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Corticosteroids deeply depress the in vitro steroidogenic capacity of Eurasian perch ovary at the end of the reproductive cycle

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Corticosteroids, steroidogenesis, gonadotropins, thyroid hormones, ovarian tissues, eurasian perch.

Abstract

Corticosteroids play positive or negative role in the reproductive mechanisms of many fish species but the physiological contexts relating to such biphasic actions are not well defined. In the present study we investigated to what extent corticosteroids (cortisol-Co, 11-deoxycorticosterone-DOC) hormones may interfere with the steroidogenic capacity of Eurasian perch ovarian tissues, and we tested whether the negative effects of corticosteroids may be mitigated by potential stimulating endocrine factors, namely insulin-like growth factor-1 (IGF), human chorionic gonadotropin (HCG) or thyroid hormones (Triiodothyronine-T3, thyroxine-T4). Ovarian tissues from six maturing fish at late vitellogenesis developmental stage (LVO) or at the start of the final meiotic oocyte maturation (FMO) were incubated during 6 h in Cortland medium containing various endocrine compounds. Both corticosteroids drastically suppressed aromatase activity (AA) and sex-steroid production, namely 17- β estradiol (E2), 17 α -20 β -dihydroxy-4-pregnen-3-one (DHP) and testosterone (T). HCG significantly prevented the suppression of both AA and sex-steroid production by low and high cortisol doses, but a lesser AA protection was observed in the case of DOC. The protection of DHP and T productions by HCG from the negative effects by the two corticosteroids was higher at FMO than at LVO stage. IGF or thyroid hormone treatments were lesser effective or ineffective in mitigating the suppression of AA or sex-steroid production by cortisol. The results suggest that an increase in cortisol or DOC such as after mild or high stress intensity may inhibit drastically the ovarian steroidogenic capacity whatever the final oocyte maturation stage in percid fish by hampering AA and sex-steroid production. That inhibition may be partly mitigated by gonadotropins but not IGF nor thyroid hormones, especially at final meiotic oocyte maturation stage.

1. Introduction

In many fish species, the achievement of the final oogenesis stages (late vitellogenesis, meiotic oocyte maturation) is associated to important stressful events, such as intensive mating behaviour, fights between dominant males, maturation checking by the breeder, or spawning migration activities that can induce high corticosteroid releases. Such increase in corticosteroid production may cause various reproductive failures depending on the stage of ovarian developmental stage at which the stress occurs (Coward et al., 1998; Schreck et al., 2001; Schreck, 2010; Small et al., 2008; Milla et al., 2009a). Specifically, cortisol has been reported to affect various reproductive processes, such as the control of vitellogenin production by estrogens in Arctic charr *Salvelinus alpinus* (Berg et al., 2004), or the testosterone (T) and estrogen (E2) production in rainbow trout *Oncorhynchus*

mykiss and tilapia *Oreochromis mossambicus* (Carragher et al., 1989; Foo and Lam, 1993; Reddy et al., 1999; Pankhurst and Van Der Kraak, 2000). Glucocorticoid increase in salmonids may also decrease gonadotropin production by inhibiting the gonadotropin releasing (GnRH) activity, and consequently reducing the steroidogenic capacity by lowering the production of gonad steroid hormones (Reddy et al., 1999; Lethimonier et al., 2004; Milla et al., 2009a; Schreck, 2010). It has been hypothesized that the negative effects of glucocorticoids may be mediated through the interactions between glucocorticoid receptors (GRs) and estrogen receptors (ERs) at different site organs involved in the reproductive mechanisms (Alspo et al., 2009; Barkataki et al., 2011; Fierro-Castro et al., 2015).

