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$PGF_{2\alpha}$ and gonadal steroid plasma levels of successful and unsuccessful spawning *Piaractus mesopotamicus* (Teleostei, Characiformes) females

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Abstract Gonadal steroid and prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$) plasma levels were evaluated in successfully (SP) and unsuccessfully ovulated (UN) female *Piaractus mesopotamicus*. Forty-one females were injected with crude carp pituitary extract (0.6 and 5.4 mg kg^{-1} with a 24-h interval between the doses) and sampled to determine the plasma concentration of 17β-estradiol (E_2), 17α-hydroxyprogesterone (17α-OHP), 17α,20β-dihydroxy-4-pregnen-3-one (DHP), $PGF_{2\alpha}$, and testosterone (T) after each injection (first—A1 and second—A2), and at the time of ovulation for SP and UN. Two clusters were obtained using multivariate analysis: 1—composed of all A1, all A2, and some UN; and 2—composed of all SP and some UN. Median values of E_2 plasma levels were similar between clusters; however, plasma levels of T, 17α-OHP, DHP, and $PGF_{2\alpha}$ of cluster 2 (predominantly formed by SP) were higher than those of cluster 1. Since cluster 2 contained all SP and females of this cluster presented higher levels of $PGF_{2\alpha}$, T, 17α-OHP, and DHP, here we evidently shown in an unprecedented manner that concomitant increased levels of these substances were associated with successful ovulation in this species, but such an increase was not determinant for successful ovulation due to the presence of some UN females in the same cluster 2. These findings highlight the unexplored potential of $PGF_{2\alpha}$ to be used as an accessory tool for inducing successful ovulation for fish farming purposes.

Keywords Pacu · Crude carp pituitary extract · Successful spawning · Gonadal steroid plasma levels · Prostaglandin $F_{2\alpha}$ · Multivariate analysis

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Introduction

Pacu (*Piaractus mesopotamicus*) is a large tropical herbivorous/frugivorous characiform fish which is cultivated in different regions of South America (Moro et al. 2013). Pacu is a total spawner in nature, simultaneously releasing all mature eggs; however, hormonal stimulation is required for spawning in captivity. The spawning rate of hypophysed females ranges from 25 to 53% and the fertility rate between 20 and 90% (Criscuolo-Urbinati et al. 2012). One of the main limitations in pacu aquaculture is associated with unsuccessful ovulation (Criscuolo-Urbinati et al. 2012), which leads to a reduction in the fish-seed production.

Induced spawning failure of fishes is common and limits management in culture (see review by Zohar and Mylonas (2001) and Mylonas et al. (2010)). Spawning is preceded by final maturation (germinal vesicle break down, GVBD) and ovulation, which, in spite of being interrelated events, are controlled by two mechanisms; while the GVBD is regulated by non-genomic actions such as maturation-inducing hormones (MIH), the ovulation is regulated by genomic mechanisms (Theofan and Goetz 1981; Pinter and Thomas 1999).

In this context, it was previously demonstrated in hormone-treated but unspawned characiform fish that a significant percentage of GVBD oocytes remained in their ovaries and were not released (Hainfellner et al. 2012; Criscuolo-Urbinati et al. 2012). Using these data, a new protocol was proposed which included prostaglandin F ($PGF_{2\alpha}$) administration together with conventional hypophysation (Criscuolo-Urbinati et al. 2012). The inclusion of $PGF_{2\alpha}$ caused a significant increase in the *P. mesopotamicus* ovulation rate with a consequent increase in the post-ovulatory follicle frequency in relation to controls (only conventional hypophysation).

Prostaglandins are potential inducers of ovulation in fish (Ogata et al. 1979; Cetta and Goetz 1982; Goetz 1983; Jalabert and Szöllösi 1975; Fujimori et al. 2011, 2012, Chourasia and Joy 2012; Joy and Singh 2013; Takahashi et al. 2013; Knight and Van der Kraak 2015), and they may act over gonadal steroid synthesis and release, especially over 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (Knight and Van der Kraak 2015), the most common maturation-inducing hormone (MIH) in fish (Nagahama and Yamashita 2008). It has been demonstrated that MIH stimulates the synthesis of PGs in the fish ovary (Kagawa et al. 2003; Knight and Van Der Kraak 2015) and DHP-treated follicles in vitro showed an increase in the production of PGE and $PGF_{2\alpha}$ in a dose-dependent manner during ovulation (Berndston et al. 1989; Bradley and Goetz 1994).

