Walquiria da Silva Pedra Parreira

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Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo (IB/USP) para obtenção do título de Mestre em Ciências, na Área de Fisiologia Geral.

Orientadora: Prof. Dra. Renata Guimarães Moreira Whitton

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Orientadora

DEDICATÓRIA

Dedico este trabalho a todos aqueles que estudam reprodução e endocrinologia de peixes e também aqueles que estudam ambos os campos em nível comparativo, cujos esforços, publicações e dados prévios, permitiu a condução deste trabalho. Dedico também a todos que de maneira direta ou indireta contribuíram para a realização desse trabalho. Por fim, desejo que este trabalho sirva como ferramenta e contribuição para estudos futuros, assim como os anteriores serviu para que este pudesse ser concluído.

E mesmo que só encontre espinhos em sua jornada, siga adiante,

pois mesmo que a estrada pareça ser muito longa e tortuosa, ela

sempre chega ao seu final e traz consigo o alívio do dever

cumprido, que apenas aqueles que não desistiram - apesar de todas

as intempéries - poderão alcançar .

(Walquiria da S.P.Parreira, 2015)

As nuvens mudam sempre de posição, mas são sempre nuvens no céu.

Assim devemos ser todo dia, mutantes, porém leais com o que pensamos e sonhamos;

lembre-se, tudo se desmancha no ar, menos os pensamentos.

(Paulo Beleki)

Não se pode aprender nada de uma lição que não seja acompanhada por dor, já que não se pode conseguir nada sem um sacrifício.

Mas quando você aguentar essa dor e a supera, as pessoas conseguem um coração forte que não perde para nada. Sim, um coração de aço.

(ArakawaHirmou)

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CORTICOSTEROIDS AS INDUCTORS OF OVULATION IN *Astyanax altiparanae* (CHARACIFORMES: CHARACIDAE) DURING ARTIFICIAL REPRODUCTION

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Stress can be defined as a condition in which the dynamic equilibrium of the body is disturbed and several studies have demonstrated the action of some corticosteroids modulating stress situations, such as reproduction. However, the role of corticosteroids in fish reproduction can be either positive or deleterious, depending on many variables. In the *Astyanax* genus, the same stress stimulus can successfully induce the reproduction in *Astyanax altiparanae*, but not in other species of the same genus. Therefore, the aim of this study was to analyze cortisol and progestogens plasma levels and receptor gene expression in the ovaries of *A. altiparanae*, a teleost species that successfully reproduce using crowding and water level drawdown as stimulus. Three experimental groups were considered: Stress (high stocking density and water level drawdown); human chorionic gonadotropin (hCG, a positive control); and Saline (a control group). Females were sampled at three different moments of the artificial reproduction experiment, initial, intermediary and at spawning. Indices of spawning performance as, fertilization rate, Gonadosomatic Index (GSI), as well as histological analysis of the ovaries were carried out. 17 α -hydroxiprogesterone (17 OHP) and cortisol (CORT) levels were measured in plasma by ELISA. Additionally, the ovarian gene expression of CORT and 17 OHP receptors (CR and PR) were carried out by Real-time quantitative PCR. Females from Stress and hCG groups released a larger volume of eggs when compared with females from the Saline (Control) group. Females from Stress group presented a decrease in GSI and ovaries mass at spawning, and many post ovulatory follicles (POFs) were present in the ovaries after spawning. At the intermediary sampling, plasma levels of CORT increased in females from the Stress group, but not in the hCG and Saline groups. 17 OHP levels did not change during the experiment. PR gene was more expressed in females from hCG group throughout the experiment, but CR gene expression did not change. We suggest a possible

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role of CORT as an inducing agent in ovulation and/or spawning of *A. altiparanae*. CR and PR did not seem to be the candidates for triggering ovulation, so the relationship between the derivative of progesterone (*17,20DHP*) should be deeper studied, as well as the role of CORT and different progestagens in *A. altiparanae* reproduction.

Resumo

O estresse pode ser definido como uma condição na qual o equilíbrio dinâmico do corpo é perturbado e muitos estudos têm demonstrado a ação de alguns corticosteroides modulando situações de stress, tal como a reprodução. No entanto, o papel dos corticosteroides na reprodução de peixes pode ser positivo ou deletério, dependendo de muitas variáveis. No gênero *Astyanax*, o mesmo estímulo de estresse pode induzir com sucesso a reprodução em *A. altiparanae*, mas não em outras espécies do mesmo gênero. Portanto, o objetivo deste estudo foi analisar os níveis de cortisol e progestágenos no plasma e a expressão dos receptores destes esteroides nos ovários de *A. altiparanae*, espécie de teleosteo que consegue se reproduzir com sucesso usando o aumento da densidade e variação no nível da água como um estímulo. Três grupos experimentais foram considerados: Estresse (alta densidade de estocagem e intenso fluxo de água); Gonadotropina Coriônica Humana (hCG, um controle positivo); e Salina (grupo controle). As fêmeas foram amostradas em três diferentes momentos do experimento de reprodução artificial, inicial, intermediário e na desova. Índices de desempenho de desova como, taxa de fertilização, Índice Gonadosomático (IGS), bem como a análise histológica dos ovários foram considerados. Os níveis de *17 α -hydroxiprogesterone* (17OHP) e cortisol (CORT) foram medidos em amostras de plasma por ELISA. Além disso, a expressão de genes de receptores de CORT (CR) e 17 OHP (PR) foram analisadas nos ovários por qPCR. Fêmeas dos grupos Estresse e hCG desovaram um volume maior de ovos quando comparadas às fêmeas do grupo Salina (controle). As fêmeas do grupo Estresse apresentaram também uma redução nos valores do IGS e na massa dos ovários no momento da desova, e muitos folículos pós - ovulatórios estavam presentes nos ovários após a desova. Na amostragem do período intermediário, os níveis plasmáticos de CORT aumentaram nas fêmeas do grupo de Estresse, mas não nos grupos de hCG e Salina. A concentração de 17OHP não se alterou durante o experimento ou entre os grupos. O PR foi mais expresso em fêmeas do grupo hCG ao longo do experimento, e a expressão do CR não

se alterou. Sugerimos um possível papel de CORT como um agente indutor na ovulação e / ou de desova de *A. altiparanae*. No entanto, CR e PR não parecem ser os candidatos para desencadear a ovulação, de modo que a relação entre receptores derivados PR (17,20DHP) devem ser mais profundamente estudados, bem como a efetiva ação de CORT e diferentes progestágenos na reprodução de *A. altiparanae*.

ABBREVIATIONS USED IN THIS STUDY

#	
17α,20β DHP	<i>17α,20β- dihidroxy-4-pregnen-3-one</i>
17 OHP	<i>17 α hidroxyprogesterone</i>
E2	<i>17β-estradiol</i>
CORT	<i>Cortisol</i>
CR	<i>Corticosteroid Receptor</i>
DA	<i>Dopamine</i>
ER	<i>Estrogen receptor</i>
FSH	<i>Follicle-Stimulating Hormone</i>
GABA	<i>Gamma-AminoButyricAcid</i>
GnRH	<i>Gonadotropin-Releasing Hormone</i>
GnIH	<i>Gonadotropin-Inhibitory Hormone</i>
HHG	<i>Hypothalamus-Pituitary-Gonads</i>
PROG	<i>Progesterone</i>
hCG	<i>Human Chorionic Gonadotropin</i>
KiSS	<i>Kisspeptine</i>
LH	<i>Luteinizing Hormone</i>
MIS	<i>Maturation Induction Steroid</i>
Neg. control	<i>Negative control</i>
PCR	<i>Polimerase Chain Reaction</i>
PERIN	<i>Perinucleolar</i>
POF	<i>Post ovulatory follicle</i>
PR	<i>Progesterone Receptor</i>
RT PCR	<i>Real time quantitative PCR</i>
VTL	<i>Vitellogenic</i>

Introduction

Reproduction and environmental modulation

Reproduction in fish is neuro-modulated by external factors such as photoperiod and temperature. Once the animal has reached the minimum biological conditions to start it, the external stimuli are received by receptor organs, converted into electrochemical signals, and then, they stimulate the synthesis and release of many hormones, including the gonadotropin-releasing hormone (GnRH), the key neurohormone controlling the Hypothalamus-Pituitary-Gonads (HPG) axis in fish, which stimulates the pituitary gonadotropic cells to initiate the synthesis and release of FSH (follicle-stimulating hormone) initiating the process that leads the conversion of cholesterol to testosterone (Millaet *al.*, 2009). This process, modulated mainly by GnRH is also mediated by kisspeptin (*Kiss*), the product of the gene *Kiss1*, also a key hormone in the regulation of reproductive functions in fish (Zohar *et al.*, 2010).

When environmental conditions and the combined hormonal stimuli lead the reproductive cycle without inhibitory effects, testosterone is transported to the granular cells, and aromatized to 17 β -estradiol (E2), which acts in the hepatocytes, stimulating the synthesis of vitellogenin, a phospholipoglycoproteins, which via the bloodstream, is captured by the oocytes via micropinocytosis, thus promoting growth of the oocyte and the incorporation of the yolk. In response to a sharp production of E2, there is a negative feedback to FSH and together with the action of GnRH stimulates the secretion of LH, which, once bounded to its receptors in the granulosa cells, initiating the process of final maturation and ovulation (Lubzenset *al.*, 2010).

This whole cycle can be regarded as a very sensitive stage to environmental stimuli and may even present differences according to the stress levels of individuals that have undergone variants, such as reproductive migration in some species, temperature, photoperiod or rainfall that can reduce circulating levels of sex steroid hormones. The stimuli capable of generating high levels of stress can also produce different responses in different stages of maturation, affecting the quality of gametes and influencing the offspring (Weedelar-Bonga, 1997; Lubzenset *al.*, 2010).

Stress and reproduction

According to Wendeelar-Bonga (1997), “*stress can be defined as a condition in which the dynamic equilibrium of the body, or homeostasis, is threatened or disturbed due to the stressors called stimulus action*”. The action of stressors is dual: they can produce effects that threat or disrupt the homeostatic balance, as may, however, cause a number of behavioral and physiological responses as compensatory and / or adaptive action, enabling the animal to overcome the threats.

If an animal is subjected to an intense and constant stress, the physiological response may lose its adaptive value, causing permanent damage to their health and well-being (Carmichael, 1984). The central adaptation to stress is the relocation of substrates for activities that require enhancement to restore homeostasis, such as respiration, locomotion, hydromineral balance and tissue repair, instead of high energy demand activities such as growth and reproduction (Mommsen *et al.*, 1999). Such dynamics can significantly reduce animal performance, both during forward recovery phase to an acute stress and in chronic stress (Mommsen *et al.*, 1999). Faced with such situation, an animal can quit a programmed step, such as reproduction, leading to immediate survival. This situation is often seen in fish farms where the maintenance of animals in captivity, management, among other routine activities may interfere with the reproductive performance of many species. As an example, the case of *Anguilla anguilla*, whose oocyte development is prevented in captivity (Milla *et al.*, 2009).

The modulation of stress reactions is directly related to the action of corticosteroid hormones, and several studies have shown a possible duality of functions. Stressful situations can inhibit the reproductive performance of fish (Small, 2004; Milla *et al.*, 2009) causing adverse effects in this process, due to the action on the HHG axis; for example, the effects of cortisol, the main corticosteroid in teleosts, in the production of gonadotropins and vitellogenesis (Small, 2004; Milla *et al.*, 2009). When exposed to severe stressful situations or long term, fish have disrupted their reproductive processes due to decreased pituitary activity, especially in relation to the synthesis and release of FSH and LH (Carragher *et al.*, 1989; Norris and Hobbs, 2006), and consequently to a decreased synthesis

of sex steroid hormones. This reduction in the concentration of steroids may occur due to the competition for common substrate (cholesterol) of sex steroids and corticosteroids (Fig. 1) (Pickering *et al.*, 1987; Milla *et al.*, 2009), and may also result in reducing the size of eggs and quality of larvae (Campbell *et al.*, 1994 Mylonasetal., 2010). In this sense, hormonal interference is needed to provide the final maturation and ovulation in teleosts, a routine practice in fish farming (Zohar and Mylonas, 2001).