Despite numerous studies which support a negative role of corticosteroids at final oocyte maturational stage, positive interactions between some corticosteroid increases and reproductive events are also reported. Indeed, positive effects of in vitro cortisol doses on oocyte maturation have been reported in some fish species (Goetz, 1983; Milla et al., 2009a; Crossin et al., 2016). In the cyprinid *Chalcalburnus tarichi*, it has been reported that 11-deoxycortisol, a cortisol precursor, maybe more potent in inducing steroids than the maturation-inducing steroid (MIS, 17 α -20 β -dihydroxyprogesterone, Unal et al., 2008; Milla et al., 2009a). Moreover, a previous in vivo study demonstrated that injections of supra-pharmaceutical doses (5 mg kg⁻¹body weight) of cortisol accelerated the ovulation time and enhanced the ovulation rate in Nile tilapia (*Oreochromis niloticus*) (Gennotte et al., 2012). Apart from cortisol, 11-deoxycorticosterone (DOC) may also modulate the final oocyte developmental stages in fish, but the impact of DOC on reproductive processes has received little attention. Some available results indicate that DOC is not efficient to induce oocyte maturation and hydration in female trout, contrary to cortisol which may participate in the hydration process (Milla et al., 2006).

The differential effects of corticosteroids on reproductive processes may be related to adequate threshold ratio between corticosteroids and other endocrine hormones, or to the ontogeny of ovarian sensitivity throughout the reproductive cycle. Indeed, previous reports described a surge in plasma cortisol around the ovulation time in various fish species such as rainbow trout, brown trout *Salmo trutta*, goldfish *Carassius auratus* and common carp *Cyprinus carpio* (see review in Milla et al., 2009a), in parallel to the time peaks of GnRHs and GTHs as well as other paracrine or exocrine factors. It has been largely demonstrated that administration of GnRHs, GTHs or human chorionic gonadotropin at vitellogenesis developmental stage are efficient for inducing effective steroidogenesis and final oocyte maturation in various fish species (Pankhurst et al., 1995; Weber and Sullivan, 2005; Nagahama and Yamashita, 2008; Das et al., 2014), despite the possible cortisol release induced by anesthesia and manipulations (Iwama et al., 1989; Zahl et al., 2012; Topic Popovic et al., 2012). Conversely, in other cases, the efficiency of ovulatory treatments depends on the features of applied external stressors and the ovarian developmental stage (Haddy and Pankhurst, 2000; Pankhurst and Van Der Kraak, 2000; Kahl et al., 2001). Previous studies found the highest GTH-stimulated E₂ and T production at peak-vitellogenic ovarian stage and the

lowest during post-vitellogenic stage in rainbow trout (Reddy et al., 1999) suggesting a change in the follicular sensitivity to gonadotropic response. But, in the latter study, cortisol (at 100 ng/ml) suppressed both basal and GTH-stimulated steroidogenic capacity for peak-vitellogenic follicles, while no effects were observed at other stages of ovarian follicles.

Somatotropin factors such as IGF-1 have been also used for stimulating steroidogenesis and final oocyte maturation in some fish species but with species-specific related efficiency (Weber and Sullivan, 2005; Das et al., 2014; Zhou et al., 2016). Little information is available about the interaction between reproductive mechanisms and other endocrine actors such as thyroid hormones, so their interaction with high corticosteroid release is not yet characterized. Nevertheless, it was reported that the levels of thyroid hormones increased with oocyte size throughout the ovarian cycle (Weber et al., 1992). But an in vitro study showed negative impact of high T₃ on GTH stimulation and response in terms of germinal vesicle breakdown (GVBD) in rainbow trout (Sullivan et al., 1989).