To address successful ovulation in fish, it is necessary to consider that other substances besides DHP and PGs, especially other gonadal steroids, are also directly or indirectly associated with this process. It has already been demonstrated that successful ovulation is preceded by a decrease in 17β-estradiol (E_2) levels just before spawning (Fostier et al. 1978; Levavi-Zermonsky and Yaron 1986; Asturiano et al. 2002; Lubzens et al. 2010; Gohin et al. 2011), and E_2 treatment in vitro significantly inhibited the MIH output of the follicles (Jalabert and Fostier 1984), maintaining oocyte meiotic arrest (Pang et al. 2008; Pang and Thomas 2010). Moreover, a testosterone (T) peak close to the spawning period has been reported in *Stizostedion vitreum* (Malison et al. 1994), *Oncorhynchus tshawytscha* (Slater et al. 1994), and *Salmo trutta* (well summarized from literature data in the introduction of Hoogenboom et al. (2012)).

Therefore, there are a large number of studies showing that PGs and gonadal steroids have integrated stimulatory and regulatory actions in the fish ovulation process; however, because most of these data were obtained in vitro, specific approaches in vivo are necessary to address,

with a comprehensive design, the possible relationships between the profile of PGs and gonadal steroids with successful and unsuccessful ovulation (Knight and Van der Kraak 2015) to improve routine protocols for fish induced spawning. Thus, considering the previously positive outcome obtained by use of synthetic $PGF_{2\alpha}$ in *P. mesopotamicus* ovulation (Criscuolo-Urbinati et al. 2012) and aiming to further improve induced breeding protocols, the main objective of this study was to assess whether increases in endogenous $PGF_{2\alpha}$ levels would result in successful ovulation in this species. Furthermore, using multivariate analysis, we evaluated if females that successfully spawn show a clear, recognizable, and constant pattern of T, E2, 17α-hydroxyprogesterone (17α-OHP), DHP, and $PGF_{2\alpha}$ plasma concentrations at the predicted time of ovulation.

Materials and methods

Fish were treated and handled according to protocols accepted by the "Comissão de Ética no Uso de Animais from the Faculdade de Ciências Agrárias e Veterinárias/Unesp" (Protocol No. 017713/14 - Jaboticabal, SP, Brazil).

Handling and maintenance of the fish

Four-year-old mature *P. mesopotamicus* breeders, obtained by induced breeding, were reared at 0.25 fish/m^3 density at the Centro de Aquicultura da UNESP – CAUNESP, Universidade Estadual Paulista – UNESP (Jaboticabal, SP, Brazil) in 200-m^3 outdoor earthen ponds supplied with approximately 20 L/min inflowing water. Fish were fed with a commercially feed (Guabi, Campinas, SP, Brazil—32.0% crude protein, 6.5% fat, 10.0% ash, and 7.0% crude fiber) twice a day to apparent satiety (1–4% of biomass).

Breeder selection for hormonal induction

Forty-one females were selected by external characteristics (swollen abdomen and enlarged urogenital papilla) and by the degree of development of the intra-ovarian oocytes sampled with a plastic catheter (≥ 30% oocytes with germinal vesicles shifted towards the periphery) according to Romagosa et al. (1990). Breeders were transported to the laboratory and given hormonal injection as described by Criscuolo-Urbinati et al. (2012). Briefly, females received two doses of crude carp pituitary extract (0.6 and 5.4 mg/kg with a 24-h interval between the doses), and males a single dose of 2 mg/kg at the time of the resolving dose for females. Females were sampled four times (n = 5–9): at the first dose (A1); at the second dose (A2); the spawned females (SP) with up to 340 accumulated thermal units (ATU); and unspawned females (UN), which had not spawned before 340 ATU [According to Criscuolo-Urbinati et al. 2012), pacu spawns within the range of 260–340 ATU)]. ATU was calculated as the sum of the water temperature (°C) over time (h) after the second dose.