Figure 1: Synopses of the probable pathways of corticosteroid biosynthesis in female teleost. Solid black arrows show that the enzyme catalyzing the steroid metabolism is active in the gonad; the grey arrows indicate that the enzyme required for that step is expressed in the gonad but its activity needs to be confirmed. Unfilled arrow indicates lack of clear information. (Adapted by Millaet *et al.*, 2009).

Conversely, in cases such as eel (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*), glucocorticoids, hormones commonly associated with stress, were used to induce a LH peak in the early sexual maturation (Millaet *et al.*, 2009). *In vitro* studies have shown the ability of corticosteroids to induce oocyte maturation in freshwater species such as catfish *Heteropneustes fossilis*, supporting the idea that corticosteroids can have direct and / or indirect action to stimulate the final maturation (Rahman *et al.*, 2001).

The role of steroid hormones in fish farming

The attempt to make fish farming a more profitable activity and the need to understand the mechanisms behind reproductive impairment in cultivated species triggered many studies trying to measure the influence of stress in the final stages of reproduction in teleost species in which disruptions in captive breeding have been reported (Milla *et al.*, 2009). Additionally, when in captive conditions (in fish farms), the natural challenges for fish are mainly related with the management imposed by the activity, such as the maintenance and transportation of breeding/juveniles, prophylactic and/or effective treatments, high densities of storage, among others. The sum of the physiological changes triggered by chemical, physical and/or biological challenges, are commonly referred to as stress responses (Wedemeyer *et al.*, 1990). In this context, the literature has shown that within fish farms, stressful situations, even though routine, such as the handling of animals, can directly affect reproductive performance, the quality of spawning or even development of the progeny (Campbell *et al.*, 1994).

Diadromous fish species need to migrate in order to find a satisfactory environment to spawn. But, restricted in tanks, they need to be manipulated and in some cases, hormonal manipulations are necessary to trigger the final steps of the reproductive cycle. The investigations of these fish species are based mainly on the studies of the plasma profile of sex steroids, an information that according to some authors, could indicate a failure in the final maturation and consequently a dysfunction in the production of progestogens (Zohar and Mylonas, 2001; Baldisserotto and Gomes, 2005; Mylonas *et al.*, 2010). In this sense, hormonal therapies are required to provide the final maturation and ovulation in Neotropical migratory species. Thus, several recent studies have attempted to measure the influence of captivity during the reproductive cycle in Neotropical fish species with blocked reproduction in captivity, mainly the potamodromous species, as *Prochilodus lineatus* (Hainfellner *et al.*, 2012; Souza *et al.*, 2014), *Salminus hilarii* (Honji *et al.*, 2011, 2013; Moreira *et al.*, 2015), *Steindachneridion parahybae* (Honji *et al.*, 2013, 2015) and *Piaractus mesopotamicus* (Kuradomiet *et al.*, 2015) among others.

Corticosteroids receptors and reproduction

In some fish species, as eel and brown trout, glucocorticoids were used to induce LH synthesis at first sexual maturation, supporting the idea that corticosteroids may exert direct and/or indirect action in stimulating the final maturation (Millaet *al.*, 2009). This feature is based on recent studies that point to a possible promiscuous action between progestagen and glucocorticoid receptors (EickandThornton, 2011), which could establish a link between understanding the mechanism of hormone action in ovulation and spawning of fish species in captivity, diminishing the use of synthetic hormones. Therefore, studies with species in which reproductive response to stress conditions are positive become interesting for understanding the mechanisms of adjusts of this animal, as well as the route of stimulation of ovulation by means of corticosteroids. Based on this context, there is a native potamodromous fish species *A. altiparanae* that is a good model for these studies, because crowding and/or water level drawdown stimulates ovulation and/or spawning in this species (Chehadeet *al.*, 2015), making possible the understanding of the relation between corticosteroids and reproduction in captivity teleost species, since studies with neotropical species are still scarce.

Experimental species

The yellow tail "Iambari", *Astyanax altiparanae* (Britski, 1964) Characiformes, Characidae family, is considered one of the most important fish species in the food chain of the rivers of South America, where it plays a secondary role in relation to those identified as noble species (for example: *Pseudoplatystomacorruscans*, *Pseudoplatystomafasciatus*, *Salminusbrasiliensis*, *Bryconamazonicus*, *Brycon insignis*, among others) (Baldisserotto and Gomes, 2005). This species belongs to a large group of migratory species, which represents the largest number of fish species in Brazil (Britski, 1972). The genus *Astyanax* can be considered the most representative of the Tetragonopterinae subfamily, being dominant in South America, with approximately a hundred species (Garutti and Britski, 2000).

This genus, in general, is composed by reophilic species that are small and characterized by having small oocytes, high relative fecundity, rapid development, external fertilization and no parental care (Suzuki *et al.*, 2005). *A. altiparanae* is known by the high biological plasticity, and is becoming a potential fish model for scientific studies about

reproduction and stress modulation, since it presents satisfactory results for reproduction in conditions considered stressful for other species within the same genus. *A. altiparanae* females can be induced to ovulation by water level drawdown and consequently increasing stocking density (Chehade *et al.*, 2015). Considering that cortisol levels increase at stressing situations and the known fact of receptor promiscuity between corticosteroids and progestogens, we hypothesize that this ovulation/spawning protocol can be considered a stressor in *A. altiparanae* females, triggering cortisol release and that, this corticosteroid can act as the Maturation Induction Steroid (MIS) in *A. altiparanae*, binding to progesterone receptors (PR).

Objectives

The main objective of this study is to gain some expertise on the main physiological steps involved in *A. altiparanae* reproduction, in which a supposed stress situation triggers ovulation and spawning. This information is important in the fields of reproductive physiology, genetics and endocrinology of reproduction, which are important areas of fish breeding technology that still needs to be developed in Brazil.

2.1 Specific objectives:

- Evaluate the reproductive performance of *A. altiparanae*, using different artificial reproduction protocols, including crowding as the ovulation inducer;
- Evaluate the role of corticosteroids and progestogens during the artificial reproduction of *A. altiparanae*;
- Evaluate the role of corticosteroid and progestogens receptors in triggering ovulation and spawning in *A. altiparanae*.

MATERIAL AND METHODOS

*Experimental procedure for captive breeding of *Astyanax altiparanae**

The experimental design was carried out at the Fish Farm of the Electric Company of São Paulo - CESP, Paraibuna (São Paulo, Brazil) (Fig.2). In December and January 2013 we sampled adult males and females (Fig.3) of *A. altiparanae*, born, raised and collected in tanks in the Hydrobiology and Aquaculture Unit of CESP. Broodstocks were kept in 4 tanks (200m²), fed with commercial diet with 15% of crude protein, distributed in the amount of 1.5% of the total biomass per day, divided in two daily treatments (morning and afternoon), 3 times a week (Monday, Wednesday and Friday) throughout the year. During the experiment, in January 2013, 225 males and 155 females with total length of 12 to 16 cm and body mass of 34 to 45g were selected in nurseries by trawl and transported with buckets and aerators to indoor tanks with 0.86m³ located in the laboratory, according to the method described by Chehadeet *al.*, (2015) for induced spawning. It should be emphasized here that the procedures to sacrifice performed in this study are according to the protocol 171/2012, the Institute of Biosciences of the University of São Paulo Animal Ethics Committee.

Figure 2: Fish Farm used for the experiments- Paraibuna - CESP.

Figure 3: Female of *Astyanax altiparanae* (total length).

The animals were divided into three tanks of 865 L (0.86m³) (75 males and 50 females/tank) supplied at a flow rate of 10L of water/minute. At the time of distribution of animals in experimental tanks, an initial sampling was carried out (time 0h) of 5 females, free of any hormonal or manipulative action. The experiment was divided into initial sampling of biological material (0h induction time of reproduction), 8h (initial), 12h (intermediate) and 24h after induction (spawning) calculated based on the previously knowledge of spawning timing of the group induced to spawn by water level drawdown (Chehadeet *al.*, 2015). After the initial sampling of females, the animals were divided in the tanks and given the following treatments:

-Stress (E): for animals whose spawning was performed only by a high density stimulus, coupled with the increased water flow into the tanks being supplied by a recirculation of 10 liters per minute, while the water level in the tank was kept low (water level drawdown). This group corresponds to the protocol routinely successfully developed at CESP;

-hCG (H): animals with a spawning protocol associated with hormonal stimulation with human chorionic gonadotropin (hCG), without interference from high density as the water volume of the tank was not lowered and the water flow was not incremented;

-Saline (S):the animals underwent the same manipulation of injections, but containing physiological saline (0.9% NaCl).

The hCG (Ovidrel[®]) was administered in females from the H group in the concentration of 5UI/g as a single dose in the lower region of the pectoral fin, quickly, not

allowing the animal to remain for a long period out of the water. The application was about twelve hours (12h) after the beginning of the experimental group E, due to the faster action of this glycoprotein. The saline was applied to the females of S group, in the same region of the fin, using the same volume used for hCG (180 μ L/female). Soon after this procedure, all the females were placed in their tanks, together with the males. During the period of twenty four hours after the induction, females were collected in each experimental group, respecting the proposed periods (initial, intermediate and spawning), to be possible to follow the time of ovulation in the females from the different groups. The males did not receive any treatment, since they produce sperm in captivity.

After spawning, the eggs were siphoned from the tank bottom to be quantified and transferred to conical incubators (200L), with 1620 liters per hour of water circulation. After hatching, the larvae were maintained for a few days in the incubators until they were transferred to the outdoor tanks (200 m²). To assess the viability of the eggs in each experimental group three aliquots of eggs were collected per group to estimate the percentage of fertilized eggs (number of fertilized eggs x 100/ total number of eggs) (Vazzoler, 1996). To measure the amount of eggs, they were siphoned, allocated in 1L beakers, allowed to separate the water phase, and then, the number of eggs were counted in subsamples and extrapolated for the total volume of water. To calculate the efficiency of each treatment, a calculation was performed based on the values obtained in the fertilization rate (average weigh between the values obtained on the fertilization rate) plus the standard deviation within the group.

Monitoring the physicochemical parameters of water

To ensure that the water conditions were established during the experiment, pH, dissolved oxygen, temperature, total dissolved solids (TDS) were monitored throughout the experiment to exclude a possible abiotic interference on the results. Data were collected over the 24 hour experiment with a Multiparameter probe (YSI, Pro Plus) and the pH was monitored with a pH meter Gehaka.

Sampling

At the time of each sampling (initial, intermediate and final), the animals were anaesthetized with benzocaine (1g benzocaine in ethanol 92%), which solution was then diluted with 10L water. After anesthesia, the blood was collected from the caudal vasculature

(Fig. 4) using disposable 1 ml syringes, heparinized (Hepamax[®]) and centrifuged for five minutes at 3000 rpm to separate plasma from the cellular fraction of blood.

Figure 4 - Blood collected from the caudal vasculature of *A. altiparanae*.

Plasma was aliquoted in *eppendorfs* and frozen in liquid nitrogen until transportation to a -80⁰C freezer. After blood collection, fish were measured, weighed, the ovaries were removed (Fig. 5) and weighed to calculate the gonadosomatic index $GSI = (\text{total mass of the ovaries} / \text{total body weight}) \times 100$] (Vazzoler, 1996).

Figure 5: Ovaries collected at the beginning and after 24 hour of the experiment.

After this procedure, a sample of the middle third of the ovarian lobes were collected and fixed in Bouin's solution to ascertain the presence of postovulatory follicles (POF) and other stages of development of gametes. The histological method (i.e., the presence of POF)

was used as a tool to calculate the percentage of the response to ovulation with the different induction protocols. A small piece of ovary was also collected and conserved in *RNAlater*, in a ratio of ½ centimeter of animal tissue, dipped in 10 ml *RNAlater* to be used in the molecular analysis.