Eurasian perch *Perca fluviatilis* is a uniparous reproductive model which has been characterized as a valuable candidate species for diversification of European freshwater aquaculture (Jourdan et al., 2000; Wang et al., 2006). Its gametogenesis is induced by decreasing photothermal environmental conditions from August, and its final vitellogenesis overcomes during the stressful winter conditions from January to mid-April. As a newly aquaculture species, Eurasian perch has been found more sensitive to stress induced by rearing conditions than aquaculture fish species such as rainbow trout (Jentoft et al., 2005). But the interaction between corticosteroids and sex hormones is not yet clarified in percid fish species. Therefore, the objectives of the present study were (1) to determine to what extent corticosteroids (cortisol and DOC) interfere with the in vitro steroidogenic capacity of Eurasian perch ovarian tissues; (2) to test whether this interference may be mitigated by metabolic endocrine factors, namely insulin-like growth factor-1 (IGF), human chorionic gonadotropin (HCG) and thyroid hormones (T₃, T₄); (3) to compare the ovarian sensitivity to these hormones between late vitellogenesis and final oocyte maturation.

2. Material and Methods

2.1. Fish and rearing conditions

The experimental manipulations were performed according to the official guidelines for the use and care of laboratory animals and in compliance with the Belgian (n° 10138 KE) and European policies on animal welfare. A group of Eurasian perch females were reared in outdoor pond conditions at a private fish farmer (Belgium) to induce ovarian maturation. Fifty gravid females (body weight: 210–350 g) were captured in early February and transferred to the indoor facilities of the University of Namur (Belgium) where they were stocked in 500-L tank connected to a recirculating aquaculture system (RAS). This RAS was adapted to mimic the progressive natural increase in photothermal conditions from February to April in

order to achieve the final oocyte maturation. Fish were fed twice a day (except on Sunday) with a commercial feed (Coppens 18.7 MJ kg⁻¹, 46% crude protein, 15% crude lipid) at apparent satiation.

2.2. Ovarian tissue culture and endocrine treatments

After one month of habituation to the RAS conditions, six maturing females were randomly selected for dissection either during late vitellogenesis stage (LVO) in early March or at the start of the final meiotic oocyte maturation (FMO) in early April. Just after the dissection, ovaries were stored in a Cortland medium previously modified and adapted for high perch

oocyte viability with only 2% picnotic oocytes after a 6-h incubation time (see in Milla et al., 2009b). Then, a pool of six pieces of ovarian tissues (each composed about 12 oocytes) was incubated in large 12-multiwell plates during 6 h at 12 °C in either control medium or in medium containing various endocrine compounds in six technical replicates per treatment. The corticosteroid doses were chosen according to the known level in Eurasian perch given that the plasma concentration of cortisol is more or less 10-fold higher than DOC level (Milla et al., 2010; Mathieu et al., 2013):

1. Control Cortland medium only (CT) vs Control Cortland medium + ethanol hormone vehicle (CTet).
2. Potential stimulating factors: IGF-I (IGF) vs hCG (HCG) vs T3 (T3-20, T3-100) vs T4: (T4-20, T4-100)
3. Corticosteroids: Cortisol (Co10, Co100, Co1000) vs DOC (D1, D10, D100)
4. Mixture of corticosteroids and potential stimulating factors at the same above doses:
 - a. HCG-Co10, -Co100, -Co1000 vs IGF-D1, -D10, -D100 vs IGF-Co10, -Co100, -Co1000.
 - b. T3-20/Co100, T3-100/Co1000 vs T4-20/Co100, T4-100/Co1000.

In summary, thirty-three pure or mixture endocrine compounds were tested at various doses representing basal, high and supraphysiological levels depending on the corticosteroid type as shown in Table 1.

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), and the purity ranged between 97% and 98%. For HCG (50 U.I./ml) and IGF (100 nM), only one dose was tested because we have already demonstrated in a previous study that such doses were effective for accelerating ovarian follicular growth and enhancing ovarian steroid production in Eurasian perch females reared under natural photothermal conditions (Milla et al., 2009b). After 6 h of incubation, the culture medium and ovarian piece tissues were removed and stored at -80 °C until analyses. In vitro ovarian steroidogenic capacity was evaluated in terms of aromatase activity (AA) in the cultured ovarian piece tissues, and of sex-steroid (testosterone-T, estradiol-E2, and 17 α -20 β -dihydroxy-4-pregnen-3-one, DHP) contents in the culture medium.

