Comparative Biochemistry and Physiology, Part A 163 (2012) 272–277

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Plasma steroid hormone levels in female flounder *Platichthys flesus* and the influence of fluctuating hydrostatic pressure

A. Damasceno-Oliveira [a,b,*], B. Fernández-Durán [a,b], J. Gonçalves [a,b], E. Couto [c], A.V.M. Canário [c], J. Coimbra [a,b]

[a] CIIMAR/CIMAR-LA — Centro Interdisciplinar de Investigação Marinha e Ambiental, Laboratory of Ecophysiology, University of Porto, R. dos Bragas, 177, 4050-123 Porto, Portugal
[b] ICBAS — Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Lg. A. Salazar, 4099-003 Porto, Portugal
[c] CCMAR/CIMAR-LA — Centro de Ciências do Mar, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

ARTICLE INFO

Article history:
Received 24 October 2011
Received in revised form 2 August 2012
Accepted 2 August 2012
Available online 10 August 2012

Keywords:
Platichthys flesus
Hydrostatic pressure
Vertical migrations
Sex steroids
Reproduction

ABSTRACT

The reproductive cycle in teleosts is timed to guarantee that eggs hatch in the right place at the right time, with environmental factors playing important roles in entraining and controlling the entire process. The effects of some environmental factors, like temperature and photoperiod, are now well understood. There are only a few studies regarding the effects of hydrostatic pressure (HP) on the reproductive cycle, in spite of its importance as a ubiquitous factor in all biological environments and affecting all living organisms. Hydrostatic pressure is of particular importance in fish because they can also experience rapid and cyclic changes in HP due to vertical movements in the water column. The aim of the present research was to investigate the effects of vertical migrations on the reproductive steroids of maturing female flounder. After a 14 day exposure to cyclic hydrostatic pressure (with a period of 12.4 h and with a maximum peak of 800 kPa of absolute hydrostatic pressure), fish showed significantly lower plasmatic concentrations of "5β,3α" steroids, metabolites of the putative maturation-inducing steroid in flounder (17α,20β-dihydroxy-4-pregnen-3-one). Results indicate that environmentally realistic cyclic changes of hydrostatic pressure can influence the metabolism of reproductive steroids. This suggests a physiological role of tidally-associated vertical migrations, affecting oocyte maturation and retarding the reproductive cycle in this species until the spawning ground is attained.

1. Introduction

Marine fish regularly perform migrations in search for food, to overwinter or to spawn. During these movements, fish are faced with changes in environmental factors such as temperature, light, salinity or hydrostatic pressure (HP). These environmental cues activate specific sensory modalities, entraining and regulating several physiological mechanisms, including the reproductive cycle (Redding and Patiño, 1993).

It has been hypothesized that HP can affect the brain-pituitary-gonad (BPG) axis directly, implicating a role in the regulation of the gonadal development and maturation in different teleost fishes (Dufour et al., 2005; Jalabert, 2005). In some species, maturation occurs after movements between habitats and after being challenged with changes in environmental HP conditions, either increased HP and/or HP cyclic fluctuations (Tesch, 1978, 1989; Metcalfe et al., 1994; McCleave and Arnold, 1999). Fontaine et al. (1985) suggested that a long immersion (i.e., 4.5 megapascals, MPa, for 3 months) stimulates the pituitary gonadotropic function of the female European eel (*Anguilla anguilla*). In the same species, it is also known that HP can affect brain catecholamine content (namely epinephrine and dopamine) (Sébert et al., 1986). In European flounder (*Platichthys flesus*) application of cyclic hydrostatic pressure simulating vertical migrations can also modify brain neurotransmitters content (Damasceno-Oliveira et al., 2006; Damasceno-Oliveira et al., 2007). Furthermore, flatfish have been referred as particularly influenced by HP cycles (Gibson, 1982, 1984) and, although lacking a swim bladder, they can have a pressure sensitivity threshold as low as 10–20 cm (Tytler and Blaxter, 1973) making them a good model to study HP effects. However, studies are still scarce, and to our knowledge, there is only one report on the effects of HP on components of the BPG other than the brain (Sébert et al., 2007).