Hormonal analysis

The gonadal steroid 17 α -hydroxyprogesterone (17OHP) and cortisol (CORT) were measured in *A. altiparanae* plasma by the method of enzyme-linked immunosorbent assay (ELISA) using a commercial kit (*IBL*[®]). Afterwards, a plasma extraction protocol, separating the protein and lipid fractions (to use only the lipid fraction, which contains the steroid hormones) was applied. Various dilutions were initially tested to ensure that the values obtained for both hormones fit the kit standard curve. To perform the extraction, the samples were removed from the -80°C freezer, plasma proteins were precipitated with ethyl ether in the ratio 1:3 (plasma: ethyl ether) and centrifuged at 1800 rpm for 9 minutes at 4°C. Immediately, after being centrifuged, the samples were placed in the -80°C freezer for 7 minutes to ensure that all the protein was decanted. Finally, the supernatant was transferred to another clean tube, dried with nitrogen and kept at 4°C until use. After the tests, a dilution factor was established for each hormone, 1: 4 for CORT and 1: 3 for 17 OHP. The steroid analysis was performed according to the manufacturer's instructions, using plasma samples in duplicate. The plates were read in a Spectra Max 250 spectrophotometer (*Molecular Devices*) at a wavelength of 450nm.

Histological analyses of ovaries

We removed the gonads from the fish, fixed them for 20-24 hours in Bouin's solution, and dehydrated them with an increasing series of ethanol dilutions. Subsequently, the ovaries were transferred to xylene (*dimethyl benzene*) and infiltrated with Paraplast (View Plast, Erviegas Surgical Instruments Ltd.), following the methodology proposed by Behmeret *al.*, (1976). Then 5 μ m sections were performed with a microtome (Leica RM / 2255 equipped with support for disposable blades) and mounted on slides with polylysine (*Poly Lysine Solution* INS *Sigma* *Diagnostics*, St. Louis MO USA.) diluted at 1: 5 in distilled water, to assist the fixing of the cutting blades. Ovaries sections were stained with hematoxylin-eosin, and then again dehydrated and finished with cover slip and Damar gum. We analyzed and

documented the prepared sections in a computerized system for image capturing (transmitted light microscope - Leica DM 1000; camera - Leica DFC 295, and image capture program - Leica Application Suite Professional, LAS V3.6).

A pattern was established using four fields of view in each set of four sections per slide in a total of five blades per animal for analysis, a total of eighty fields of view per animal. The sections were analyzed and documented, using a computerized image capture (transmitted light microscope Leica DM 1000, equipped with camera Leica DFC 295, and image capture program *Leica Application Suite Professional*, LAS V3 .6). From the distribution and observed cell types, we assembled a table to identify/quantify the maturity stage of groups throughout the experiment. This method was performed according to Gomes *et al.*, (2014). For each symbol (+) added, giving a greater frequency of a cell type, observed within the group, at different times throughout the experiment, as follows: (-) absent; (+) present; (++) frequent; (++++) predominantly, to cells observed in the fields analyzed in the respective group. The classifications of gamete maturation stages as well as the identification of postovulatory follicles were performed according to Grier (2007).

Molecular analysis

Assuming that there was no previous data about the target genes (Cortisol and Progesterone receptors) in *A. altiparanae*, the polymerase chain reaction (PCR) for amplification of receptors of cortisol (CR) and progesterone (PR) cDNA from *A. altiparanae* was performed using degenerate primers (more details about the techniques on the annex section). For this goal, we used the preserved tissue on *RNA later* to extract DNA and estimated which species could be close enough to start the analysis. The molecular techniques described below were initiated at Dr. Carlos Strussmann's laboratory, at the Department of Applied Marine Bioscience, at Tokyo University of Marine Science and Technology, Japan (FAPESP/BEPE 2013/26563-1). The analysis was carried out using GENETYX[®], an analysis program responsible for selecting species for alignment, and creation of BLAST (Fig. 6). Based on this program, we designed the degenerate primers for the CR based on the nucleotide sequences of *Salmo trutta* (accession number: AY863149), *Danio rerio* (access number: BX276106), *Astyanax mexicanus* (access number: XM_007247018.1) and *Dicentrarchus labrax* (accession number: AY619996).

Figure 6: GENETYX© choosing the closest sequences for alignment, separating by size and percentage match for analyses with Cortisol receptor.

Considering that the sequence of nitrogenous bases for CR is very conserved among species, compatible species were found in the BLAST genetic alignment. The same situation was found for Beta-Actin, where *Danio rerio* and *Odontheistesbonariensis* (access number: EF026002.1 and EF044319.1 respectively) where used to design the primers. Unlike the genes mentioned above, the PR was relatively complex, since the number of inserts present in the sequence provided sixty four combinations failures before of functional primers for receptor I, show magnification in the samples. For PR we used an alignment among *Anguilla japonica* (AB032075), *Danio rerio* (NM_001166335) and *Astyanax mexicanus* (XM_007231093). Otherwise, for the receptor associated with the MIS (Maturation Inducing Steroid), 17α - 20β DHP (17α , 20β - dihydroxy-4-pregnen-3-one), the alignment showed a common profile among the species, to be more conserved than the Receptor I. We used the following species: *Carassiusgibelio* (Access number: AB934933), *Danio rerio* (Access number: FJ409244), *Astyanax mexicanus* (Access number XM_007231093). After analysis with the GENETYX© program, we have chosen the degenerate primer sequence for the PCR,

Figure 7: Alignment made by GENETYX[®] for *Astyanax altiparanae* progesterone receptor.

Using the computer program for alignment and genomic analyses, Genetix[®], the best regions were chosen and the sequence for the primers was designed. The functional primers is described below (Table 1). For CR gene we designed two sequences named DGCRfw1 and DGCRfw2, sequences forward and one reverse named DGCRrv1. For PR gene, we designed two combinations of forward and reverse primers (Forward/Reverse A and Forward and Reverse B) that gave us results. To study the MIS receptor, we designed two forwards and one reverse primer named MIH Forward/Reverse A. For Beta-actin gene, used as a reference gene, we designed primer sequences of Forward and Reverse named Beta forward/reverse.

Table 1: Degenerated primers used for the target genes in PCR analyses.

<i>Primer name</i>	<i>Sequence forward</i>	<i>Sequence reverse</i>
DGCRfw1	TGTGGCAGCTGCAAGGTCTTCTT	-
DGCRfw2	GCCTTATGACCACCCTCAACA	-
DGCRrv1	-	TGCCGGTTCTGGCTGGAGTTC
Forward A	AGTTCTGACCTGCGGAAGCTGC	
Reverse A	-	CTCATCTCGTCAAACCTGGCCCTGACTCT
Forward B	ATCCACGATGCAGTCGTTTC	
MIH Forward A	TGCTACCAGGCAGGGATGATC	
MIH Reverse A	-	CCACTCCTTTCTCCTTTA
Beta-actinForward	CAAGGCCAACAGGGAAAA	
Beta-actin Reverse	-	TCTCTGCCAGCCAGATCC

The polymerase chain reaction (PCR) is a rapid procedure allowing the *in vitro* amplification of specific fragments of DNA, starting from a minimal amount of target DNA or RNA converted to cDNA previously. For the application of the technique, three segments of nucleic acids are needed: a double-stranded DNA to serve as template and the construction of two oligonucleotides (primers) specific - simple and tape the two complementary DNA template to strips - designed so flanking the sequence to be amplified. In addition, DNA polymerase is necessary, dNTPs, buffer, and salts (MgCl). The technique consists of repetitive cycles; each cycle is divided into the steps shown in Fig. 8 (Ausubelet *al.*, 2003).

Step 1 (94-96°C) - Denaturation of DNA at high temperature, due to the disruption of hydrogen bonds that bind the two strands.

Step 2 (30-60 ° C) - Annealing of primers at specific positions (this temperature is defined in terms of the nucleotidic sequence of primers).

Step 3 (72-75°C) - Extension of the sequence to be amplified by the action of DNA polymerase.

Figure 8: Scheme polymerase chain reaction. The process involving all three steps may be repeated 20 to 40 cycles, it is possible to increase, in each cycle twice the pre-existing DNA concentration (Ausubelet *al.*, 2003).

- For the genes studied the programs described below were outlined.

Each program is under the reason Temperature/Time:

- CR: Number of cycles : 35

94°C - 2:00 min;

94°C - 0:30 min;

55°C - 0:30min;

72°C - 1:00 min;

72°C – 2:00 min

4°C – α

- MIS Receptor: Number of cycles : 35

94°C - 2:00 min;

94°C - 0:30 min;

55°C - 0:30min;

72°C - 1:00 min;

72°C – 1:30 min

4°C - α

▪ PR: Number of cycles: 35

94°C - 2:00 min;

94°C - 0:30 min;

52°C - 0:30min;

72°C - 1:00 min;

72°C – 2:00 min

4°C - α

▪ Beta-Actin: Number of cycles: 35

94°C - 2:00 min;

94°C - 0:30 min;

58°C - 0:30min;

72°C – 0:30 min;

72°C – 1:00 min

4°C - α

The agar gel electrophoresis is generally the next step, when the amplification cycle ends. Used to visualize the result of the amplification of a specific sequence, is a simple and efficient method. The addition of fluorescent dye (ethidium bromide or SYBR[®]), which intercalates between the bases of DNA and the use of ultraviolet radiation allow visualization and photography (Souza, 2003). After checking the result, we cut the bands and made purification of the genetic material. This technique permitted separation, identification and purification of DNA molecules, preparing for cloning and sequence step (details for the protocols on annex 1,2 and 3). The molecular cloning is by definition, the diffusion of identical DNA molecules and is based on natural propagation or cell genetically identical to the original individuals.

The experiment of gene cloning is to introduce the gene into bacterial cells and isolate them in colonies (Nascimento *et al.*, 1999). In the first step there was the link between a DNA fragments called insert, containing the gene of interest with another DNA molecule, the vector. In the second step, the recombinant DNA molecule was transported into a host cell, usually a bacterium, where the transformation process takes place. The cell that received the recombinant DNA is called transformed cell which undergoes many cycles of division to produce multiple copies of the recombinant DNA. The procedure described followed the commercial kits for purification, ligation, transformation and cloning adapted by Promega® industrial protocol.

For ligation and after transformation, the manufacturing process consisted in introducing an exogenous DNA into host cells can be bacteria, most commonly used bacterial cell is *Escherichia coli* cell (Brown, 2003). For this project, we used the pGEM®-T Easy Vector. All vector models for cloning contain three common features: selective brand, origin of replication and multiple cloning sites. The selective mark refers to genes that confer resistance to antibiotics and provide selection of transformed clones those unprocessed. For this selection it was added to the culture medium appropriate antibiotic in concentrations which enable to differentiate (sensitive to the antibiotic) non-transformed cell of the transformed cell (antibiotic-resistant).

The procedure for ligation and transformation are briefly described below. Additional details are present on the annex pages.

REACTION COMPONENT	STANDARD REACTION	POSITIVE CONTROL	BACKGROUND CONTROL
2x Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®- T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl	-	-
Control insert DNA	-	2µl	-
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl

Nuclease-free water to a final volume	10µl	10µl	10µl
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1. We briefly centrifuged the pGEM®-T Easy Vector tubes to collect the contents at the bottom of the tubes.
2. Set up ligation reactions as described below. We vortex the 2X Rapid Ligation Buffer vigorously before each use.
3. The reactions were mixed (Fig. 9) by pipetting. The reactions were incubated for 1 hour at room temperature. Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.

Transformation

We prepared two LB/ampicillin/IPTG/ X-Gal plates to each ligation reaction, plus two plates for determining transformation efficiency (pos and background control). The plates were equilibrated to room temperature prior to plating. The tubes were centrifuged containing the ligation reactions to collect contents at the bottom of the tube. Add 2µl of each ligation reaction to a sterile 1.5ml microcentrifuge tube on ice. We set up another tube on ice with 0.1ng uncut plasmid for determination of the transformation efficiency of the competent cells. So, we removed the tube(s) of frozen JM109 High Efficiency Competent Cells from -70°C storage and placed them in an ice bath until just thawed (about 5 minutes). Mixed the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile. Carefully we transferred 50µl of cells into each tube prepared (100µl cells for determination of transformation efficiency). (Avoid excessive pipetting, as the cells are very fragile). Gently flicked the tubes to mix and place them on ice for 20 minutes. Heated shock the cells for 45-50 seconds in a water bath at exactly 42°C. Immediately returned the tubes to ice for 2 minutes. Incubate for 1.5 hours at 37°C with shaking (~150rpm).