2.3. Histological examination

3. Results

3.1. Aromatase activity

As expected, the proportion of advanced maturational oocytes, namely late vitellogenic oocytes and final meiotic maturational oocytes were lower ($P < 0.05$) at LVO stage (49% and 22%) than at FMO stage (16% and 79%). HCG induced a higher AA stimulation action relative to values of control CTet at FMO stage than at LVO stage ($P < 0.001$, Table 2, Fig. 1a). IGF was lesser effective than HCG in stimulating AA, and a higher significant magnitude response was also observed at FMO (+123%, $P < 0.001$) stage than at LVO ($P < 0.05$) stage. Thyroid hormones were not effective in stimulating AA whatever the dose and the oogenesis stage (Table 2, Fig. 1a). Even, a trend of decrease in AA was induced by both T3 doses

Histological examination was done at LVO and FMO stages to ascertain for the proportions of advanced maturational oocytes. Therefore, after classic fixation of ovarian piece tissues in Bouin (75% picric acid, 20% formol, 5% acetic acid) and staining with the trichrome method, the proportions of advanced maturational oocytes were determined as late vitellogenic oocytes and final meiotic maturational oocytes at each oogenesis stage.

2.4. Sex-steroid and aromatase activity assays

Levels of sex-steroids were determined in the incubation culture medium by radio immunoassay (RIA) after two extractions with cyclohexane/ethyl acetate according the method of Fostier and Jalabert (1986) adapted by Mandiki et al. (2005). Two sets of assays were done for each hormone and the intra- and interassay coefficients of variation were 4.2% and 4.9%, 4.7% and 5.5%, 3.8% and 4.5%, 5.0% and 6.2% for T, E2, 11KT and DHP respectively. The detection limits of the assays ranged from 5.5 to 10 pg/ml.

Aromatase activity was measured as the specific release of tritiated water produced by the conversion of [1β -H 3]-androstenedione to estrone, as previously described by Noakson et al. (2001), Gonzalez and Piferrer (2002), and adapted as summarized in our previous reports (Mandiki et al., 2005; Milla et al., 2009b). Values were expressed in fmol/min/mg proteins after determination of proteins in the homogenate pellet by the Lowry method. The intra-assay coefficient of variation was 4.85%.

2.5. Statistical analyses

After checking of the normality and homogeneity of variances, the effects of sampling time and endocrine treatment on AA or production of sex-steroids were analyzed by a two-way analysis of variance (ANOVA) followed, when significant, by LSD tests. The level of significance used in all tests was $P < 0.05$, 0.01, 0.001. Statistical analyses were performed using Statistica software (StatSoft, Tulsa, OK, USA). The pool of ovarian tissues (repetition) composed of ovarian piece tissues from the six sampled fish at each oogenesis stage was used as statistical unit and data were log-transformed in the cases of percentage values.

during both oogenesis stages, and a significant decrease was observed with both T4 treatments at FMO ($P < 0.05$).

Cortisol and DOC treatments induced a drastic suppression of AA compared to the control CTet whatever the oogenesis stage (Table 3, Fig. 1b, and $P < 0.001$). AA was almost totally suppressed by high and supraphysiological corticosteroid doses. Moreover, AA values after HCG and corticosteroid treatments together were markedly lower than those obtained after pure HCG treatment, indicating that corticosteroid doses significantly inhibited HCG stimulation action (Fig. 1c, $P < 0.01$). Conversely, the comparison between HCG + corticosteroids with pure corticosteroid treatments indicated that HCG significantly protected from the negative effects of

basal (Co10) and high (Co100) cortisol doses, especially at LVO stage (Table 4 and $P < 0.05, 0.01$). Such protection of AA by HCG was lower in the case of low and high DOC doses (Table 4).

