2). These results allowed us to suggest that a signal of abundance of nutrients flew from the hypothalamus to the liver. The vagus nerve is the most important link between the brain and the liver with both afferent and efferent fibers belonging to the sympathetic and parasympathetic systems (3).
Therefore, the aim of the present study was to evaluate if the effect of central AMPKα2 on liver metabolism and food intake might be mediated by vagal connection between the brain and liver.

Materials and Methods

Rainbow trout of about 100g body mass were obtained from a local fish farm (A Estrada, Spain) and were maintained for 1 month in 100 litre tanks under laboratory conditions and a natural photoperiod in dechlorinated tap water at 15 °C. On the day of treatment administration 24 h fasted fish were anaesthetized in water containing 2-phenoxyethanol (0.02% v/v). Then, we administered by i.c.v. injections adenoviral vectors containing green fluorescence protein (GFP) alone (CONTROL) or linked to a constitutive inactive isoform of AMPKα2 (DN-AMPKα2) inducing the inhibition of AMPK expression in rainbow trout hypothalamus. Simultaneously, we sectioned the vagal nerve by bilateral incision (VGX) or made the same incision without sectioning the nerve (SHAM) (4). Fish were returned to their experimental tanks and food intake was recorded during 19 days. On the day 19 after i.c.v. treatment fish which were not fed, were anesthetized in their holding tanks with 2-phenoxyethanol (0.02% v/v), and blood, hypothalamus and liver were removed. The whole brain from four fish per group were removed and immediately fixed for immunohistochemical analysis in order to verify the presence of the adenovirus in the hypothalamic III ventricle (Fig.1). Parameters were assessed as described previously (2).

Results and Discussion

Food intake levels (Fig.2) decreased in AMPKα2-DN group with respect to GFP in SHAM, but it did not reach statistic difference in the VGX group. As it occurs in mammals, food intake in the VGX groups was lower than those of SHAM groups, for both GFP and AMPKα2-DN treatments.

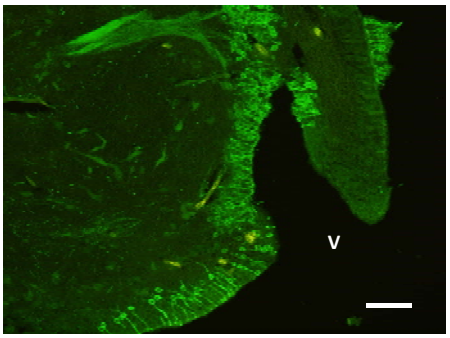


Figure 1. Immunohistochemical validation of treatment with adenoviral vectors expressing DN-AMPKα2 isoforms tagged with GFP. Photomicrographs of transverse sections through the III ventricle (V) of rainbow trout brain showing GFP immunoreactivity (GFP-ir) 19 days after i.c.v. administration. Scale bar: 100µm.

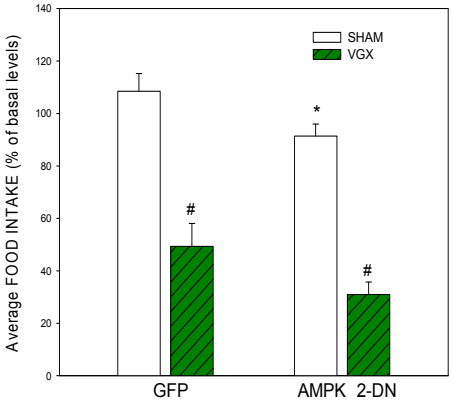


Figure 2. Average daily food intake registered in rainbow trout from day 7 to day 18 after i.c.v. administration of adenovirus tagged with GFP or AMPKα2-DN in SHAM or VGX fish. Food intake is displayed as the percentage of food ingested with respect to baseline levels (calculated as the average of food intake the 7 days previous to experiment). Each value is the mean ± SEM of n = 12 fish per treatment. *, significantly different ($P<0.05$) from GFP within SHAM or VGX; #, significantly different ($P<0.05$) from SHAM within GFP or AMPKα2-DN.

In accordance, the mRNA abundance of *npy* decreased after AMPKα2-DN treatment compared with GFP both in SHAM and VGX groups (Fig. 3A). Similarity, the mRNA abundance of *agrp1* decreased after AMPKα2-DN treatment compared with GFP in SHAM group but not with AMPKα2-DN treatment in VGX group (Fig. 3B). *cartpt* mRNA abundance increased after AMPKα2-DN treatment compared with GFP in SHAM group (Fig. 3C). Finally, no significant changes occurred for *pomca1* mRNA abundance in any of the experimental settings (Fig. 3D).

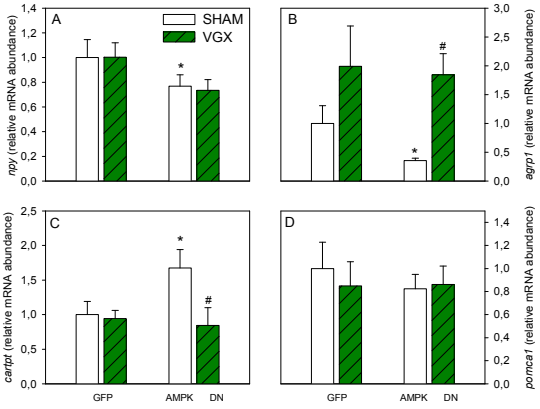


Figure 3. mRNA abundance of *npy* (A), *agrp1* (B), *cartpt* (C), and *pomca1* (D) in the hypothalamus of rainbow trout 19 days after i.c.v. administration of adenovirus tagged with GFP or AMPKα2-DN in SHAM or VGX fish. Gene expression are normalized by *actb* and *eef1a1* expression. Each value of is the mean ± SEM of n = 6 fish per group. For symbols details see Fig. 2

Levels of plasma metabolites (glucose, lactate, fatty acid, triglyceride and amino acid) were affected by inhibition of hypothalamic AMPKα2 expression and, in general, VGX treatment did not impair such effects (data not shown). In liver, the central inhibition of AMPKα2 elicited reduced capacities for synthesis and use of glucose and lipid, and reduced use of amino acid. Furthermore, several of the peripheral effects induced by central inhibition of AMPKα2 are blocked by vagotomy (data not shown). In conclusion, these results support that actions of hypothalamic AMPKα2 on liver metabolism are mediated by the autonomic nervous system, thus providing new evidence regarding the involvement of central AMPKα2 in regulation of energy homeostasis in fish.

Acknowledgements: Supported by grants from Spanish Agencia Estatal de Investigación and European Fund of Regional Development to JLS (AGL2016-74857-C3-1-R). M.C-S was recipient of a postdoctoral fellowship (Xunta de Galicia). S.C. was recipient of a predoctoral fellowship (Program FPU) from Spanish Ministerio de Educación, Cultura y Deporte.

References

1. López M. (2017) Hypothalamic AMPK: a golden target against obesity? *Eur J Endocrinol* 176, R235-R246.
2. Conde-Sieira M., Capelli V., Álvarez-Otero R., Comesaña S., Liñares-Pose L., Velasco C., López M., Soengas, JL. (2019). Differential role of hypothalamic AMPKα isoforms in fish: an evolutive perspective. *Mol. Neurobiol.* 56, 5051-5066.
3. Cork S.C. (2019). The role of the vagus nerve in appetite control: implications for the pathogenesis of obesity. *J Neuroendocrinol* 30: e12643.
4. Seth H., Axelsson M. (2010) Sympathetic, parasympathetic and enteric regulation of the gastrointestinal vasculature in rainbow trout (*Oncorhynchus mykiss*) under normal and postprandial conditions. *J Exp Biol* 213, 3118-3126.

ARE VEGETABLE OILS A GOOD ALTERNATIVE IN A SCENARIO OF GLOBAL WARMING FOR OPTIMAL ENDOCRINE GROWTH REGULATION OF GILTHEAD SEA BREAM?

S. Balbuena-Pecino[1], N. Riera-Heredia[1], E. Gasch-Navalón[1], A. Sánchez-Moya[1], R. Fontanillas[2], J. Gutiérrez[1], E. Capilla[1], I. Navarro[1]

[1]*Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Spain.*
[2]*Skretting Aquaculture Research Centre, Stavanger, Norway.*

Replacement of fish oil (FO) by plant-derived oils has emerged as an approach to minimize economic and environmental problems, achieving a more sustainable aquaculture. Furthermore, the upward trend of the seawater temperature has encouraged improving knowledge of its consequences on fish. The aim of this study was to evaluate whether a partially substituted FO diet by palm oil (P) at two different temperatures (21 and 28 ºC), and diets containing the oils (P, rapeseed (R) or both (PR)) at 28 ºC may influence musculoskeletal development in gilthead sea bream (*Sparus aurata*) juveniles. The mRNA levels of growth hormone (GH)/insulin-like growth factors (IGFs) system-, osteogenic- and myogenic-related genes in bone and/or white muscle were analyzed. Fish maintained at 28 ºC presented significantly higher somatic growth parameters. In fish fed P diet, high temperature down-regulated *igf-1*, the receptor *igf-1rb*, and the binding proteins *igfbp-4* and *igfbp-5b* in both, bone and muscle tissues, and the receptor *igf-1ra* in muscle as well, suggesting hormonal adjustments to a rapid growth situation. Concerning dietary composition effects, juveniles fed with the PR diet presented in bone, increased expression of *igf-1* and its receptors compared to the P diet-fed group, while *igfbp-5b* expression was highest in the fish fed the R diet. Contrarily, in white muscle, most of the genes of the GH/IGFs axis remained unaltered among groups, indicating that this tissue appears to be less sensitive to changes in the dietary fatty acids profile. Overall, the PR seems to be the most beneficial diet in terms of promoting an optimum endocrine environment for musculoskeletal growth in a situation of elevated temperature.

Introduction

Nowadays, aquaculture plays a crucial role facing the growing demand for global fish consumption in an increasing world population. Additionally, the need to ensure a more sustainable production makes necessary exploring alternative protein and lipid sources in fish feeds while focusing on the impact of climate change. In the last years, reduction and replacement of fishmeal (FM) and fish oil (FO) in diet formulations by vegetable-derived products has emerged as a viable approach (1). Palm (P) and rapeseed (R) oils are two of the most common plant oils used for fish feed formulation, which are rich in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFAs), respectively (2). However, the potential effects of global warming should be studied.

Materials and Methods

Gilthead sea bream juveniles were maintained at the animal facilities of the Faculty of Biology of the University of Barcelona. Fish were distributed into one

400-L and two 200-L tanks per condition under a 12 h light/12 h dark photoperiod. For 2 months (October-December), three different partially substituted FO diets (60-65% content in vegetable oils, Skretting ARC, Stavanger, Norway), were administered to four groups of fish. Two of them were fed with a diet containing P, but each was held at a different temperature, 21 or 28 ºC. The two groups left were reared at 28 ºC and received a diet containing either R or a combination of P and R oils (PR). Fish were fed with a constant rate of 2.5% body weight adjusted each week. Juveniles were anesthetized (MS-222) and subsequently sacrificed by a blow to the head. Samples of white muscle and vertebral bone were collected and immediately frozen in liquid nitrogen and stored at -80 ºC. After performing the RNA extraction from ~100 mg of each tissue and the cDNA synthesis, the mRNA levels of the genes of interest were examined in a CFX384^{TM} real-time system (Bio-Rad). The expression level of each target gene analyzed was calculated using the Pfaffl method relative to the corresponding reference genes.

Results and Discussion

Elevated temperature significantly increased somatic growth while reduced hepatosomatic and viscerosomatic indexes (Table 1).

Table 1. Growth parameters and somatic indexes of fish fed with the experimental diets P at 21 ºC or P, R or PR at 28 ºC for 2 months.

	21 ºC	28 ºC		
	P	**P**	**R**	**PR**
IBW (g)	24.21 ± 0.57	22.89 ± 0.40	22.80 ± 0.75	22.24 ± 0.77
FBW (g)	54.32 ± 1.78*	68.02 ± 2.34	63.33 ± 2.09	62.21 ± 3.80
WG (%)	124.3 ± 5.19*	197.5 ± 13.27	177.8 ± 2.33	179.2 ± 7.62
SGR (%/days)	1.52 ± 0.04*	2.05 ± 0.08	1.93 ± 0.02	1.94 ± 0.05
BL (cm)	15.06 ± 0.02*	16.55 ± 0.11	16.37 ± 0.07	16.23 ± 0.22
CF (%)	1.59 ± 0.06	1.5 ± 0.04	1.44 ± 0.03	1.45 ± 0.03
HSI (%)	1.62 ± 0.05*	1.05 ± 0.12	0.90 ± 0.03	0.90 ± 0.05
VSI (%)	7.79 ± 0.51*	5.94 ± 0.17	6.33 ± 0.2	6.34 ± 0.19
MFI (%)	1.64 ± 0.16	1.17 ± 0.15	1.49 ± 0.03	1.43 ± 0.13

Data are shown as Mean ± SEM (n = 3 tanks). Asterisks indicate significant differences between fish fed with P diet at different temperatures. No differences were observed among diets at high temperature ($p<0.05$). Initial body weight (IBW); final body weight (FBW); weight gain (WG) [(FBW−IBW/IBW)·100]; somatic growth rate (SGR) [((ln FBW−ln IBW)/time)·100], where time was 53 days; body length (BL); condition factor (CF) [(FBW/BL^3)·100]; Hepatosomatic index (HSI) [(liver weight/FBW)·100]; Viscerosomatic index (VSI) [(viscera weight/FBW)·100]; Mesenteric fat index (MFI) [(mesenteric fat weight/FBW)·100]. Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

In fish fed P diet, high temperature down-regulated the expression of most of the GH/IGFs-related genes (*igf-1*, *igf-1rb* and the binding proteins *igfbp-4* and *igfbp-5b* in both bone and muscle tissues, and the receptor *igf-1ra* also in muscle) (Fig. 1A, B), suggesting hormonal tunings to compensate a rapid growth situation. In addition, significantly lower gene expression values were found in the bone resorption activity-related genes *ctsk* and *mmp9* in the P diet group at 28 ºC (data not shown), suggesting a negative developmental condition in the

skeleton. With regards to changes in the dietary fatty acids profile, bone from juveniles fed with PR diet showed increased expression of *igf-1* and its receptors in comparison with the P-diet group (Fig. 1C), while the white muscle appeared to be less sensitive, since the vast majority of genes, including myogenic-related ones (data not shown), were not affected, with the exception of *igfbp-4* (Fig. 1D).

