

Cortisol is responsible for positive and negative effects in the ovarian maturation induced by the exposure to acute stressors in Nile tilapia, *Oreochromis niloticus*

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Abstract The aim of the present study was to evaluate the effect of acute stress and cortisol injection on oocyte final maturation process in female Nile tilapia (*Oreochromis niloticus*). Handling followed by a prophylactic treatment (0.3 mL L⁻¹ H₂O₂, 5 g L⁻¹ NaCl solution during 30 min) and an environmental change (transfer from a 2 m³ fibreglass square tank to 50 L aquaria) were used as acute stressors and compared to a single cortisol injection (0.5 or 5 mg kg⁻¹ body weight). For both acute stress and cortisol injection (0.5 mg kg⁻¹ body weight), serum cortisol level was significantly increased from 2.3 to

134.1 ng mL⁻¹ 1 h post-stress/injection and returned to a resting basal value 24 h after the stress/injection. In fish injected with 5 mg kg⁻¹ body weight cortisol, mean serum cortisol level reached a peak up to 2500 ng mL⁻¹ 1 h after injection. 63 % of the females (mean body weight: 242 ± 4 g) submitted to the acute stress ovulated within 72 h after the stress. In the same way, cortisol injection (5 mg kg⁻¹ body weight) at the 10th day of the maturation cycle led to a twofold reduction of the time before ovulation compared to vehicle injected control fish. Relative and total fecundity were significantly decreased in females submitted to an acute stress or cortisol injected at 5 mg kg⁻¹ body weight, but not fertilization or hatching rates. In conclusion, acute stress and cortisol induction exert both positive and negative effects on the final reproductive process in *O. niloticus*, and cortisol is the endocrine mediator causing these changes.

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Introduction

Stress, experienced in the wild or in culture conditions, affects all life functions of fish: growth, immunology, disease resistance and reproduction (Iwama et al. 1997; Pankhurst 2011; Schreck et al. 2001). Stress induces three levels of response: a primary physiological response, which consists in the initial

neuroendocrine response, with a release of catecholamine and the stimulation of the hypothalamic-pituitary-interrenal axis inducing the release of corticosteroid hormones; a secondary response, with physiological adjustments linked to metabolism, respiration, acid–base status, immune function and cellular responses; and a tertiary response expressed by a decrease of growth, inhibition of reproduction, modification of behaviour or decrease of disease resistance (Barton 2002; Schreck et al. 2001). Under culture conditions, fish encounter many acute and chronic stressors such as crowding, confinement, handling and other husbandry routines, which may affect these three levels of response.

In fish, the main corticosteroids are cortisol, cortisone, 11-deoxycortisol and corticosterone, cortisol being one of the most commonly used welfare and stress physiological indicator in fish (Barton 2002; Milla et al. 2009). Effects of stress and corticosteroids on fish reproduction have been widely studied (Contreras-Sanchez et al. 1998; Milla et al. 2009; Schreck et al. 2001) and can affect all the levels of the reproductive axis, from neuroendocrine axis to gamete quality and embryo/larval development (Campbell et al. 1992; Iwama et al. 1997; Schreck 2010). Briefly, depending on the intensity/duration of the stressor and the stage of development, the reproductive cycle of females exposed to a stressor or corticosteroid treatments could be negatively or positively affected in term of reproductive performances (Contreras-Sanchez et al. 1998; Milla et al. 2009; Schreck 2010). For examples, negative effects as the occurrence of follicular atresia, disruption or modification of vitellogenesis process (in salmonids), advance or delay in oocyte maturation and ovulation, decrease of fertilization and hatching rates, modification of the spawning behaviour and subsequently low larval quality were reported in many fish species (Schreck et al. 2001; Milla et al. 2009; Schreck 2010). On the other hand, some paradoxical positive effects of stress on reproduction were also punctually reported as the reduction of the interspawning period (Milla et al. 2009; Stratholt et al. 1997) or the enhancement of vitellogenesis (Ding et al. 1994; Shankar and Kulkarni 2006). However, it is not clear whether these stress effects are evoked or not by the transient or prolonged cortisol elevation in the blood because few studies have investigated in parallel the consequences of

stress exposure and cortisol administration on the reproductive features.