IGF showed low or no-protection of AA against the negative effects of basal and high cortisol doses whatever the oogenesis stage (Table 4, Fig. 1c). Neither HCG nor IGF was able to mitigate the negative effects of supraphysiological cortisol dose of 1000 ng/ml on AA (Table 4).

No protection of AA by all thyroid hormone treatments was observed against the negative effects of all cortisol doses whatever the oogenesis stage (results not shown).

3.2. Sex-steroid production

a. Estrogen production

HCG treatment significantly ($P < 0.001$) increased E2 production relative to values of control CTet, during both oogenesis stages (Table 2, Fig. 2a). IGF treatment was lesser effective than HCG in stimulating E2 production, especially at FMO stage. As for AA, thyroid hormones did not stimulate E2 production, and a decrease was observed for most of the tested doses whatever the oogenesis stage (Table 2, Fig. 2a, and $P < 0.05$).

Corticosteroid treatments induced a significant dose related suppression of E2 production (Fig. 2b, Table 3, $P < 0.05-0.001$). Relatively to HCG alone, the corticosteroids combined with HCG induced a decrease of E2 production (Fig. 2C). The magnitude of that reduction was lower compared to that observed for suppression in ØValues after HCG associated to basal (D1) and high (D10) DOC doses indicated that HCG significantly decreased the inhibition of E2 production by DOC, especially at FMO stage (Table 4). In contrast, no HCG mitigation was observed against the basal and high cortisol doses at that FMO stage. IGF seemed more effective than HCG in preventing the negative effects of basal and high cortisol doses but neither HCG nor IGF did not prevent E2 production from the negative effects of supraphysiological cortisol dose of 1000 ng/ml (Table 4).

b. Testosterone production

HCG and IGF treatments induced significant increase in T production relative to values of control CTet at FMO stage not at

LVO stage (Table 2, Fig. 3a, $P < 0.01$). Thyroid hormone treatments slightly decreased T production whatever the oogenesis stage or hormone dose (Table 2, Fig. 3a, and $P < 0.05$).

All the tested doses of corticosteroids significantly ($P < 0.05$) suppressed T production with a lower amplitude reduction compared to that observed for AA (Table 3, Fig. 3b). The suppression amplitude of T production was slightly more elevated at FMO stage (-45% to -62%) than at LVO stage (-30% to -52%). HCG treatment significantly mitigated the negative effects of basal and high cortisol or DOC on T production, especially at FMO stage (Table 4, Fig. 3c, and $P < 0.01, 0.001$). But HCG could not prevent the negative effects of supraphysiological cortisol (Co1000) or DOC (D100) on T production, except for a slight protection at FMO stage. IGF was ineffective in preventing the suppression of T production by cortisol treatments during both oogenesis stages.

c. DHP production

HCG treatment induced a significant increase ($P < 0.001$) in DHP production relative to values of control CTet with the highest amplitude at FMO stage (Table 2, Fig. 4a). IGF stimulated DHP production only at FMO stage ($P < 0.01$), and with a lower amplitude response comparing to HCG. T3 and T4 significantly decreased ($P < 0.05$) DHP production for all the tested doses whatever the oogenesis stage (Table 2, Fig. 4a).

All corticosteroid doses significantly ($P < 0.05-0.001$) suppressed DHP production in dose related manner with the lowest amplitude reduction observed at FMO stage (Table 3, Fig. 4b). The addition of corticosteroids to HCG decreased the production of DHP relatively to the results obtained with HCG alone (Fig. 4C). HCG significantly prevented the suppression of DHP production by basal and high cortisol doses only at FMO stage (Table 4, $P < 0.01, 0.001$). Such HCG protection was also observed against basal and high DOC doses with a higher amplitude at FMO than at LVO stage. No protective effect of HCG was observed for cortisol or DOC supraphysiological dose. IGF did not prevent the negative effects of cortisol, except for basal dose (Co10) at FMO stage (Table 4).

