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Research paper

Hormonal and fitness consequences of behavioral assortative mating in the convict cichlid (*Amatitlania siquia*)

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ABSTRACT

In monogamous species, partner compatibility is a key factor influencing pairing and reproductive success. In pairs with biparental care, studies have mostly focused on behavioral compatibility because it is likely to encourage the coordination of parental care within pairs, leading to a better reproductive success. Behavior modulation, throughout the breeding season and as a function of the social context, is under the regulatory feedback control of endocrine mechanisms. From this link, the idea of hormonal partner compatibility as a key component of pair cohesion and maintenance has recently emerged. Here, we investigated the link between partner behavioral assortment and their hormonal response to the pairing context. We formed assortative and disassortative pairs of convict cichlids based on their behavioral type (proactive or reactive) and took hormone and fitness measurements. Testosterone, 11-ketotestosterone, 17β-estradiol and cortisol levels were measured from fish-holding water before and after pair formation. We found no relationship between the behavioral type of individuals and their pre-pairing hormone levels. Only the level of cortisol was affected by the partner but independently of its behavioral type. Reproductive success was not affected by the level of hormonal similarity within pairs, but we found that the variation in 11-ketotestosterone similarity between the isolated context and the pairing context was related to spawning size, and the variation in cortisol similarity to the number of fry. Behavioral compatibility does not reflect hormonal compatibility in the convict cichlid, but the relationship between reproductive success and the flexibility of hormonal similarity between partners suggests hormonal adjustment within pairs in this species.

1. Introduction

In socially monogamous pairs, partner compatibility plays a key role in reproductive success. Compatibility occurs at different levels, inducing long-term consequences for both parents and offspring. For example, females can have fitness benefits from choosing mates with whom they are genetically compatible, i.e. genetically dissimilar males or those males with whom they share the fewest alleles across loci (Foerster et al., 2003; Kamiya et al., 2014; Mays and Hill, 2004; Thünken et al., 2012). Genetic diversity between partners will thus convey a heterozygote advantage to the offspring which results in a direct genetic benefit. However, social mates not only contribute genes to their offspring but they also often provide parental care. In species that require biparental care, behavioral compatibility between partners is therefore a crucial post-copulatory determinant of offspring fitness (Ihle et al., 2015). Behavioral similarity within pairs increases parental synchrony, which results in better reproductive success and offspring condition in birds and fish (Both et al., 2005; Burtka and Grindstaff, 2015; Gabriel and Black, 2012; Laubu et al., 2016; Schuett et al., 2011; Spoon et al., 2006). On the contrary, behavioral dissimilarity could be advantageous in pairs with parental division of labor as it would reduce sexual conflict, avoid redundancy in parental tasks, and increase complementarity between the partners (Groothuis and Carere, 2005; Wolf and Weissing, 2010, 2012). For example, by choosing a dissimilar partner, proactive (i.e. aggressive, fast explorer and bold) males can obtain the best care giver and reactive (i.e. non aggressive, slow explorer and shy) females the best food exploiter and territory defender. Throughout the breeding season and as a function of the social context, the modulation of these behavioral responses is under the regulatory feedback control of endocrine mechanisms (Adkins-Regan, 2005). From this link, the idea of hormonal partner compatibility as a key component of pair cohesion and maintenance has recently emerged (Hirschenhauser, 2012).

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Hormonal compatibility is likely to act at two levels. Firstly, individuals adapt their hormone levels to their partner to increase coordination in reproductive physiology and hormonally-mediated behaviors. In humans, three months after initiation of a new relationship, the two partners not only had higher oxytocin (OT) levels than singles, but there was also no within-pair difference in these levels. In addition, the OT levels were reported as positively correlated with the expression of affiliative interactions (Schneiderman et al., 2012). In mammals, rapid and long-lasting increases in OT levels were found in both partners during pair formation (Barth et al., 1997; Wallner et al., 2006). The positive within-pair testosterone co-variation (TC) found in pairs of Greylag geese, *Anser anser*, was maintained year-round, including during the non-breeding phases, and resulted in long-term pair-bond maintenance and better reproductive success for the TC-matched pairs (Hirschenhauser et al., 1999; Weiβ et al., 2009). Given that within- and among-individual variations in socio-sexual behavior are often mediated by hormonal mechanisms (Bonier et al., 2011; Cantarero et al., 2015; Hau and Goymann, 2015; Rodgers et al., 2013, 2006), hormonal compatibility may thus reflect behavioral synchrony and therefore be a measure of pair-bond quality.

Secondly, the modulation of hormone levels is affected by the social context and between-partner interactions. Social instability or the need to compensate for the partner's poor performance in nest defense or food provisioning can induce a hormone-specific response (Almeida et al., 2014; Snekser and Itzkowitz, 2014; van Breukelen and Itzkowitz, 2011). In socially monogamous species, separation from the pair partner (due to the death or disappearance of one of the partners for instance) is a relevant social stressor that is known to cause an increase in activation of the hypothala mic–pituitary–adrenal (HPA) axis (Carter et al., 1995; Hennessy, 1997). This stress response continues, sometimes over a period of several weeks, even when an unfamiliar partner is proposed to the individual after separation from their previous mate (Griffith et al., 2011; Remage-Healey et al., 2003). In monogamous species, mate quality is likely dependent on its ability to provide efficient parental care in terms of territory monopolization, nest defense and provisioning (Kelly and Alonzo, 2009; Royle et al., 2012). However, these behaviors do not always positively co-vary. For instance, aggressive males efficiently defend the nest against predators or competitors but invest less in food provisioning (Duckworth, 2006). In great tits, *Parus major*, fast-exploring birds manage to access and defend high quality territories but increase risk-taking behaviors thereby exposing them to predator attacks, whereas slow-exploring individuals invest more in offspring-oriented parental care (Both et al., 2005). Similarly, although pairing with an aggressive partner seems to be beneficial for territory defense, it may also increase within-pair aggressiveness. This additional social stressor is likely to induce a stress response in the mate suffering from agonistic interactions. In this pairing context, to maintain minimum reproductive success, an individual mated with an absent or poor caregiving partner has to, at least partially, increase its number of visits, feedings or aggressiveness towards intruders to maintain better fitness (Hunt et al., 2014; Johnstone and Hinde, 2006; Markman et al., 1995; Sanz et al., 2000; Schuett et al., 2011). Because behavioral flexibility is mediated by hormonal mechanisms, it is likely that the behavioral adjustment between partners also reflects an adjustment at the hormonal level. For now, we are not aware of any studies linking behavioral assortment between partners with a fine-tuned hormonal response to the pairing context.

We investigated the effect of behavioral assortment between partners on their hormonal response and fitness consequences in the convict cichlid, *Amatitlania siquia* (previously *Cichlasoma nigrofasciatum, Archocentrus nigrofasciatus*; alternately *Amatitlania nigrofasciata*; Schmitter-Soto, 2007a,b). This territorial and monogamous fish species with biparental care is a particularly important model study species in behavioral and evolutionary ecology to address questions on reproductive behavior, foraging behavior, habitat selection, territoriality, aggression, chemical ecology and physiology, among others (e.g. Grant and Guha 1993; Wisenden 1995; Brown et al., 2006; Dechaume-Moncharmont et al., 2013). Its reproductive behavior is well known with females usually taking care of the brood while males defend the territory against both predators and competitors (Lavery and Reebs, 1994), but both members of the pair can defend the breeding territory when faced with a high risk of predation (Itzkowitz et al., 2002; Laubu et al., 2016). In addition, the endocrine mechanisms mediating these socio-sexual behaviors have been well described in a phylogenetically close species, the monogamous cichlid *Cichlasoma dimerus* (Ramallo et al., 2014). Their affiliative, aggressive and reproductive behaviors (nest digging and courtship) are regulated by androgen and estrogen receptor pathways depending on the social context (i.e. challenging *vs*. reproductive context; Sessa et al., 2013; van Breukelen, 2013). Males showed an increase in their levels of circulating 11-ketotestosterone (11-KT) and testosterone (T) during courtship, while they decreased during the parental care and non-breeding phases, both in the field and laboratory (Birba et al., 2015; van Breukelen et al., 2015). In a competitive context, the most aggressive males had the highest T and 11-KT levels but the lowest E2 levels (Ramallo et al., 2015). Finally, recent findings regarding the fitness consequences of behavioral assortment showed that disassortative pairs of convict cichlids can quickly adjust their behavioral type after pairing, thereby increasing their behavioral similarity (Laubu et al., 2016). The disassortative pairs that managed to converge not only improved their reproductive success, but also raised it up to the level of the assortative pairs. Using a similar design based on the formation of behavioral assortative and dissortative pairs of cichlids, we measured the circulating levels of cortisol (CORT), 17β-estradiol (E2), T and 11-KT, an oxidized form of T that has been shown to act as the active androgen in this species (O'Connell et al., 2012), before and after pair formation. Because T, 11-KT and E2 regulate courtship and parental care in cichlids, and CORT is involved in the mobilization of energy stores to cope with a demanding and/or risky situation, hormone responses were expected to vary with the pairing combinations based on the behavioral types. One could predict from previous results on behavioral matching that first assortative pairs also show a hormonal similarity, and second their reproductive success should increase with the degree of hormonal similarity. Conversely, in disassortative pairs, individual hormonal adjustment, resulting in differences in CORT levels between partners, may indicate a stress response in at least one partner or the need to compensate for the difference in behavioral type between partners.