In teleosts, as in all vertebrates, the gonads produce steroid hormones which regulate gamete growth and maturation. A major role in the synthesis of sex steroids is played by thecal and granulosa cells surrounding the oocytes. Thecal cells synthesize 17α-hydroxyprogesterone (17-P) and testosterone (T) which are subsequently converted, respectively, to 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 17β-estradiol (E2) in the granulosa cells (Yoshikuni and Nagahama, 1991). 17,20β-P has been proposed as the most probable maturation-inducing steroid (MIS) in several flatfish species such as dab, *Limanda limanda* (Canario and Scott, 1990), plaice,

* Corresponding author at: Rua dos Bragas, 177, 4050-123 Porto, Portugal. Tel.: +351 223401800; fax: +351 223390608.
E-mail address: aol@ciimar.up.pt (A. Damasceno-Oliveira).

1095-6433/$ – see front matter
http://dx.doi.org/10.1016/j.cbpa.2012.08.001

Pleuronectes platessa (Canario and Scott, 1990; Vermeirssen et al., 1998), Dover sole, *Solea solea* (Scott and Canario, 1992) and Atlantic halibut, *Hippoglossus hippoglossus* (Vermeirssen et al., 2000), however, often the blood plasma levels of 17,20β-P are very low or undetectable (Scott and Canario, 1992). In contrast, 17,20β-P metabolites, such as 5β-pregane-3α,17,20β-triol (3α,17,20β-P-5β) have been identified in the urine and plasma of mature female plaice in relatively high levels (Canario and Scott, 1989; Scott and Canario, 1992; Scott et al., 1998). High levels of the 11-deoxycortisol metabolite 3α,17,21-trihydroxy-5β-pregnan-20-one (3α,17,21-P-5β) were also found in plasma (Inbaraj et al., 1997; Scott et al., 1997). However, all the 5β-reduced preganes identified in plaice have little oocyte maturation-inducing activity (Canario and Scott, 1990).

The present study was designed to test the effects of cyclic hydrostatic pressure on gonadal maturation and steroid levels in female flounder and if those effects are dependent on the phase of ovary maturity. For that purpose, and after urine steroid profiling, fish were subject to environmentally realistic cyclic hydrostatic pressure and the effect on reproductive parameters (i.e., oocyte maturation, E2, T, 17,20β-P and 5β-reduced metabolites) was analysed.

2. Materials and methods

2.1. Fish

Maturing but not ovulating European flounder, *P. flesus* Linnaeus, females were caught in the lower Douro River estuary (Portugal) in water depths of <5 m and transported in oxygenated 80-litre tanks to the laboratory (at the Centre for Marine and Environmental Research, Portugal). Fish were transferred and held in 2000-litre tanks with sand-substrate 3–4 days prior to the start of the experiments. Four other females were captured and reared under the same conditions for 2–3 months except they were fed on live *Nereis diversicolor*. These females were used in a preliminary screening to evaluate steroids potentially involved in oocyte hydration and ovulation, after histological examination to confirm the presence of hydrated oocytes. All animals were maintained and sampled in compliance with the Council of the European Communities Directive 86/609/EEC.

2.2. Experimental procedure

The experiment was repeated twice in order to assess the effects of bi-daily fluctuations in hydrostatic pressure on gonadal maturation and steroid levels in female flounder. This work sought to determine if the environmentally realistic fluctuating hydrostatic pressure could influence the final phase of ovarian maturity in flounder (i.e. on oocyte maturation, experiment 2). Additionally, this work also sought to define if the effects are restricted to that particular phase or, on the other hand, other phases are influenced, namely the late vitellogenesis phase, revealing a general effect on steroidogenesis (experiment 1).

In experiment 1 twelve females with an average body mass of 541 ± 37.9 g (mean ± s.e.m.) and in experiment 2 ten females with an average body mass of 546 ± 41.4 g (mean ± s.e.m.) were used. In the present study, fish were sorted by using a procedure based on the method for staging maturing plaice described by Scott et al. (1998). In experiment 1, females with protuberant ovaries but with their distal ends only distinguished by touch were selected. In experiment 2, females with totally swollen ovaries and with their distal ends easily observed near the caudal peduncle were selected. In both experiments, only fish in which hydrated oocytes could not be expressed by applying gentle pressure, and thus had not yet started ovulating, were used.