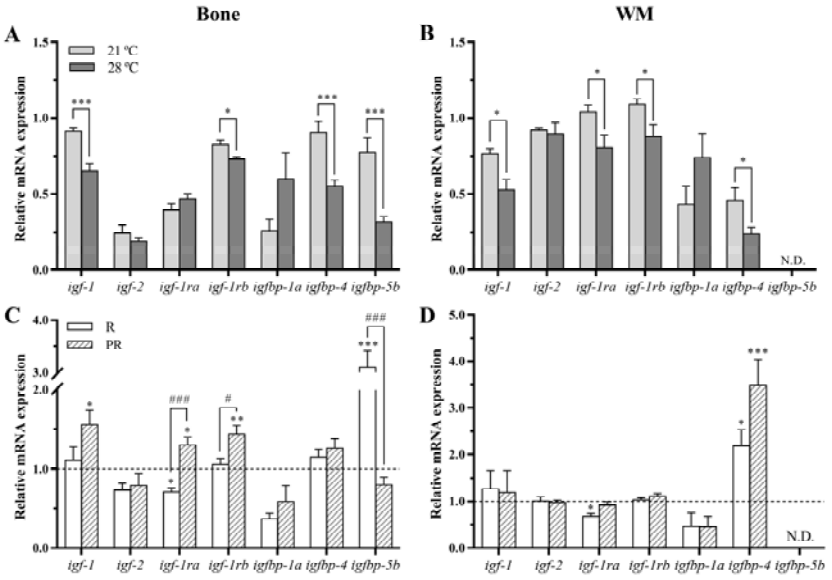


Figure 1. Relative mRNA expression of GH/IGFs axis-related genes in (**A**, **C**) bone and (**B**, **D**) white muscle (WM) of (**A**, **B**) fish fed with P diet at 21 ºC and 28 ºC or (**C**, **D**) fish fed with the experimental diets R or PR respect to fish fed with P diet (dotted line) and reared at 28 ºC expressed as fold change. Data are shown as mean + SEM (n=8-10 for bone, or n=6-8 for WM). Significant differences between temperature groups are indicated by asterisks ($p<0.05$). Significant differences between fish fed with R or PR diets compared to those fed with P diet are indicated by asterisks, and between animals fed R and PR diets with a hash ($p<0,05$). Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

To sum up, PR diet seems to be for gilthead sea bream the most beneficial one in terms of promoting an optimum endocrine environment for musculoskeletal growth at high temperature conditions. Moreover, these results demonstrate the importance, for proper growth and physiological status, of taking into account the possible global world temperature increases, when the substitution of raw materials in new feeds for this species is evaluated.

Acknowledgements: Supported by the Ministerio de Ciencia, Innovación y Universidades (MICIUN) AGL2014-57974-R, AGL2017-89436-R and, Generalitat de Catalunya 2017SGR-1574. S.B-P and N.R-H are predoctoral fellows from the MICIUN (PRE2018-085580 and BES-2015-074654).

References

1. FAO. The State of World Fisheries and Aquaculture – (SOFIA) (2018).
2. Sánchez-Moya A., García-Meilán I., Riera-Heredia N., Vélez E.J., Lutfi E., Fontanillas R., Gutiérrez J., Capilla E., Navarro I. (2020). *Aquaculture.* 519, 7349881. doi: 10.1016/j.aquaculture.2019.734881

ORAL PRESENCE OF SPECIFIC AMINO ACIDS IN RAINBOW TROUT IS INVOLVED IN CENTRAL REGULATION OF FOOD INTAKE

S. Comesaña[1], M. Conde-Sieira[1], C. Velasco[1], R. Álvarez-Otero[1], J.L. Soengas[1], S. Morais[2]

[1]Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía and Centro de Investigación Mariña, Universidade de Vigo, Spain.
[2]Lucta S.A., Innovation Division, UAB Research Park, Eureka building, 08193 Bellaterra, Spain.

Nutrients from the ingested food interact with sensors along the mammalian digestive tract. These sensors, through vagal afferent nerves, can activate brain areas related to the control food intake (FI). In the oral cavity, as a first stop of the food, umami taste receptors can detect the presence of different amino acids and inform the hypothalamus. The integration of this information in this brain area could result in changes in FI. However, taste signalling pathway and its relation with FI control has not been studied in fish. Therefore, the aim of this study is to analyze, in rainbow trout, possible activation of the umami taste signalling pathway caused by the presence of specific amino acids in the oral cavity, and its effect on FI. Thus, an oral administration was carried out with distilled water alone (control) or containing 40 µmol/mL of L-leucine, L-valine, L-proline or L-glutamate. After 10, 20 and 30 minutes, samples of hypothalamus and tongue were taken. mRNA abundance of parameters related to FI control were evaluated in brain, while in tongue, parameters related to taste signalling pathway were assessed. The oral administration was repeated to evaluate FI 1, 2 and 3h post-treatment. In general, only oral administration of proline decreased FI. However, in hypothalamus all studied amino acids, especially after 30 min, changed different parameters related to the control of FI. In tongue all amino acids regulated the expression of genes related to the taste signalling pathway after 10 and 20 min. The presence of amino acids in the oral cavity might be involved in the control of FI by hypothalamus.

Introduction

The impact of amino acids on fish satiety/hunger mechanisms is relatively unknown. The few available studies in fish regarding this issue focused on post-absorptive signalling (1). In mammals, most studies addressing pre-absorptive (peripheral) satiety signals focus on those originated in the gut, whereas few of them tried to elucidate the role of signals from the oral cavity (2). Nutrients from the ingested food interact with specific receptors along the digestive tract to produce the sense of taste (3). These receptors of the oral cavity transduce taste stimuli into electrochemical signals and transmit them through afferent nerve fibres to brain areas related to the control of food intake (4). However, taste signalling pathway and its relation with food intake control has not been studied in fish.

The aim of this study is to explore whether the presence of specific amino acids in the oral cavity might be detected by oral taste receptors, which could act as a

pre-absorptive satiety signal communicating with the brain, more specifically the hypothalamus, to regulate food intake.

Materials and Methods

Rainbow trout of about 85 g body mass were obtained from a local fish farm (A Estrada, Spain) and were maintained for 1 month in 100 litre tanks under laboratory conditions and a natural photoperiod in dechlorinated tap water at 15 °C. On the day of experiment fish were lightly anaesthetized with 2-phenoxyethanol, and weighed. An oral administration was carried out with distilled water alone (control) or containing 40 µmol/mL of L-leucine, L-valine, L-proline or L-glutamate. After 10, 20 and 30 minutes, samples of hypothalamus and tongue were taken. In hypothalamus, mRNA abundance of neuropeptides and other parameters related to food intake control were evaluated (*agrp1*, *npy*, *pomca1*, *cartpt*, *foxo1*, *mtor*). Parameters related to taste signalling pathway were assessed in tongue (*tas1r1*, *tas1r2*, *gnat3-like*, *plcb2*). The oral administration was repeated to evaluate food intake 1, 2 and 3h post-treatment.

Results and Discussion

In general, only oral administration of proline decreased food intake (Fig.1). However, in hypothalamus all studied amino acids, especially after 30 min, changed different parameters related to the control of food intake (Table 1). In tongue all amino acids regulated the expression of some genes related to the taste signalling pathway after 10 and 20 min (Table 2).

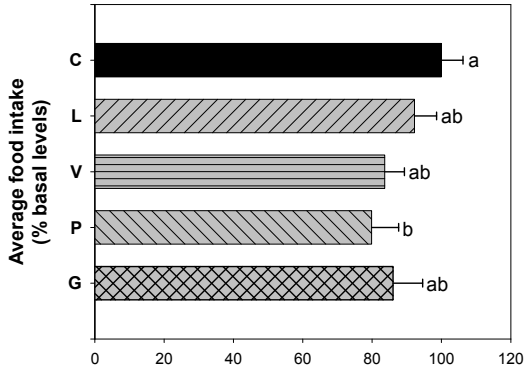


Figure 1. Average food intake in rainbow trout of 1, 2 and 3 h after oral administration of distilled water alone (Control, C) or containing L-leucine (L), L-valine (V), L-proline (P) or L-glutamate (G). Different letters indicate significant differences ($P<0.05$) from different groups.

The present study provides, for the first time in teleost fish, information about pre-absorptive regulation of food intake by amino acids. The presence of leucine, valine, proline or glutamate in the oral cavity appears to be detected by taste receptors in tongue. The signalling originated in tongue presumably arrives to the brain since changes in food intake regulatory mechanisms in hypothalamus were observed. However, only proline decreased food intake. This satiety signal is probably occurring also for the other amino acids assessed though probably higher concentrations are required to achieve a comparable effect.

Table 1. mRNA levels of neuropeptides, the transcription factor *foxo1* and the integrative sensor *mtor*, all of them involved in the regulation of food intake in hypothalamus of rainbow trout 10, 20 and 30 min after oral administration of distilled water alone (Control, C) or L-leucine (L), L-valine (V), L-proline (P) or L-glutamate (G).

		10 min	20 min	30min			10 min	20 min	30min
	C	1,00 ± 0,14	1,00 ± 0,40	1,00 ± 0,15 a		C	1,00 ± 0,32	1,00 ± 0,24 a	1,00 ± 0,10 a
	L	0,71 ± 0,12	0,55 ± 0,16	1,15 ± 0,31 ab		L	0,57 ± 0,09	0,93 ± 0,31 a	1,05 ± 0,04 a
agrp1	V	0,80 ± 0,14	0,85 ± 0,19	1,29 ± 0,14 ab	***pomca1***	V	1,00 ± 0,19	1,94 ± 0,20 b	1,19 ± 0,16 a
	P	0,88 ± 0,22	0,66 ± 0,11	1,60 ± 0,19 b		P	0,67 ± 0,15	1,71 ± 0,33 ab	2,29 ± 0,31 b
	G	0,61 ± 0,20	0,87 ± 0,22	1,15 ± 0,23 ab		G	0,61 ± 0,13	1,32 ± 0,25 ab	1,15 ± 0,18 a
	C	1,00 ± 0,12 a	1,00 ± 0,21	1,00 ± 0,21		C	1,00 ± 0,04	1,00 ± 0,09 a	1,00 ± 0,05 a
	L	0,74 ± 0,12 ab	1,09 ± 0,18	0,96 ± 0,19		L	1,01 ± 0,12	1,10 ± 0,11 ab	1,04 ± 0,03 a
npy	V	0,87 ± 0,09 ab	1,14 ± 0,23	1,12 ± 0,17	***foxo1***	V	1,02 ± 0,10	1,05 ± 0,09 ab	1,32 ± 0,12 b
	P	0,70 ± 0,05 b	1,15 ± 0,34	1,20 ± 0,09		P	1,11 ± 0,06	1,11 ± 0,12 ab	1,32 ± 0,08 b
	G	0,91 ± 0,20 ab	1,23 ± 0,21	0,85 ± 0,11		G	1,03 ± 0,12	1,44 ± 0,17 b	1,24 ± 0,15 ab
	C	1,00 ± 0,14	1,00 ± 0,14	1,00 ± 0,14 a		C	1,00 ± 0,06	1,00 ± 0,09	1,00 ± 0,04 a
	L	0,91 ± 0,09	1,18 ± 0,17	1,13 ± 0,16 ab		L	0,99 ± 0,10	1,12 ± 0,10	1,11 ± 0,03 a
cartpt	V	0,70 ± 0,14	1,09 ± 0,16	1,43 ± 0,10 ab	***mtor***	V	1,10 ± 0,12	1,20 ± 0,13	1,49 ± 0,12 b
	P	0,73 ± 0,11	1,23 ± 0,13	1,58 ± 0,10 b		P	0,97 ± 0,04	1,14 ± 0,07	1,41 ± 0,08 b
	G	0,71 ± 0,13	1,51 ± 0,25	1,40 ± 0,13 ab		G	0,96 ± 0,14	1,30 ± 0,14	1,33 ± 0,07 b

Each value is the mean ± SEM. Results are referred to control group and are normalized by *ef1a* and *b-actin* expression. Different letters indicate significant differences (P<0.05) from different groups at the same time. No comparison between times. *agrp1*, agouti-related protein 1; *npy*, neuropeptide Y; *cartpt*, cocaine and amphetamine regulated transcript; *pomca1*, pro-opiomelanocortin a1; *foxo1*, forkhead box protein O1; *mtor*, mechanistic target of rapamycin.