2. Material and methods

2.1. Study animals and laboratory conditions

Male and female cichlids were obtained from an aquarium fish wholesaler (Amazon Fish, Pfaffenhoffen, France) and then maintained at the animal facility in the Biogeosciences laboratory at the University of Burgundy (Dijon, France) at least three months prior to the start of the experiment. All of the fish were individually marked with passive integrated transponder (PIT) tags and housed in same-sex tanks (52–96 L), with ten fish per tank, at 25 ± 1 °C and with a 12:12 h Light:Dark cycle. The fish were fed daily with Cichlid XL flakes (Tetra, Germany). Four to five days before the start of the behavioral tests, they were isolated in a 20-liter tank ($40 \times 20 \times 25$ cm). Each tank was equipped with a PVC tub (8 cm in diameter) serving as a shelter, an air stone and gravel substrate

(Fig. S1). To prevent food dispersion throughout the tank, a piece of polystyrene formed the boundary of a 10-cm wide feeding area at the front end of the tank and the surface of the water. Visual isolation was achieved by opaque plastic dividers between the tanks. After the behavioral tests, the fish were once again housed in same-sex tanks. All of the procedures described below were approved by the French Ministry of Research and Higher Education and the University of Burgundy Ethics Committee.

2.2. Profiling of individual behavioral types

The personality assessment followed the procedure detailed in Laubu et al. (2016). In summary, we recorded the behavioral responses of 108 fish (44 males and 64 females) using four repeatable tests (Laubu et al., 2016; Schweitzer et al., 2015): food neophobia, territory maintenance, aggressiveness and exploration. Except for the exploration test, each focal fish was tested in its 20-liter individual tank. Food neophobia was assessed by recording the latency of the fish to consume a novel food type. Two different novel foods (Tetra Delica Krill Shrimp Freeze Dried Treat or two Power Pellets, a green and a red one, Aquadistri B.V.) were used with all fish in a randomize order to avoid familiarization. To stimulate territory maintenance activity, gravel from the floor was shoveled into the shelter and subsequently 20 min. focal observations were performed, recording the time spent carrying the gravel away. In the aggressiveness test, we staged intra-sexual agonistic encounters between two isolated individuals of the same sex, and suddenly disturbed the agonistic interaction by dropping a 5 g glass marble into the tank from a height of 30 cm (Arnott and Elwood, 2009; Schweitzer et al., 2015). The marble landed behind a narrow screen of opaque tape on the wall separating the two fish so that only the focal fish was startled. Interactions were recorded for 15 min. after recovery from this startle stimulus. The same procedure was repeated for a second startle response. Interactions were terminated 15 min. after recovery from the second startle stimulus by placing the opaque divider back between the two adjacent tanks. The number of agonistic displays (frontal and lateral displays, gill extensions and attempted bites) provides a measure of the intensity of the aggressive behaviors of the focal individual, and the time taken to resume the contest provides a measure of its aggressive motivation (Arnott and Elwood, 2009; Schweitzer et al., 2015). The exploration test was carried out in a 500-liter tank divided into two compartments, a small acclimation compartment (ca. one fourth of the total tank volume) separated from the large "exploration" compartment by an opaque plastic partition that could easily be remotely removed by the experimenter using a system of pulleys. The novel environment contained gravel substrate, two plastic plants and six PVC tubs (8 cm in diameter, commonly accepted as artificial nests by breeding convict cichlids) to provide shelters to explore. The fish was first transferred to the acclimation compartment for 5 min. The partition was then removed and the fish was allowed to explore the large compartment for 15 min. The total distance covered by the focal fish was recorded using an automated trajectometry software (Ethovision, Noldus, The Netherlands) and was used as a measure of the exploration activity of the individuals.

For a given focal fish, boldness toward a novel food, maintenance and exploratory activity were tested within one day in this order, while the aggressive test was conducted the next day. The four tests were repeated one week later to estimate the behavioral repeatability. Following the tests, the standard length and body weight of the fish were measured (to the nearest 0.1 cm and 0.01 g, respectively) before they were returned to their same-sex housing tanks.

The four behavioral traits were repeatable and correlated, thus defining a behavioral syndrome (see Tables S1 and S2 for detailed values; Sih et al., 2004). It was summarized in a one-dimension behavioral score (alongside a reactive-proactive continuum) which allows us to assign each fish to a discrete behavioral type (proactive or reactive). In summary, for each fish, a synthetic behavioral score was calculated from the four behavioral traits using a principal component analysis (PCA; as in Réale et al., 2007). The first two principal components (PC1 and PC2) of the PCA respectively explained 39% and 25% of the total variance. As the PC1 component has an eigenvalue of 1.54 which satisfies to the requirements for dimensionality reduction in PCA (Manly et al., 2004), this axis was retained as the synthetic personality score. It was mostly defined by the exploration and aggressiveness scores, for all of the subsequent analyses, the PC1 axis characterized the reactive-proactive continuum, with positive values indicating proactive individuals (highly aggressive and explorative individuals) and negative values indicating reactive individuals (less aggressive and less explorative individuals; for further details and values see Laubu et al., 2016). For each fish, the PC1 value defined its behavioral scores. The two sexes were evenly distributed along this score.

2.3. Pair formation

Fifty days after the behavioral testing, thirty males (mean standard length ± standard deviation: 62 mm ± 11 mm) and thirty females (48 ± 5 mm) were chosen from their behavioral score in order to form either assortative or disassortative pairs. To prevent frequent injury between partners in the case of experimentally forced pairs, we designed a procedure to control for the behavioral type of the partners while letting the individual choose their partner. We used a 450-liter tank divided into four distinct territories, each freely accessible. Each territory contained an artificial nest (8 cm diameter PVC tube), gravel substrate, a plastic plant and a rock. For each pairing combination, three or four males with a similar behavioral type were first introduced into the tank for 72 h in order to let them establish their territory. We then introduced three or four females, respectively, with a similar behavioral type. For example, the introduction of four reactive males was followed by the introduction of four proactive females in order to form four mismatched pairs including one proactive female and one reactive male. After the introduction of the females, we let the individuals choose their partners. When a pair was formed in a territory (partner's close proximity near the nest, no agonistic interactions between them and nest defense against conspecifics), the pair was moved into a 54-liter breeding tank until the end of the experimental period. Each tank was visually isolated from the other tanks and contained an artificial nest, gravel substrate, an air stone and a heater to maintain the water at 25 °C. This procedure was used to let the individuals have the opportunity to choose between several potential partners while achieving the desired pairing combinations (Fig. S2). Hence, all of the fish managed to find a partner, and we observed no injury among them.