The Nile tilapia (*Oreochromis niloticus*) is one the most cultured fish species worldwide. It easily reproduces in captivity, without any hormonal stimulation. Under constant photothermal conditions (26–28 °C and 14 h light/10 h dark photoperiod), females spawn regularly throughout the year. However, because of its asynchronous oocyte development, and the failure of hormonal stimulation to trigger spawning, it is difficult to synchronize reproduction in tilapia (Foo and Lam 1993a; Srisakultiew and Wee 1988). Nevertheless, sudden environmental change (mainly a cooling period followed by an increase of water temperature) may improve spawning synchronization in tilapiine species (Coward et al. 1998; Srisakultiew and Wee 1988). Acceleration of the final maturity and the induction of the spawn were previously observed on *Tilapia zillii*, which synchronously spawn within 1–2 days after their transfer into individual aquaria (Coward et al. 1998).

The objectives of the present study were to assess the capacity of acute stress occurrence to synchronize ovulation in Nile tilapia and to determine whether these effects are mediated by cortisol elevation. To address these questions, we investigated the impact of acute stress and single cortisol injection on the induction of ovulation, fertilization and hatching rates in female Nile tilapia.

Materials and methods

Fish

Nile tilapia *O. niloticus* (Lake Manzala strain) originated from the Research and Education Centre in Aquaculture (CEFRA), University of Liège (Belgium). Twenty-month-old broodstock fish (males and females) were maintained in a 2 m³ (4 m²) fibreglass square tank in a recirculating system at 27–28 °C, 14 h light/10 h dark and at a stocking density of 30 kg m⁻³. Fish were fed at satiation with special broodstock commercial tilapia diet (32 % proteins, 6 % lipids, Coppens—The Netherlands). Experiments were carried out according to the guidelines of the University of Liège ethical committee and the European animal welfare recommendations.

Experiment 1: Effect of acute stress on oocyte maturation cycle

Acute stress consisted of netting followed by prophylactic treatment and breeders transfer from the stock tank to another rearing facility. Fish were netted from the stock tank and anaesthetized with 2-phenoxyethanol (Sigma) at 0.4 mL L^{-1} . Sixty females (mean body weight: $242 \pm 4 \text{ g}$) were selected after gentle stripping and oocyte emission. Because of their asynchronous oocyte development strategy, females were at different stages of sexual maturity (Foo and Lam 1993a). In order to avoid pathogens transfer between rearing systems, fish were treated in $0.3 \text{ mL L}^{-1} \text{ H}_2\text{O}_2$, $5 \text{ g L}^{-1} \text{ NaCl}$ solution during 30 min before their transfer into individual 50 L aquaria at $27 \text{ }^\circ\text{C}$ with a 14 h light/10 h dark photoperiod and oxygen above 6 ppm.

After their transfer, female ripeness was checked daily as described by Myers and Hershberger (1991). Ovulation time was assessed by genital papilla distension and behaviour change (nesting activity) just before spawning. Time between stress and ovulation was recorded. Ovulation was also followed in 28 unmanipulated females (second maturation cycle in aquarium) as a control. Eggs were collected by stripping, weighted in order to determine the total and relative fecundity and artificially fertilized with 0.3 mL of sperm originating from three different males. Spermatozoa activation was checked under microscope. Fertilized stripped eggs were incubated in 1.5 L Zug bottle at $28 \text{ }^\circ\text{C}$. Egg quality was assessed by fertilization rate (2 h after fertilization), hatching rate and survival rate at 10 days post-fertilization (dpf). Quality of the first stripped eggs, following an acute stress, was compared to quality of eggs stripped after the following maturation cycles.

Blood was sampled from 42 fish for cortisol level analysis before and after the exposure to acute stress. Fish were netted and anaesthetized in 2-phenoxyethanol (Sigma) (0.4 mL L^{-1}), and blood (1 mL) was rapidly ($<5 \text{ min}$) sampled by caudal venipuncture on eight stressed fish at 1, 6, 24 h after acute stress application and on eight fish at the time of ovulation following the acute stress. Ten fish from the stock tank were used to determine the basal cortisol level.