Table 2. mRNA levels of parameters involved in taste signalling pathway in tongue of rainbow trout 10, 20 and 30 min after oral administration of distilled water alone (Control, C) or L-leucine (L), L-valine (V), L-proline (P) or L-glutamate (G).

		10 min	20 min	30min			10 min	20 min	30min
	C	1,00 ± 0,08 ab	1,00 ± 0,06 a	1,00 ± 0,08		C	1,00 ± 0,08 a	1,00 ± 0,04 a	1,00 ± 0,04
	L	1,21 ± 0,09 a	1,00 ± 0,04 ab	0,95 ± 0,09		L	1,28 ± 0,07 b	1,14 ± 0,07 ab	1,01 ± 0,08
tas1r1	V	1,05 ± 0,09 ab	1,12 ± 0,06 ab	1,17 ± 0,10	***gnat3-like***	V	1,39 ± 0,24 ab	1,18 ± 0,05 b	1,09 ± 0,15
	P	1,06 ± 0,15 ab	1,03 ± 0,09 ab	0,99 ± 0,11		P	1,35 ± 0,13 b	1,20 ± 0,10 ab	0,86 ± 0,07
	G	0,93 ± 0,02 b	1,22 ± 0,07 b	1,02 ± 0,10		G	1,43 ± 0,11 b	1,29 ± 0,09 b	1,07 ± 0,08
	C	1,00 ± 0,06 a	1,00 ± 0,13	1,00 ± 0,08		C	1,00 ± 0,13	1,00 ± 0,14 a	1,00 ± 0,12
	L	1,61 ± 0,12 b	1,21 ± 0,09	0,98 ± 0,12		L	0,84 ± 0,05	0,79 ± 0,06 a	0,81 ± 0,02
tas1r2	V	1,44 ± 0,23 ab	1,17 ± 0,16	0,85 ± 0,26	***plcb2***	V	0,83 ± 0,03	0,87 ± 0,10 a	0,90 ± 0,29
	P	1,35 ± 0,23 ab	1,05 ± 0,06	1,01 ± 0,07		P	0,88 ± 0,20	0,63 ± 0,05 b	0,85 ± 0,09
	G	1,41 ± 0,11 b	1,11 ± 0,12	1,02 ± 0,14		G	0,73 ± 0,08	0,76 ± 0,09 ab	0,92 ± 0,12

Each value is the mean ± SEM. Results are referred to control group and are normalized by *ef1a* and *b-actin* expression. Different letters indicate significant differences (P<0.05) from different groups at the same time. No comparison between times. *tas1r1*, taste receptor type 1 subunit 1; *tas1r2*, taste receptor type 1 subunit 2; *gnat3-like*, gustducin α 3-like; *plcb2*, phospholipase C β2.

Acknowledgments: Supported by LUCTA and Spanish Agencia Estatal de Investigación and European Fund of Regional Development (AGL2016-74857-C3-1-R and FEDER). SC was recipient of a predoctoral fellowship from Spanish Ministerio de Educación, Cultura y Deporte (FPU16/00045).

References

1. Soengas J.L., Cerdá-Reverter J.M., Delgado M.J. (2018). *J. Mol. Endocrinol.* 60, R171-R199.
2. Fromentin G., Darcel N., Chaumontet C., Marsset-Baglieri A., Nadkarni N., Tomé, D. (2012). *Nutr. Res. Rev.* 25, 29-39.
3. Han P., Bagenna B., Fu M. (2019). *Int. J. Food. Sci. Nutr.* 70, 125-135.
4. Kasumyan A. O. (2019). *J. Fish Biol.* 95, 155-178.

HEPATIC GLUCOSE AND LIPID METABOLISM IS MODIFIED BY PALMITOYLETHANOLAMIDE TREATMENT IN GOLDFISH (*Carassius auratus*)

M. Gómez-Boronat[1], E. Isorna[1], M. Conde-Sieira[2], M.J. Delgado[1], N. de Pedro[1], J.L. Soengas[2]

[1] Dpto. de Genética, Fisiología y Microbiología; UD Fisiología Animal; Fac. de Biología; Universidad Complutense de Madrid. Spain.
[2] Lab. de Fisioloxía Animal; Dpto. de Bioloxía Funcional e Ciencias da Saúde; Fac. de Bioloxía; Universidade de Vigo. Spain.

In last years the relevance of N-acylethanolamines (NAEs) as metabolism regulators in mammals has emerged, however the role of these local regulators in fish is not clear. The aim of this study was to investigate whether the NAE palmitoylethanolamide (PEA) is involved in the regulation of hepatic metabolism of glucose and lipids in fish. Therefore, we studied the effect of a 10-day PEA treatment on body weight of goldfish (*Carassius auratus*). Furthermore, we evaluated the effects of acute (6 hours) or chronic (10 days) treatment with PEA on the levels of metabolites in plasma and liver, and enzyme activities related to glucose and lipid metabolism in the liver of goldfish. We also determined the hepatic mRNA abundance of *pparα*, *bmal1a*, and *rev-erbα* in both acute and chronic conditions. Firstly, 10-day PEA treatment significantly reduced body weight gain compared to control fish. Regarding hepatic glucose metabolism, PEA induced a significant reduction in the gluconeogenic potential, and an increase in glycogenolytic potential, supporting that PEA enhanced the glucose utilization. In addition, a lipogenic effect was induced by PEA treatment, similarly to the oleoylethanolamide effects in this teleost. The increase in the hepatic expression of *bmal1a* and *rev-erbα* after PEA treatment might be responsible for the metabolic changes observed in PEA-treated goldfish. Altogether, our results indicate that PEA can be involved in the regulation of liver energy metabolism in fish.

Introduction

Palmitoylethanolamide (PEA) is an N-acylethanolamine (NAE) mainly synthesized in the gastrointestinal tract (1), that binds to the peroxisome proliferator-activated receptor α (PPARα) to regulate different functions in mammals, including energy homeostasis (2,3). Previous results of our laboratory demonstrated anorectic effects in goldfish treated with PEA or another similar NAE, the oleoylethanolamide (OEA). Moreover, it has been shown that OEA regulates body weight (bw) and hepatic metabolism in this teleost species (4). But metabolic effects of PEA in fish need to be further examined. Therefore, the first objective of this work was to study the possible role of PEA on bw in fish, using the cyprinid *Carassius auratus* as a teleost model. Secondly, we studied the implications of this lipid-derived messenger in the regulation of hepatic glucose and lipid metabolism.

Materials and Methods

The experiments were performed with goldfish (10-15 g bw) kept under a 12L:12D photoperiod (lights on at 8 am) and daily fed with 1% bw. Fish were anesthetized with MS-222 (160 mg/l) before handling, and when sacrificed a

dose of anaesthetic 320 mg/ml was used. All procedures comply with Spain and EU current legislation (RD53/2013, EU63/2010).

Acute PEA treatment. Goldfish (*n* = 10 fish/group) under a 48-h fasting period were intraperitoneally (IP) injected with 10 µl/g bw of vehicle (5% Tween80, 5% polyethylene glycol, 90% teleost saline) alone or containing PEA (20 µg/g bw). After 6 h, blood was collected to assess metabolite levels and then animals were sacrificed. Liver was quickly collected to assess metabolite levels and enzyme activities by colorimetric assays (4), and gene expression of *pparα* (main receptor for PEA), and *bmal1a* and *rev-erbα* (clock genes also related with metabolism) were quantified by RT-qPCR (4).

Chronic PEA treatment. Goldfish (*n* = 10 fish/group) were IP injected with 10 µl/g bw of vehicle alone or containing PEA (20 µg/g bw) for 10 days and changes in bw were quantified. After last injection, animals were food deprived for 48 h and then, blood and liver were collected to measure metabolite levels, enzyme activities, and gene expression as previously described (4).

Statistical differences of bw gain were determined by Student *t*-test, while the rest of parameters here analysed were determined by one-way ANOVA followed by SNK *post hoc* test.

Results and Discussion

Changes in bw gain after 10-day PEA treatment are shown in Fig. 1. This NAE significantly impaired the increase in bw gain observed in control fish, similar as described in mammals (2).

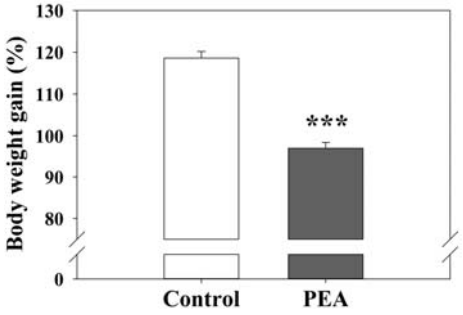


Figure 1. Body weight gain after chronic PEA (20 µg/g bw) treatment. Data are expressed as mean + SEM (*n*=10/group). *t*-test, ***$p < 0.001$.

Table 1. Metabolic parameters in goldfish liver after acute or chronic PEA (20 µg/g bw) treatment.

LIVER	Acute		Chronic	
	Control	PEA	Control	PEA
Triglyceride	0.84 ± 0.04	0.61 ± 0.06 **	0.73 ± 0.27	0.52 ± 0.02
PEPCK	0.36 ± 0.22	0.83 ± 0.24	4.07 ± 1.79	0.11 ± 0.01 *
PFK	10.77 ± 1.06	14.68 ± 0.82 **	15.75 ± 3.37	13.24 ± 1.31
PFK CAR	0.57 ± 0.09	0.94 ± 0.11 **	1.69 ± 0.48	2.32 ± 0.91
GPase	36.30 ± 0.82	43.83 ± 1.01 ***	32.75 ± 3.33	43.15 ± 2.33 *
G6Pase	0.15 ± 0.08	0.12 ± 0.06	0.06 ± 0.01	0.15 ± 0.03 *
FAS	0.12 ± 0.07	0.19 ± 0.06	0.50 ± 0.09	0.86 ± 0.10 *

Data represent mean ± SEM of liver triglyceride (µmol/g) and enzyme activities (mU/mg protein). One-way ANOVA and *post hoc* analysis (SNK test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control group) were used. FAS, fatty acid synthase; GPase, glycogen phosphorylase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PFK CAR, phosphofructokinase coactivation ratio.

Plasma metabolites were not significantly different in any parameter assessed due to the treatments with PEA (data not shown). In the liver, only the triglyceride levels were reduced after acute PEA treatment (Table 1). Most changes were found in hepatic enzyme activities (Table 1). PFK, PFK CAR, and GPase activities were increased after acute PEA treatment; whereas PEPCK activity was reduced and GPase, G6Pase, and FAS activities were increased after chronic treatment with PEA. These results suggest that the gluconeogenic potential is reduced and the glycogenolytic capacity is increased, enhancing the liver glucose utilization. Related to lipid metabolism, PEA seems to induce the lipogenic potential. PEA treatment did not modify the hepatic expression of the receptor *pparα* (Fig. 2A). On the other hand, PEA induced both *bmal1a* expression after chronic treatment (Fig. 2B), and *rev-erbα* expression after both acute and chronic treatments (Fig. 2C). These changes could be responsible for the PEA-induced metabolic changes.

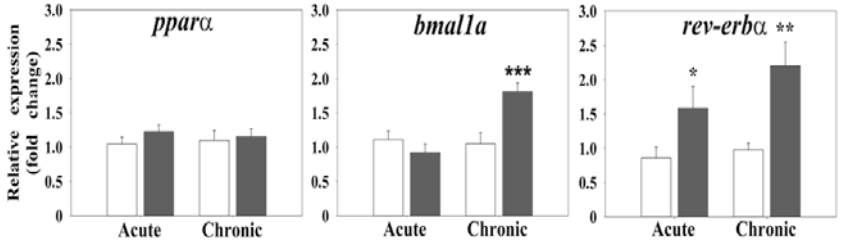


Figure 2. mRNA levels in liver of goldfish after acute (6 h) and chronic (10 days) treatments with PEA (20 µg/g bw). Data are expressed as mean + SEM (*n*=10/group). Control, white bars; PEA, grey bars. Asterisks show significant differences by one-way ANOVA and *post hoc* analysis (SNK test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to control group).

In summary, PEA modifies body weight and hepatic metabolism of glucose and lipids, suggesting a role for this lipid-derived signal in the regulation of energy homeostasis in fish.

Acknowledgements: Supported by the Spanish MINECO (AGL2016-74857-C3-1-R and FEDER, and AGL2016-74857-C3-2-R). M.G-B and M.C-S were recipients of fellowships from Spanish MINECO (FPI Program; BES–2014–068103) and Xunta de Galicia (ED481B2018/018), respectively.

References

1. Astarita G, Rourke BC, Andersen JB, Fu J, Kim JH, Bennett AF, Hicks JW, Piomelli D. (2006). Postprandial increase of oleoylethanolamide mobilization in small intestine of the Burmese python (*Python molurus*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290, R1407-R1412.

2. Mattace Raso G, Santoro A, Russo R, Siomeli R, Paciello O, Di Carlo C, Diano S, Calignano A, Meli R. (2014). Palmitoylethanolamide prevents metabolic alterations and restores leptin sensitivity in ovariectomized rats. *Endocrinology* 155, 1291-1301.