Four pairing combinations were formed: eight pairs with two reactive partners, seven pairs with two proactive partners, seven pairs with one reactive female and one proactive male, and eight pairs with one proactive female and one reactive male. Within a given sex, individuals from the assortative and disassortative pairs did not differ in standard length (males: $t = -0.30$, $p = 0.77$, Cohen's $d = -0.11$, 95% CI = [-0.89; 0.65], females: $t = 0.40$, $p = 0.69$, Cohen's $d = 0.15$, 95% CI = [-0.60; 0.96]). Assortative and disassortative pairs did not differ in contrast between the length of the male (LM) and the length of the female (LF) defined as $|LM\text{-}LF|/(LM + LF)$ ($t = 0.40$, $p = 0.69$, Cohen's $d = 0.15$, 95% CI = [-0.60; 0.94]). The behavioral score between partners did not significantly differ in the assortative pairs ($n = 15$, $t = -0.09$, $p = 0.92$, Cohen's $d = 0.03$, 95% CI = [-0.79, 0.76]), whereas it was contrasted in the disassortative pairs ($n = 15$, $t = -2.90$, $p = 0.008$, Cohen's $d = 1.10$, 95% CI = [0.31, 2.32], Fig. S1).

2.4. Water sample collection and hormone assays

We used the non-invasive technique to measure multiple hormone concentrations in the holding water described in Kidd et al. (2010). Measurement of fish hormone in water offers the following advantages: no anaesthetic, no bleeding and minimal handling stress likely to disturb pair behavior and bias fitness measurements, the possibility of making non-lethal measurements on small fish, repeated measurements on the same fish, and allowing concurrent monitoring of behavior and physiology (Gabor and Contreras, 2012; Scott and Ellis, 2007). Previous studies also provide convincing evidence that the water concentration is a good proxy for plasma concentration (Ellis et al., 2004; Félix et al., 2013; Gabor and Contreras, 2012; Sebire et al., 2007; Wong et al., 2008). Sample collection was performed in two different contexts: at the end of the personality testing period (isolated context) and 24 h after pair formation (pairing context). All of the samples were collected between 9:00 and 11:00 am. The glassware was rinsed prior to use with 100% ethanol and the beakers were then filled with 300 or 500 ml (for body sizes smaller or larger than 6.7 cm, respectively) of filtered unused water. Each fish was removed from its tank, by netting, individually placed in the beaker for 30 min. and at the end of the water sample collection, the fish was reintroduced into its tank. The water sample was then filtered to remove particulate matter and the hormones were extracted. Hormones from each water sample were collected by processing the liquid through a Sep-Pack Plus C18 cartridge (Waters Corp.) connected to a 12-port vacuum manifold, followed by 6 ml of Millipore ultrapure water. Each C18 cartridge was primed by flushing 6 ml of 100% ethanol and then 6 ml of Millipore ultrapure water through the cartridge. The cartridge was then stored at −20 °C until eluted. The cartridges were thawed and then eluted with 4 ml of 100% ethanol into 13 × 100 mm glass tubes using the vacuum manifold. The eluted samples were then completely dried under nitrogen gas flow at room temperature. The sample dried pellets were stored at 820 °C until the enzyme immunoassays (EIA) were performed.

Three assay systems used in this study were purchased from ELS (Enzo Life Sciences, Inc.): T (# 900-065), E2 (# 900-008) and CORT (# 900-071). The 11-KT assay system was purchased from Cayman Chemical (# 582751). The subsequent description of the EIA protocol is identical for all three ELS assays and the manufacturer's instructions were followed unless stated otherwise. In order to take simultaneous measurements of multiple waterborne hormones from the same sample, we used a universal assay buffer for the T and E2 EIAs. The buffer consisted of 0.5 M Tris pH 7.0, 0.15 M NaCl, 1% BSA and Millipore ultrapure water. For the 11-KT kit, the manufacturer's instructions were followed as written. Sensitivities and cross-reactivity values for each assay kit are shown in supplementary Table S3. We ran the samples in duplicate at a dilution of 1:4 for T and CORT, 1:2 for E2, and 1:8, 1:16 or 1:100 for 11-KT, respectively, for females and males with a body size smaller or larger than 6.7 cm in the respective assay buffers. The intra- and inter-assay coefficients of variation were respectively 8.7% and 10.8% for CORT, 9.1% and 10.5% for 11-KT, 9.9% and 6.8% for T, and 10.8% and 5.6% for E2. Plates were read at 405 nm using a Spectra Max® Plus 384 microplate reader (Molecular Devices, LLC).

2.5. Assessment of the reproductive success

The breeding tanks were visually inspected twice a day for spawning. Reproductive success was measured as the number of eggs and the number of fry. The number of eggs was counted just after spawning. To prevent any parental effects on the offspring traits resulting from parental care, the eggs were subsequently gently removed from the tank using a tiny brush and placed in self-constructed cichlid egg tumblers until hatching. The egg tumblers were made of transparent plastic tubes (34 cm in height and 5 cm in diameter, Fig. S3) vertically fixed in a 90-liter tank while submerged in a water bath of 25 °C. Each tumbler was equipped with an air stone that blew air into the tube, resulting in continuous gentle tumbling movements of the eggs. At a given time, an egg tumbler only held the eggs from one pair. After hatching, the number of fry was counted, and 10 fry per pair were randomly chosen for morphometric measurements: total length (TL), myotome height (above anal opening, MH), snout-vent length (SV), eye diameter (ED), yolk sac length (YSL), yolk sac height (YSH), yolk sac perimeter (YSP) and yolk sac area (YSA). The yolk sac volume (YSV) was estimated using the formula: $\left(\frac{\pi}{6}\right) \times (YSL \times YSH^2)$ (Bagarinao, 1986). Each fry was placed on a moistened glass microscope slide and turned on its side to measure all of the morphometric variables using a stereomicroscope (NIKON SMZ1500) with a magnification of 10X.

2.6. Statistical analysis

All of the statistical analyses were performed using the R 3.2 Software (R Core Team, 2015). When required, the behavioral scores were log-transformed to normality. The relationships between the individual behavioral score and hormone levels were assessed by Pearson's correlation tests. To avoid type I error inflation, we used the Benjamini-Hochberg correction for multiple tests (Benjamini and Hochberg, 1995). The difference in individual hormone levels between the isolated and pairing contexts was assessed using a mixed-effect linear model with individual identity as a random variable using the "lme4" packages (Bates et al., 2015). We used linear models to analyze the effect of the male and female behavioral scores on their hormone concentrations in the pairing context and on the variation in their hormone concentrations between the isolated and pairing contexts (Δhormone = hormone concentration in the pairing context – hormone concentration in the isolated context). Starting from a full model including all the variables listed above, we identified the covariates with a significant effect on the response variable by sequentially comparing the nested sub-models (i.e. the two models including or not a given covariate) and backward stepwise elimination of non-significant variables or interactions (Hegyi and Laczi, 2015). For covariates with a significant effect, we performed *post-hoc* pairwise comparisons. In order to limit the number of correlations and to avoid problems arising from multicollinearity, the morphometric measurements of the fry were summarized in two synthetic dimensions using a PCA (Fig. S4). The PCA enabled us to distinguish two principal axes that together explained 82.1% of the variance. PC1 axis explained 49.8% of the variance and was characterized by the yolk parameters at hatching (Pearson's correlation coefficient between PC1 and the measurement, YSA: $r = 0.45$, YSL: $r = 0.38$, YSV: $r = 0.45$, YSH: $r = 0.41$, YSP = 0.45). PC2 axis explained 32.3% of the inertia and was principally characterized by the body parameters at hatching (MH: $r = 0.43$, SV: $r = 0.47$, ED: $r = 0.50$ and TL: $r = 0.52$). We used PC1 and PC2 for the subsequent analyses. Finally, hormonal similarity (S) was calculated for each measured hormone independently following the within-pair similarity index defined by Laubu et al. (2016). It was based on the relative difference between the hormone concentration in males (C_M) and females (C_F): $S = |C_M - C_F|/(C_M + C_F)$. A value of S close to 0 corresponds to similar partners and a value close to 1 indicates dissimilar partners. We subsequently investigated the extent to which the differences in fitness were associated with the within-partner variation in the hormone levels (Δhormone) or the variation in the hormonal similarity (ΔS = S in pairing context – S in isolated context) using mixed-effect models with pair identity as a random variable.