Experiment 2: Effect of cortisol injection on the final stages of reproduction

In this experiment, cortisol injection was used to mimic blood cortisol surge that is generally associated with an acute stress. Forty females were transferred into experimental aquaria as described in experiment 1 and allowed to naturally spawn first (Foo and Lam 1993a). After the spawn, eggs were immediately and gently removed from the mouth of the females. Ten days after the first spawn, when secondary oocytes are supposed to complete vitellogenesis (Hussain 2004), fish were anaesthetized with 2-phenoxyethanol (Sigma) at 0.4 mL L^{-1} and weighted. Seven females were injected with 0.5 mg kg^{-1} body weight of cortisol (Sigma, hydrocortisone), and 21 females were injected with 5 mg kg^{-1} body weight of cortisol. Cortisol was previously dissolved in saline solution (0.9 % NaCl) and injected intraperitoneally. Control fish ($n = 11$) received an injection of saline vehicle only. Time between injection and the following ovulation was measured, and quality of stripped eggs was assessed as in experiment 1.

In order to assess the blood cortisol increase after injection, 82 other individuals were used to determine serum cortisol level. Blood was sampled on 10 fish before the cortisol or vehicle injection (0 h, control). Twenty-four individuals were injected with vehicle, 24 received a 0.5 mg kg^{-1} body weight cortisol injection, and 24 other fish received a 5 mg kg^{-1} body weight cortisol injection. For each treatment, eight individuals were blood-sampled at 1, 6 and 24 h after injection.

ELISA cortisol analysis

After centrifugation (4,500 rpm, 20 min, $10 \text{ }^\circ\text{C}$), serum was stored at $-20 \text{ }^\circ\text{C}$ until analyses. Serum cortisol assay was carried out by a competitive ELISA following manufacturer instructions (BioSource, Nivelles, Belgium). Twenty microlitres of each cortisol standard, control and serum samples (each in duplicate) were dispensed into different wells of a 96-well plate. Two hundred microlitres of cortisol horseradish peroxidase conjugate were added into each well. After thorough mix for 10 s, 60 min incubation, and three times washing with solution provided in the kit, 100 μL of substrate solution containing tetramethylbenzidine were added. Then, the plate was incubated

for 15 min at room temperature, and the reaction was stopped by adding 100 μL of H_2SO_4 0.5 M. The optical density was read at 450 nm with a microtiter plate reader. The detection limit was 2 ng mL^{-1} , the inter-intra variability was 6.5–7.7, 3.2–7.0 %, and the recovery range was evaluated at 85–111 %, depending on doses.

Statistics

Statistical analysis was performed using Statview 5.0.1 (SAS Institute Inc., USA). Differences among mean values were tested using an analysis of variance (ANOVA) for the reproductive parameters and a Mann–Whitney test for cortisol levels. Data are reported as mean \pm standard error of the mean (SEM).

Results

Effect of acute stress

After application of the acute stress, 63 % of the 60 females ovulated within the following 72 h, and 85 % within 1 week. All the females ovulated within 2 weeks (Fig. 1). The time course between exposure to stressor and ovulation ranged from 729 $^{\circ}\text{C h}$ (27 h

at 27 $^{\circ}\text{C}$) to 9,180 $^{\circ}\text{C h}$ (14 days at 27 $^{\circ}\text{C}$) with a mean value of $2,886 \pm 341$ $^{\circ}\text{C h}$. In the control group, only 7 % of the 28 females ovulated within 72 h, 53 % within 1 week and 78 % within 2 weeks.

Acute stress (handling followed by prophylactic treatment and transfer) induced a significant ($p < 0.05$) rapid increase of serum cortisol, from 2.6 ± 1.7 ng mL^{-1} before the stress (basal level) to 131.1 ± 63.7 ng mL^{-1} 1 h post-stress (Fig. 2). After this peak, cortisol level significantly decreased and returned to a resting basal value 24 h after the stress. The cortisol level slightly increased at the time of ovulation (28.0 ± 15.5 ng mL^{-1}), but the reached level was not significantly different from the level measured in pre-initial controls or 24 h after the stress.