3. Zambrana-Infantes E, Rosell del Valle C, Ladrón de Guevara-Miranda D, Galeano P, Castilla-Ortega E, Rodríguez de Fonseca F, Blanco E, Santín LJ. (2018). Palmitoylethanolamide attenuates cocaine-induced behavioral sensitization and conditioned place preference in mice. *Pharmacol. Biochem. Behav.* 166, 1-12.

4. Gómez-Boronat M, Velasco C, Isorna E, de Pedro N, Delgado MJ, Soengas JL. (2016). The satiety factor oleoylethanolamide impacts hepatic lipid and glucose metabolism in goldfish. *J. Comp. Physiol. B.* 186, 1009-1021.

CHARACTERISATION OF CENTRAL NERVOUS SYSTEM DEVELOPMENTAL PROCESSES REGULATED BY THYROID HORMONES IN FLATFISH METAMORPHOSIS

Aurora Olvera[1], C. Carballo[2], M. Manchado[2], and D.M. Power[1]

[1] *Centro de Ciencias do Mar, Faro, Portugal.*
[2] *IFAPA el Toruño, Puerto de Santa María, España.*

A high-throughput transcriptomic analysis of the Senegalese sole (*Solea senegalensis*) larvae exposed to the thyroid hormone (TH) synthesis inhibitor methimazol (MMI) revealed significant changes in the expression levels of 6 transcripts (TUBB6, SOX4, NOTCHL, AHNAK, CDNF, KLF9) directly related to CNS development, more specifically to neuronal differentiation and migration, as well as maturation. To analyse TH involvement in CNS remodelling during flatfish metamorphosis, we designed an experimental paradigm involving 3 groups; i) a standard normal larvae group; ii) a hypothyroid, MMI-treated pre-metamorphic larvae; and iii) a third group, TH-treated with tetraiodothyronine (T4); and sampled in 3 different stages up until the end of metamorphosis. By analysing gene expression, the aim of this project was to gain insight into how neurodevelopment processes changed during metamorphosis and as a consequence of TH availability.

Introduction

Vertebrate metamorphosis is an abrupt and spectacular post-embryonic transformation of a larva into a juvenile (1), it implies the remodelling of the entire body axis as well as many organs. The main trigger and regulator of vertebrate metamorphosis are the THs, which act through the binding to their specific nuclear receptors (TRα and TRβ), that function as transcription factors regulating a cascade of target genes. Flatfish metamorphosis is a great example of this TH-regulated developmental process, where the symmetric pelagic larva transforms into an asymmetric benthic juvenile (2,3). It is well known that THs are a primordial signalling system in all vertebrates and are essential for normal development, growth and maintenance of metabolism. In the central nervous system (CNS), THs are essential for brain development, maturation and maintenance, influencing a wide range of biological processes, such as myelination, neurogenesis, neuronal and glial migration, synaptogenesis, synaptic establishment, among others (4). In flatfish metamorphosis one enigmatic and non-obvious consequence is the remodelling of the CNS, and although it is well known that THs have a critical and determinant role in CNS development in all vertebrates, CNS changes during flatfish metamorphosis remains unexplored. Furthermore, by blocking sole metamorphosis with a TH synthesis inhibitor and then analyzing transcriptional profiles we found changes in expression of at least 6 transcripts directly related to neuronal differentiation, maturation and cell migration This suggests that a TH deficiency produces a failure in the CNS development in flatfish that could have highly detrimental consequences similar to those documented in mammals. To gain insight into TH involvement in this process, a new experimental set-up was carried using a TH synthesis inhibitor and direct effects of T4 on the metamorphosing larvae with particular interest in head modifications.

Materials and Methods

Newly hatched larvae [1-day post-hatch (dph)] were collected from naturally spawning *Senegalese sole* broodstock from the IFAPA Centro el Toruño. At 7 dph larva were transferred into a six well plates at 18 ºC, separated into 3 groups. A hypothyroid group was established by adding MMI to a final concentration of 0.3 mM; a hyperthyroid group was created by adding thyroxine (T4) to a final concentration of 100 nM (both treatments dissolved in DMSO); and the control group was added with DMSO 0.05 nM. Sampling of whole larvae was done at 3 different stages, pre-metamorphic (S0), initiation (S1), climax (S3) of metamorphosis and end of metamorphosis (S4). Larvae (~80 per group) were euthanised and collected in RNA later and quickly frozen at -80 ºC until processing. RNA extraction was completed with E.Z.N.A. Total RNA Kit I following the manufacturer's instructions. Specific primers were used for real-time PCR. Quantification was performed using EvaGreen chemistry and the relative ΔΔCt method, normalisation was performed using the transcript abundance of 18S ribosomal subunit (rRNA 18S). Data was analysed using a two-way ANOVA test followed by a Tukey's post-hoc test. Significance was taken at $p<0.05$.

Results and Discussion

A high-throughput transcriptomic analysis of the Senegalese sole larvae exposed to MMI revealed significant changes in the expression of 6 transcripts related to CNS development (*tubb6, sox4, notch-l, ahnak, cdnf, klf9*). These transcripts are directly related to the regulation of neuronal differentiation, maturation and migration. We questioned if as consequence of metamorphosis, CNS remodelling occurred under the regulation of THs. Having identified candidate genes for the CNS by in-depth transcriptome analysis of whole larvae we then conducted analysis to identify more precisely the change in the CNS. We did this by performing a dissection of the head and body and analysed the gene expression of *sox4* and *klf9* (neurodevelopmental-related genes); and deiodinase type 2 (*dio2*) and thyroid hormone receptor beta (*thrb*; TH-signalling pathway) (Figure 1). Expression of *dio2* was significantly up-regulated by MMI and down regulated by T4 in the body in the different metamorphic stages, which is concordant with the fact that this enzyme converts the pro-hormone T4 into the most active form of TH, T3; which is allosterically regulated by the TH availability. In the case of the head, we found no significant changes among treatments in *dio2* expression. *thrb* gene expression was significantly up regulated by T4 treatment in all the different stages in the body and in S2 and S3 in the head, as previously reported this receptor is the principal mediator of TH effects in metamorphosis (3,5). Body expression of *sox4* was up-regulated by MMI in S1; and *klf9* expression was down regulated in the head at S1 by MMMI and up regulated by T4 in the body at S4. As *klf9* and *sox4* code for transcription factors involved not only in neuronal but also cell proliferation, differentiation and migration (6,7) their altered expression by TH disruption during sole metamorphosis suggests they could be candidate effectors in CNS remodelling as well as be essential for the progression of metamorphosis.

In summary, in sole metamorphosis the TH signalling system is regulated in a tissue-specific fashion, even though it is known that THs have a critical role in regulating CNS development. How TH signalling regulates transcriptional responses in this developmental process is far from clear, the preliminary finding that some transcription factors related to CNS development are being regulated by THs gives new clues into how the remodelling of this tissue occurs during metamorphosis.

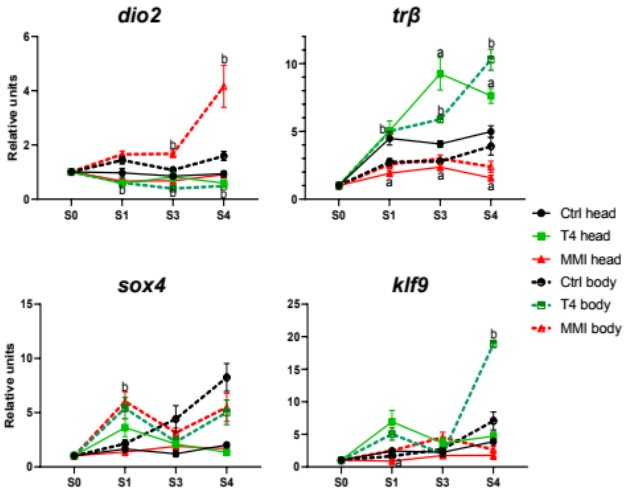


Figure 1. Relative gene expression of TH-signalling (*dio2*, *thrβ*) and CNS development components (*sox4*, *klf9*) at different metamorphic stages. Continuous line represents the head and dotted line the body in control (black), T4 (green) and MMI (red) groups. The significance (p<0.05) is denoted by an *a* in the head and *b* in the body against the respective controls in each stage. Data are shown as mean +/- SEM.

Acknowledgements: AOV funded by a CONACYT post-doctoral grant. Research supported by an EU H2020 MSCA-RISE project 691102 (Algae4A+B) and Portuguese national funds from FCT (Foundation of Science and Technology) through project UID/Multi/04326/2019 and POCI-01-0145- FEDER-007440 ref. UID/NEU/04539/2019, project RTA2017-00054-C03-01 funded by MCIU/AEI/FEDER, UE and through the European Marine Biological Research Infrastructure Cluster-EMBRIC (EU H2020 research and innovation program, agreement nº 654008).

References

1. Shi, Y.B., 2000. Amphibian metamorphosis from morphology to molecular biology. New York: John Wiley & sons, Inc.
2. Power, D.M., et al, 2001. Thyroid hormones in growth and development of fish. Comp Biochem Physiol C Toxicol Pharmacol 130, 447-459.
3. Power, D.M., et al, 2008. The molecular and endocrine basis of flatfish metamorphosis. Rew Fish Sci 16, 93-109.
4. Bernal J., 2006. Thyroid hormone receptors in brain development and function. Nat Clin Prac 3, 249-258
5. Manchado, M., et al., 2009. Molecular characterization, gene expression and transcriptional regulation of thyroid hormone receptors in *Senegalese sole*. Gen Comp Endocrinol 160, 139-147
6. Yin K.J., et al, 2015. Krûpple-like factors in the central nervous system:novel mediators in Stroke. Metab Brain Dis 30, 401-410.
7. Taboada X., Viñas A., Adrio F., 2017. Comparative expression patterns of sox2 and sox19 genes in the forebrain of developing and adult turbot (*Scophthalmus maximus*). J Comp Neu 526, 899-919.

IMMUNOSTIMULATORY EFFECTS OF OESTROGEN ON THE DEVELOPING IMMUNE SYSTEM OF JUVENILE SEA BASS

C. Moreira[1], M. Paiola[1], A. Duflot[1], R. del Pozo[3], M. C. Piazzon[3], B. Fouz[4], I. Varó[3], A. Sitjà-Bobadilla[3], T. Knigge[1], P. Pinto[2], T. Monsinjon[1]

[1] UMR-I 02 Environmental Stress and Aquatic Biomonitoring (SEBIO), University of Le Havre Normandy, F-76600 Le Havre, France; [2] Centro de Ciências do Mar (CCMAR), Universidade do Algarve, 8005-139 Faro, Portugal; [3] Instituto de Acuicultura Torre de la Sal, CSIC, 12595 Torre de la Sal, Castellón, Spain; [4] Departamento de Microbiología y Ecología, & Estructura de Recerca Interdisciplinar en Biotecnologia i Biomedicina (ERI BIOTECMED). Universitat de València. Burjassot, Valencia. Spain.

Environmental endocrine disruptors raise major health concerns as they can affect several physiological processes, including the immune system. Oestrogenic endocrine disrupting chemicals (EEDCs) have been suggested to increase the prevalence of autoimmune diseases and cancer, especially when exposed during development. Given the conservation of the thymus and its oestrogenic modulation in jawed vertebrates, EEDCs are also expected to affect immune system development and thus immunocompetence establishment in teleost fish. Hence, we exposed European sea bass (*Dicentrarchus labrax*) fingerlings to 20 ng/L of exogenous oestradiol (E_2) at two different stages of development of the thymus: from 47 to 54 days post-hatch (dph) and from 60 to 90 dph. After depuration, fish from both groups were challenged by intracoelomic injection with *Vibrio harveyi* at 213 dph. The bacterial challenge indicated that exposure to E_2 from 60 to 90 dph have long lasting effects on immunocompetence, as it improved the fish's resistance to vibriosis.

Introduction

Oestrogens play a fundamental role in many physiological processes in vertebrates, including immune system development and functioning. If the animals are exposed during critical ontogenic stages, exogenous oestrogens may irrevocably affect the immune system with long-lasting impact on immunocompetence.

In mammals, oestrogens are related to several diseases such as allergies and cancer (1). In fish, oestrogens also modulate the immune system, including the thymus, thereby affecting immunocompetence *i.e.,* fish's capacity to fight against pathogens (2, 3, 4). Hence, the aim of this study was to evaluate effects of an environmental concentration of oestradiol, at two different stages of thymus ontogenesis, on the immunocompetence establishment of juvenile sea bass exposed to a bacterial challenge.

Materials and Methods

D. labrax fingerlings from 'Les Poissons du Soleil' (France) were acclimatised for seven days at the Instituto de Acuicultura de Torre de la Sal (IATS-CSIC, Spain), and thereafter exposed to 20 ng/L of waterborne E_2 at two key stages of thymus development: Group I was exposed when organ regionalization begins, from 47 to 54 days post-hatch (dph), whereas group II was exposed when thymus regionalization is already in progress, from 60 to 90 dph (3). A solvent control with methanol was conducted for each exposure group. Water and liver

samples were collected and E_2 was quantified by a radioimmunoassay (RIA). Following depuration, both groups were subjected simultaneously to a bacterial challenge at 213 dph by intracoelomic injection with 2.6 x 10^4 CFU/fish of a virulent strain of the emergent pathogen *Vibrio harveyi* (5) and the cumulative mortality was registered over one week. An independent Student *t*-test was conducted to compare controls and treated groups in RIA's results and Log-rank (Mantel-Cox) test was performed for the survival data.