To produce solid medium the components necessary are described below (Figs 10a,b):

LB MEDIUM	1000 ML	500ML
YEAST EXTRACT	5g	2,5g
BACTO TRYPTONE	10g	5g
NaCl	5g	2,5g

AGAR	15g	7,5g
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Figure 10a : Calculation for the medium producing before autoclave

Autoclave and transfer to the incubater at 60°C. So add;

LB MEDIUM	1000ML	500ML
AMPICILLIN (50ng/ml)	500µl	250µl
IPTG (100Mm)	5ml	2.5ml
X-GAL	4.0ml	2.0ml

Figure 10b: Calculation for the medium producing after autoclave.

We plated 100µl of each transformation culture onto duplicate LB /Ampicillin/ IPTG/X-Gal plates (if a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1.000 x gfor 10 minutes, resuspend in 200µl SOC medium, and 100µl plated on each of two plates). The plates were incubated overnight (16-24 hours) at 37°C. Longer incubations or storage plates at 4°C (after 37°C overnight incubation) were used to facilitate blue color development (Fig. 11). White colonies generally contain inserts; however, inserts may also be present in blue colonies. White clones were picked up by sterile tooth-picker into universal tube with 5ml LB plus Ampicillin (final concentration 100µg/ml) media, incubated overnight at 37C, 150rpm. Then we seek to 1 ml culture into 1.5ml tube for following PCR checking if successful insert and the remains are stored at 4°C.

Figure 11: Plates after 21hours of incubation for the cloning experiment to the CR. White colonies possibly mean positive inserts.

After the incubation, we selected the bigger white colonies for each sample and then picked up to a new plate, named master plate. The master plate should be incubated for 16~24hours. Then, the sample was homogenized and after, put on the heat bath at 95°C for 1~5 minutes. This procedure made sure that the bacteria cell was broken. Then, we used this material to run a PCR for confirmation for all samples you are cultivating (Fig. 12).

Figure 12: Results for the PCR to confirm the efficiency of the inserts for CR in *Astyanax altiparanae*.

After confirming the insertion, the master plate was incubated overnight, and then the colonies removed with a tip for 10 ~100 μ l, in a sterile tube with 2~3ml of liquid media. Then, the samples were incubated in a heat bath/shaker for 16~24hours at 37°C and 136 rpm. After this period, the samples were ready to be purified and sent for sequence in a specialized industry. The protocol used for purification is described on the annex (annex 6).

Real-time quantitative PCR

The real-time quantitative PCR primers for PR and CR receptors genes (forward and reverse) were designed using the software Perl Primer v1.1.17. The gene efficiency was optimized to be between 95 and 105% using the standard curve with five different dilutions of a pool of cDNA samples from all experimental animals. The real time PCR was performed using 1X SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μ M primer (forward and

reverse) and an equivalent of 5 ng cDNA. The reaction was incubated for 2 min at 50 ° C, 10 min at 95 ° C followed by forty cycles of 15 sec at 95 ° C and 40 s at 60 ° C. At the end of these cycles an analysis of the melt curve was carried out to test the specificity of the reaction. The reaction was performed in triplicate in a thermocycler ExicyclerTM96 through Exicycler Diagnosis3 software. The EF1 α gene was used as an endogenous reference gene for analyzing the relative expression of target genes. Gene expression was calculated using the Ct value, which was normalized by a reference sample (cDNA pool), standardized by EF1 α .

Statistical analyses

Data of plasma concentration of steroid hormones were expressed as mean $\pm$ standard error of the mean (SEM). Comparisons of steroid plasma levels and relative gene expression of CR and PR among the experimental groups (stress, saline and hCG) in *A. altiparanae* were performed by analysis of variance (ANOVA *One Way*) considering the time of the experiment (initial, intermediate or spawning). Tukey was used as a post-hoc test. The analyses were performed using the *Sigma Stat* (version 3.5) software.

Results

The experiments carried out were divided into two parts:

In the first part, we describe the results obtained on the hormonal profile and histological analysis, it showed the efficiency of the induction protocol, considering the results of the spawning females in the stress group. For these analyzes, the cortisol assay results showed statistical significance, while the histological examination revealed the presence of mature cells and then to experiment, the presence of post-ovulatory follicles in two of the three groups, confirming the expected in the drawing trial

In the second part of this work, we performed a molecular analysis of CR and PR in cells from the ovaries. These results aimed to elucidate the expression of Membrane receptor signaling for CR and PR, and Beta-actin in *A. altiparanae*.

In conventional PCR, the results were obtained using beta-actin gene as a normalizer.

However, for the RT PCR analysis, the gene set for use, due to better results in the optimization tests, was Ef1a. The results showed statistical difference for the gene expression of the related Progesterin receptor.

Reproductive performance

Regarding the behavior of the animals, there was a period of approximately 24hours from the beginning of the experiment, when it was observed the pairing of animals in the Stress group, a behavior that was followed by the positive response in all experimental groups. Taking into account the results obtained in the morphological analysis and the values of GSI, it can be inferred that animals from the Stress group ovulated in the period that comprised the second half of the experiment, responding rapidly to stress controlled, which may be considered a positive result to the proposed protocol. The values of GSI and gonad mass decreased significantly ($P < 0.001$ and $P = 0.005$, respectively) in the animals from the Stress group at the time of spawning. The body mass did not change in any experimental group (Table 2). The fertilization rate of the eggs did not vary in females from the different

experimental groups ($P = 0.518$) and the percentage of responses remained unchanged ($P = 0.346$) (Table 3).

Table 2: Body and gonadal weight, and GSI *Astyanax altiparanae* females of different experimental groups (mean $\pm$ standard deviation). Different letter means statistic difference ^{a, b}

Group	Gonad Mass (g)	Body Mass (g)	GSI
Stress			
Initial	8,72 $\pm$ 4,70 ^a	37,33 $\pm$ 5,57 ^a	23,54 $\pm$ 9,23 ^a
Intermediate	10,36 $\pm$ 2,05 ^a	43,10 $\pm$ 4,17 ^a	23,85 $\pm$ 2,56 ^a
Spawning	2,45 $\pm$ 4,37 ^b	41,91 $\pm$ 7,37 ^a	5,45 $\pm$ 10,35 ^b
Saline			
Initial	11,91 $\pm$ 3,91 ^a	48,33 $\pm$ 9,41 ^a	24,54 $\pm$ 2,53 ^a
Intermediate	12,53 $\pm$ 5,51 ^a	55,37 $\pm$ 6,08 ^a	21,12 $\pm$ 11,39 ^a
Spawning	11,98 $\pm$ 4,86 ^a	68,96 $\pm$ 8,16 ^a	21,93 $\pm$ 11,48
hCG			
Initial	11,98 $\pm$ 5,99 ^a	56,78 $\pm$ 6,94 ^a	24,54 $\pm$ 12,02 ^a
Spawning	10,99 $\pm$ 6,38 ^a	46,97 $\pm$ 8,77 ^a	18,99 $\pm$ 10,09 ^a

Table 3: Volume of eggs, fertilization rate and percentage of response to spawning *Astyanax altiparanae* in the different experimental groups

	hCG	Saline	Stress
Eggs volume (mL)	1100	300	2750
Fertilization rate (%)	72 $\pm$ 9,5	68 $\pm$ 29,7	70 $\pm$ 25,0
Percentage of response (%)	72 $\pm$ 1,5	68 $\pm$ 1,9	70 $\pm$ 2,6

Physicochemical parameters of water

The statistical test showed differences only for pH values, that was more acidic in the water of the Stress group when compared to other groups ($P = 0.001$). The analysis of water quality parameters showed no statistical differences among the three experimental groups for TDS ($P = 0.398$), dissolved oxygen ($P = 0.519$) and temperature ($P = 0.436$) values, that were not statistically different (ANOVA).

The results can be explained by the different procedures used in each experimental group, as the case of reducing the the water pH in the tank of animals from the Stress group. Since the density was higher, the production of organic compounds and the consequent reduction in pH of the water tank can be supported.

Histological analysis

From the observation made macroscopically, we found that the animals were sexually mature. From the middle third of the ovarian samples it was possible to discern and account for the distribution of cell types in the different parts in the ovaries of each individual and thus group the cell types by qualitative and subjective method to identify the maturity stage of groups throughout the experiment (Fig. 14).

The analysis of the gonads of the animals from the initial group was considered an initial reference for what would be observed in other animals throughout the experiment. Before the pre-established results for the genus *Astyanax*, it could be seen the final stage of animals sampled in the maturation experiment in the course of hours. It was observed the predominance of oocytes in an advanced stage of development, whose cytoplasm was filled with yolk granules, which have rounded shape (Fig. 13). This oocyte maturation stage is classified as vitellogenic, the stage considered as the starting point for the animals initially used.

Figure 13: *Astyanax altiparanae* female from the control group, named Saline. Time zero of the experiment. Oocyte at the vitellogenic state. (Increased 20X)

Qualitatively, the cells observed on the slides allowed the classification of the groups in relation to the time of sampling and their evolution throughout the experiment (Fig.15). Using this criterion, the most frequently observed cells have been described, such as the presence of vitellogenic oocytes along the cortical cellular oocytes. Additionally to these oocyte developmental stages, it was determined the presence of POFs (postovulatory follicles), a determining factor to estimate the efficiency of the protocol applied.

In general the animals from the initial group had vitellogenic oocytes in greater quantity than cortical alveolar and were not detected POFs. In females from the Saline group it was possible to estimate the predominance of vitellogenic oocytes, however the presence of POFs was slight, even at the time of spawning. The animals collected from approximately 24 hours divided between ovulated and vitellogenic (Fig. 16), predominating the second group, corroborating the data presented in the reproductive performance results.

Given the large number of vitellogenic oocytes it can be observed troublesome form of connective tissue stroma and may be concluded that the animal was in an advanced stage of maturation, since the number of oocytes at early stages of development (cortical-alveolar and perinucleolar) was low and it was displayed among the vitellogenic oocytes. However, oocyte nucleus migration was not registered in the periphery of the cell, a possible indication that the animal still need more time to ovulation or that this event may not occur without an additional stimulus.

hCG				SALINE			STRESS			INITIAL CONTROL		
	Vit	POF	Cort	Vit	POF	Cort	Vit	POF	Cort	Vit	POF	Cort
0h										++	-	+
Initial	-	-	-	+++	-	+	+++	-	+	-	-	-
Interm	++	++	+	++	-	+	+++	+	+	-	-	-
Spawn	+	+++	+	++	+	+	+	+++	+	-	-	-

Figure 15: Summary table of the frequency of the different stages of development of the oocytes and the presence of postovulatory follicles in relation to the number of hours after the experiment with *Astyanax altiparanae* in the different experimental groups, hCG, Saline and Stress. (-) Absent; (+) Present; (++) Frequent; (+++) Predominant; Vitellogenic (Vit); Follicle after ovulation (POF); Cortical Alveolar Follicle (Cort).

Figure 16: *Astyanax altiparanae*. a - ovaries of females from the Stress group in the initial collection. Mature and perinucleolar oocytes (arrowhead) (Bar = 45µm. Increased 20X); b - ovaries from the Stress group in the intermediary period; POF presence recorded in the half of the experimental period (bar = 90µm. 10X Increase); c - Ovaries from the Stress group at spawning. Presence of POF and cortical alveolar oocytes (Bar = 90µm, Increase 10x); d - ovaries from the Saline group during the initial collection (bar = 45µm increase 20x.), e - ovaries from the Saline group during intermediate collection, presence of perinucleolar and vitellogenic oocytes (Bar = 90µm Increased 10X.); f - ovaries from the Saline group at the spawning period. Presence of vitellogenic oocytes, and POF (Bar = 45µm increase 20X); g - ovaries after ovulation induction with hCG. On the arrow's head, a vitellogenic oocyte among the postovulatory follicle (POF). (Bar = 45µm, enlarged 20X); h - ovary of the hCG group at spawning. Presence of POF in large quantities (Bar = 90µm magnification 10x).