All thyroid hormone treatments did not protect sex-steroid production against the negative effects of corticosteroid treatments whatever the oogenesis stage (results not shown).

4. Discussion

4.1. Corticosteroids drastically depressed steroidogenic capacity

In the present study, the *in vitro* effects of cortisol and DOC doses on steroidogenic capacity were evaluated on Eurasian perch ovarian piece tissues at late vitellogenesis stage (LVO) characterized by a high proportion of late vitellogenic oocytes, and at the start of the final meiotic oocyte maturation (FMO) with a high proportion of oocytes at final maturation. For each corticosteroid type, the basal and high doses were comparable to baseline and poststress circulating levels usually observed in various fish species including percoid fish (Milla et al., 2010,

2015; Pankhurst, 2011; Douxfils et al., 2011, 2012, 2014; Mathieu et al., 2013, 2014).

The results demonstrated that all tested doses of cortisol or DOC suppressed drastically AA and sex-steroid production at the two late oogenesis stages. That suppression could prevent the establishment of the final oocyte maturation in contrast to data from previous studies that cortisol and DOC are effective in inducing *in vitro* oocyte maturation in several fish species including close species to Eurasian perch such as yellow perch *Perca flavescens* (Goetz, 1983; Pankhurst et al., 1995; Milla et al., 2009b). It has been also shown that 11-deoxycortisol, a

cortisol precursor, may be more potent in stimulating final oocyte maturation than some maturation steroids such as the maturation-inducing steroid DHP in the cyprinid species *Chalcalburnus tarichi* (Unal et al., 2008; Milla et al., 2009a). An *in vivo* study also demonstrated that injections of high supraphysiological doses of cortisol had positive effects on ovulation timing and reproductive efficiency in Nile tilapia (Gennotte et al., 2012). But a negative relationship is generally observed between chronic stress-induced sustained glucocorticoid increase and various reproductive mechanisms or reproductive efficiency (Pankhurst and Van Der Kraak, 2000; Wingfield and Sapolsky, 2003; Small et al., 2008; Schreck, 2010). It was also shown that cortisol implants associated to a sustained increase in plasma cortisol impaired reproductive success in Pacific salmon (*Oncorhynchus gorbusha*) (McConnachie et al., 2012). But the latter authors demonstrated that a transient plasma cortisol increase relative to acute stress events, or a suppression of plasma cortisol increase by metyrapone implants did not alter reproductive success in Pacific salmon. So, the interactions between corticosteroids and reproductive mechanisms in fish may be species specific or related to the amount of corticosteroids released in the blood plasma, as reported for other animal species (Crossin et al., 2016).

The relationship between corticosteroids and reproductive mechanisms has been reported to vary with changes in the oocyte sensitivity through the ovarian cycle. Indeed, some *in vitro* studies showed that cortisol at 100 ng/ml in the incubation medium significantly suppressed E2 and T productions of rainbow trout oocytes at peak-vitellogenic developmental stage, while no marked effects were observed at post-vitellogenic developmental stages or preovulatory (Reddy et al., 1999). In the present study, the suppression of DHP by basal and high cortisol or DOC doses was more important at LVO stage than at FMO stage, and the opposite was observed for T production. This finding indicates that the negative action of corticosteroids on steroidogenic capacity may be related to the oocyte sensitivity, but also to the interaction between corticosteroids and the pathway relays of each sex-steroid production. The low negative effects of high cortisol doses observed at FMO stage may be related to a programmed genetically habituation to hypercorticolemic conditions since post-vitellogenic and preovulatory developmental stages are characterized by a transient increase in plasma cortisol levels in various fish species (Vijayan et al., 1997; Reddy et al., 1999; Milla et al., 2009a; Gennotte et al., 2012). It has been also reported that DHP production pathways are optimized in some fish species at final meiotic oocyte developmental stage and the pre-ovulation time (Nagahama and Yamashita, 2008; Moreira et al., 2014; Moreira et al., 2015). That pathway optimization may account for the reduction in the negative effect of corticosteroids on DHP production. Some authors reported a correlation between an increase in cortisol secretion and DHP production at the final stage of oocyte maturation of some fish species, and hypothesized a corticosteroidogenesis process in some fish species (Hwang et al., 2012).