3. Results

3.1. Hormonal and behavioral profiles

Males and females differed in their pre-pairing 11-KT (Welch Two Sample *t*-test: $t = 8.16$, $df = 58.6$, $P < 0.001$, Cohen's $d = 1.81$, 95% confidence interval (95% CI) = [1.39; 2.35]) and E2 ($t = -6.68$, $df = 104.6$, $P < 0.001$, Cohen's $d = -1.22$, 95% CI = [−1.64; −0.86]) concentrations, whereas there was no difference between the two sexes in their pre-pairing T ($t = -0.55$, $df = 64.4$, $P = 0.58$, Cohen's $d = 0.11$, 95% CI = [−0.26; 0.51]) and CORT ($t = 0.60$, $df = 96.7$, $P = 0.55$, Cohen's $d = -0.11$, 95% CI = [−0.57; 0.27]) concentrations (Fig. 1). There was no relationship between each of their single pre-pairing hormone levels and their behavioral type (Table 1).

3.2. Hormonal response to pairing

After pair formation, all of the fish showed an increase in their hormone concentrations (mixed-effect linear model, $\chi^2_1 \geqslant 12.7$, $P < 0.001$ in all cases, Fig. 1), except for the CORT concentration which remained constant in the females ($\chi^2_1 = 2.14$, $P = 0.14$). Not only was the pairing context expected to induce a hormonal response in the partners, but individuals may also differ in their physiological response depending on their own behavioral type or the behavioral type of their mate. Females paired with proactive males had higher 11-KT concentrations ($r = 0.44$, 95% CI = [0.018; 0.72], $P = 0.040$). No other hormone level was affected by the partners' behavioral scores (see Table S4 for the summary of the model selection procedures). Similarly, the variation in the hormone levels of the male and female cichlids between the isolated and pairing contexts did not depend on their own behavioral score or the behavioral score of their mate (Table S4). However, there was a significant negative relationship between the partners in the variation of their CORT concentration after pair formation ($r = -0.48$, 95% CI = [−0.77; −0.01], $P = 0.045$, Fig. 2). An increase in the CORT concentration in one mate was correlated to a decrease in the CORT concentration in the other one, regardless of the partners' behavioral type.

3.3. Fitness consequences

At the individual level, there was a significant relationship between the increase in the T concentrations of the females and PC1 characterizing the yolk parameters of the fry (linear models correcting for female and male body lengths: $F_{1,17} = 11.54$, $P = 0.003$, effect size $\beta = 0.20$, 95% CI = [0.17; 0.98]). Females that had a larger increase in T concentrations between isolated and pairing contexts had fry with larger yolk parameters. In the pairing context, fitness was not affected by hormonal similarity between partners ($p \geqslant 0.11$ in all cases). However, the spawning size was related to the variation in 11-KT similarity ($\Delta S_{11\text{-KT}}$) between the isolated and pairing contexts (linear models correcting for female and male body lengths: $F_{1,16} = 5.36$, $p = 0.035$, effect size $\beta = -182.8$, 95% CI = [−351.03; −14.53], Fig. 3a). Pairs that showed an increase in their 11-KT similarity between the isolated and pairing context had a lower number of eggs than pairs that showed a decrease in their 11-KT similarity. A reversed relationship was found between the number of fry and the variation in CORT similarity (ΔS_{CORT}) between the isolated and pairing contexts ($F_{1,13} = 4.95$, $p = 0.046$, effect size $\beta = 149.2$, 95% CI = [3.04; 295.39], Fig. 3b).

4. Discussion

We found no relationship between the individual behavioral type and their pre-pairing hormone levels. Only the level of CORT was affected by the partner but independently of its behavioral

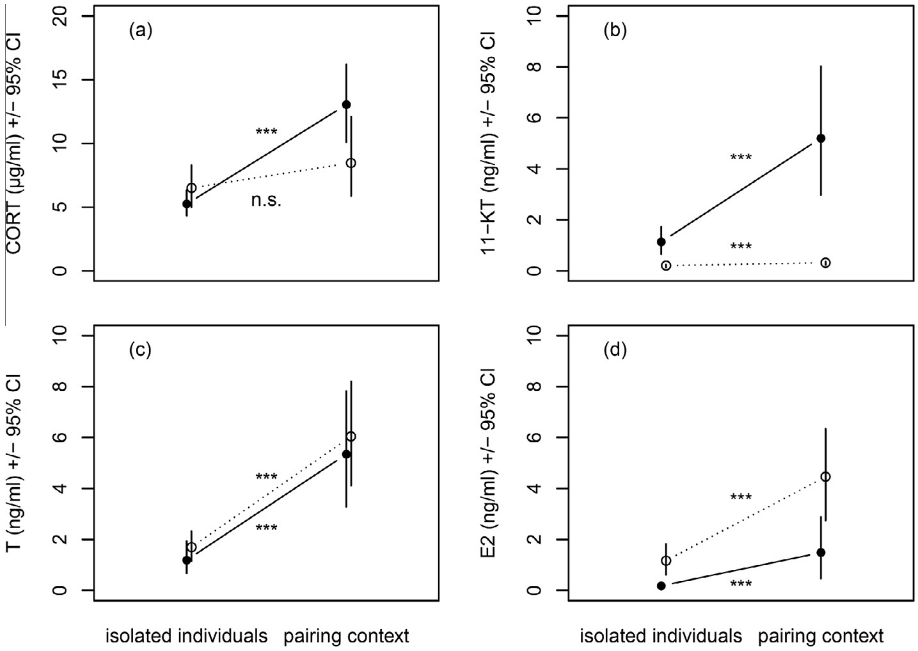

Fig. 1. Mean (±95% CI) pre-pairing and post-pairing concentrations of male (solid line) and female (dotted line) convict cichlids. Pre- and post-pairing samples used to measure the CORT (a), 11-KT (b), T (c) and E2 (d) levels were collected in isolated individuals and 24 h after pair formation, respectively (mixed-effect linear model: $^{***}P < 0.001$, n.s., not significant).

Table 1
Results from Pearson's correlation tests between the behavioral score and the pre-pairing hormone concentrations in male and female cichlids.

Hormone	Sex	*r*	95% CI	*P*	*P* adjust [a]
CORT	Male	0.05	−0.27; 0.36	0.76	0.94
	Female	0.02	−0.23; 0.28	0.85	0.94
11-KT	Male	0.07	−0.24; 0.36	0.66	0.94
	Female	−0.20	−0.42; 0.05	0.11	0.44
T	Male	0.05	−0.25; 0.35	0.74	0.94
	Female	−0.12	−0.36; 0.13	0.33	0.88
E2	Male	0.26	−0.04; 0.52	0.09	0.44
	Female	0.01	−0.24; 0.25	0.94	0.94

[a] P-values after Benjamini and Hochberg's (1995) correction for multiple tests.

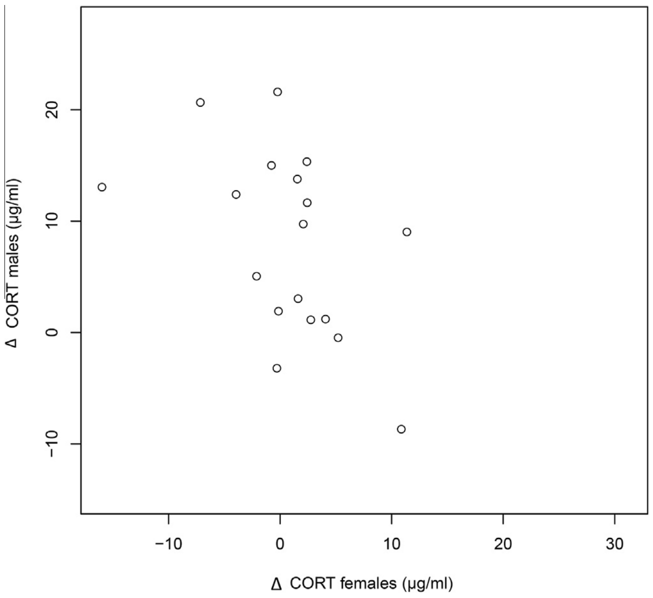

Fig. 2. Relationship between male and female cichlids in terms of the variation in the CORT concentrations (μg/ml) between the isolated and pairing contexts.

type; such as the increase in CORT concentrations after pairing in one mate was associated with a decrease in the other one and the other way around. The level of hormonal similarity within the pair did not affect reproductive success. However, our results suggest that the flexibility in 11-KT and CORT concentrations between partners, in response to pair formation, is likely to influence reproductive success. Indeed, the spawning size was related to the change in 11-KT similarity, and the number of fry was related to the change in CORT similarity.