Collection procedures and biological material storage

For sample collection, fish were anesthetized with 100 mg/L of benzocaine; blood samples were collected from the caudal vein using heparin-treated syringes. The serum was separated

by centrifugation at 1500×*g* for 15 min at 4 °C and then stored in liquid nitrogen until measurement of the steroid hormone concentrations. For PG dosage, 10 μM indomethacin was added to the blood immediately after collection to inhibit PG synthesis [commercial enzyme-linked immunosorbent assays (ELISA) kits; Cayman Chemical Company, Ann Arbor, MI, USA)]. Plasma levels of T (AccuBind™, Monobind Inc., Lake Forest, CA, USA), E_2, 17α-OHP (DRG Instruments GmbH, Marburg, HE, DE), DHP, and $PGF_{2\alpha}$ (Cayman Chemical Company, Ann Arbor, MI, USA) were quantified by ELISA using commercial kits. The absorbance measurements were performed in a microplate reader (Epoch 2, Biotek Instruments Inc., Winooski, VT, USA); all of the samples were read in duplicate. For validation of the kits, intra- and inter-assays were performed, and we obtained the respective following variations: 0.04 to 17.31% and 3.65 to 18.42% for E_2; 0.05 to 19.84% and 12.84 to 21.28% for T; 0.34 to 17.73% and 1.87 to 13.60% for 17α-OHP; 0.53 to 18.70% and 2.96 to 17.57% for DHP; and 0.87 to 19.64% and 0.30 to 20.08% for $PGF_{2\alpha}$, respectively.

Multivariate statistical analysis

The statistical analyses were performed using statistical software (STATISTICA; StatSoft, Inc., Tulsa, OK, USA) or Excel (Microsoft, Redmond, USA). Three multivariate statistical methods were applied in order to classify fish into groups: hierarchical cluster analysis, non-hierarchical cluster analysis (*k*-means), and principal component analysis. All multivariate analyses were performed after the standardization of variables, of which the mean and variance were 0 and 1, respectively.

The hierarchical cluster analysis was performed by calculating the Euclidean distance between accesses to the set of five variables (T, E_2, 17α-OHP, DHP, and $PGF_{2\alpha}$ plasma levels) and using Ward's algorithm for obtaining the similar access groups. The result of the analysis was presented as a graphic (dendrogram). Fish identification of the groups was also made by the *k*-means method with the number of clusters observed by hierarchical cluster analysis.

In the principal component analysis (PCA), the initial set of five variables (T, E_2, 17α-OHP, DHP, and $PGF_{2\alpha}$ plasma levels) came to be characterized by two new latent variables, which allowed its location in two-dimensional figures (sort of the access by principal components). The appropriateness of this analysis was verified by the amount of the total information of the original variables retained by the principal components showing eigenvalues higher than one.

The average hormone levels between the groups formed by non-hierarchical cluster analysis were compared using the Mann-Whitney *U* test with a significance level of 5% and expressed in median.

Results

Multivariate statistical analysis

Hierarchical cluster analysis

The dendrogram obtained by the hierarchical cluster analysis is shown in Fig. 1. It is accepted that a cluster division may be done if an expressive variation is obtained in the amounts of