Relative and total fecundity were significantly ($p < 0.05$) decreased in females submitted to the acute stress by comparison with unstressed control fish (Table 1). Stressed females ovulated significantly ($p < 0.05$) bigger eggs (6.4 ± 0.3 mg) than control females (5.0 ± 0.2 mg). Mean fertilization and hatching rates were not significantly ($p > 0.05$) different between control (93.0 ± 2.0 and 63.2 ± 3.8 %, respectively) and stressed females (94.5 ± 1.2 and 56.4 ± 5.1 %, respectively). Survival rates of larvae at 10 dpf were not significantly different between progenies from both groups (± 55 %).

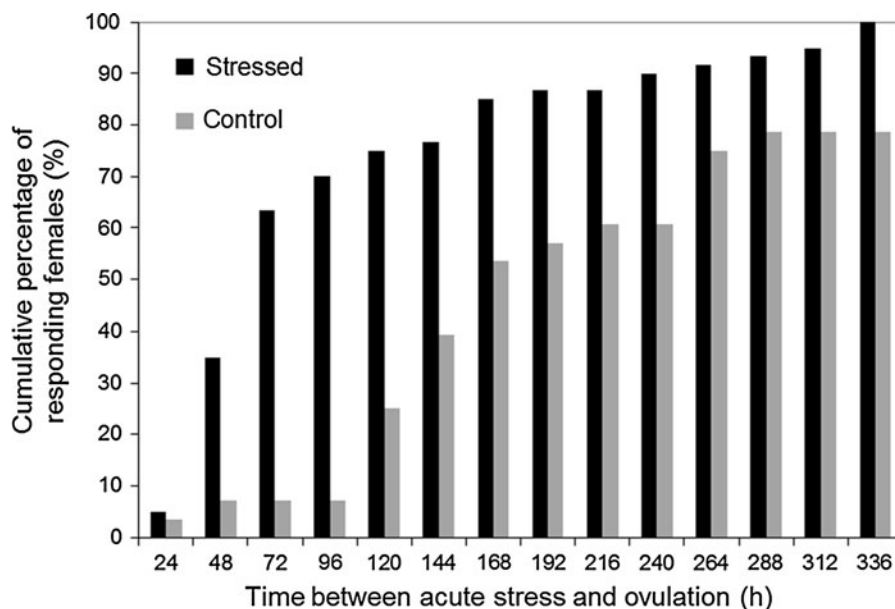


Fig. 1 Cumulative percentage of females ovulating after an acute stress (handling + prophylactic treatment + transfer, $n = 60$) compared to control fish (unmanipulated females, $n = 28$)

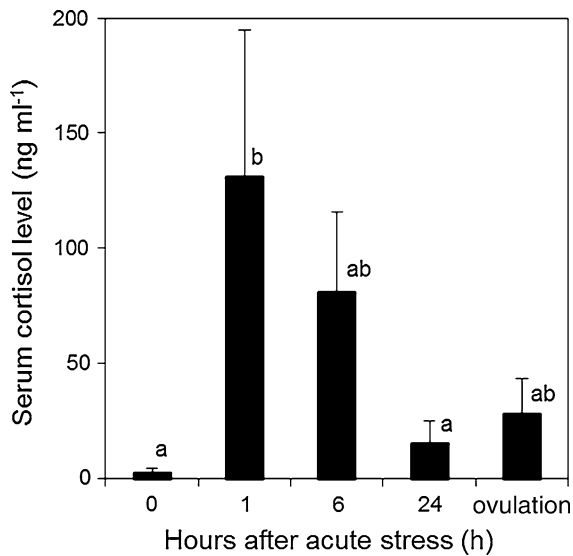


Fig. 2 Serum cortisol concentration (ng mL^{-1}) before, 1, 6 and 24 h after application of an acute stress (handling + prophylactic treatment + transfer) in female Nile tilapia. Values are means + SEM ($n = 6\text{--}10$). Values with different letters are significantly ($p < 0.05$) different

Effect of cortisol injection

When females were injected on the 10th day of the maturation cycle, ovulation occurred more rapidly in 5 mg kg^{-1} body weight cortisol injected fish than in control fish (after $2,902 \pm 473$ and $4,861 \pm 714^\circ\text{C h}$, respectively; $p < 0.05$) (Fig. 3). However, injection of 0.5 mg kg^{-1} body weight cortisol did not significantly affect ovulation time. For each treatment, some females did not ovulate within the 14 days following injection: 1 female (9 %) did not ovulate in the control group, and 2 (28 %) and 5 (24 %) females did not