Based on the extensive literature reporting a relationship between physiology and behavior, especially the fact that specific behavioral responses are mediated by changes in the hormonal profile (Adkins-Regan, 2005; Ketterson et al., 2009; Killen et al., 2013) and that the selection of lines based on their hormonal levels induced differences in the behavioral type in many species (Cockrem, 2013; Jones et al., 1992, 1999; Martins et al., 2007; Øverli et al., 2002), we expected that the behavioral type would be predicted by the hormonal profile in this species of cichlids. The recent pace-of-life syndrome (POLS) hypothesis also predicts these relationships between behavioral types and physiological traits. Between-individual variation in behavioral traits such as exploration, aggressiveness, or risk-taking are expected to correspond to a suite of metabolic, hormonal and immunity traits that have coevolved with inter-individual variation in life-history strategies related to different ecological conditions (Biro and Stamps, 2008; Réale et al., 2010). For instance, the POLS hypothesis predicts that the most exploratory, active, or aggressive individuals (i.e. proactive individuals) should show a higher metabolism (Careau et al., 2011; Montiglio et al., 2014), a higher energy intake (Carter et al., 2010; David et al., 2011) and a higher growth rate (Biro et al., 2014), but will have a higher short-term, rather than long-term, reproductive success (Patterson and Schulte-Hostedde, 2011). Contrary to these predictions which are based on poorly investigated premises, we observed a lack of relationship between the behavioral type and physiological traits. We propose two ways to explain these results. First, the behavioral type may not be reflected by the hormone concentrations in undisturbed individuals (pre-pairing concentrations) but rather by the change in the hormonal profile in response to a given situation. This could be measured as a reaction norm (Dingemanse and Wolf, 2013;

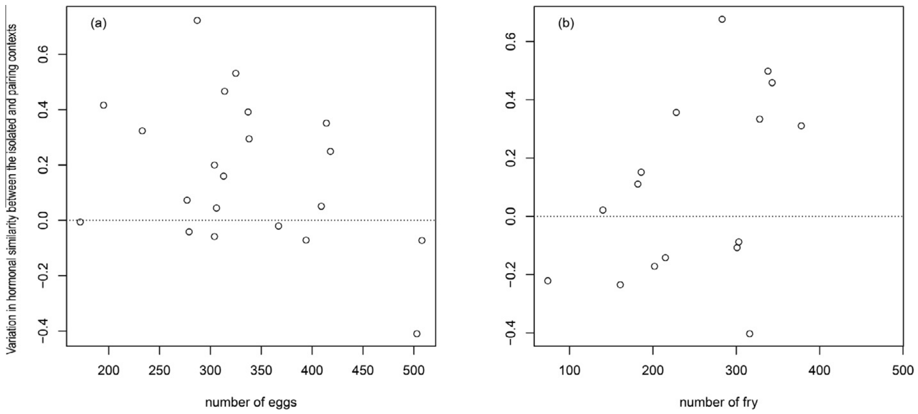

Fig. 3. Fitness consequences of the variation in hormonal similarity between the isolated and pairing contexts. (a) Pairs with little change in 11-KT similarity had more eggs, while (b) pairs with higher change in CORT similarity had more fry. A value close to 0 corresponds to no change in hormonal similarity, while positive and negative values indicate an increase and a decrease, respectively, in hormonal similarity between the isolated and pairing context.

Dingemanse et al., 2009; Royle et al., 2014). Such reaction norms have been found for CORT responses in birds (Cockrem, 2013) and fish (Fürtbauer et al., 2015). Based on this assumption, proactive fish may be less sensitive to stressors or they may be bolder, regardless of the intensity of the stressful event (novelty *vs*. predation risk). In contrast, reactive fish may show more flexibility in their hormonally mediated behavioral response and have a lower increase in its CORT level when a novel object is present while still being more stressed or shyer than proactive conspecifics in this scenario. The reaction norms along the environmental gradient (isolated *vs*. pairing context here) may thus demonstrate more plastic phenotypes for CORT levels in the reactive fish, while they still have higher individual CORT levels than proactive fish. This suggests that if the underlying hormonal differences can predict different behavioral types, this should be indicated by differences in hormonal plasticity to cope with a given situation and not by differences in individual baseline hormone concentrations (Hau and Goymann, 2015; Rensel and Schoech, 2011; Wingfield, 2013). In our study, both male and female cichlids showed an increase in hormone concentrations, except for the female CORT levels, in response to pair formation. This is consistent with the need to mobilize resources and generate hormonally-mediated sexual behavior and parental care in the pairing context. Previous findings reported an increase in the 11-KT concentrations of male cichlids in socio-sexual contexts (Sessa et al., 2013), with more specifically higher levels during courtship and a subsequent decrease in 11-KT levels during the parental care and non-breeding phases (van Breukelen et al., 2015). Previous studies have shown that females expressed the highest level of aggressiveness during the pre-spawning phase, which was associated with elevated T and E2 plasma levels (Ramallo et al., 2014). However, the change in hormone levels in response to pair formation did not depend on the individual behavioral scores of our fish in contrast with the POLS hypothesis. Our results do not support the existence of a relationship between behavioral type and hormonal profile in either the isolated or pairing context in convict cichlids. It would have been interesting to test the hormonal responses of the partners to additional situations (e.g. predation risk, social instability, etc.), but these experimental tests would raise considerable methodological problems. If these additional tests had been carried out before spawning, they would have most probably disturbed the pairs and negatively affected their reproductive success. On the contrary, the tests could have been performed quickly after spawning but we had to remove the eggs from the nest in order to standardize the fitness measurements. As this egg removal event could indicate a reproductive failure in this species, it is likely that the hormonal profile of the parent would have been affected. We therefore decided to limit our behavioral type or hormonal measurements to the strict minimum.