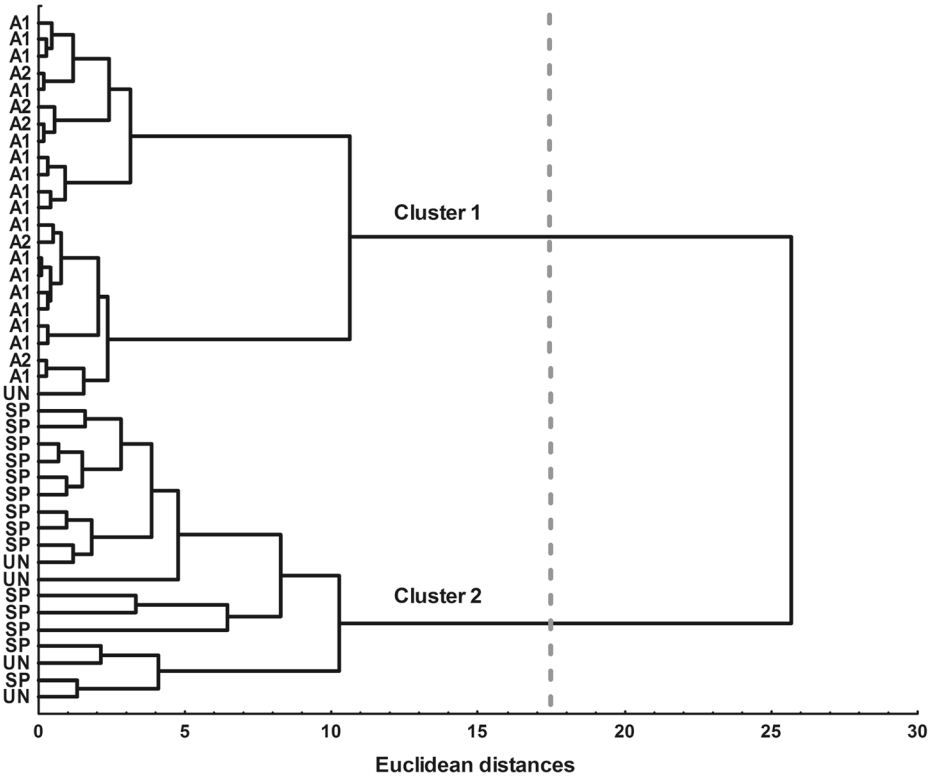


Fig. 1 Dendrogram of *P. mesopotamicus* hypophysed females resulting from hierarchical cluster analysis showing the formation of groups according to plasma concentrations of testosterone, 17β-estradiol, 17α-hydroxyprogesterone, and prostaglandin $F_{2\alpha}$. Fish at the time of the first dose (0.6 mg/kg) of crude carp pituitary extract (A1), at the time of the second dose (5.4 mg/kg) of crude carp pituitary extract (A2), and at the time of spawning: spawned females (SP) and unspawned females (UN)

Euclidean distance among the sampling unit to the set of variables. Therefore, herein, the observation of the Euclidean distance axis allowed the division of the sampling units in two clusters: cluster 1 composed of A1, A2, and UN fish; and cluster 2 of SP and UN fish.

Non-hierarchical cluster analysis

In this analysis, two clusters for application of the *k*-means clustering were considered. The pacu females (sampling units) were sorted into two clusters, where cluster 1 was composed of all A1 and A2 fish and two UN fish and cluster 2 of all SP and three UN fish. Moreover, according to the ANOVA (Table 1), the variables T, 17α-OHP, DHP, and $PGF_{2\alpha}$ plasma levels were important for the ranking ($P < 0.05$). Comparing the median of each variable within each group by Mann-Whitney *U* test, at 5% (Fig. 2), we observed higher values for plasma levels of T, 17α-OHP, DHP, and $PGF_{2\alpha}$ in cluster 2 (predominantly formed by SP) than cluster 1. However, E_2 plasma levels were similar between groups (Fig. 2). Plasma levels of E_2, T, 17α-OHP, DHP, and $PGF_{2\alpha}$ were 6.498 and 7.261 ng/mL; 0.3153 and 18.120 ng/mL; 1.396 and 2.704 ng/mL; 0.020 and 0.152 ng/mL; and 0.115 and 91.911 ng/mL for clusters 1 and cluster 2, respectively (Fig. 2).

Table 1 Analysis of variance for each variable of the groups formed by *k*-means non-hierarchical cluster analysis

Hormones	Comparison	Sum of squares	Degrees of freedom	*F*	*P*
Testosterone	Between groups	19.325	1	36.454	0.000
	Within groups	20.675	39		
Estradiol	Between groups	3.101	1	3.277	0.078
	Within groups	36.899	39		
17α-Hydroxyprogesterone	Between groups	18.559	1	33.758	0.000
	Within groups	21.441	39		
17α,20β-Dihydroxy-4-pregnen-3-one	Between groups	14.892	1	23.133	0.000
	Within groups	25.108	39		
Prostaglandin $F_{2\alpha}$	Between groups	14.120	1	21.278	0.000
	Within groups	25.880	39		

Principal component analysis

The PCA allowed one single distribution of the fish because, according to Table 2, only two eigenvalues were higher than one, with the highest eigenvalue being 2.329 (principal component 1—PC1) and the second highest eigenvalue being 1.026 (principal component 2—PC2). The amount of total information of variables retained by both PCs was 67.095% (46.571% from PC1 + 20.524% from PC2). With PC1 and PC2, a bidimensional ordination of the fish and variables was performed, allowing the construction of a biplot graphic (Fig. 3) where the ordination of the fish by PC1 and PC2 confirmed the ordination of the fish in two groups, similar to the sort obtained by hierarchical and non-hierarchical cluster analysis (Fig. 1).