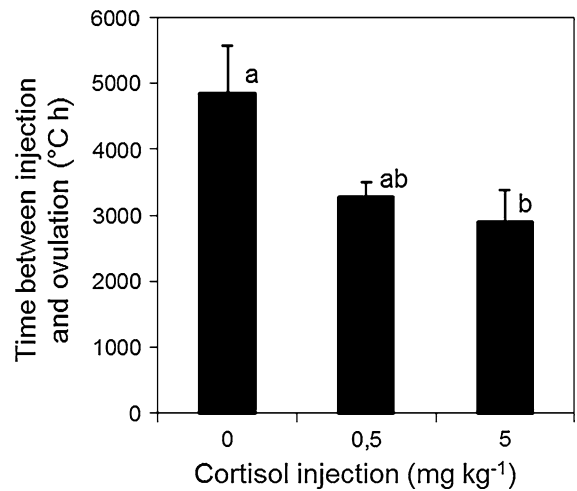


Fig. 3 Time ($^\circ\text{C h}$) between cortisol (0.5 mg kg^{-1} , $n = 5$; 5 mg kg^{-1} , $n = 16$) or vehicle ($n = 11$) injection and ovulation in female Nile tilapia. Fish were injected on the 10th day following the previous ovulation. Values are means + SEM. Different letters indicate significant differences ($p < 0.05$). Fish that did not respond to injection within the following 14 days are not considered in this data set

ovulate after the cortisol injection at 0.5 and 5 mg kg^{-1} body weight cortisol doses, respectively.

Injection of 0.5 mg kg^{-1} body weight cortisol or saline induced a similar increase in serum cortisol, from $2.3 \pm 1.3 \text{ ng mL}^{-1}$ before injection to 134.1 ± 33.8 and $90.5 \pm 34.7 \text{ ng mL}^{-1}$, respectively, 1 h after injection (Fig. 4). In fish injected with 5 mg kg^{-1} body weight cortisol, mean serum cortisol level reached $2,547.4 \pm 471.7 \text{ ng mL}^{-1}$ 1 h after injection. This value is significantly higher than the level measured in control fish and fish injected with 0.5 mg kg^{-1} body weight cortisol. Six h and 24 h after injection, all the groups showed similar intermediate serum cortisol levels,

Table 1 Egg characteristics of control females, females submitted to an acute stress (handling + prophylactic treatment + transfer) and saline- (control) and cortisol-injected females

Female groups	Number of females	Egg weight (mg)	Relative fecundity (g eggs kg female^{-1})	Total fecundity (eggs female^{-1})	Fertilization rate (%)	Hatching rate (%)	Survival rate at 10 dpf (%)
Control	12	5.0 ± 0.2^a	7.6 ± 0.5^a	$2,058 \pm 242^a$	93.0 ± 2.0	63.2 ± 3.8	55.6 ± 3.2
Stressed	6	6.4 ± 0.3^b	5.2 ± 0.6^b	$1,022 \pm 126^b$	94.5 ± 1.2	56.4 ± 5.1	55.4 ± 4.5
Control (saline) injected	4	5.0 ± 0.3^a	8.0 ± 0.4^a	$2,001 \pm 136^a$	94.3 ± 1.4	64.9 ± 5.1	52.5 ± 2.1
Cortisol injected (5 mg kg^{-1})	11	6.3 ± 0.2^b	5.9 ± 0.3^b	$1,249 \pm 82^b$	89.1 ± 1.9	65.6 ± 4.8	52.8 ± 3.9

Values are means \pm SEM. In each column, mean values with different superscript letters are significantly different ($p < 0.05$)