Another possibility would be that the consequences of behavioral assortment do not rely on individual hormonal profiles but on hormonal compatibility between partners. Both males and females, from fish to humans, are known to respond with hormone changes to behavioral interactions in courtship, mating and competition contexts (Adkins-Regan, 2005; Antunes and Oliveira, 2009; Lieberwirth and Wang, 2014; Oliveira, 2004). These hormone changes mediate both the anticipation of a challenging situation (searching for a good partner, accessing and defending a territory or parenting) and the expression of the appropriate behavior in the right situation (e.g. displaying courtship when encountering a potential mate, agonistic interaction with intruders or parental care to offspring). In the specific pairing context, because hormones regulate pairing and parental behavior in cichlids (van Breukelen, 2013; van Breukelen et al., 2015) and behavioral compatibility has been shown to improve reproductive success in several monogamous species including convict cichlids (Both et al., 2005; Laubu et al., 2016; Schuett et al., 2011), it is likely that hormonal compatibility also has fitness consequences. Hormonal compatibility may occur either through within-pair positive co-variation in the seasonal transitions of hormone patterns or depending on the degree of hormonal similarity between partners. While the fitness consequences of the matching hormonal profile over the season is not limited to monogamous species (see Hirschenhauser, 2012 for similar results in the Japanese quail), the benefit of increasing hormonal similarity between partners during the breeding period has been only studied in two species. In great tits, Ouyang et al. (2014) only investigated their corticosterone levels and regardless of the behavioral type of the partners. Pairs that increased their hormonal similarity within a season (from prebreeding to breeding) had the highest reproductive success. Pairs with more similar baseline corticosterone levels and higher reproductive success were also more likely to remain together after the breeding season. In Eastern bluebirds, androgen and corticosterone similarity within pairs was not related to fledging success (Burtka et al., 2016). This lack of fitness consequences may be explain by the fact that they studied well-established pairs which could have previously increased their similarity according to the recent findings of Laubu et al. (2016). In our study, hormonal similarity did not affect reproductive success and is not related to behavioral assortment between partners. However, we found significant relationships between the variation in 11-KT similarity and spawning size, and the variation in CORT similarity and the number of fry. The increase in 11-KT similarity in the pairing context was related to better fitness, whereas the increase in CORT similarity was related to less fry. Such hormonal plasticity may be consistent with our hypothesis, suggesting that the mate may adapt its hormone levels both to its partner and in response to the social context. 11-KT mediates both gametogenesis and spawning behavior in both males and females of some fish species. It (or its precursor the luteinizing hormone) may also act as a pheromonal signal that helps synchronize spawning. It is thus likely that similar levels of 11-KT in the two partners help coordinate spawning leading to a larger amount of eggs. The decrease in CORT similarity in the pairing context is also consistent in pairs with parental division of labor as in our convict cichlids. They may have been physiologically prepared for these roles at the time of water collection. If both partners had extremely high or extremely low CORT levels, it is reasonable to predict that the fitness of the fry would have suffer, while differential investment in circulating hormones may promote a better coordination between partners. The main investment of isolated fish is searching for a partner and defending their territory, whereas pairing and pre-spawning individuals must divide their time between securing a reproductive site and anticipating parental displays (Itzkowitz et al., 2002; Snekser and Itzkowitz, 2014; Snekser et al., 2011). We cannot conclude that this hormonal adjustment between partners occurred in our pairs of cichlids but the evidence of flexibility in hormonal similarity in the pairs of cichlids and its link with their reproductive success show hormonal changes between partners in response to pair formation and suggest long-term fitness consequences. An additional result at the individual level also suggests that there could be a hormonal adjustment between partners after pairing regardless of their behavioral type. After pair formation, the increase in the CORT concentration in one mate is related to the decrease in the CORT concentration in the other one. Different levels of investment in socio-sexual behaviors between partners may again explain this result, as well as previous observations in pairs that females will often push and butt males away from the nest or block his access to them (Laubu et al., 2016). Further work is required to determine the relationship between the variation in the hormonal levels of the partners and their coordination in socio-sexual behaviors.

Our results suggest that behavioral type and hormonal compatibility in this monogamous species are not related and may act via different pathways. They also show a relationship between reproductive success and the flexibility of hormonal similarity between partners. This questions the existence of a potential hormonal adjustment between partners in response to pairing that emphasizes the need for further studies regarding the relationships between the behavioral coordination of the partners throughout the breeding period and their hormonal similarity in monogamous convict cichlids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.10.010.

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Hormonal and fitness consequences of behavioral assortative mating in the convict cichlid (*Amatitlania siquia*)

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Supplementary Material

Four supplementary figures

Fig. S1: Set-up used for the behavioral tests.

Fig. S2: Formation of the 30 pairs used in the study based on their behavioral type along the proactive-reactive continuum

Fig. S3: Artificial egg tumblers

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Four supplementary tables:

Table S1: Repeatability of the behavioral traits

Table S2: Correlation between the behavioral traits used to define the proactive-reactive behavioral types

Table S3: Sensitivities and cross reactivity values for non-target substances for each EIA system

Table S4: Summary of model selection procedure for assessing the effect of the male behavioral score (BS_M) and the female behavioral score (BS_F) on the hormone concentrations of the partners

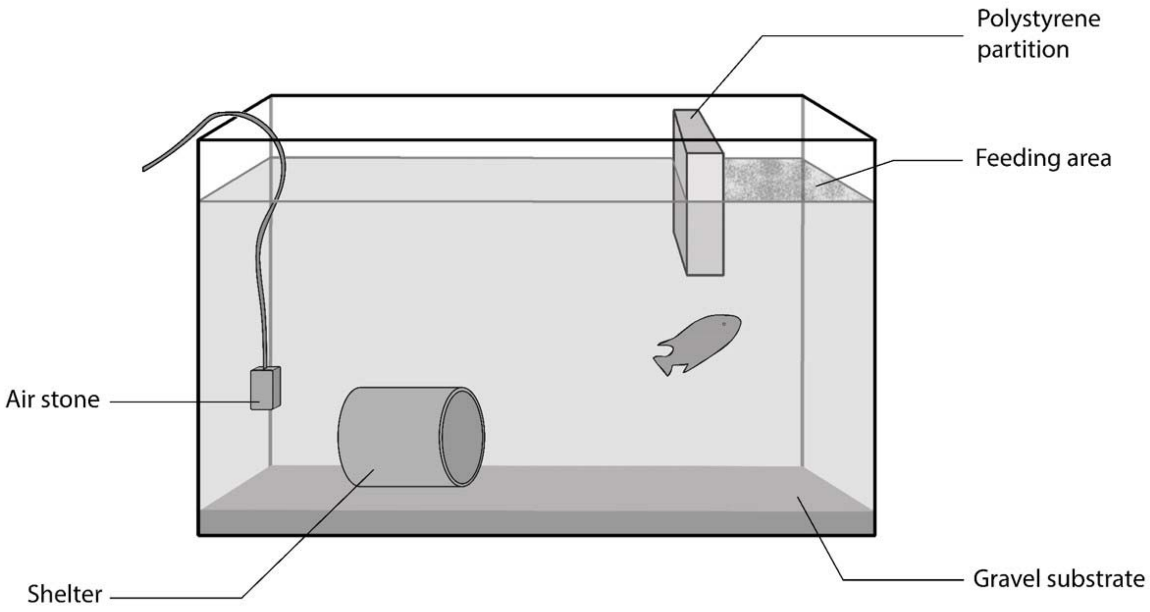

Figure S1. Set-up used for the behavioral tests. Four to five days before the start of the behavioral tests, each fish was isolated in a 20-liter tank (40 x 20 x 25 cm) equipped with a PVC tub (8 cm in diameter) serving as a shelter, an air stone and gravel substrate. To prevent food dispersion throughout the tank, a piece of polystyrene formed the boundary of a 10-cm wide feeding area at the front end of the tank and the surface of the water.