From the visualization of histological sections it was observed that the animals from all experimental groups were at an advanced stage of maturity, however, the number of animals that reached ovulation until the end of the experiment varied. The group that received hCG presented individuals at different stages of oocyte development, plus POFs on the first

sampling of the experiment (Fig. 16g), without, however spawning before the end of the experiment. This observation suggests a period of about 12 hours between ovulation and spawning, which was also observed in the Stress group.

In the sampling realized at the time of spawning it was observed completely ovulated ovaries, and the presence of POFs was more frequent in the Stress and hCG groups (Figs. 16c, h) compared with the Saline group. These data corroborated the GSI values, which decreased in females from the Stress group at the end of the experiment, but did not change in the animals of Saline group. Taking into account the different experimental groups, the qualitative comparison among the groups suggests the greater presence of POFs in the Stress and hCG groups in the 24 hour period of the experiment (Figs. 16c, h). Still within the Stress group, during the initial and intermediate periods the ovaries were full of vitellogenic oocytes, which have been somewhat replaced by POFs at spawning (Figs. 15 and 16).

Hormonal analysis

Whereas one of the main objectives of the work was to evaluate possible hormonal variations during the experiment, we have chosen to present the results over the time for the analyzed steroids. There was no statistical difference in the plasma CORT concentration throughout the experimental period considering each experimental group (Fig. 17) ($P = 0.711$, 0.775 and 0.411 for Saline, hCG and Stress groups, respectively). However, analyzing the different experimental groups at each period it was observed that in the intermediate period, about 12 hours after the beginning of the experiment, plasma CORT levels were higher in females from the Stress group, when compared with females from the other groups ($P = 0.018$), showing a peak. At the initial and spawning period of experiment, plasma showed no difference among the groups ($P = 0.946$ and $P = 0.472$, respectively).

In the 17 OHP plasma levels (Fig.18) there was no difference among the groups over the 24 hours, with the concentration unchanged within the groups ($P = 0.643$, Saline group; $P = 0.519$, Stress group; and $P = 0.669$, hCG group). The comparison among the different times also showed no significant differences ($P = 0.989$, $P = 0.091$ and $P = 0.196$, respectively, for the initial, intermediate and spawning periods).

Figure 17: Plasma concentration of cortisol in *Astyanax altiparanae* females during the experiment. ^{ab} Different letters mean significant differences among the different experimental groups in a given period (Mean $\pm$ SEM). ($p < 0.05$)

Figure 18: Plasma concentration of 17 α OHP in *Astyanax altiparanae* females during the experiment (Mean $\pm$ SEM).

Cortisol receptor and β -actin expression PCR results

After analysis of GENETYX[®] program and subsequent cloning, the purified samples for CR gene allowed us to obtain sequences with approximately 800bp (Fig. 21), while Beta-actin consensus (21a) revealed had $\approx$ 300bp (19b). The results for CR running are demonstrated below (Fig. 19). To obtaining the results we realized tests to determine the best conditions for reaction optimization of time and voltage to the gel running, conditions adapted for expression of each gene of interest. The results for optimization are present below (Fig. 20).

Figure 19: Results for the CR gene expression on the all groups during the experiment. To obtain these results, the samples ran at 8 μ l for 11 minutes at 135V. On the asterisk (*) A = animal control; H= hCG group; S = Saline group and E = Stress group.

When we saw the first results we could realize that this gene has a positive expression in all experiment, such as positive control. Then we optimized the PCR (Fig. 20), modifying the time for the running, and then, we could visualize a faith expression on the beginning and most strong expression in the end.

Figure 20: Optimized PCR result for CR in the Stress group. The circle indicates the stronger samples expressed for the target of study. For this running we used 8µl for 16 minutes at 135V.

After cloning four samples, and sequencing made by industrial company, we analyzed the results obtained and compared with other known sequences by GENETIX[®]. The sequence for the CR is represented below (Fig. 21).

```

“TGTGGCAGCTGCAAGGTCTTCTTCAAAAGAGCTGTGGAAGGGCAACACAACCTACCTGTGTGCTGG
GAGGAATGACTGCATTATCGATAAGATCCGCCGGAAGAACTGTCCGGCCTGCCGCTTCCGCAAGTG
TCTCATGGCCGGGATGAACCTAGAGGCACGCAAAGTTAAGAACTAAAGGGCTTAAAGGGTGTGC
AGCAGCCAAATGCAGCTGATCTGAGCCCAGTTCCAGTGCAGGAGGTGCGGGCGCTGGTGCCCAAG
GCCATGCCACAGCTCGTGCCACCATGCTGTCCCTGCTCAAAGCCATCGAGCCAGAGACCATTAT
GCTGGATACGACAGCACACTGACCGACACCTCCACTCGCCTAATGACCACCCTCAACAGGCTGGGC
GGCCGCCAGTTCGTCTCCGCAGTCAAGTGGGCCAAAACCTCTGCCAGGTTTATAGGAACCTTCACCTG
GATGATCAGATGACTCTACTGCAGTCCTCTGGCTCTTCTCATGTCTTCGGCCTGGGCTGGAGGT
CCTACCAGCAGTGCAATGGAAACATGCTCTGCTTTGCTCCAGATCTGGTGATCAATGAGGAGAGGA
TGAAACTGCCCTACATGGGTGATCAGTGTGCACAGATGCTGAAGATCTCCAATGAGTTTGTGAGGC
TGCAGGTGTCCAACGAGGAATACCTGTGCATGAAGGTCCTGCTGCTGCTCAGCACCGTGCCAAAGG
ACGGTCTGAAGAGCCAGGCTGTGTTTCGATGAGATCAGGATGTCCTACATTAAAGAACTGGGCAAA
GCCATTGTCAGGAGAGAGGAGAACTCCAGCCAGAACCGGCA”

```

Figure 21: Partial sequence for CR in *A. altiparanae* after purification and sequencing.

As the same way, we purified two samples for Beta-actin, which showed us good results on sequencing. When compared with *O. bonarienses*, species used for studies on the

collaborator laboratory, we detected that the sequence is smaller than the one described for *pejerrey* (Figs. 22a, b; 23a,b and c).

11f	1	-----AGGCTGAG-AGC-CCCAGTCCTGCTGACC	27
9f	1	-----ATCAGAG-AGC-CCCAGTCCTGCTGACC	26
10r	1	ATCACACCCCTGTACAATGAGCTGAGAGTTGCCCTGAGGAGCACCCAGTCCTGCTGACC	60
11f	28	GAGGCCCCCTGAACCCCAAGGCCAACAGGGAAAAGATGACTCAGATCATGTTTGAGACC	87
9f	27	GAGGCCCCCTGAACCCCAAGGCCAACAGGGAAAAGATGACTCAGATCATGTTTGAGACC	86
10r	61	GAGGCCCCCTGAACCCCAAGGCCAACAGGGAAAAGATGACTCAGATCATGTTTGAGACC	120
11f	88	TTCAACACACCAGCCATGTATGTGGCCATCCAGGCCGTGCTGTCCCTGTACGCCTCTGGT	147
9f	87	TTCAACACACCAGCCATGTATGTGGCCATCCAGGCCGTGCTGTCCCTGTACGCCTCTGGT	146
10r	121	TTCAACACACCAGCCATGTATGTGGCCATCCAGGCCGTGCTGTCCCTGTACGCCTCTGGT	180
11f	148	CGTACCACTGGTATCGTCATGGACTCCGGTGACGGTGTGACCCACACAGTGCCCATCTAT	207
9f	147	CGTACCACTGGTATCGTCATGGACTCCGGTGACGGTGTGACCCACACAGTGCCCATCTAT	206
10r	181	CGTACCACTGGTATCGTCATGGACTCCGGTGACGGTGTGACCCACACAGTGCCCATCTAT	240
11f	208	GAAGGCTACGCTCTGCCCCATGCCATCCTCAGGCTGGATCTGGCTGGCAGAGACCTCACA	267
9f	207	GAAGGCTACGCTCTGCCCCATGCCATCCTCAGGCTGGATCTGGCTGGCAGAGACCTCACA	266
10r	241	GAAGGCTACGCTCTGCCCCATGCCATCCTCAGGCTGGATCTGGCTGGCAGAGACCTCACA	300
11f	268	GACTACCTCATGAAAATCCTGACTGAGAGGGGTTACAGCTTCACCACCACAGCCGAGCGT	327
9f	267	GACTACCTCATGAAAATCCTGACTGAGAGGGGTTACAGCTTCACCACCACAGCCGAGCGT	326
10r	301	GACTACCTCATGAAAATCCTGACTGAGAGGGGTTACAGCTTCACCACCACAGCCGAGCGT	360
11f	328	GAGATCGTGC GCGACATTAAGGAGAAGCTGT- GTTACGTTGCTTTGGACTTTGAGCAAGA	386
9f	327	GAGATCGTGC GCGACATTAAGGAGAAGCTGT- GTTACGTTGCTTTGGACTTTGAGCAAGA	385
10r	361	GAGATCGTGC GCGACATTAAGGAGAAGCTGT- GTTACGTTGCTTTGGACTTTGAGCAAGA	419
11f	387	AATGGGAAC TGCTGCCTCCTCTTCATCATTGGA AAAAAAGAGCTA	430
9f	386	AATGGGAAC TGCTGCCTCCTCTTCATCATTGGA AAAAAAGAGCTA	429
10r	420	AATGACTC-----	427

Figure 22a: Results for the consensus among the cloned samples of *A.altiparanae*, for the gene of interest, Beta-actin receptor in the ovary.

ATCAGAGAGCCCCAGTCCTGCTGACCGAGGCCCCCCTGAACCCCAAGGCCAACAGGGAAAAGATG
 ACTCAGATCATGTTTGAGACCTTCAACACACCAGCCATGTATGTGGCCATCCAGGCCGTGCTGTCC
 CTGTACGCCTCTGGTTCGTACCACTGGTATCGTCATGGACTCCGGTGACGGTGTGACCCACACAGTG
 CCCATCTATGAAGGCTACGCTCTGCCCCATGCCATCCTCAGGCTGGATCTGGCTGGCAGAGACCTC
 ACAGACTACCTCATGAAAATCCTGACTGAGAGGGGTTACAGCTTCACCACCACAGCCGAGCGTGA
 GATCGTGC GCGACATTAAGGAGAAGCTGTGTTACGTTGCTTTGGACTTTGAGCAAGAAATGGGAAC
 TGCTGC CTCCTCTTCATCATTGGA AAAAAAGAGCTA

Figure 22b: Partial sequence for Beta-actin expressed in the ovary of *A.altiparanae*

Figure 23a: First results for Beta-actin expression in hCG group (H). The higher expression demonstrates the good design of the primers sequence. (For this running were used 7 μ l at 12 min on 135V)

Figure 23b: Optimized results for Beta-actin after tests with time and volume of PCR products. For this expression we used 5,5 μ l, for 16 minutes at 135V.

Figure 23c: Optimizing the time for running gel and voltage had been adjusted and in the end, 5,5 μ l, for 16 minutes at 135V show us the best results for Cortisol and Beta-Actin expression. In the picture, the hCG group for both genes; (*a) represents the expression for CR and (*b) for Beta-actin.