The graphical representation and the correlation of the variables in the PC (Fig. 3 and Table 2) allowed characterization of the variables that best discriminate in the group formation. PC1 correlated more with the variables T (− 0.736), 17α-OHP (− 0.851), DHP (− 0.819), and $PGF_{2\alpha}$ (− 0.598) responsible for the SP female discrimination in the PC1 left side (negative correlation). Thus, the SP females could be characterized as the fish that have higher levels of T, 17α-OHP, DHP, and $PGF_{2\alpha}$ compared to females collected at other periods of hormonal induction (A1 and A2). On the other hand, UN females did not form a defined group because their hormone concentration profiles were distributed in clusters 1 and 2. In the PC2, only the variable E_2 showed correlation (0.947), but the fish distributed at the top and bottom of the biplot graph by E_2 do not seem to be associated with spawning and/or sampling time during the hormonal induction.

Discussion

In this study, we observed that successful ovulation in *P. mesopotamicus* was associated with increased $PGF_{2\alpha}$ in the plasma at the time of spawning and $PGF_{2\alpha}$ values in cluster 2 (containing all SP females) were 90× higher than those in cluster 1 (91.911 vs 0.115 ng/mL). Increase in $PGF_{2\alpha}$ plasma levels at spawning was associated with ovulation in hormonally induced *Misgurnus anguillicaudatus* (Ogata et al. 1979) and *Heteropneustes fossilis* (Chourasia and Joy 2012), as well as with spontaneously spawned *Danio rerio* (Lister and Van Der Kraak 2008). However, in the present study, even together with a DHP peak, we