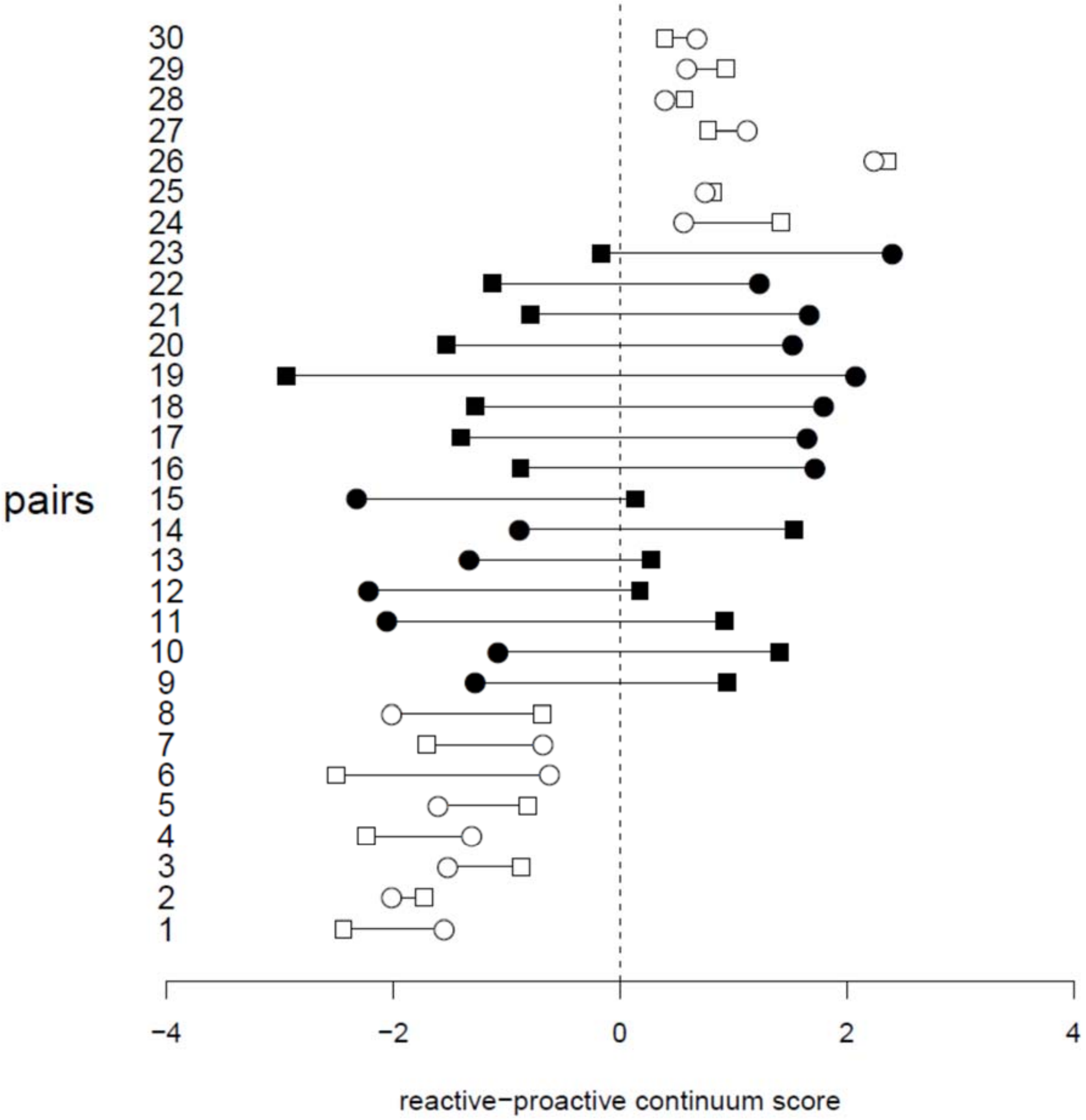

Figure S2. Formation of the 30 pairs used in the study based on their behavioral type along the proactive-reactive continuum. Positive scores indicated proactive individuals (highly aggressive and explorative individuals) and negative scores indicated reactive individuals (less aggressive and less explorative individuals). Assortative pairs: open dots, and disassortative pairs: solid dots. Circles indicate females and squares indicate males.