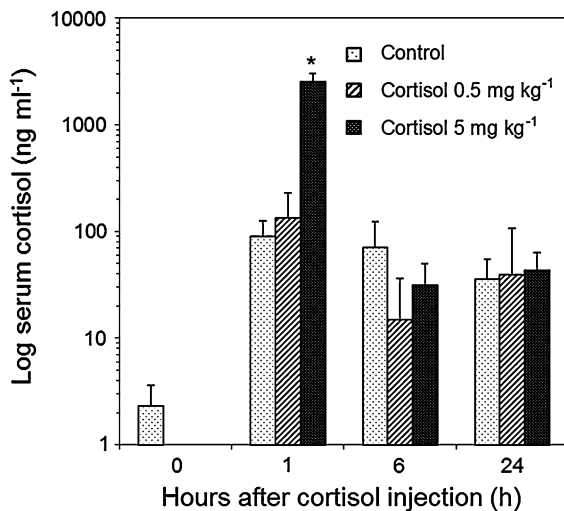


Fig. 4 Serum cortisol concentration after intraperitoneal injection of saline vehicle only (control) or 0.5 and 5 mg kg⁻¹ cortisol solution in female Nile tilapia. Values are mean \pm SEM ($n = 8$). *Significantly different from other values

between 14.8 ± 7.5 and 70.4 ± 53.4 ng mL⁻¹. Except from resting values measured before injection, all the values showed a large interindividual variability.

Cortisol-injected females ovulated significantly ($p < 0.05$) bigger eggs (6.3 ± 0.2 mg) than saline-injected ones (5.0 ± 0.3 mg). On contrary, cortisol injection at 5 mg kg⁻¹ body weight significantly ($p < 0.05$) decreased the relative and total fecundity (Table 1). Neither fertilization nor hatching rate (89.1 ± 1.9 and 65.6 ± 4.8 %, respectively) was affected by this cortisol injection. Survival rates of larvae at 10 dpf were not significantly different between progenies (± 52.5 %). Unfortunately, egg batches from 0.5 mg kg⁻¹ cortisol-injected fish were lost during incubation due to a technical problem that occurred in the hatchery.

Discussion

Acute stress advances oocyte maturation in Nile tilapia

Depending on the intensity, the time of application and duration of the stress, the female reproductive process in fish could be negatively or positively influenced by stress (Pankhurst 1998; Schreck 2010). Our results suggest that acute stress accelerates the final stages of

oogenesis in Nile tilapia and reduces the time course before ovulation. Schreck et al. (2001) reported that when Nile tilapias are stressed during late vitellogenesis, the spawn occurs immediately. Similar conclusions were drawn by Coward et al. (1998) on *T. zillii* that synchronously spawned within 24–48 h after their transfer into individual aquaria. Such a shortening of the maturation process was also mentioned in salmonids. In coho salmon and rainbow trout, Stratholt et al. (1997) and Contreras-Sanchez et al. (1998) observed that females submitted to a chronic stress during the final maturation process, ovulated on average 2 weeks before unstressed fish. Thus, we suggest that acute stress speeds up the achievement of the final stages of ovarian maturation in all tilapia species and possibly in other fish families.

Cortisol is one of the endocrine mediators of the induction of ovulation post-stress

In our study, the acceleration of the final oocyte maturation soon after the transfer into individual aquaria was associated with an increase of serum cortisol level. This serum cortisol profiles, marked by a significant peak within 1 h after the application of the stress, is a usual response to acute stress widely described in many fish species (Barton 2002; Pankhurst 2011; Schreck et al. 2001). This rapid increase of cortisol after a stress, with comparable concentrations, was also reported in stressed Nile tilapia (Correa et al. 2003) and in *Oreochromis mossambicus* juveniles submitted to an acute stress of handling (Barcellos et al. 1999; Foo and Lam 1993b), confinement (Vijayan et al. 1997) or an acute stress of salinity (Kammerer et al. 2010). Comparative cortisol increase at the time of ovulation and spawning was also reported in salmonids, goldfish and common carp (Milla et al. 2009; Schreck 2010; Schreck et al. 2001; Stratholt et al. 1997). Small (2004) observed an increase of spawn number in cortisol-fed channel catfish and speculated that cortisol could act as a maturation-inducing agent in the channel catfish. In our study, injection of supra-physiological doses of cortisol (5 mg kg⁻¹ body weight) accelerated ovulation, suggesting that this cortisol administration could have the same effect on final oocyte maturation than the cortisol elevation observed in fish following acute stress. However, given that the cortisol release was supra-physiological, we cannot rule out any indirect

effect of cortisol on metabolism, which in turns may interfere with reproductive processes. At any case, we can speculate that the effect of acute stress on spawning induction is at least partly explained by the increase of cortisol level and that cortisol may be directly or indirectly implicated in the achievement of maturing process in Nile tilapia.