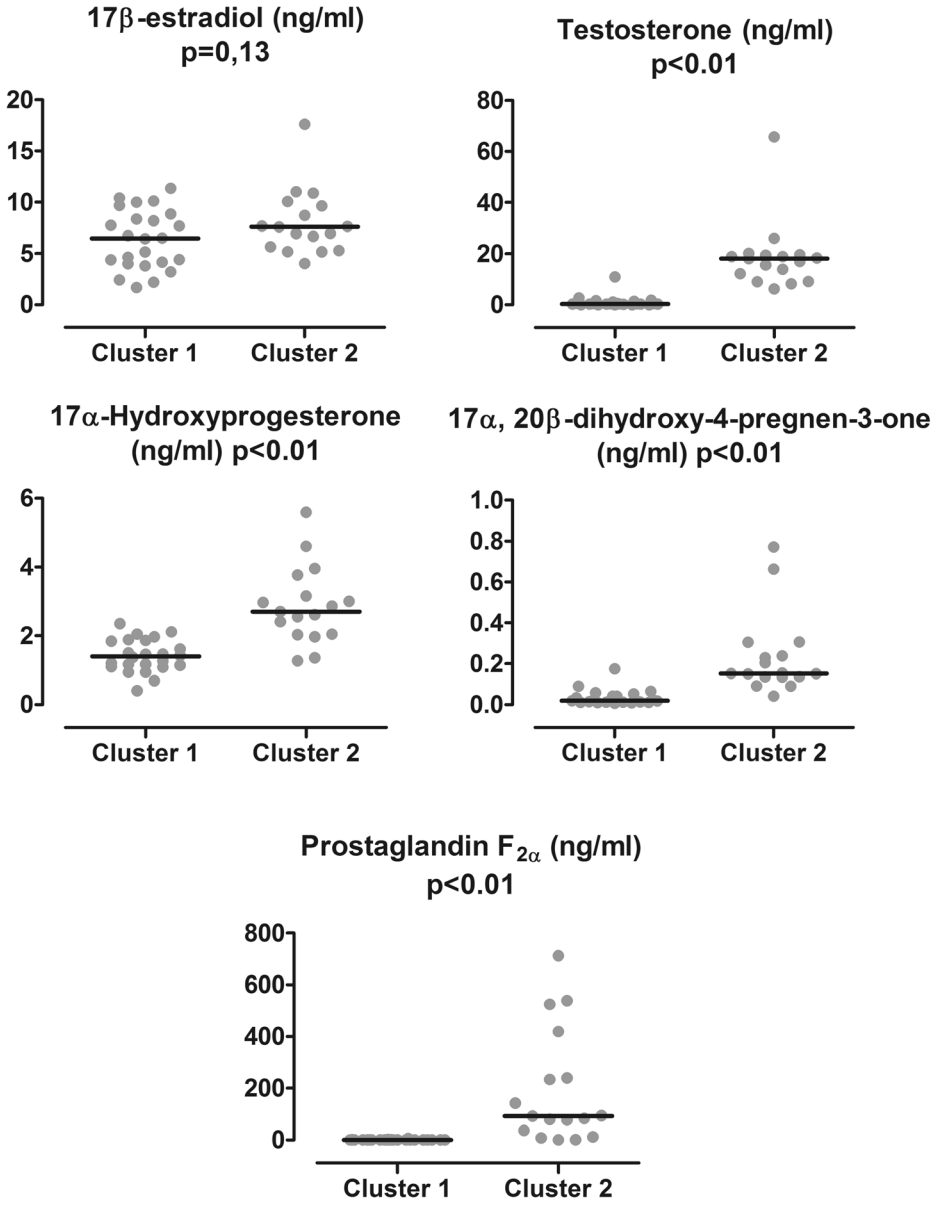


Fig. 2 Plasma concentrations of testosterone, 17β-estradiol, 17α-hydroxyprogesterone, and prostaglandin $F_{2\alpha}$ of the clusters ranked by cluster analysis by *k*-means method. Values are presented as median (-) and distributions of data (●). *Significant difference between groups ($P < 0.05$, Mann-Whitney *U* test). Cluster 1 was predominantly composed of fish at the time of the first dose (0.6 mg/kg) of crude carp pituitary extract, at the time of the second dose (5.4 mg/kg) of crude carp pituitary extract, and at the time of spawning: unspawned females (UN), and cluster 2 was composed of at the time of spawning: spawned females and UN fish

showed that a $PGF_{2\alpha}$ peak at the time of spawning was not associated with successful ovulation in all treated *P. mesopotamicus* females; plasma levels of these substances were similar among a few UN and all SP females of cluster 2.

Table 2 Details of the principal component analysis of the fish undergoing hormonal induction collected at different times

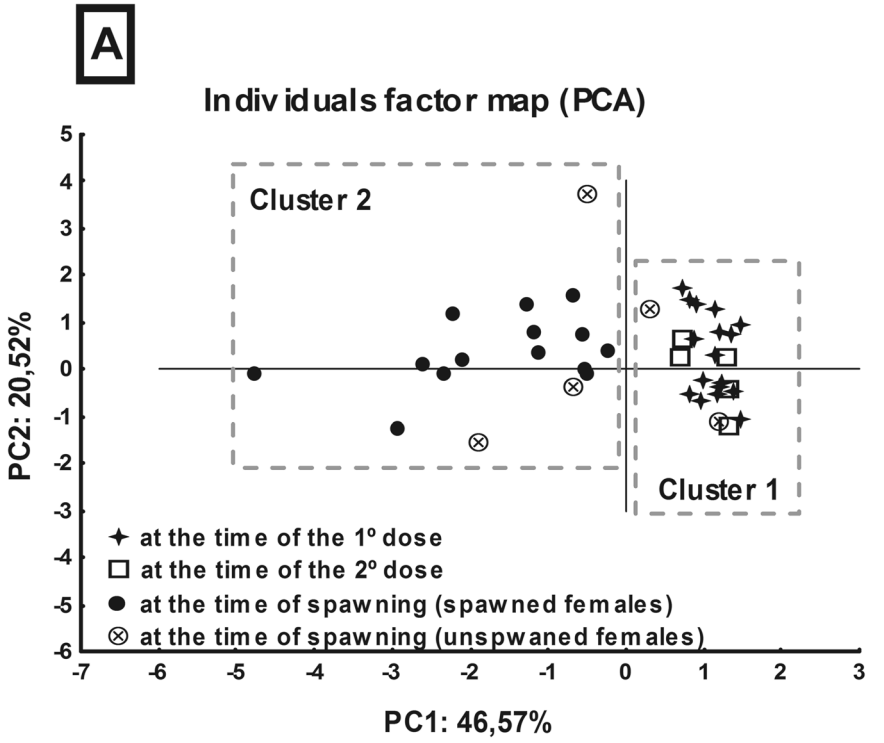
	Principal component 1	Principal component 2
Explained variance % (eigenvalue)	46.571 (2.329)	20.524 (1.026)
Variable correlation		
Testosterone	−0.736	0.103
Estradiol	−0.184	0.947
17α-Hydroxyprogesterone	−0.851	0.053
17α,20β-Dihydroxy-4-pregnen-3-one	−0.819	−0.132
Prostaglandin $F_{2\alpha}$	−0.598	−0.314