Results and Discussion

The measured oestrogen concentration of the water samples was 20.22 ± 5.66 ng E_2/L during the exposures, whereas the control groups were situated below the detection limit. The liver samples corroborated the efficacity of the exposure, with exposed animals having a statistically higher concentration of oestradiol compared to the control samples (Fig. 1).

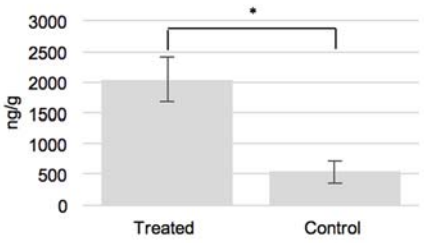


Figure 1. Oestradiol (E_2) concentrations in the liver of E_2-treated (20 ng E_2/L) and control fish (*Dicentrarchus labrax*) after 30 days of exposure. Means ± standard error of the means; *, significantly different between Control (n=7) and Treated (n=10) at $p = 0.0078$.

During the bacterial challenge, E_2 exposure reduced mortality in fish from group II (p=0.0157). This finding suggests that E_2 had immunostimulating effects in older fish when exposed at latter stage of thymus organization. In treated group I, one replicate showed an exceptionally high mortality of 90%. This could, however, not be confirmed by the other replicate, which had a mortality not different from the control (Table 1). In contrast, previous studies with juvenile fish of different species suggested immunosuppression following oestrogen exposure at early stages, with the survival rates being reduced following bacterial challenge in E_2 exposed fish (6, 7, 8, 9). Nevertheless, these studies used higher oestrogen concentrations, whereas lower physiological concentrations of oestrogens were found to be immunostimulatory. This interpretation is further corroborated by several studies, which reported that oestrogen can upregulate various immune parameters in fish and mammals, such as respiratory burst or immunoglobulin levels (10). Moreover, studies in mammals have also demonstrated both, immunosuppressive and stimulating effects of oestrogens (2). The present results provide further support for the importance of oestrogens during thymus development and the resulting consequences for immunocompetence establishment in fish.

Table 1. Total mortality (%) recorded over a week during a bacterial challenge with *Vibrio harveyi* juvenile sea bass (*Dicentrarchus labrax*) after exposure to 20 ng/L oestradiol.

	Control Group I		Treated Group I		Control Group II *		Treated Group II *	
Replicate / Bacterial Challenge	1	2	1	2	1	2	1	2
2.6 x 10^4 CFU/fish	50.0 (n=10)	50.0 (n=10)	90.0 (n=10)	50.0 (n=10)	71.4 (n=7)	85.7 (n=7)	28.6 (n=7)	42.9 (n=7)

* significantly different at p=0.0157 between the treated and the control fish from group II

Acknowledgements: This work was supported by ANR(FR) through project ETaT (ANR-15-CE32-0014), FCT(PT) through project UID/Multi/04326/2019 and researcher contract to PP (DL57/2016/CP1361/CT0015) and by EU H2020 mobility programs AQUAEXEL2020 (grant 652831) and ASSEMBLE PLUS (contract SR02022018976f).

References

1. Adeel, M., Song, X., Wang, Y., Francis, D., & Yang, Y. (2017). Environmental impact of estrogens on human, animal and plant life: a critical review. *Environment International 99*, 107-119.
2. Rehberger, K., Werner, I., Hitzfeld, B., Segner, H., & Baumann, L. (2017). 20 Years of fish immunotoxicology–what we know and where we are. *Critical reviews in toxicology*, *47*(6), 516-542.
3. Seemann, F., Knigge, T., Olivier, S., & Monsinjon, T. (2015). Exogenous 17 β-oestradiol (E2) modifies thymus growth and regionalization in European sea bass *Dicentrarchus labrax*. *Journal of fish biology*, 86(3), 1186-1198.
4. Paiola, M., Knigge, T., Duflot, A., Pinto, P. I., Farcy, E., & Monsinjon, T. (2018). Oestrogen, an evolutionary conserved regulator of T cell differentiation and immune tolerance in jawed vertebrates?. *Developmental & Comparative Immunology*, *84*, 48-61.
5. Fouz, B., Llorens, A., Liz, D., Iser-Subich, M., Amaro, C. (2017) Emergence of a new serovar of *Vibrio harveyi* pathogenic for European seabass (*Dicentrarchus labrax*). XVIII Conference "Diseases on fish and shellfish" (Ireland).
6. Dong, M., Seemann, F., Humble, J. L., Liang, Y., Peterson, D. R., Ye, R., et al. (2017). Modification of the plasma complement protein profile by exogenous estrogens is indicative of a compromised immune competence in marine medaka (*Oryzias melastigma*). *Fish & Shellfish Immunology 70*, 260-269.
7. Wenger, M., Sattler, U., Goldschmidt-Clermont, E., & Segner, H. (2011). 17Beta-estradiol affects the response of complement components and survival of rainbow trout (*Oncorhynchus mykiss*) challenged by bacterial infection. *Fish & Shellfish Immunology 31*(1), 90-97.
8. Wenger, M., Krasnov, A., Skugor, S., Goldschmidt-Clermont, E., Sattler, U., Afanasyev, S., & Segner, H. (2012). Estrogen modulates hepatic gene expression and survival of rainbow trout infected with pathogenic bacteria *Yersinia ruckeri*. *Marine Biotechnology 14(5)*, 530-543
9. Thilagam, H., Gopalakrishnan, S., Bo, J., & Wang, K. J. (2009). Effect of 17β-estradiol on the immunocompetence of japanese sea bass (*Lateolabrax japonicus*). *Environmental Toxicology and Chemistry, 28(8)*, 1722-1731.
10. Nalbandian, G. and S. Kovats. (2005). Understanding sex biases in immunity: effects of estrogen on the differentiation and function of antigen-presenting cells*. Immunological Research 31*: 91-106.

EXPRESSION OF STRESS RELATED GENES IN THE HPI-AXIS OF THE ANTARCTIC FISH *Notothenia rossii* AT DIFFERENT THERMAL REGIMES

Sandra C Silva, Pedro M. Guerreiro, Bruno Louro, Adelino VM Canário

Centre of Marine Sciences, University of Algarve, Faro, Portugal.

Antarctica's extreme temperatures and geographic isolation resulted in a strong selective pressure for the development of a stenothermal fish fauna. With an initial pressure for the development of close-to-zero-living adaptations, "irreversible" gene loss occurred, and afterwards the environmental stability and low population connectivity culminated in a relatively low genetic variation background. In a Climate Change context is imperative to study how Antarctic fish will cope with the warming effects. The objective of this study was to evaluate the expression plasticity of target genes associated with the stress response at the different levels of the hypothalamic-pituitary-interrenal (HPI) axis in *Notothenia rossi* experimentally acclimated to natural (2ºC) and increased temperatures (5 and 8ºC) and subsequently exposed to a standard stress test (chasing+netting+air exposure). Our results point to warmer waters will impact fish by overloading the regulatory mechanisms of HPI-axis indicated mainly by glucocorticoids receptors desensitization of liver cells.

Introduction

Global climate change prediction points to an increase in water temperatures with unique effects in polar regions. In Southern Ocean (where Antarctica is inserted) the water temperature is stable along the year, rounding -0.5 up to -1.9ºC. Notothenoidei are endemic of Antarctica and the dominant suborder. Its isolation lasts for 10 to 15 million years after a wide-scale extinction and geographic speciation[1]. Being Antarctica Peninsula (Figure 1) the fastest warming region of the Antarctica continent, it is the doorway of studies about the relationship between climate and molecular adaptation.

Figure 1. Google Maps, 2020. Antarctica, 1:1.500. Google Maps [online] [Accessed 3 November 2020].

The "Principle of Allocation" proposed by Levins, 1968 predicts that animals living in a narrow tolerance window specialized themselves to optimize performance. Antarctic fishes are considered as the ultimate thermal specialists with the development of antifreeze glycoproteins, cold adapted enzymes and ubiquitously expressed heat shock proteins, to mention few. According to this principle the thermal specialization is accompanied by a loss of capacity to adapt to new environments. However the discussion of the "thermal specialization paradigm" argues that, at least some Antarctic fish, retained the capacity to compensate for chronic temperature change[2].

In a global warming perspective, this study allowed to test the hypothesis that Antarctic *Notothenia rossi* fish has lost its ability to respond to increasing water temperatures. To do so, the expression plasticity of some genes involved on the activation of the hormonally mediated stress response was evaluated along the HPI-axis.

Materials and Methods

The experiment was conducted during a scientific campaign to King George Island, Antarctica, in 2016. Fishes were caught in the wild and transferred into laboratory tanks and kept at environmental water temperatures (~2ºC) for two weeks. The animals were therefore allocated in six different groups and acclimated for 10 days to the final temperature condition (1.5-2ºC; 4.5-5ºC and 7.5-8ºC experimental groups, Figure 2). At final temperature, the control group of each condition was sacrificed. The other groups were stressed by chasing+netting+60 seconds air exposure and returned to tank. Ninety minutes after, all the fish were sacrificed and tissue samples of pituitary, head kidney and liver collected. At CCMAR, Faro laboratory total RNA was extracted, cDNA synthetized, and RT-qPCR performed.

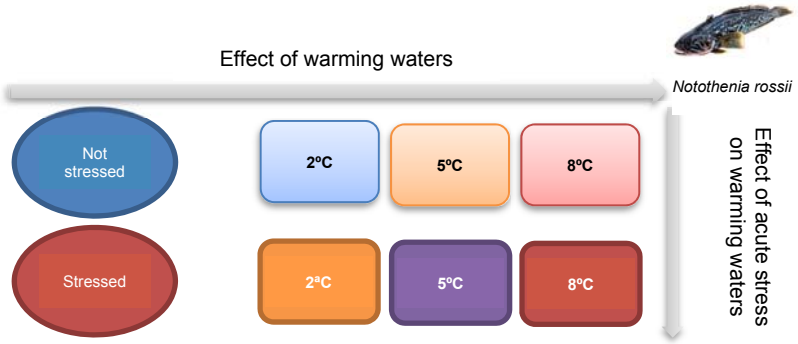


Figure 2. Schematic representation of the experiment with *Notothenia rossii* and clarification of the main expected outcomes after differential gene expression studies.

Results and Discussion

Warmer water temperature had a moderate impact in the expression of POMCs genes, the precursors for ACTH, in pituitary (Figure 3A). Only POMCb was upregulated with the increased temperatures in no stressed group set, perhaps because the timing for glucocorticoids production was gone and they are now available in bloodstream until the end of its half-lives. Important changes were observed in cortisol receptors (MR and GR) mainly in the liver as a peripheral tissue responsible for metabolic actions (Figure 3C). In fact, GR and MR belongs to two different systems of glucocorticoids receptors: MR responds to small increases in glucocorticoids baseline having stimulating effects while GR responds to huge and persistent glucocorticoids concentration with harmful effects. The downregulation of both receptors is part of receptor desensitization process that, by reducing the number of available receptors, reduces cells' sensitive to glucocorticoids. This mechanism of turning off the hormonal signalling is a sign of an overloaded HPI response.

Adaptation to a new environment will only occur if evolutionary processes are faster than environmental change. With the effects of climate changes on polar regions predicted for a near future, these preliminary results point to the necessity for a thorough consideration about *Notothenia rossii* as an important piece of global biodiversity that probably will threatened.

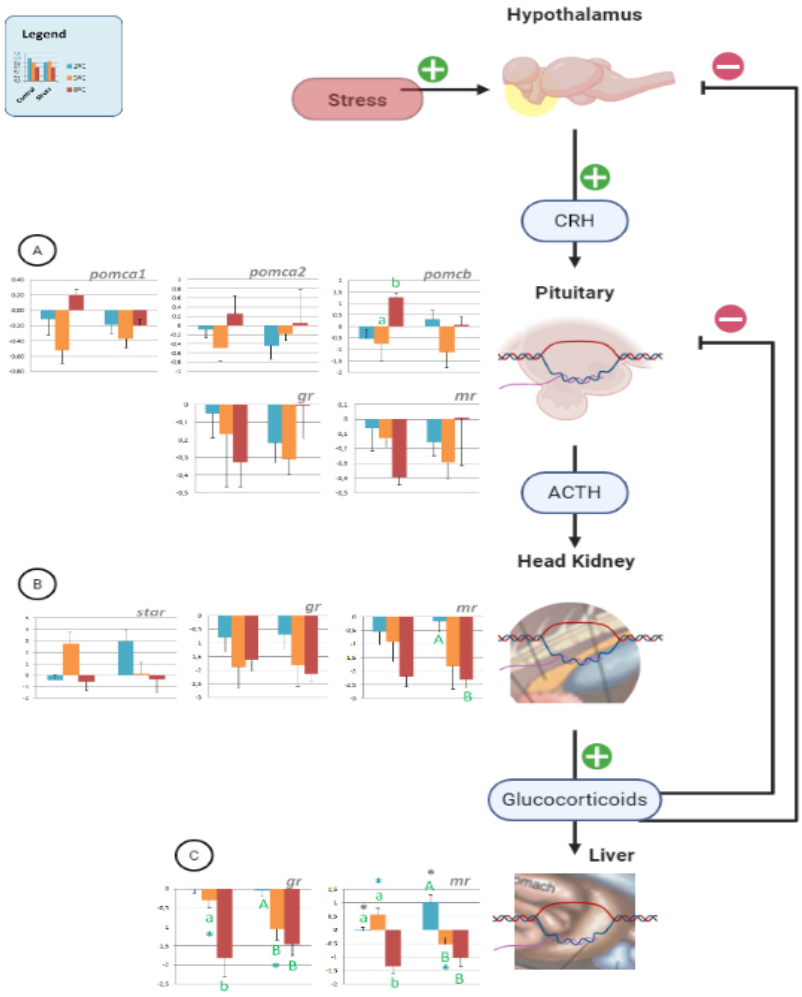


Figure 3.: Expression data in Y axis represented in Log2 of Fold Change in relation to control (2ºC). Gene expression was normalized against the geometric mean of 3 reference genes (18S, Ef1α and βActin). Small letters mean statistical differences in control group. Capital letters mean statistical differences in stressed group. * indicates differences between groups (same temperature). Two-way ANOVA, $P<0.05$. HPI-axis scheme created with BioRender.com

Acknowledgements: This work was supported by FCT grants PTDC/BIAANM/3484/2014 and CCMAR/Multi/04326/2013. We thank the crees of the Henrik Arctowski (PL) and Great Wall (CN) Antarctic stations and the PROPOLAR for their logistic support.