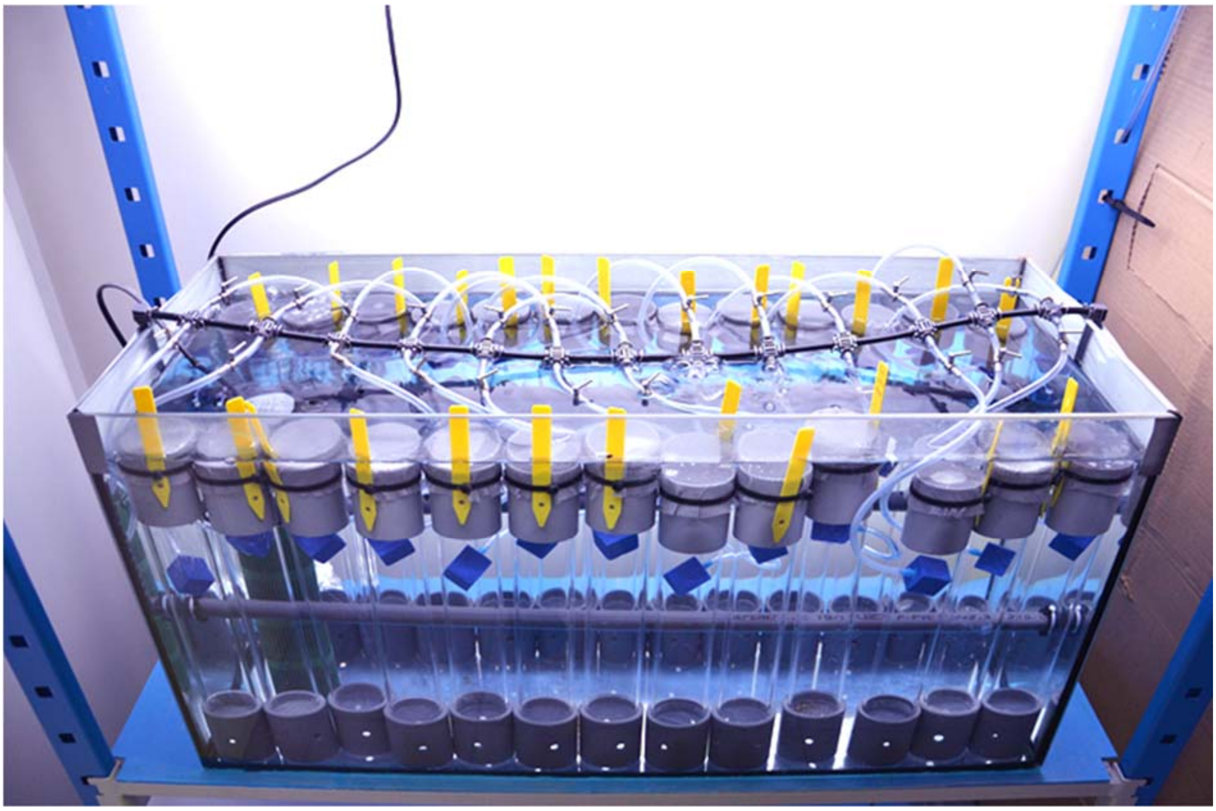

Figure S3. Artificial egg tumblers

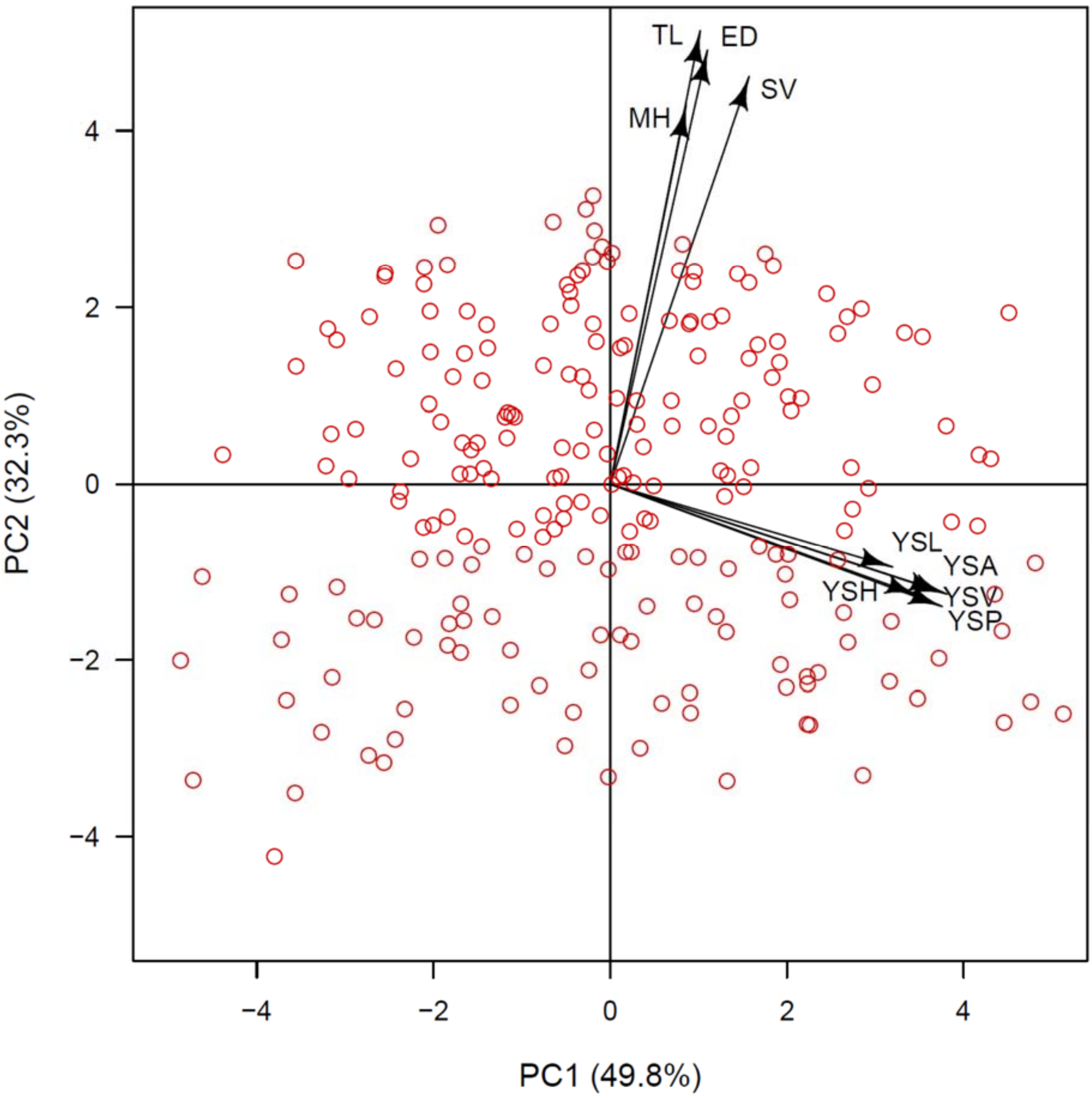

Figure S4. The morphometric measurements. Scatter plot of the principal component analysis based on the morphometric variables measured in fry. The two synthetic dimensions used to test the influence of hormonal similarity on the morphometry of fry were derived from the PC1 and PC2 values which characterized the yolk parameters (YSA, YSL, YSV, YSH and YSP) and the body parameters (MH, ED and TL, see methods for detailed results) at hatching, respectively.

Table S1. Repeatability of the behavioral traits adapted from Laubu et al. (2016). Repeatability was assessed in the 108 fish by the intra-class correlation coefficient and 95% confidence intervals (in brackets) for exploration and aggressiveness. Rank repeatability based on Spearman's correlations were used to analyze the two other behavioral traits (which did not meet the assumptions for parametric estimation of repeatability), and 95% confidence intervals (in brackets) were computed by bootstrapping. All behavioral traits were strongly consistent over a one-week interval.

Behavioral traits	Repeatability
Exploration	R = 0.50 [0.37; 0.61]
Food neophobia	ρ = 0.42 [0.23, 0.59]
Territory maintenance	ρ = 0.57 [0.40, 0.71]
Aggressiveness	R = 0.24 [0.04; 0.41]

Table S2. Correlation between the behavioral traits used to define the proactive-reactive behavioral types (adapted from Laubu et al. 2016). Spearman correlation (ρ) with 95% confidence interval between the four behavioral traits depending on sex (female F: n=64; male M: n=44). Correlations still significant after correcting for multiple comparisons (following Benjamini-Hochberg procedure, see Schweitzer et al. 2015) are figured in bold.

	Sex	Food neophobia	Territory maintenance	Aggressiveness
Exploration	M	-0.32 [-0.58, -0.01]	**-0.39 [-0.60, -0.12]**	**0.42 [0.15, 0.63]**
	F	0.09 [-0.19, 0.33]	**0.45 [0.22, 0.64]**	**0.35 [0.10, 0.58]**
Food neophobia	M		-0.05 [-0.30, 0.21]	-0.13 [-0.45, 0.19]
	F		0.19 [-0.06, 0.43]	-0.22 [-0.42, 0.0]
Territory maintenance	M			-0.13 [-0.48, 0.21]
	F			**0.39 [0.17, 0.56]**

Table S3. Sensitivities and cross reactivity values for non-target substances for each EIA system. All values are according to the manufacturers' specifications except the cross-reactivity values for 11-KT (in bold) which was derived in the Kidd *et al.*'s study (2010).

Assay	Sensitivity	Cross reactivity
Testosterone (T)	5.67 – 2,000 pg/ml	T = 100%; 19-hydroxytestosterone = 14.64% Androstendione = 7.20% **11-KT < 5.00%** all else ≤ 0.72%
17β-estradiol (E2)	28.5 – 30,000 pg/ml	E2 = 100% Estrone = 4.64% all else ≤ 0.53%
Cortisol (CORT)	56.72 – 10,000 pg/ml	CORT = 100% Prednisolone = 122.35% Corticosterone = 27.68% 11-deoxycortisol = 4.00% Progesterone = 3.64% All else ≤ 0.85%
11-ketostestosterone (11-KT)	0.78–100 pg/ml	11-KT = 100% Adrenosterone = 2.90% T = <0.01% all else ≤ 0.01%

Table S4. Summary of model selection procedure for assessing the effect of the male behavioral score (BS_M) and the female behavioral score (BS_F) on either the hormone concentrations of the cichlid partners in the pairing context, or on the variation of concentration between the contexts (Δhormone = concentration in the pairing context – concentration in the isolated context).

		Male				Female			
	Model	F	df	*P*	AIC	F	df	*P*	AIC
CORT	BS_M x BS_F	0.019	1	0.89	163.4	0.495	1	0.49	168.4
	$BS_M + BS_F$	1.882	2	0.18	161.4	0.447	2	0.64	166.9
	BS_M	0.308	1	0.58	162.9	0.060	1	0.81	165.9
	BS_F	3.576	1	0.072	159.7	0.872	1	0.36	165.0
Δ_{CORT}	BS_M x BS_F	0.360	1	0.56	153.3	0.520	1	0.48	151.8
	$BS_M + BS_F$	0.770	2	0.48	151.7	0.362	2	0.70	150.5
	BS_M	0.300	1	0.59	151.2	0.651	1	0.43	148.5
	BS_F	1.287	1	0.27	150.1	0.074	1	0.79	149.2
11-KT	BS_M x BS_F	0.488	1	0.49	39.7	2.622	1	0.12	5.4
	$BS_M + BS_F$	1.030	2	0.37	38.3	2.251	2	0.13	6.4
	BS_M	0.457	1	0.51	38.0	**4.713**	**1**	**0.042**	**4.4**
	BS_F	1.645	1	0.21	36.8	0.072	1	0.79	9.0
$\Delta_{11\text{-}KT}$	BS_M x BS_F	1.224	1	0.28	159.9	0.216	1	0.65	7.3
	$BS_M + BS_F$	0.168	2	0.85	159.3	0.169	2	0.84	5.6
	BS_M	0.046	1	0.83	157.6	0.086	1	0.77	3.9
	BS_F	0.304	1	0.59	157.3	0169	1	0.61	3.7
T	BS_M x BS_F	0.158	1	0.69	28.1	0.024	1	0.88	37.3
	$BS_M + BS_F$	0.190	2	0.82	26.3	1.677	2	0.21	35.3
	BS_M	0.146	1	0.71	24.6	3.353	1	0.082	33.3
	BS_F	0.245	1	0.62	24.4	7.10^{-4}	1	0.98	36.9
Δ_T	BS_M x BS_F	0.061	1	0.81	147.8	0.025	1	0.88	146.9
	$BS_M + BS_F$	0.557	2	0.58	145.8	0.719	2	0.50	145.0
	BS_M	0.205	1	0.65	144.9	1.407	1	0.25	143.0
	BS_F	0.645	1	0.34	144.1	0.032	1	0.86	144.5
E2	BS_M x BS_F	0.014	1	0.91	45.9	0.012	1	0.91	39.8
	$BS_M + BS_F$	1.238	2	0.31	43.9	1.324	2	0.29	37.8
	BS_M	2.379	1	0.14	42.0	1.458	1	0.24	37.0
	BS_F	0.090	1	0.77	44.5	1.596	1	0.22	37.4
Δ_{E2}	BS_M x BS_F	0.654	1	0.43	125.0	0.020	1	0.89	145.1
	$BS_M + BS_F$	0.433	2	0.65	123.8	0.471	2	0.63	143.1
	BS_M	0.631	1	0.44	122.1	0.785	1	0.39	141.2
	BS_F	0.240	1	0.63	122.5	0.159	1	0.69	142.0