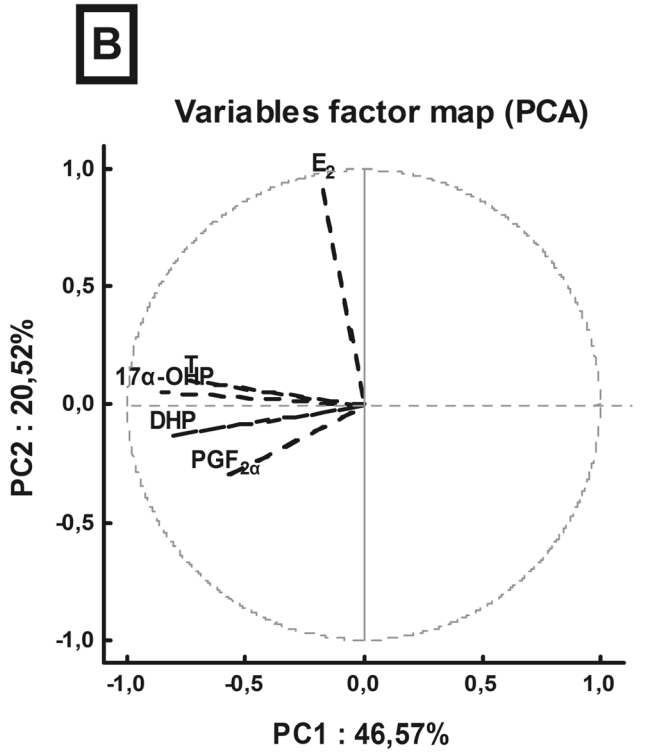
The required levels of $PGF_{2\alpha}$ to trigger ovulation may vary in *P. mesopotamicus* females; Criscuolo-Urbinati et al. (2012) previously obtained 100% ovulation in $PGF_{2\alpha}$-treated *P. mesopotamicus* females; thus, it is possible that the level of $PGF_{2\alpha}$ required to promote ovulation in all females (considering individual variations) has been reached only because exogenous $PGF_{2\alpha}$ had been applied. In this case (Criscuolo-Urbinati et al. 2012), the percentage of ovulation in hypophysed-only females was 25, 53, and 83.3% (3 trials). In addition, it must also be considered that, in the present study, the same CPE dose provoked a great heterogeneity in the levels of $PGF_{2\alpha}$ in females analyzed because, regardless of successful or unsuccessful spawning, SP and UN coefficients of variation were 122.88 and 135.26, respectively.

Considering the $PGF_{2\alpha}$ and gonadal steroid profiles together, the data obtained in this study were in accordance with the current literature because successful ovulation in *P. mesopotamicus* was associated with concomitant peaks of $PGF_{2\alpha}$ and DHP. Both of these substances are known to have intrinsic connections and to be among the main promoters of fish ovulation (Berndston et al. 1989; Bradley and Goetz 1994; Kagawa et al. 2003; Nagahama and Yamashita 2008; Lubzens et al. 2010; Knight and Van Der Kraak 2015). Therefore, the higher DHP levels of cluster 2 females (7.6× higher than cluster 1) may have contributed to a $PGF_{2\alpha}$ peak in this group, as it is known that DHP stimulates the synthesis of PGs in fish ovaries (Kagawa et al. 2003; Knight and Van Der Kraak 2015) and mature follicles (Berndston et al. 1989; Bradley and Goetz 1994).