References

1.Carney Almroth, B. *et al.* Warmer water temperature results in oxidative damage in an antarctic fish, the bald notothen. *J. Exp. Mar. Bio. Ecol.* **468**, 130–137 (2015).
2.Seebacher, F., Davison, W., Lowe, C. J. & Franklin, C. E. A falsification of the thermal specialization paradigm: Compensation for elevated temperatures in Antarctic fishes. *Biol. Lett.* **1**, 151–154 (2005).

STRESS EFFECTS ON DAILY PROFILE OF CORTISOL BIOSYNTHESIS PATHWAY IN RAINBOW TROUT: ROLE OF CORTISOL

Fatemeh Naderi, M. Chivite, R.M. Ceinos, M.A. López Patiño, C. Otero-Rodiño, J.M. Miguez

Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da saúde, Universidade de Vigo, Spain.

Cortisol plays a key role in mediating the physiological response to stress in vertebrates, including fish. Its biosynthesis includes the steroidogenic acute regulatory (StAR) protein, cytochrome P450 cholesterol side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD) and 11β-hydroxylase (11βH), and takes place within the head kidney. However, little is known about stress effects on the functioning of this tissue and whether cortisol mediates such effect. Then, we evaluated the daily profile of mRNA abundance of parameters related the cortisol biosynthesis in head kidney of rainbow trout (*Oncorhynchus mykiss*), the impact of stress, and the role played by cortisol as mediator of such response. Four groups of trout were used: *1)* normal stocking density (NSD, control group), *2)* high stocking density, for 72 hours (HSD, stressed group), *3)* NSD implanted with mifepristone (general glucocorticoid receptors antagonist), and 4*)* HSD+mifepristone. Fish of each group were sampled every 4-h along the 24-h LD cycle. Cortisol plasma levels and the mRNA abundance of *StAR*, *11βH*, *3βHSD* and *P450ssc* were assessed in head kidney samples. Daily rhythms of *StAR, P450scc, 3βHSD* and *11βH* mRNA abundance were demonstrated with peaking levels at day time. Stress enhanced the expression of those enzymes, but mifepristone prevents such effect. These results confirm a key role played by cortisol as mediator of the response to stress in trout head kidney, but also an important role of the glucocorticoid as input of the head kidney circadian system might be expected.

Introduction

Cortisol plays a key role in mediating the physiological response to stress in vertebrates, including fish (1). Cortisol synthesis is under the control of the HPI/HPA axis and takes place in the adrenal gland of mammals and the interrenal cells of fish, which locate at the head kidney. The biosynthesis process is well conserved among vertebrates and it involves the microsomal enzymatic pathways, including the steroidogenic acute regulatory (StAR) protein, cytochrome P450 cholesterol side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD) and 11β- hydroxylase (11βH) within the head kidney (2). Although cortisol levels in blood display marked daily rhythms in fish, changes in the biosynthesis machinery in head kidney at daily level are poorly known, as well as the influence that stress exerts on them (3). Then, our aim was to evaluate the daily profile of mRNA abundance of the enzymes involved in cortisol synthesis in head kidney of rainbow trout (*Oncorhynchus mykiss)*. Additionally, we evaluated the impact of stress on such rhythms, and the role of cortisol as a possible mediator of the stress effects.

Materials and Methods

Rainbow trout weighing 90 ± 5 g were obtained from a local hatchery (A Estrada, A Coruña, Spain) and transferred to facility of Vigo University. Fish were kept in

continuously renovated fresh water tanks (120L, 12.5 ± 1ºC) under 12L:12D photoperiod. Daily feeding time was scheduled at zeitgeber time (ZT) 2 (ZT0 = light on), with a commercial dry pellet diet. After two-week acclimation trout were deeply anaesthetized by addition of 2-phenoxyethanol (0.2% v/v-Sigma Aldrich) in tank water. Once they were anesthetized, individual blood samples were collected and fish were rapidly sacrificed. A sample of head kidney was collected under sterile conditions, according to previously described (4), immediately frozen in liquid nitrogen, and stored at -80°C until qPCR assayed for mRNA abundance of cortisol biosynthetic pathway (*StAR, P450scc, 3βHSD* and *11βH*). Plasma samples were obtained by blood centrifugation, and then were immediately frozen and stored at -80º C until assayed. Cortisol levels and mRNA abundance assays were carried out as previously described (5).

Results and Discussion

Plasma cortisol displayed a significant daily rhythm in control fish with peaking values at first hours of day (Table 1). Exposure of rainbow trout to stress for 72h increased mean values of plasma cortisol along the day. In addition, cortisol increased following treatment with the GR antagonist, mifepristone, which agrees with previous studies in this species (6). This could be due to altered negative feedback control of released cortisol from the interrenal cells of the head kidney. Indeed, the enhancing effect was more robust in trout receiving mifepristone and after forwards subjecting to stress.

Table1: Parameters provided by the cosinor analysis defining the daily rhythm of mRNA abundance for genes of cortisol biosynthesis at the head kidney of rainbow trout.

	Data	Control	Stress	Mifepristone	Mife+Stress
Cortisol	Mesor	29.01	40.36	50.55	192.16
	Amplitude	12.33	16.41	16.50	52.24
	Acrophase (ZT/C)	1.5	12.47	12.26	8.28
	N/S	0.19	0.35	0.22	0.18
StAR	Mesor	3.31	4.69	4.15	4.15
	Amplitude	2.48	2.21	1.95	1.95
	Acrophase (ZT/C)	8.11	7.21	8.06	8.06
	N/S	0.15	0.15	0.14	0.14
11βH	Mesor	1.44	2.56	1.91	1.91
	Amplitude	0.45	1.23	0.52	0.52
	Acrophase (ZT/C)	8.44	9.03	8.49	8.49
	N/S	0.18	0.15	0.15	0.15
3βHSD	Mesor	1.89	3.04	2.21	2.21
	Amplitude	0.82	1.45	0.76	0.76
	Acrophase (ZT/C)	9.34	8.49	10.20	10.20
	N/S	0.20	0.16	0.15	0.15
P450ssc	Mesor	1.24	2.09	1.63	1.63
	Amplitude	0.25	1.02	0.47	0.47
	Acrophase (ZT/C)	8.17	8.52	8.27	8.27
	N/S	0.18	0.15	0.16	0.16

Data represent the mesor (mean value of the rhythm), the amplitude, the acrophase (time of peak), and the noise/signal (N/S) ratio. N/S<0.3 means that a significant rhythm exists.

Daily rhythms of *StAR, P450scc, 3βHSD* and *11βH* mRNA abundance in head kidney were documented (Fig 1, Table1), with peaking values at the second half of the light phase, which is in line with that reporteded in mammals (7, 8). These data indicate that there is a time lag (approximately 16-20 h) between the rates of gene expression of biosynthetic enzymes and those of plasma cortisol levels, suggesting that translational (pre-, co-, post-) regulatory processes are involved in the temporary availability of the functional enzymatic proteins (9).

Stress increased mean daily levels, and amplitude of the mRNA abundance of enzymes involved in cortisol biosynthesis (Table1), suggesting that enhanced cortisol synthesis exists all along the day within head kidney of stressed fish. Based on the protective effect that mifepristone exerts on the stress-related

response, our data suggest that released cortisol plays a role in mediating the effect of stress on the daily profile of its biosynthesis in trout by exerting a modulatory feedback action at this tissue level.

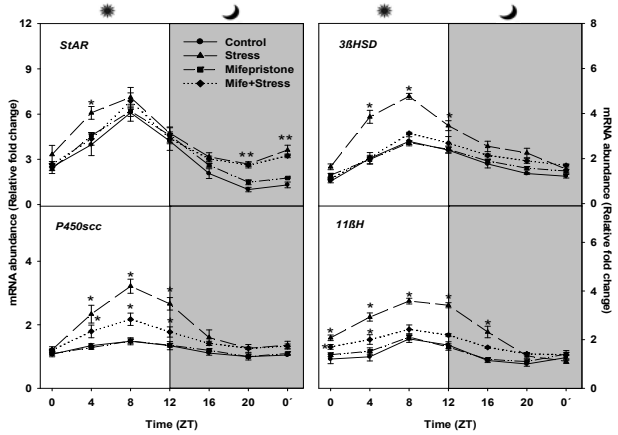


Figure 1. Daily profile of mRNA abundance of cortisol biosynthesis enzymes (*StAR, 11βH, 3βHSD* and *P450ssc*) in head kidney of rainbow trout subjected to the different experimental conditions. * P<0.05 respect to control at the same ZT period.

Acknowledgements: Funded by a Research grant from Spanish Agencia Estatal de Investigación (AEI) and European Fund of Regional Development (AGL2016-74857-C3-1-R and FEDER).

References

1. Wendelaar-Bonga S.E. (2011). Hormonal responses to stress. In: A.P.Farrell. (Ed.) Encyclopaedia of Fish Physiology. *Academic. Press, San Diego, USA*. 1515-1523.
2. Mommsen T.P., Vijayan M.M., Moon T.W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish. Biol.* 9: 211-268.
3. Sánchez-Vázquez F.J., López-Olmeda J.F., Vera L.M., Migaud H., López-Patiño M.A., Míguez J.M. (2019) Environmental cycles, melatonin and circadian control of stress response in fish. *Front. Endocrinol.* 10:279.
4. Doyon C., Gilmour K.M., Trudeau V.L., Moon T.W. (2003). Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic area of socially subordinate rainbow trout. *Gen Comp Endocrinol*.133:260-71.
5. Naderi F., Hernández-Pérez J., Chivite M., Soengas J.L., Míguez J.M., López-Patiño MA. (2018).Involvement of cortisol and sirtuin1 during the response to stress of hypothalamic circadian system and food intake-related peptides in rainbow trout, *Oncorhynchus mykiss*. *Chronobiol Int*. 35:1122-1141.
6. McDonald M.D., Wood C.M. (2004). The effect of chronic cortisol elevation on urea metabolism and excretion in the rainbow trout (*Oncorhynchus mykiss*). *J. Comp. Physiol. B* 174:71-81
7. Park S.Y., Walker J.J., Johnson N.W., Zhao Z., Lightman S.L., Spiga F. (2012). Constant light disrupts the circadian rhythm of steroidogenic proteins in the rat adrenal gland. *Mol Endocrinol*. 22:114-23.
8. Lemos D.R., Downs J.L., Urbanski H.F. (2006). Twenty-four-hour rhythmic gene expression in the rhesus macaque adrenal gland. *Mol Endocrinol.* 20:1164-76
9. Kiriakidou M., McAllister J.M., Sugawara T., Strauss J.F. (1996). Expression of steroidogenic acute regulatory protein (StAR) in the human ovary. J *Clin Endocrinol Metab*. 81:4122-8.

CORTISOL PROFILES AND INTERRENAL SENSITIVITY OF THE ANTARCTIC FISH Notothenia rossii TO TEMPERATURE CHALLENGE O

Pedro M. Guerreiro, B. Louro, S.C. Silva, A. Alves, E. Couto, A.V.M. Canário

Comparative Endocrinology and Integrative Biology, Centre of Marine Sciences - CCMAR, University of Algarve, Faro, Portugal.

Antarctic fish evolved in a stenothermal environment and are subject to very small fluctuations in temperature throughout their life (normally between –1°C to 2°C). In Antarctic Peninsula the rate of climate change is up to ten times faster than the world's average. As fish in these waters face a warmer future their ability to adapt to such changes is uncertain. If on one hand, it has been shown that they lack an inducible heat shock response, and suggested that their metabolic/enzymatic rates must be slow, on the other, they have relatively high CTmax and it appears that they possess the genomic tools that allow for warmer acclimation, as several close relatives broke from Antarctica to warmer waters in a relatively close past. We aimed at evaluating the mechanism and capabilities of the HPI axis in Antarctic fish and we show the profiles of cortisol and energy metabolites in plasma in fish acclimated to different temperatures before and after stress and the cortisol in medium fractions collected from perifusions of the *ex vivo* interrenal tissue of these fish.