4.2. HCG and IGF induced variable protective effects of steroidogenic processes from corticosteroid suppression

HCG and IGF have been found to be potent inducers of ovarian steroidogenesis both *in vitro* and *in vivo* for a variety of fish species (Weber and Sullivan, 2000, 2005; Dabrowski et

al., 2002; Milla et al., 2009a, b; Das et al., 2014; Muruganankumar et al., 2016). In the present study, pure HCG treatment induced a significant increase in AA and sex-steroid production with the highest amplitude response at FMO stage. That gonadotropin stimulation action was suppressed by all the tested corticosteroid doses. But, HCG treatment markedly protected AA and sex-steroid production against the negative effects of basal and high cortisol doses. That protection of AA by HCG was lower against DOC than cortisol, indicating a stronger inhibition by DOC. Previous studies demonstrated that the synthesis of sex-steroids in the fish ovary is stimulated by gonadotropin binding to ovarian GTH receptors which activate various pathway relays such as expression of G-proteins, increase in cAMP and the expression of other steroidogenic genes including steroidogenic acute regulatory (StAR), 17 β -hydroxysteroid dehydrogenase type 3 and aromatase P450 (Ings and Van Der Kraak, 2006; Alspo et al., 2009; Barkataki et al., 2011; Fierro-Castro et al., 2015; Liu et al., 2017). Besides the latter previous hypothesis on the inhibition of gene expressions, the results of the present study suggest that the activities of ovarian steroidogenic enzymes may be also suppressed as evidenced by a drastic reduction in the enzymatic P450 aromatase activity and E2 production by both cortisol and DOC whatever the oogenesis stage. It was recently demonstrated that the inhibition of ovarian P450arom gene expression at vitellogenic stage was associated to a suppression of ovarian aromatase activity in common carp, indicating that the processes controlling the steroidogenic enzyme activities and their gene expressions may be closely related in fish (Das et al., 2016).

It has been shown that cortisol and potentially DOC have different receptor networks in fish, such as rtGR1 and rtGR2 (glucocorticoid receptors 1 and 2) and rtMR (mineralocorticoid receptors) in rainbow trout (Milla et al., 2006). The latter authors also reported that exposure of final meiotic oocytes to cortisol stimulates hydration process while DOC is not effective in inducing this process. Thus, the lower protection of AA by gonadotropin from DOC compared to cortisol inhibition observed in the present study may indicate that the glucocorticoid and mineralocorticoid receptors act differentially through the ovarian gonadotropin receptor networks, and that DOC may have more suppressive impact on these relays. In the present study, it seems that cortisol and DOC physiological doses may have the same negative impact on steroidogenic capacity. Indeed, cortisol induced lesser inhibition than DOC for HCG-stimulated AA. But cortisol induced a higher suppression than DOC concerning the stimulation of E2 production by HCG at FMO stage. Moreover, the protective action of T and DHP production by HCG from cortisol and DOC doses was comparable at FMO stage. A previous *in vitro* study on rainbow trout has shown that cortisol at 100 ng/ml suppressed both basal and gonadotropin-stimulated production of T and E2 of peak-vitellogenic oocytes, while no apparent effect was observed for post-vitellogenic oocytes (Reddy et al., 1999). These latter observations may indicate a decrease in the responsiveness of ovarian gonadotropin receptors to corticosteroids in relation with a progressive activation of glucocorticoid receptors relative to an increase of cortisol in oocytes or in the blood at final oocyte maturation stages as previously observed in some fish species (Milla et al., 2009a; Li et al., 2012).