In each experiment, fish were randomly selected and transferred to a hyperbaric chamber (with a volume of 153 L), part of a computer-controlled pressurizing system enabling the prolonged simulation of cyclic or constant hydrostatic pressure. In a previous study (Damasceno-Oliveira et al., 2004), we demonstrated that by using this flow-through pressurized system both experimental (HP) and control fish were submitted to similar conditions of photoperiod, temperature, dissolved gases and water quality. Furthermore, it was also demonstrated that exposing flounder (*P. flesus*) to hydrostatic pressure conditions herein described do not cause a significant stimulation of the hypothalamus-pituitary-interrenal axis, with no elevation on plasma or biliary levels of corticosteroids (including cortisol) or their main metabolites (Damasceno-Oliveira, 2007). We used this system to simulate a tidally associated vertical migration to a maximum depth of 80 m, and with a semi-diurnal pressure cycle of 600 kilopascals (kPa) amplitude (between 200 and 800 kPa) and 12.4 h period. Six female flounder in experiment 1 and 5 female flounder in experiment 2 were submitted to these experimental conditions. Control fish (n = 6 in experiment 1 and n = 5 in experiment 2) were maintained at 110 kPa (c. 1 atm) of absolute hydrostatic pressure (i.e., simulating sea surface conditions) in a similar hyperbaric chamber. The water temperature was maintained at 14.5 ± 0.5 °C and a 12 h : 12 h light/darkness cycle was used for the 14 day duration of the experiment. All experiments were ended between 10:00 and 14:00 h, with the pressurized system being decompressed at 350 kPa·min^{-1}. All the fish were rapidly netted and anesthetized by immersion in benzocaine (60 mg·L^{-1}).

2.3. Sampling

Immediately after anesthesia, flounder were weighed, measured and blood was collected from the caudal vessels with heparinized syringes. Blood was transferred to heparinized tubes and centrifuged (10 min, 800 g). Plasma was collected and stored (−80 °C) in Eppendorf tubes until steroid assays were performed. Immediately following blood collection, fish were killed by severing the spinal cord. The gonads were dissected out and a section of the mid-part of the left ovary was fixed in Bouin, embedded in paraffin, sectioned at 7 μm with a Reichert–Jung (model 2030) microtome, stained with haematoxylin and eosin and examined by light microscopy. Ovarian developmental stage was classified according to the criteria of Janssen et al. (1995) and Monteiro (2000).

2.4. Thin-layer chromatography (TLC)

A preliminary screening evaluated the occurrence of potential maturation-inducing steroids in female maturing/ovulating female flounder. Urine from 4 mature females with hydrated oocytes was collected by urinary bladder puncture with a syringe and pooled (50 μL × 4). Urine samples were first extracted with Sep-Pak cartridges C_{18} preactivated with methanol (2 × 2 mL) followed by 2 mL of distilled water. The free and conjugated steroids were eluted with ethanol, centrifuged for 4 min at 2000 g, collected into glass tubes and evaporated on a speed vacuum concentrator at 40 °C.

For the assay of free steroids, the dry residue was redissolved in 200 μL of distilled water and extracted with diethyl ether (2 × 3 mL). The organic phase was dried at 30 °C under a stream of nitrogen and the residue redissolved in 70 μL of dichloromethane. For the assay of sulphated steroids, the aqueous phase was dried and 1 mL of trifluoroacetic acid (TFA)/ethyl acetate (1/100, v/v) was added to each glass tube containing the dry residue and tubes were placed in a water bath for 24 h at 40 °C with agitation. After this period, the solution was evaporated at 40 °C under a stream of nitrogen and the residue was redissolved in 500 μL sodium acetate buffer (0.1 M, pH 5.0). Free steroids generated by solvolysis of the sulphates were extracted with diethyl ether (2 × 3 mL). The extract was evaporated at 30 °C under a stream of nitrogen and the residue redissolved in 70 μL of dichloromethane. The sodium acetate phase was incubated overnight at 37 °C with 10 μL of bovine liver β-glucuronidase (Sigma). Free steroids generated by enzyme hydrolysis of the