Introduction

The stress response is crucial to the adaptive success, as it elicits a coordinated set of compensatory behavioral and physiological processes that enable the animal to overcome the threats to its homeostatic equilibrium. However, during intense and/or prolonged stress the stress response may lose its adaptive value and become dysfunctional, affecting negatively functions such as metabolism, growth, reproduction or immunity (1,2). In Antarctic fish, studies on stress response have focused mainly on the demonstrated lack of increase in the gene expression or protein levels of the inducible heat shock proteins (HSPs) during thermal shock or acclimation (3). The Hypothalamus-Pituitary-Interrenal (HPI)-axis, as the primary responder to stress has been relatively neglected and it was initially hypothesized that such endocrine response should be low and slow, since the enzymatic machinery required to synthesize and release cortisol (and catecholamines) would be hindered by the very low temperatures (4), and thus just a few studies have addressed the role of the HPI-axis and reported cortisol levels in Antarctic nototheniids (5,6). Using an array of drugs, blockers and agonists of the HPI-axis, in control and stress conditions, we have previously observed this fish is capable to mount an endocrine response to temperature and salinity, and that the HPI-axis is fully functional with swift cortisol synthesis, operational receptors and efficient feedback mechanisms. Here we aimed to evaluate the impacts of acute stress in the amplitude and duration of the cortisol profiles under normal temperature and to assess the effects of long term acclimation to different temperatures on the acute stress response *in vivo* and *ex vivo*, to test the sensitivity of the HPI-axis during chronic stress.

Materials and Methods

Fish, *Notothenia rossii*, were collected by boat, using hook and line, from depths between 5-20 meter, in the waters of Maxwell bay, surrounding the Great wall Station, in King George Island. Fish were swiftly caught (ca. 1 fish every 2-3

minutes), placed in tanks aboard, with continuously renewed, cold (1.5-2ºC) and highly oxygenated seawater and then transferred to tanks in an open circuit inland with pumped seawater at natural temperature and oxygen conditions. Upon acclimation two sets of experiments were performed. In the first, four groups of fish (n=6) were selected and placed in tanks with flowing natural seawater and left undisturbed for 72 hours. One group was not manipulated and served as the control. In the other three groups, fish were exposed to a standard stress test (SST: chasing+netting+1min air exposure) and were then returned to the respective tank and sampled after 1, 4 and 24h. In the second, six groups of fish (n=6-9) were acclimated to the experimental temperatures (1.5, 4.5 and 7.5ºC), two groups per temperature, for 10 days. At this time point the control group of each temperature was sacrificed. The other group was stressed (SST), returned to the tank and sacrificed 90 minutes after. Blood was collected and the interrenal tissue was placed in ice-cold Ringers and then distributed in a perfusion system and bathed in a continuously flow of oxygenated Ringers. Fractions were collected every 20min for 240min. A spike with 10^{-7}M ACTH was done at 100min to evaluate the interrenal response. Cortisol was measured by radioimmunoassay and glucose and lactate determined using colorimetric kits.

Results and Discussion

The cortisol profile to a SST appears similar to those of a temperate fish, with a peak between 1 and 4 hours and a subduction to basal levels between 24-48 hours. Lactate showed similar profile reflecting the energy-demanding SST, but curiously there were no changes in plasma glucose during this period after SST.

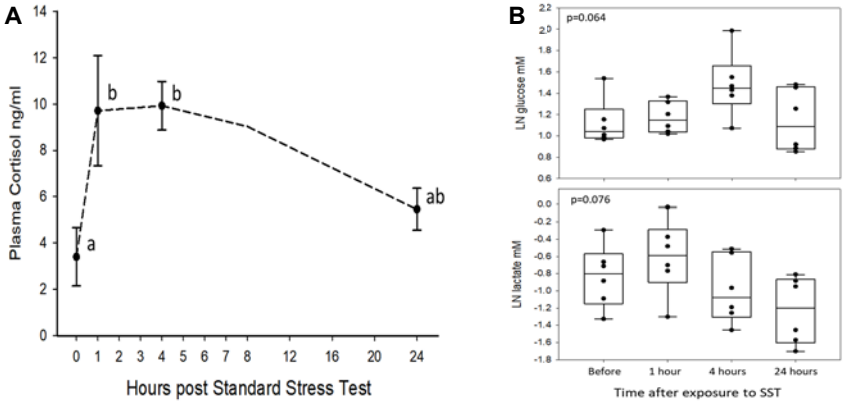


Figure 1. A - Cortisol profile during a 24-hour period in the plasma of *N. rossii* acclimated to control temperatures before and after a SST. **B** – LN transformed data for glucose (top) and lactate (bottom) for the same fish. Different letters indicate statistical differences between time-points (p>0.05, n=6).

Acclimation to increased temperature clearly influenced cortisol, glucose and lactate responses to a SST, and at higher temperatures cortisol levels in the non-stressed group were as high as those measured in fish exposed to the SST. As seen in the time-course, SST at low temperature had no effect over glucose release. Contrary to previously suggested (4) these fish show a clear and swift cortisol response, even if circulating levels are low, but comparable to other cold waters fish or with slow metabolism (1,2), although below other reported nototheniids (4,5,6). Interrenal sensitivity was very variable but tissues from warmed fish showed elevated cortisol release and low reaction to ACTH,

suggesting low sensitivity and/or exhaustion, in line with the data collected *in vivo*. It remains to be seen what effects warming may have on other functions, but it is reasonable to expect reduced scope for other physiological processes.

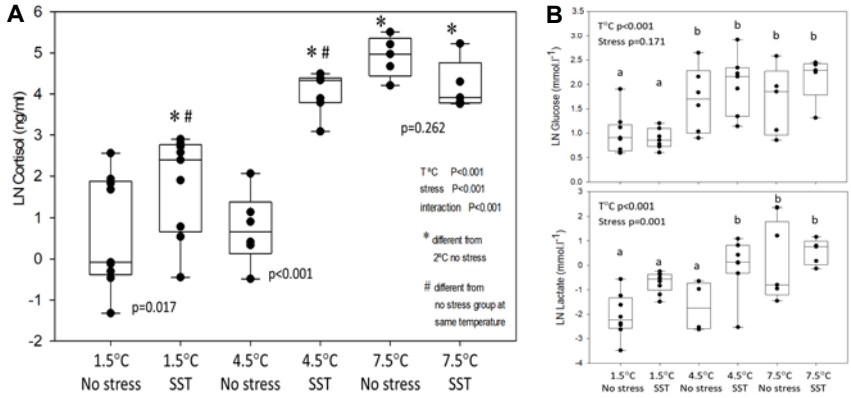


Figure 2. A - Cortisol levels in *N. rossii* previously acclimated at different temperatures and subjected to an acute SST. **B** – LN transformed data for glucose (top) and lactate (bottom) for the same fish. Letters indicate statistical differences between groups; * different from overall control; # different from paired no-SST (p>0.05, n=6-9)

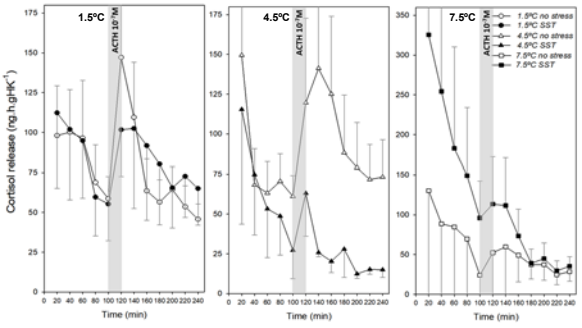


Figure 3. Cortisol release by the interrenal tissue of *N. rossii ex vivo* after acclimation of fish to SST at various temperatures. The gray shade indicates a 20-min exposure of the tissue to 10^{-7}M ACTH. Note the amplitude of the spike and recovery after that point and the difference in scales between groups (n=6-8).

In conclusion, the cortisol response in *N. rossii* follows a general pattern described for other fish (1,2), responding in a similar time frame and with the same mechanisms, to a comparable thermal stimulus. Exposure to increased temperature, as a chronic stress, results in maladaptation in nototheniids, with impacts for the survival of this group of highly specialized fish.

Acknowledgments: We thank the Henrik Arctowski (PL) and Great Wall (CN) Antarctic stations and the PROPOLAR for their logistic support. FCT grants PTDC/BIAANM/3484/2014, CCMAR/Multi/04326/2013, UID/Multi/04326/2019.

References

1. Wendelaar Bonga SE (1997). Physiol Rev. 77(3):591-625.
2. Iwama GK, Afonso LOB, Vijayan MM. In: Evans DH, Claiborne JB, editors. The physiology of fishes. Boca Raton: CRC Press; 2005. p. 320.
3. Place SP, Zippay ML, Hofmann GE (2004). Am J Physiol. 287(2): R429-36.
4. Whiteley NM, Egginton S. (1999). J Exp Biol. 202(Pt 24):3623-9.
5.Ryan, S.N. (1995) i. Experientia 51, 768–774.
6. Hudson, H.A., Brauer, P.R., Scofield, M.A. et al. (2008). Polar Biol 31, 991–997

ACCLIMATION TO DIFFERENT ENVIRONMENTAL SALINITIES MODIFIED THE EXPRESSION OF SEVERAL ADENOHYPOPHYSEAL HORMONES IN GREATER AMBERJACK, *Seriola dumerili* (Risso, 1810)

Liliana Rodríguez-Velásquez [1], N. Gilannejad [2], M. Alameda [1], Barany A. [1], J. Roo [3], J.L. Muñoz [4], J.M. Mancera [1], G. Martínez-Rodríguez [2]

[1]Department of Biology, Faculty of Marine and Environmental Sciences, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI-MAR), University of Cádiz, 11510 Puerto Real, Cádiz, Spain. [2]Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas (ICMAN-CSIC), 11510 Puerto Real, Cádiz, Spain. [3]Universidad de Las Palmas de Gran Canaria (ULPGC). [4]Centro IFAPA "El Toruño", Junta de Andalucía.

The impact of different salinities in the physiology of greater amberjack (*Seriola dumerili*) juveniles was examined. Higher plasma glucose concentrations were observed at 15 and 36 ppt, when compared to 22 ppt. Lactate showed an inverse linear relationship with environmental salinity (15 < 22 < 36 ppt). Relative expression levels of gene transcripts showed significantly lower *gh* levels at higher salinities, whereas *prl* showed an inverse relationship with salinity increase (15 > 22 > 36 ppt).

Introduction

Greater amberjack (*Seriola dumerili*, Risso 1810) is an important commercial fish in Europe, North America, and Japan because of its high-quality meat and global commercial value (1). Thus, *S. dumerili* presents a number of features that makes it a promising and attractive species for aquaculture production, mainly due to its rapid growth (~800 g in the first year; reaching a maximum weight of 80 kg in adults), high fecundity, and worldwide market acceptance.

Materials and Methods

Juveniles of *S. dumerili* around 78 g body mass were transported from the ULPGC to the IFAPA "El Toruño" facilities, where they were randomly distributed into three independent RAS experimental groups, with different water salinities (5, 22, and 36 ppt). In each tank, the number of individuals was adjusted in order to get the same experimental density (3 g L^{-1}) and number (n = 25). During the experimental period, fish were reared under a 12D/12L photoperiod and a water temperature ranging from 17 to 22 ºC, and were fed at a daily ration of 2 % of body mass, with a commercial diet (Skretting®, Stavanger, Norway). At the end of the experimental period (77 days), all fish were anaesthetized with a lethal dose of 2-phenoxyethanol. Blood was collected and plasma was stored in aliquots at -80 ºC. Pituitary was removed and conserved in appropriate volume (1/10 w/v) of RNAlater™ (Invitrogen Life Technologies). Pituitary total RNA was extracted using the NucleoSpin® XS kit (Macherey-Nagel). Reverse transcription was performed using the qScript™ cDNA synthesis kit (Quanta BioSciences), with 50 ng of total cDNA assumed from total RNA input. Expression levels of *prolactin* (*prl*), *somatolactin* (*sl*), *growth hormone* (*gh*), *proopiomelanocortin a* and *b* (*pomca* and *pomcb*) were evaluated using real-time in a BioRad CFX Connect™. Expression of target genes were normalized to *β-actin* (*actb*) and *elongation factor 1-alpha* (*ef1a*), as internal reference genes (2).

Results and Discussion

Plasma parameters are shown in Table 1. Glucose showed significantly higher levels at 15 and 36 ppt. Lactate showed an inverse relationship with salinity (15 > 22 > 36 ppt). No significant differences were observed in either triglycerides or total proteins among salinities. Changes in mRNA levels are shown in Figure 1. Expression of *prl* showed a significant inverse relationship with environmental salinity (15 < 22 < 36 ppt). Individuals exposed to 22 ppt showed significantly up-regulated *gh* compared to 15 ppt. No significant differences were detected for *pomca*, *pomcb*, or *sl*, but an up-regulation trend was observed for *pomca* with environmental salinity increase.

Table 1. Plasma glucose, lactate, triglycerides (TAG), and total protein in greater amberjack (*Seriola dumerili*) individuals raised under different salinities (15, 22, and 36 ppt), over the experimental period (77 days). Data are presented as mean ± SEM (n = 8-12). Different letters indicate significant differences among salinities ($P < 0.05$, one-way ANOVA followed by a Tukey's *post hoc* analysis).