We also detected a significant increase in T levels in SP females (57× higher in cluster 2 than cluster 1). A specific and direct role of T in the ovulation process is unknown, but the increased levels of T in SP females may be related to high levels of $PGF_{2\alpha}$. It is known that PGs are eicosanoids produced from AA, which, under Lh action, are capable of stimulating steroidogenesis, particularly of T (Mercure and Van Der Kraak 1995). It is also known that the actions of AA on T synthesis are dependent on its conversion into metabolites by prostaglandin-endoperoxide synthase (Ptgs or cyclooxygenase) (Van Der Kraak and Chang 1990). Thus, prostaglandin synthesis may indirectly stimulate the production of T via 3′,5′-cyclic adenosine monophosphate (cAMP) (Wade and Van Der Kraak 1993; Mercure and Van

Fig. 3 Distribution of individuals (rows in the database) and direction of hormones according to principal components 1 (CP1) and 2 (CP2). **a** Principal component analysis (PCA) was performed with samples taken at the time of the first dose (0.6 mg/kg) of crude carp pituitary extract, at the time of the second dose (5.4 mg/kg) of crude carp pituitary extract, and at the time of spawning; spawned females and unspawned females, which correspond to the number of each observation in the database. **b** Testosterone (T), 17β-estradiol (E_2), 17α-hydroxyprogesterone (17α-OHP), 17α,20β-dihidroxi-4-pregnen-3-one (DHP), and prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$) plasma levels as vectors


A
Individuals factor map (PCA)
Cluster 2
Cluster 1
at the time of the 1º dose
at the time of the 2º dose
at the time of spawning (spawned females)
at the time of spawning (unspwaned females)
PC2: 20,52%
PC1: 46,57%


B
Variables factor map (PCA)
E2
17α-OHP
DHP
PGF2α
PC2 : 20,52%
PC1 : 46,57%

Der Kraak 1995). Thus, it is possible that the increased levels of T in cluster 2 may have occurred, at least in part, as a result of AA metabolism by Ptgs, which likely demonstrated increased levels because $PGF_{2\alpha}$ levels were remarkably higher (90× higher in this group than in cluster 1).

Several studies have demonstrated that successful ovulation is frequently preceded by decreases in E_2 levels (Levavi-Zermonsky and Yaron 1986; Asturiano et al. 2002; Levavi-Sivan et al. 2004; Yaron et al. 2009), as well as reduced expression levels of the gene encoding its synthesizing enzyme, *cytochrome P450 family 19 subfamily A member 1* (*cyp19a1*), prior to ovulation (Gohin et al. 2011). A "steroidogenic shift" process, involving concomitant increase and decrease of DHP and E_2, has been also previously described in several teleosts (Levavi-Zermonsky and Yaron 1986; Asturiano et al. 2002; Levavi-Sivan et al. 2004; Yaron et al. 2009) to be important for ovulation (Senthilkumaran et al. 2004). Moreover, E_2 in vitro-treated follicles had significantly inhibited MIH output (Jalabert and Fostier 1984). However, in contrast to most studies, our results were more closely related to those of Mikolajczyk et al. (2007) in goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) where E_2 plasma levels remained similar during induced spawning. Therefore, in the present study, there was not an obvious inhibitory function of E_2 on *P. mesopotamicus* ovulation.

In this study, we confirmed previous evidence (Criscuolo-Urbinati et al. 2012) showing that increased levels of $PGF_{2\alpha}$, at the time of ovulation, are associated with successful ovulation in *P. mesopotamicis* hypophysed females, but due to the presence of UN females in cluster 2 (obtained here), the endogenous levels of $PGF_{2\alpha}$ of some females (few UN of cluster 2) may be insufficient to provoke ovulation. This possibility may explain the 100% ovulation rate when exogenous $PGF_{2\alpha}$ was previously applied in this species (Criscuolo-Urbinati et al. 2012). This hypothesis should be addressed in future approaches, but if this is true, individual variations should be considered to ensure predictable ovulation.

Failure to ovulate occurs in various fish species worldwide and is a problem that affects one of the main foundations of production of any species: the constant supply of fingerlings (Zohar and Mylonas 2001; Mylonas et al. 2010). In this sense, this study reinforces the association between successful ovulation with prostaglandin levels, but also highlights potential use of prostaglandins as an auxiliary tool to obtain successful spawning for fish farming purposes. However, further progress towards predictable ovulation in *P. mesopotamicus* must also address substances other than those considered here, such as PGE_2 (Goetz and Theofan 1979; Takahashi et al. 2013), vasotocin (Joy and Chaube 2015), or melatonin (Ogiwara and Takahashi 2016), as well as other factors such as different susceptibilities of fish to reproduction management (Pottinger et al. 1992; Schreck 2010).

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