Progesterone receptor and Progesterone receptor associated to MIS -PCR results

After analysis of GENETYX[®] program and subsequent cloning, the purified samples for PR and subsequently to the derivate of progesterone, *17,20- dihydroxy-4-pregnen-3-one*, through research made in GenBank (NCBI) and then aligning made by GENETYX[®], allowed us to obtain sequences with approximately 800bp. The result for the consensus alignment is showed below (Figs. 24a, b,c,d). For those results we ran the gel using the cDNA provided by the same samples to compare the gene expression of the target receptor on the same time. So, we could observe for some samples the expression for one or other receptor indicating different conditions inside the same group. If we compare all groups for the two genes, we can observe, for Stress group, the number of samples expressing the receptor for MIS is bigger than the PR, different from the other two groups, in which the number of samples expressing a stronger band for PR is observed.

Figure 24a: Expression of the PR for the same samples, initial animals collected on the experiment. PR associated to MIS (MIH receptor), show us a lower expression. In the other hand the expression of PR can was higher in some samples. *abbreviation for “animal control”; C means sample Negative control Sample.

Figure 24b: Expression of the PR for the, hCG group. PR shows us a higher expression. In the other hand the expression of PR associated to MIS(MIH receptor) is weak in some samples. *abbreviations; H is for hCG and C means Negative Control.

Figure 24c: Expression of PR for “Saline” group. PR and MIIH receptor. We can see faint expression on some samples, for receptor associated to MIS (MIH receptor) compared to the PR during the entire experiment. *Abbreviation: “S” means “Saline”, while “C” means Negative Control Sample.

Figure 24d: Expression of PR in “Stress” group. PR associated to MIS (MIH receptor) more expressed in the end of experiment than Progesterone receptor. *Abbreviation: E means Stress group.

The result for the automated sequencing and genetic consensus was determined by GENETYX[®] (Figs. 25 and 27), revealing the conserved regions and sequence for the both receptors. Along the sequences, insertions, characteristics for the *A. altiparanae* (Figs. 25 and 27) can be observed for both sequences. After the consensus, the sequence was determined by around 800 bp (Figs. 26 and 28). For best results in the agar gel, tests with different cycles, temperature, volume of PCR products, run time and voltage were performed (adapted by Yang *et al.*, 1994 method). The results can be seen below (Fig. 29).

2f	0	-----	0
XM_007231093	1	CGCGGGGTTTCGACAGCTGTCCGCCCTTTCCGCGCCTTCGCGGGTGTACCGCCCCAGAG	60
2f	0	-----	0
XM_007231093	61	AATGTGTGTGATCTGCGGAGACGAGGCTTCAGGATGTCATTATGGAGTTCTGACCTGCGG	120
2f	1	-----AGGTCCTAGAGAGCTGTGGA--GGA--ATCACAAGTACCTGTGTGCTGG	44
XM_007231093	121	AAGCTGCAAGGTGTTCTTCAAGAGAGCTGTGGAAGGACATCACAAGTACCTGTGTGCTGG	180
2f	45	GAGAAACGACTGCATCGTGGATAAAATCAGGAGGAAAACTGCCCGCTTGTGCGGCTCAG	104
XM_007231093	181	GAGAAACGACTGCATCGTGGATAAAATCAGGAGGAAAACTGCCCGCTTGTGCGGCTCAG	240
2f	105	GAAATGCTACCAGGCAGGGATGATGCTGGGAGGTCGTAAAGCTGAAGCGGTTTGGAGTTTT	164
XM_007231093	241	GAAATGCTACCAGGCAGGGATGATGCTGGGAGGTCGTAAAGCTGAAGCGGTTTGGAGTTTT	300
2f	165	AAAGGCCATGGGTTCAGCCCATCACTGATGTTCCAGAACCCCTCTGACCCTGCCAGTCGA	224
XM_007231093	301	AAAGGCCATGGGTTCAGCCCATCACTGATGTTCCAGAACCCCTCTGACCCTGCCAGTCGA	360
2f	225	GCCCCACGCCCTTGTCCACCATCTCCTGTATGCCCGCCATCCGAGAACTGCAGTTGACTCC	284
XM_007231093	361	GCCCCACGCCCTTGTCCACCATCTCCTGTATGCCCGCCATCCGAGAACTGCAGTTGACTCC	420
2f	285	ACAGATCATCAGCATACTGGAGAGCATCGAGCCAGAGGTGGTCTACTCAGGTTACGACAA	344
XM_007231093	421	ACAGATCATCAGCATACTGGAGAGCATCGAGCCAGAGGTGGTCTACTCAGGTTACGACAA	480
2f	345	CTCCCAGCCAGACCTGCCACACTTACTACTCAACAGCCTGAACAGACTGTGCGAACGCCA	404
XM_007231093	481	CTCCCAGCCAGACCTGCCACACTTACTACTCAACAGCCTGAACAGACTGTGCGAACGCCA	540
2f	405	GCTCTGTGGATCGTCAGGTGGTCCAAGTCTTTGCCAGGTTTTCGGAATTTCGACATCAG	464
XM_007231093	541	GCTCTGTGGATCGTCAGGTGGTCCAAGTCTTTGCCAG-----	578
2f	465	TGACCAGATGACGCTAATCCAGTACTCCTGGATGAATCTGATGGTGTTTTCTTTGGGGTG	524
XM_007231093	578	-----	578
2f	525	GAGAACCTTCCAGAACGTACAGGCGATTATGTACTTCGCACCGGACCTCATCCTCAG	584
XM_007231093	578	-----	578
2f	585	CCAAGAGCGGATGAGGAGGTCCGCAATCTACGACCTGTGCCTGGCCATGCAGATTATTCC	644
XM_007231093	579	----AGCGGATGAGGAGGTCCGCAATCTACGACCTGTGCCTGGCCATGCAGATTATTCC	633
2f	645	GCAGGAGTTTGCCAGTCTGCAGGTTTCAAGAGAGGAGTTCTGTGCATGAAGGCGCTCGT	704
XM_007231093	634	GCAGGAGTTTGCCAGTCTGCAGGTTTCAAGAGAGGAGTTCTGTGCATGAAGGCGCTCGT	693
2f	705	GCTGCTCAACACCGTGCCGTTGGAGGGCTTGAAGAGTCAGGTC--AGTTGAACGAGATGGA	763
XM_007231093	694	GCTGCTCAACACCGTGCCGTTGGAGGGCTTGAAGAGTCAGGTCAGTTTGACGAGATG--A	752
2f	764	GAAAAAAGGTTGTTCTCTTCTTGGTGGGCTGTTCTCTTATTGGAAAC---	820
XM_007231093	753	GACAGAGCTACATCCGA--GAA--CTCAC--CAAGGTCATCCAGATAAAGGAGAAAGGAGT	807
2f	820	-----	820
XM_007231093	808	GGTGGGGAGCTCTCAGAGATTCTACCATCTCACCAAGCTCATGGACTCCATGCACGAGAT	867

Figure 25: Alignment between *A. altiparanae*(2f) and *A. mexicanus* (XM_007221092) represented by the Gene bank access number. Alignment made by Genetix© program showing the consensus among the two species.

ATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGG
AACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAA
AATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTTCCCC
TGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCC
CTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCCGGTGTAGGTCGTTCC
CTCCAAGCTGGGCTGTGTGCACGAACCCCCGTTTACGCCGACCGCTGCGCCTTATCCGGTAACTA
TCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGAT
TAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACAC
TAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAC
CTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTAC
GCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACTGGGTCTGACGCTCAGTGGAA
CGAAACTCACGTTAAAGGATTTTGGGTCATGAGATTATCAAAAAGGA

Figure 26: Sequence obtained after purification and sequencing of PCR products. PR sequence for deposit on the Gene Bank

Figure 27: Alignment for consensus, PR II, associated to MIH, after sequencing between *A. altiparanae* and *A. mexicanus* (analyses made by Genetix®).