Parameters	15 ppt	22 ppt	36 ppt
Glucose (mM)	12.81 ± 0.89 [a]	7.36 ± 0.77 [b]	10.44 ± 0.39 [a]
Lactate (mM)	5.20 ± 0.50 [a]	4.81 ± 0.30 [ab]	3.74 ± 0.23 [b]
TAG (mM)	2.01 ± 0.16	1.81 ± 0.20	1.87 ± 0.10
Proteins (mg dL^{-1})	295.65 ± 24.14	242.78 ± 9.17	262.30 ± 13.66

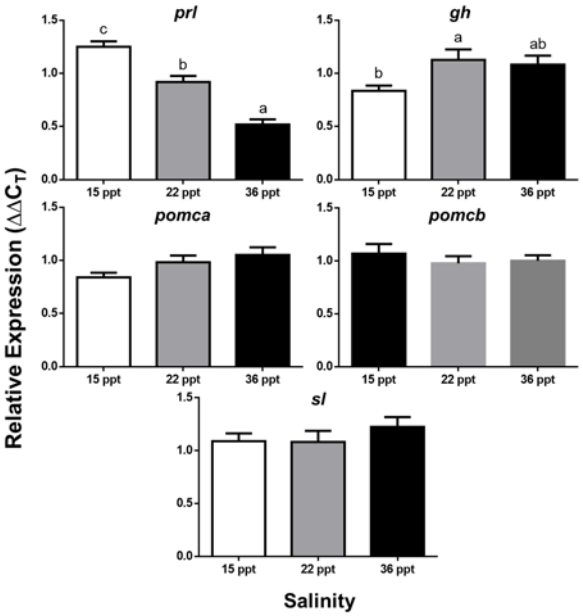


Figure 1. Relative expression of *prl*, *gh*, *pomca*, *pomcb*, and *sl* in greater amberjack (*Seriola dumerili*) individuals raised under different salinities (15, 22, and 36 ppt), over the experimental period (77 days). Data are presented as mean ± SEM (n = 12). Different letters indicate significant differences among salinities ($P < 0.05$, one-way ANOVA followed by a Tukey's *post hoc* analysis).

Our results could indicate a better culture condition under 22 ppt than at the other two salinities, supported by lowest plasma glucose levels and the highest hypophyseal *gh* mRNA expression in these individuals. However, an additional putative osmoregulatory role of GH should be taken into consideration. In contrast, lactate showed the lowest levels in animals exposed to 36 ppt. Similarly, *pomca* tendency presented an inverse relationship with lactate, suggesting a possible stress response with the increase in environmental salinity. In addition, the inverse relationship between *prl* expression and salinity confirmed the role of this hormone, in facilitating acclimation to low salinity environments (3).

Acknowledgements: This work was funded by Proyect "Diversificación de la Acuicultura Española mediante la optimización del cultivo de *Seriola dumerili*" JACUMAR 2016 (MAPAMA) and Fondo Europeo Marítimo y de Pesca (FEMP). N. Gilannejad is supported by a grant from MINECO (BES-2015-071662). A. Barany is currently supported by University of Cadiz Ph.D. scholarship (PIF UCA/REC02VIT/2014).

References

1. FAO 2016-2020. Cultured Aquatic Species Information Programme. Seriola dumerili. Cultured Aquatic Species Information Programme. Text by Jerez Herrera S., and Vassallo Agius R. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 1 January 2016.
2. Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: research0034.
3. Sakamoto T., McCormick, S. D. (2006). Prolactin and growth hormone in fish osmoregulation. General and Comparative Endocrinology, 147(1), 24–30.

SMART BIOSENSING DEVICE FOR TRACKING FISH BEHAVIOUR

E. Rosell-Moll[1], C. Piazzon[2], J. Sosa[3], M. Á. Ferrer[4], E. Cabruja[5], A. Vega[3], J. A. Calduch-Giner[1], A. Sitjà-Bobadilla[2], M. Lozano[5], J. A. Montiel-Nelson[3], J. M. Afonso[6], J. Pérez-Sánchez[1]

[1]Nutrigenomics and Fish Growth Endocrinology Group, Institute of Aquaculture Torre de la Sal (IATS-CSIC), Castellón, Spain. [2]Fish Pathology Group, IATS-CSIC, Castellón, Spain. [3]Institute for Applied Microelectronics (IUMA), University of Las Palmas de Gran Canaria (ULPGC), Las Palmas, Spain. [4]Technological Centre for Innovation in Communications (iDeTIC), ULPGC, Las Palmas, Spain. [5]Institute of Microelectronics of Barcelona (IMB-CNM, CSIC), Barcelona, Spain. [6]Aquaculture Research Group, Institute of Sustainable Aquaculture and Marine Ecosystems (IU-ECOAQUA), ULPGC, Las Palmas, Spain.

Biosensor technology for tracking individual challenged fish behaviour has the potential to revolutionize aquaculture, allowing farmers and breeders to orientate selective breeding towards more robust and efficient fish or improve culture conditions for a more sustainable and ethical production. The proposed solution within the AQUAEXCEL[2020] EU project is a stand-alone, small and light (1 g) device (AEFishBIT), based on a tri-axial accelerometer and a microprocessor. It is externally attached to the operculum to monitor physical activity by mapping accelerations in x- and y-axes, while operculum beats (z-axis) serve as a measurement of respiratory frequency. The conducted operculum attachment protocol does not show signs of tissue damage or growth impairment in active feeding gilthead sea bream. AEFishBIT offers a wide range of new information based on individual behaviour, allowing to point out the asynchrony of movements as an indirect measure of aging and adaptability to farming environment, as well as to discriminate different coping behaviour (proactive or reactive) of gilthead sea bream challenged with low water oxygen concentrations. AEFishBIT also provides reliable information of disease outcome in fish parasitized with an intestinal myxozoan, emerging as a powerful tool for sensing the quality of the environment and improving selective breeding protocols.

Introduction

The use of new technologies for individual and non-invasive monitoring of farmed fish is becoming a demand of the aquaculture industry (1). This offers the possibility of measuring variables that are directly or indirectly related with metabolic condition, health and welfare status (2). In order to track individual fish movement responses and associate them with proper farming conditions and welfare status, a biosensor device to provide reliable and simultaneous measurements of fish physical activity and respiratory frequency was designed within the AQUAEXCEL[2020] EU project. This smart biosensor (AEFishBIT) is composed of a tri-axial accelerometer, a microprocessor, a battery and an RFID tag for identification. The device is attached in the operculum using a fairly invasive procedure, and initial testing was conducted in gilthead sea bream and European sea bass in swimming metabolic chambers (3). The present work is aimed to further evaluate the impact of the biosensor attachment on fish welfare and growth, and to validate its functionality by analyzing biotic and abiotic factors in free-swimming fish.

Materials and Methods

AEFishBIT was used for recording and processing acceleration data from x-, y- and z-axes. Records of operculum breathing (z-axis) served as a direct measurement of respiratory frequency, whereas estimation of fish activity was derived from x- and y-axes signals. The final weight of the full packaged device is less than 1 g in air. The autonomy of the system in stand-alone mode is 6 h of continuous data recording with different programmable time schedules. AEFishBIT is protected by a registered patent (P201830305).

Devices were externally attached to the fish operculum using monel piercing tags with a flexible heat shrink polyethylene tube that is able to easily fit the device. The impact of this tagging procedure was evaluated in 1-year old gilthead sea bream that were fed once daily. Ten days after tagging, fish were weighed and specific growth rates (SGR) were calculated.

Functional validation was assessed in free-swimming gilthead sea bream to underline differences according to: 1) biological age (1- and 3-year old fish in 3000L tanks); 2) oxygen concentration (2-3 ppm O_2 for 2.5h in 90L tanks); 3) tank space availability (90L, 500L and 3000L tanks); and 4) disease progression in fish infected with the myxozoan parasite *Enteromyxum leei*. Devices were programmed for on-board calculation of respiratory frequency and physical activity over 2 min time windows each 15 min along two consecutive days. In all studies, rearing density was 9-14 kg/m^3, fish remained unfed over the recording time, and devices were retrieved after recording for on-board processed data download. Data analysis of physical activity and respiratory frequency was assessed through Student's t-test and Pearson coefficients. The daily rhythmicity of the time series analysis was analyzed by a simple cosinor model.

Results and Discussion

After 10 of intervention, biosensor attachment did not alter tissue integrity, feeding behavior and growth performance (SGR = 1.14 and 1.19 in tagged and non-tagged fish, respectively). Measures of respiratory frequency and jerk accelerations revealed age-related changes in basal metabolism and feeding behaviour (**Fig. 1**). According to this, the higher respiratory frequency (indirect measure of basal metabolism) in association with a more continuous physical activity are interpreted as a reliable measure of physiological age. Likewise, the occurrence of high jerk accelerations with decreasing tank size is informative of the welfare condition. In hypoxia tests, AEFishBIT also contributed to identify stress proactive fish that showed an increased size and increased physical activity for supporting escape reactions (**Fig. 2**). Finally, reduction of respiratory frequency measured by AEFishBIT was proven to provide reliable information of disease progression (parasitic enteritis) in fish parasitized with *Enteromyxum leei* (**Fig. 3**).

In summary, the present work is the proof of concept of a miniaturized device as a reliable tool for individual fish phenotyping of metabolic condition and welfare. The different behaviour patterns of free-swimming fish can be associated with a better performance or differences in stress and disease resilience. This opens new research opportunities for individual fish phenotyping of productive traits through the production cycle, but also for selective breeding in combination with other omics approaches (e.g transcriptomics, methylomics, metabolomics, microbiomics).

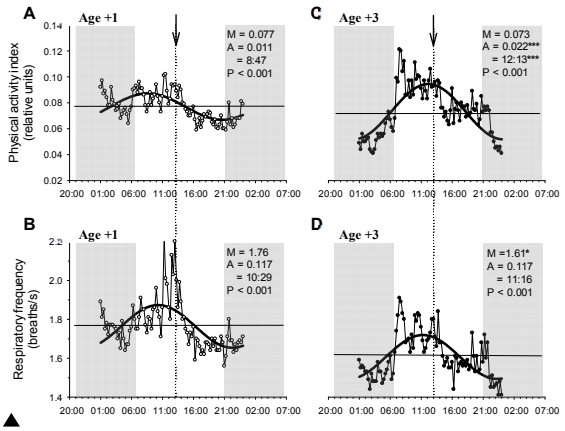


Figure 1. Biological age. Jerk accelerations (**A**, **C**) and respiratory frequency (**B**, **D**). Cosinor adjust of consensus data over the course of light and dark phases. Mesor (M), amplitude (A), acrophase (ϕ), and p-value (P) for the cosinor adjust are indicated. Asterisks represent significant differences between age +1 and +3 fish (*, P < 0.05; ***, P < 0.001, t-test). Arrow and dotted vertical line mark habitual feeding time. Mesors are represented by horizontal straight lines.

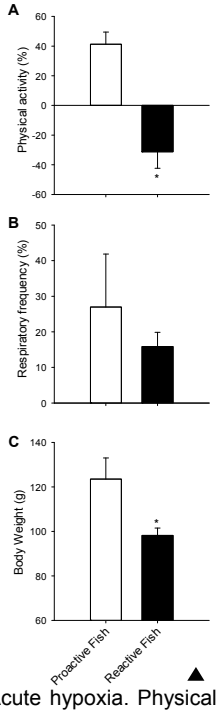


Figure 2. Acute hypoxia. Physical activity (A) and respiratory frequency (B) and body weight of proactive and reactive fish. Means ± SEM are represented. Changes of activity and respiratory frequency are calculated as a percentage of initial measures. Asterisks represent significant differences between proactive and reactive fish (P < 0.05, t-test).

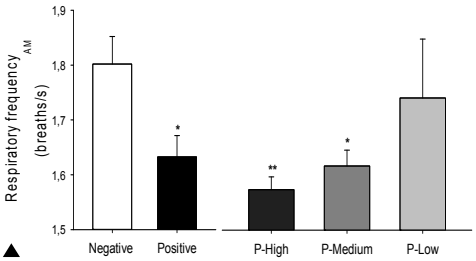


Figure 3. Parasite infection. The reduction of respiratory frequency (basal metabolism) correlates with the different progression stages of parasitic enteritis (P-, parasitized fish with different levels of intensity of infection). Means ± SEM are represented (n = 2-10). Asterisks represent differences with uninfected (Negative) group (P < 0.05, t-test).

Acknowledgments: The study has received funding from the European Union's Horizon 2020 research and innovation programme, GA no 652831 (AQUAEXCEL[2020]).

References

1. Føre M., Frank K., Norton T., Svendsen E., Alfredsen J. A., Dempster T., et al. (2018). Precision fish farming: A new framework to improve production in aquaculture. Biosystems Engineering 173, 176-193.
2. Neethirajan S., Tuteja S. K., Huang S. T., and Kelton D. (2017). Recent advancement in biosensors technology for animal and livestock health management. Biosensors and Bioelectronics 98, 398-407.
3. Martos-Sitcha J. A., Sosa J., Ramos-Valido D., Bravo F. J., Carmona-Duarte C., Gomes H. L., et al. (2019). Ultra-low power sensor devices for monitoring physical activity and respiratory frequency in farmed fish. Frontiers in Physiology 10, 667.

AUTHOR INDEX

www.aiecweb.org

www.ccmar.ualg.pt/page/aiec-2019

https://www.facebook.com/AIEC-Asociación-Ibérica-de-Endocrinología-Comparada-194675647226941