Which are the physiological mechanisms triggered by exposure to acute stress and cortisol?

In fish, corticosteroids display biphasic (positive and negative) effects on fish reproduction in females mainly during the meiotic oocyte maturation (Milla et al. 2009). In *Oreochromis aureus*, cortisol had an acute effect on hepatic vitellogenin gene expression supporting a direct effect of cortisol on vitellogenesis (Ding et al. 1994). In *Notopterus notopterus*, cortisol administration induced the enhancement of vitellogenesis during the pre-spawning season (Shankar and Kulkarni 2006). This enhancement of vitellogenesis would therefore shorten the interspawning interval (Tacon et al. 2000). In tilapia, the occurrence of oogenesis acceleration after stress or cortisol administration seems to be linked to changes in serum testosterone (T) and estradiol (E2) levels, but the reported data are quite controversial (Foo and Lam 1993a; Coward et al. 1998; Tacon et al. 2000) and mechanisms still unclear. Contreras-Sanchez et al. (1998) suggested that the reduction of ovulation time under stressful conditions late during the maturation process may occur as a switch in reproductive strategy when environmental conditions are not stable. We therefore hypothesise that acute stress in relation to cortisol elevation would accelerate the final oocyte maturation process and ovulation in Nile tilapia. On the other hand, we also observed a slight proportion of female that did not ovulate at all after the injection of cortisol, (28 and 24 % for 0.5 and 5 mg kg⁻¹ cortisol, respectively) suggesting that cortisol can also inhibit ovulation or prevent the earlier stage of final oocyte maturation of these females. This difference of response is probably due to differences in stages of oocyte development of the female at the time of cortisol injection. This confirms the biphasic effect of cortisol on the reproductive cycle in Nile tilapia.

Early ovulation could be a direct consequence of the activation of progestogen receptors by corticosteroids. However, if binding of corticosteroids to

progestogen receptors was demonstrated for 11-deoxycorticosterone and 11-deoxycortisol in other species, no evidence of such receptor cross-reactivity exists for cortisol (Milla et al. 2009). Considering this effect in an adaptive point of view, early ovulation could be an advantageous reproductive tactic under stressful conditions, as egg retention can be energy consuming. In our study, the acceleration of the ovulation process could also be triggered by the modification of the social structure in the broodstock tanks. Indeed, *O. niloticus* displays a social hierarchy with dominant and subordinated females (Binuramesh et al. 2005). Thus, we also speculate that isolation of females from the social group suppresses the inhibition induced by the presence of the dominant female and allows the subordinated ones to continue their ovulation process, thus reducing the duration of the final stages before ovulation.

Comparison of stress and cortisol effects on reproductive performances

Depending on fish species, fecundity, as well as egg size, hatching rate and survival of new-hatched larvae could be significantly decreased after a stress exposure (Campbell et al. 1992; Contreras-Sanchez et al. 1998; Milla et al. 2009; Soso et al. 2008). In *O. mossambicus*, chronic stress induced by long-term (18 days) cortisol implant caused negative effects on female reproduction, with a significant decrease of GSI and oocyte diameter (Foo and Lam 1993b). In our study, relative and total fecundity were decreased by acute stress and single cortisol injection suggesting that high levels of cortisol are responsible for this loss in fecundity. But surprisingly, egg size was significantly increased for both stressed and cortisol injected females, this discrepancy highlighting the biphasic implications of cortisol in female fish reproduction. Nevertheless, as fertilization, hatching and survival rates of progenies were not significantly affected by the acute stress nor by cortisol injection, we can conclude that even if the weight of the ovary and the eggs were affected, acute stress did not reduce the quality of gametes produced in Nile tilapia.

In conclusion, our results showed that acute stress both positively and negatively affect oocyte final maturation process and ovulation in Nile tilapia, with the acceleration and synchronization of ovulation, a decrease of fecundity and an increase of egg size.

These modifications of the final maturational process seem to be caused by a cortisol elevation.

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