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GGGCTTAGCTAGCGGTTTGGAGTTTTAAGGCCATGGGTCTCAACCCGTCCTGATGTTCCAGAACC
CTCTGACCCTGCCGGTCGAGCCCCACGCCTTGTCCACCATCTCCTGTATGCCCGCCATCCGAGAACC
GCAGTTGACTCCACAGATCATCAGTATACTGGAGAGCATCGAGCCAGAGGTGGTCTACTCAGGTTA
CGACAACTCCCAGCCAGACCTGCCACACTTACTGCTCAACAGCCTGAACAGACTGTGCGAACGCCA
GCTCCTGTGGATCGTCAGGTGGTCCAAGTCTTTGCCAGGTTTTCGGAATTTGCACATCAGTGACCAG
ATGACGCTAATCCAGTACTCCTGGATGAATCTGATGGTGTTCCTTTGGGGTGGAGAACCTTCCAGA
ACGTCACAGGCGATTATATGTACTTCGCACCGGACCTCATCCTCAGCCAAGAGCGGATGAGGAGGT
CGCCAATCTACGACCTGTGCCTGGCCATGCAGATTATCCGCAGGAGTTTGCCAGTCTGCAGGTTTC
ACGAGAGGAGTGCTGTGCATGAAGGCGCTCGTGCAGATCAACACCGTGCCGGTGTAAGGATTGA
AGAGACAGGGCCAGTTTGACCAGATGACACAAAATTTCCCCCGATAGGGTTGCATGGTCATCCAGA
ACCACGAAAACCTCTTCCTATCTCCTAAGATAGACAAGATAAGCTCGCAGGCGTTATATGTGGACC
AGCCTACAAGCCACCGGGCGAAGCTCGTGCCTGGATGTTATCGCATCCTTCTCAGCACCTGGCCAC
AGAGAGAGAGTGAGGCACTAGTTCA

```

Figure 28: Sequence obtained after purification and sequencing of PCR products for PR II. PR associated to MIS (MIH receptor) sequence.

Figure 29: Comparison among groups, revealing the expression for the two PR and their derivate hormone (MIS). We can realize, for most part of animals tested the expression for PR is easier visualized, than the receptor associated to MIS. However, the expression for the derivate receptor in animals of the Stress group is relevant for this project hypothesis. On the asterisk “*A” = animals control; “*H”= hCG group; “*S” = Saline group and “*E” = Stress group. To obtain this optimized results we used 5,5µl for 20 minutes at 135V.

Real-time quantitative PCR

For the RT-PCR analysis, aEfa1 gene (kindly donated by Dr. M.IBorella), specific for the genus *Astyanax*, was used as a reference gene. This gene was used for normalizing the results observed in the absolute quantification for CR and PR genes. The results, demonstrated by the Melting Curve and the Flowchart for Efa1 gene expression (Figs 30 and 31), CR (Figs. 32 33, 34 and 35) and PR (Figs. 36, 37, 38 and 39) are demonstrated below.

Figure 30: Melting curve for the reference gene Efa1a

Figure 31: Flowchart for the reference gene Efla

Figure 32: Melting curve for CR gene expression in the Stress group samples.

Figure 33: Flowchart for CR gene expression in the hCGgroup samples.

Figure 34: Flowchart for CR gene expression in the Saline group samples.

Figure 35: Flowchart for CR gene expression in the Stress group samples.

Figure 36: Melting curve for PR gene expression in the hCG group samples.

Figure 37: Flowchart for PR gene expression in the hCG group samples.

Figure 38: Flowchart for PR gene expression in the Saline group samples.

Figure 39: Flowchart for PR gene expression in the Stress group samples.

The data were then normalized and after statistical analysis, the results showed an increase in PR gene expression in females from hCG group before and after spawning, when compared with females from Stress and Saline group (Fig.40). On the other hand, CR gene expression did not change among experimental groups or throughout the experimental period (Fig.41).

Figure 40: Absolute quantification values normalized, for PR in *Astyanax altiparanae* females during the experiment. ^{ab}Different letters mean significant differences among the different experimental groups in a given period ($P=0,025$).

Figure 41: Absolute values normalized, for CR in *Astyanax altiparanae* females during the experiment. The results did not show significant differences among the different experimental groups in a given period($P=0,972$).

Discussion

Fertilization rates, histological and hormonal analyses

The objective of this study was to test the combined effects of high density and increased water flow as stress factors, able to induce ovulation in *Astyanax altiparanae* and relate the results to the possible physiological causes. The protocol for the captive breeding of *A. altiparanae* known as "stress inducing" was successful providing a greater volume of eggs in the stress group compared with other experimental groups. Regarding the results found in the other groups we can infer that the mechanical action of induction by itself (needle punching and saline injection) was not able to accelerate ovulation or even increase the success of spawning as can be observed in the GSI calculated values between the first and the second period within that group.

In the hCG group, considered as the positive control for the induction of ovulation in, denote the ability of the synthetic hormone to induce ovulation and spawning, as described by Baldisserotto (2002), which reports that the ability of hCG to induce ovulation may be as effective as hormones commonly used as ovulation induction promoter in fish farming, such as GnRH or LH. Therefore, in *A. altiparanae* it was possible to obtain a larger amount of eggs, i.e., ovulation has been possible with inducing agents, such as hCG or stress, being connected to the stimuli by agglomeration and increase of the water flow.

Also in relation to the results obtained in the reproductive performance of animals, possible changes caused by abiotic factors can be excluded, which mostly showed no significant differences, except for the pH. The differences in the pH values can be attributed to the difference in the volume of water in the Stress group as well as differences in water flow in the tank. In this regard, it is notable that the exclusion of abiotic factors as determinants of the results ensures merit to the experimental protocol to induction, and ensures that the results obtained in the groups are purely statements of physiological modulation of animals to stimuli, as quoted by Aragão and Araújo (2006), to highlight the importance of removing the interference of bias in experimental protocols.

It is known that genre *Astyanax*, ovarian development is similar to other teleosts where the ovaries are paired organs developed as elongated and leaf-like changes in color and

thickness sustained throughout the reproductive cycle (Drummond, 1996). The gametes pass through the following phases: Initial, Previtellogenic and Vitellogenic. Evaluating the results of morphological analysis, we conclude they refer to mature cells or vitellogenic at time zero (0h) and post-vitellogenic follicles at the end of the experiment, indicating positive response to ovulation and spawning, coinciding with the descriptions of the genre (Pereira-Filho, 2000).

Regarding hormonal profiles, several studies have shown a duality function among the possible actions of corticosteroids, being able to inhibit the reproductive performance of fish (Small, 2004; Milla et al, 2009). These authors reported that corticosteroids can cause adverse effects on reproduction and its action throughout the hypothalamic-pituitary-gonadal (HPG) axis. Foo and Lam (1993) argue in their study, that CORT administration delayed the reproductive process and the quality of spawning in tilapia (*Oreochromis mossambicus*).

Unlike in the study presented by these authors, stress conditions stimulate increased corticosteroid production, but did not decrease or blocked the activity of other hormones, such as progesterone, for example. This behavior may be related to high reproductive plasticity presented by this species on the reproduction. From the results obtained using the stress protocol, we can infer that the modulating action of CORT did not inhibit the reproductive performance of *A. altiparanae* females.

In *A. altiparanae*, we could observe the positive action of corticosteroids in the final maturation, considering that the animals showed good spawning results, corroborating Kubokawa et al., (1999), which attributed to CORT an important role in the maturation of Perciformes and on metabolic parameters presented by the animals during this period.

Since the 1980s, in some species of teleost as *Oncorhynchus mykiss*, *Salmo trutta*, *Carassius auratus* and *Cyprinus carpio* a peak of plasma CORT was detected at the time of ovulation (Cook et al., 1980; Pickering and Christie, 1981; Bry, 1985; Kime and Dolben, 1985), a response that was found in *A. altiparanae* females from the Stress group, which denotes a common profile for this species with the experiments carried out in recent decades. Additionally, it is already known the ability of teleost gonads (testes and ovaries) in synthesizing corticosteroids (Milla et al., 2009).

Stolte et al. (2008) suggested that corticosteroid receptors could be regulated in accordance with different types of stress in *Cyprinus carpio* and that the period of greater activity of the stressor was accompanied by behavioral changes in animals due to high

sensitivity to stress. Among the aforesaid cases, deaths have been reported in individuals with high plasma concentrations of corticosteroids. Furthermore, Berg *et al.*, (2005) described the action of corticosteroids in blocking gonadal progestins receptors, which may be related to a negative action in the maturation and subsequent reproduction of some species.

The progestagen *17 α OHP* is known as a precursor steroid in the maturation of oocytes in fish and its synthesis proceeds, as in all steroids, from cholesterol. *17 α OHP* is synthesized from progesterone and it is subsequently converted into *17 α ,20 β -dihydroxy-4pregnen-3one*, known as maturation induction steroid (MIS) in granulose cells (Lubzens *et al.*, 2010; Munakata and Kobayashi, 2010). During the experiment with *A. altiparanae* it can be observed homogeneous concentration of this hormone in plasma, throughout the experimental period in all experimental groups.

Considering that *17 OHP* is converted to MIS near the ovulation, we might initially expect high levels of this hormone and differences among the experimental groups. However, the results were quite different than expected, as this hormone did not change during the experiment. As *17 OHP* is rapidly converted into other hormones (MIS, androstenedione and corticosteroids, mostly) we could infer that it could have been used both in the production of MIS as expected by the work of Peter and Yu (1997), and diverted to the CORT pathway production, as suggested in studies as Munakata and Kobayashi (2010). According to Planas *et al.*, (1992) MIS is very critical at the moment of "germinal vesicle breakdown" (VGBD), preparing the oocytes for fertilization, a period in which insufficient levels result in possible failure in the reproductive process.

One hypothesis for the results that have been generated can be the plasticity of *A. altiparanae* in the stressful conditions of captivity, so the animals would be able to use the stress situation as a potentiating agent of spawning. The results may be related as suggested by Copley (2003) with regard to "promiscuous" of steroid receptors, suggesting here that CORT can act as MIS due to its ability to bind to the receptors of the progestogen, amplifying action and reducing the effective action of other hormones. The contrary is also known; in *Oryzias latipes* progestins can activate glucocorticoid receptors (Miyagawa *et al.*, 2015). In *A. altiparanae* we can infer a positive action of corticosteroids in the maturation, because the animals showed good spawning results, corroborating Kubokawa *et al.*, (1999), who attributed to CORT an important role in the maturation of Perciformes.

According to the data presented, CORT levels fluctuated throughout the 24 hours of experiment, showing a peak in the intermediate period of approximately 12 hours in the Stress group, when various POFs were already found in the ovaries of these animals. This peak of CORT at ovulation has also been reported in other classes of vertebrates, however, the understanding of the role of this hormone and if the increased levels can be considered as cause or consequence of the ovulatory process is still speculative (Millaet *al.*, 2009). This peak can be correlated with ovulation, a process that demands high energy expenditure and resulting in the production of steroid inducer of final maturation (MIS), or even that CORT itself can also act as MIS. In “pacu”, *Piaractusmesopotamicus*, a species from the same family of *A. altiparanae*(Characidae) CORT levels were higher in females at the advanced stage of maturity, as well as the time of ovulation with LHRH (Gazolaet *al.*, 1996), confirming the importance of CORT in the ovulation process. In our hypothesis, the results obtained in the animals from the Stress group allow us to infer, in this case in particular, that CORT can be considered an important stimulus for ovulation of this species in captivity. The use of this protocol can spare the use of synthetic hormones in breeding protocol of this species, allowing positive results in a short time.

Molecular analyses

Some physiological effects of steroid hormones are too rapid to be mediated by the classical genomic mechanism of steroid action involving activation of nuclear steroid receptors (Yang *et al.*, 1994). Rapid, non-genomic steroid actions initiated on the cell surface have been described in a wide variety of animal, tissue, and cell models (Revelli *et al* 1998). Corticosteroid signaling is mediated by the glucocorticoid receptor (CR) and mineralocorticoid receptor (MR), which are ligand-activated transcription factors. CRs and MRs possess the four functional domains common to nuclear hormone receptors: A/B, C (DNA-binding), D (hinge region), and E (ligand-binding) domains. The C and E domains are highly conserved across vertebrates (Mommssen *et al*, 1999, Pascual-Le *et al.*, 2005).

In teleosts, CR and its ligand CORT are best known for their roles in the stress response and, specifically, modulate aspects of intermediary metabolism, growth, behavior, and immune function (Charmandari, 2005). But, when considering the role of CORT in reproduction, there is not a consensus among the researches about positive or deleterious effects (Milla *et al.*, 2009). Depending on the period and the intensity of stressor application,

it may cause follicular atresia, advance or delay oocyte maturation and ovulation or affect egg size, fertilization success, spawning behavior and progeny quality (Clearwater and Pankhurst, 1997; Coward *et al.*, 1998; Schreckel *et al.*, 2001; Okumura *et al.*, 2002). Furthermore, the presence of corticosteroid receptors in reproductive tissues including ovaries and testis, as the corticosteroid receptors gene expression (gluco- and mineralocorticoid receptors) found in the male/ female gonads where the transcripts were detected in the ovary (Sturm *et al.*, 2005; Milla *et al.*, 2006) and corticosteroid receptors (Colombe *et al.*, 2000; Filby and Tyler, 2007; Milla *et al.*, 2008) leads to the consideration that corticosteroids can exert positive or inhibitory effects on reproductive functions, as hypothesized here for *A. altiparanae*, with the possibility of induction of spawning mediated by stressful stimuli.

When we evaluated the performance of reproduction of *A. altiparanae* by counting the number of fertilized eggs, and measured the levels of CORT and 17 α OHP, we can infer that stress triggered a modulation in the production of steroids, mainly CORT and rapidly, since in hours, the animals had conditions to spawn when stressed, whereas without any stimulation there was a delay to spawn.

Considering the higher CORT levels at the intermediate period of females from the stress group, it was clear the importance to evaluate the expression of CR gene, as well as understand the pattern of expression of progesterone receptor (PR) gene, since the literature suggests a higher expression of CR and a low expression of PR as a compensatory effect derived from a "diversion of resources" for the production of CORT, since the synthetic pathway of both hormones is similar.

To determine the possibility of the ovulation stimuli by CORT, we chose to observe the expression of membrane receptors involved, which could signal a stimulation of the pathway and its consequent production, explaining the results in hormonal assays. However, we found that the CR gene is expressed throughout the experimental period, even in animals experimentally not stressed, Saline control and hCG females, a pattern that can be considered a characteristic of this species.

Analysis of the data showed, however, that the gene expression compared CR was similar among the female of all groups, however, there is a tendency of an increase in CR gene expression in the intermediate period of females from the stress group, coinciding with the time of CORT peak. We know that animals from the Stress group had the best results for reproductive performance, then the chance for positive and stimulating role of CORT in *A.*

altiparanae reproduction may suggest a truly positive mechanism, differing from studies where the peak of CORT can be deleterious for ovulation, as Brown (2003) say in his study with *Salvelinus fontinalis*. This kind of situation was reported in another species, as rainbow trout, where females undergoing acute stress produce small amounts of eggs (Campbell et al., 1992).

These results could be explained by some types of interactions between the CR and the Progesterone receptor (PR). However, the strong answer in ovaries for PR expression did not show great results for spawning, such as the “Serum” (control) group results, for example. So, when we saw the optimized PCR results, we observed that very clear bands for PR expression did not so stronger on the Stress member group.

This was one point to suppose the substrate that has been used for CORT production. Another interesting fact about the result on the Stress group is while PR for 17OHP was not producing stronger bands, the derivative, associate at 17,20 β DHP hormone, had produced sensitivity for expression on the tissue analyzed. This is an important step to elucidate the relation between those two hormones on the reproduction and associate an important and positive role for the CORT on this group.

Focusing on CORT during reproduction, the role of this steroid must be undertaken with higher caution regarding the potential interaction between plasma CORT levels and mating activities (Greenwood *et al.*, 2003). In consequence, blood sampling should be rapidly carried out to avoid any CORT contamination due to a stressful physiological state. Regarding the other corticosteroids, if the effects of stress on their blood plasma release remain undetermined, one should pay the same attention to them. On the other hand, reproduction often corresponds to a fasting period, or is at least accompanied with a decrease of feeding. As the plasma glucocorticoid levels are linked to the fish nutritional status, it may therefore be difficult to distinguish between the glucocorticoid fluctuations linked to reproductive physiological state and those linked to the alteration of the energetic metabolism, providing the necessary adjustment to cause a physiological stimulus for spawning, even without the strong expression of derivatives of progesterone (Zhu *et al.*, 2003)

What can be inferred by these results is that there would be no "diversion" of resources for production of CORT and consequently a low expression for progestagen receptor and its derivatives, but that the gene expression of progesterone and CORT receptors remained high

at the same time, which suggests another factor stimulating spawning of animals, that did not show strong bands for the expression in all animal for progesterone on the conventional PCR.

We suggest that somehow the same period of gene expression of CORT and PR associated to MIS (*17,20-DHP*) receptors has allowed the animals to ovulation and subsequent spawning, even with animals in the group showing no different results in the conventional PCR analysis for PR (Control and Stress groups).

When we look at the results found in the conventional PCR and compared with the values found for the gene expression of the same targets genes, we realize that the answer to the reproductive behavior shown by these animals may depend on a combination of factors, which may be associated with another pathway for steroid synthesis. Analyzing the values obtained, we suggest that the PR may represent significant differences to influence the spawning in the other two groups, since no animals in the control group or animals treated with stressor stimulus demonstrated any differences in their hormonal profiles, for example. When comparing the results between the groups, taking into account the division of animals sampled before and after spawning, the statistical difference observed, occurred only in the animals of hCG group. This result lies within expectations, since the group had a hormone stimulating factor. Compared with the other groups PR gene expression was sharp in hCG group, but no statistical significant differences were found in Stress and Saline groups, which would be an indicator that 17 OHP is not really necessary to spawning in these animals. This corroborates the findings that another steroid, in this case, CORT can be somehow modulating ovulation and spawning in animals, but not through CR.

Cortisol is the main corticosteroid in teleosts, secreted in response to stressor exposure and plays a key role in stress adaptation (Mommensen *et al*, 1999). But, when we analyzed the CR gene expression, interestingly, the profile of the saturation curve suggests a low gene expression in the ovaries, demonstrating a different approach from those results obtained in the gel bands by conventional PCR. So, when analyzing the values found for CR, gene expression there were no statistical differences to confirm our hypothesis that CORT could exert a stimulatory effect in ovulation (acting as MIS) observed in the reproduction. However, the results obtained in plasma of females from group Stress (peak of CORT into ovulation) indicate an undeniable role of this steroid in the behavior and/or spawning animals. Given these results, we can infer other possibilities, considering that this receptor represents one of the largest and possibly most ancient classes of receptors, responding to a diverse array of

signaling molecules (Bockaert and Pin, 1999). One possibility is that the receptor gene expression does not necessarily correspond to the active form of the encoded protein (Millaet *al.*, 2009).

Moreover, when testing some steroid combinations, a synergy in the maturational response was demonstrated between corticosteroids and MIS since the 80s. For example, *in vitro* treatments with CORT increased the sensitivity of oocytes to MIS in rainbow trout (Jalabert and Fostier, 1984). We can link these results within that study, when observe the gene expression results in *A. altiparanae* treated with hCG. Thus, although the MIS receptor is identified and the partial sequence for the associated receptor described, as the first results for these genes for this species, therefore, these results support that corticosteroids might be directly and/or indirectly involved in final oocyte maturation control.

Finally, we suggest that the ovaries of *A. altiparanae* are able to respond to the corticosteroids stimulation and CORT, the main corticosteroid in fish, displays direct and positive effects in relation to reproduction in this species. Additionally, the available data indicates that corticosteroids may participate in the modulation of reproductive endocrine control, and together with progesterone, may prove to be a possible trigger, allowing satisfactory results in captive breeding.

We believe that the relationship between the progesterone derivatives (*17,20DHP*) must be further studied in order to determine the actual level of output required for stimulation of the animals, as well as the role of the actions of corticosteroids and progesterone together in end of the steroidogenic pathway. This step can be considered as satisfactory taking into account the limited knowledge about the sequences or gene expression of steroid receptors on global genetic databases. We would like to highlight the importance of these results for future research, assuming that hormone receptors and their interaction are impacting on the reproduction of the species of vertebrates.

In conclusion, the results here found contribute to elucidate the physiological basis that drives the reproductive plasticity of this species. The ability of *A. altiparanae* to reproduce under stress conditions triggers new ideas about hormonal pathways involved in fish ovulation and spawning. Additionally, this proposal provided important data about genetic sequences of CR and PR and the interaction between them during the experiments. Therefore, we do not rely only on steroid plasma profile to understand the physiological

modulation of ovulation under stress conditions, starting a deeper understanding of this species physiology.

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Annex section

Annex 1- Extraction and production of Genomic DNA

To run the assay the following solutions were prepared:

a - Saline Buffer

0.4M NaCl (5M 8ml of NaCl)

10mM Tris-HCl pH8.0 (1ml Tri-HCl (1M pH8)

2 mM EDTA pH 8 (400 0.5M EDTA pH8)

DW up to 100ml

b-20% SDS

c - Proteinase K (20 mg / ml)

d - RNase (10ml / ml)

e - 6M NaCl

f - Isopropanol

g –Ethanol 70%

h- TE 7,0

- 1- First, to dry the samples
- 2- Homogenize in 300µl of saline buffer
- 3- Add 30µl of SDS 20% and 5µl of Prok
- 4- Incubate at 65°C for 1h or 37°C overnight
- 5- Add 270µl of NaCl 5M and vortex for 1 min
- 6- Centrifuge at 10.000g for 10 min
- 7- Transfer the supernatant to a new tube with 300µl of Isopropanol. Mix by inversion.

- 8- Incubate at -20°C for 30 seconds
- 9- Centrifuge at 10.000g for 5 minutes at 4°C
- 10- Discharge supernatant and wash the pellet in 600µl of EtOH 70%
- 11- Centrifuge at 10.000g for 5 minutes at 4°C.
- 12- Dry the pellet
- 13- Add TE buffer (20~50µl)
- 14- After this proceeding the sample will be ready for use on the PCR.

Annex 2 – RNA extraction and cDNA synthesis

Add 1000µl of TRIzol® Reagent (BioTechniques) into a 1.5ml tube ,

Add tissue and homogenize

Incubate at room temperature for 5 minutes,

Add 200µl of Chloroform and shake by hand vigorously for 15 seconds,

Centrifuge at 12.000xg for 15 minutes at 4°C,

Transfer the supernatant (DNA + RNA) to a new 1.5ml tube,

Add 500µl of Isopropanol and vortex,

Incubate at room temperature for 10 minutes,

Centrifuge at 12.000xg for 10 minutes at 4°C,

Discharge the supernatant,

Add 500µl of 70% EtOH

Centrifuge at 12.000xg for 5 minutes at 4°C

Discharge the supernatant and let the RNA dry for 5~10 minutes

Re-suspend the pellet in DEPC water or TE Buffer determining the concentration of RNA using the NanoDrop (NanoPhotometer®) to determinate the volume of water or TE.

Annex 3 – Removing Genomic DNA, DNase treatment

Add 1µg of RNA into a 0.5ml tube

Add 1µl of 10x DNASE I Reaction Buffer and 1µl of DNase I Amp Grade,

DEPC water to 10µl,

Incubate at 25°C for 15 min,

Add 1µl of 25mM EDTA,

Heat for 10 minutes at 65°C to inactivate the DNase

Cool down at 4°C,

The RNA will be ready for reverse transcription

Annex 4 – DNA synthesis

Add: 1µl of Oligo (dt) 10mM

1µl of DNTP (10mM) mix

2µl of DEPC water,

Centrifuge briefly (spin down)

Heat the mixture to 65°C for 5 minutes and then cool down on ice for at least 1 minute,

Add: 4µl of 5X First- Stand Buffer

1µl of 0.1 DTT

0.5µl of RNase OUT

0.5µl of SSIII RT (Super Scribe III)

Centrifuge briefly,

Incubate at 50°C for 60 minutes,

Inactivate at 70°C for 15 minutes,

Store at -20°C until use

After this process the sample will be ready for use on the PCR.

Annex 5 – Extracting DNA from the Agar gel

First estimate the µg of DNA present on the cut band.

1. Determine weight of agarose gel band slice in a 1.5- 2.0 ml tube.
2. Add 1/2 volume of ULTRA MELT and 4.5 volumes of ULTRA SALT (example: if TBE GEL = 0.1grams, add 0.05ml (50µl) ULTRA MELT and 0.45ml of ULTRA SALT)
3. Mix well
4. Incubate at 55°C to melt agarose gel. Mix occasionally by shaking thoroughly. Completely melt gel before proceeding (approximately 5 minutes).
5. Re-suspend ULTRA BIND by vortexing at highest speed with ULTRA BIND tube in a horizontal position until homogeneous (around 1 minute)
6. Add ULTRA BIND: 5 plus 1µl per µg of DNA you expect to recover (e.g. for 0-1µg of DNA use 6µl)
7. Incubate 5 minutes at room temperature. Mix several times during this binding step by flicking the tube for small volumes or shaking and inverting for large volumes.
8. Centrifuge 5 seconds. Remove the supernatant and set aside.
9. Ensure 250ml of 100% ethanol was added to the ULTRA WASH before using for the first time. Re-suspend the pellet in 1ml of ULTRA WASH/EtOH by vortexing 5-10 seconds. Soak for 5 minutes instead.
10. Centrifuge 5 seconds. Discard the supernatant.
11. Centrifuge 5 seconds again. Remove all traces of Ultra Wash by aspirating with a narrow pipet tip.
12. Re-suspend the pellet in water or TE (10mM Tris, 0.1mM EDTA). Use a volume twice that of the Ultra Bind silica used before and re-suspend by pipetting until homogeneous. Don't vortex. Disrupt pellet with the pipet tip and then pipet up and down to make sure the entire pellet is re-suspended.

13. Incubate up to 5 minutes at room temperature.
14. Centrifuge 1 minute.
15. Remove the supernatant immediately and transfer it to a new tube. If white silica is carried over, spin again and transfer the supernatant to a new tube. The DNA will be ready to use for directly sequencing or use for cloning.

Annex 6 – Cloning purification

1. First at all, make sure place the Binding Buffer on ice before starting the procedure.
2. Prepare the incubated tube, centrifuge at 13.000xg for 30 seconds. Discard the supernatant.
3. Add 250µl Suspension Buffer + RNase to the centrifuge tube containing the bacterial pellet.
4. Re-suspend the bacterial pellet and mix well.
5. Add 250µl of Lysis Buffer. Mix gently by inverting the tube 3~6 times. Is important do not vortex. Incubate for 5 minutes at any temperature between +15°C and +25°C.
6. Then, treat the lysed solution add 350µl chilled Binding Buffer. Mix gently by inverting the tube 3 to 6 times and then incubate on ice for 5 minutes.
7. Centrifuge for 10 minutes at 13.000 x g.
8. After the centrifugation, insert one High Pure filter tube into one Collection Tube.
9. Transfer the entire supernatant into upper buffer reservoir of the Filter tube and insert the entire higher pure tube assembly into a centrifuge.
10. Centrifuge for 1 minute at full speed.
11. After this centrifugation the Filter tube from the Collection Tube should be removed and the flow through liquid should be discarded. The Filter tube should be re-inserted on the same collection tube.
12. So, add 500µl of Wash Buffer I to the upper reservoir of the Filter Tube.
13. Centrifuge for 1 minute at full speed and discard the flow through
14. Add 700µl of Wash Buffer II to the upper reservoir of the Filter Tube. Centrifuge for 30-60 seconds at full speed and discard the flow through
15. After discarding, centrifuge the entire High Pure assembly for additional 1 minute. So, discard the Collection Tube.

16. Insert the Filter Tube into a clean, sterile 1.5 ml centrifuge tube.
17. Add 100 μ l of Elution Buffer or double dist water to the upper reservoir of the Filter Tube.
18. Centrifuge the tube assembly for 1 minute at full speed.
19. Then, the micro-centrifuge tube now contains the eluted plasmid DNA