In the present study, the IGF dose (100nM) showed poor protective action against the negative effects of basal and low cortisol doses whatever the oogenesis stage. This maybe

related to the fact that pure IGF treatment was not highly effective in stimulating steroidogenic processes compared to pure HCG treatment. The latter observation suggests that the efficiency of IGF to stimulate steroidogenic processes may depend on the experimental context such as population features because a higher response in terms of E2 and T productions was observed in our previous *in vitro* study using Eurasian perch ovarian tissues at the same oogenesis stage, and the same IGF dose (Milla et al., 2009b). Other studies also reported that the effectiveness of IGF to stimulate sex-steroid production may vary from none to potent effects between fish species (Weil et al., 1999; Weber and Sullivan, 2005; Weber et al., 2007; Das et al., 2014; Zhou et al., 2016). Despite that species related differences in fish, the triggering action of IGF for ovarian growth and sex-steroid production is likely regulated through an abundant presence of receptors in both follicular layers (Kagawa et al., 1995; Maestro et al., 1997; Das et al., 2014; Zhou et al., 2016; Mukherjee et al., 2017) by which IGF may also act to interfere in the stimulatory action of gonadotropins. A high variation in the affinity of IGF follicular receptors throughout the ovarian developmental stages has been reported in relation to a higher sensitivity of oocytes at final stages than vitellogenic stage (Nagahama and Yamashita, 2008; Zhou et al., 2016). Such increase in positive regulation may explain the highest IGF stimulation response observed in the present study at FMO stage.

4.3. Thyroid hormones had no positive effects on ovarian AA and sex-steroid production

The two doses (20 and 100 ng/ml) of triiodothyronine (T3) or thyroxine (T4) tested in the present study induced a slight decrease in AA or sex-steroid production whatever the oogenesis stage. Therefore, no protective actions on steroidogenic processes were observed for the mixture treatments composed of thyroid hormones and cortisol doses. There is no large consensus of a positive role of thyroid hormones on reproductive mechanisms in fish even if a relationship between that endocrine system and reproductive status has been suggested in some fish species (Cyr and Eales, 1996; Comeau and Campana, 2006). Recent studies demonstrated that thyroid hormones play important roles in

gonadal maturation and functions in fish through various actions such as an impact on the expression of various steroidogenesis genes, namely cytochrome P450 aromatase A (cyp19a), 11b-hydroxysteroid dehydrogenase (11bhsd), and on sex-steroid hormone synthesis in some fish species (Duarte-Guterman et al., 2014; Yu et al., 2015). It can be also hypothesized that T3 and T4 may play a positive action on the oocyte development since an increase in the secretion of these hormones has been described in parallel to the increase in oocyte size throughout the ovarian cycle in tilapia (Weber et al., 1992). But there is no information concerning such profiles at oocyte developmental stages for Eurasian perch. Previous *in vitro* studies showed that low concentrations of T3 ranging between 0.5 and 50 ng/ml may stimulate the final oocyte maturation of rainbow trout by potentiating the steroidogenic responses, while higher T3 concentrations may induce negative impact in terms of occurrence of germinal vesicle breakdown in the same species (Sullivan et al., 1989). Thus, it is possible that the lack of positive effects by thyroid hormonal treatments may be related to high unsuitable doses for Eurasian perch oocytes in the present study. But another *in vitro* study using lower T3 dose (10 ng/ml) than in the present study did not show any positive effect on basal or gonadotropin-stimulated T or E2 production by rainbow trout ovarian follicles, and no beneficial effects of gonadotropin over the suppressive effect of cortisol were observed (Reddy et al., 1999). So, the interactions between thyroid hormones and reproductive processes may not be only species related but may also vary according to the experimental conditions. In conclusion, the present results suggested that an increase in cortisol and DOC secretions such as those observed after mild or high stress intensity may inhibit drastically the ovarian steroidogenic capacity whatever the final oocyte maturation stage in percid fish by hampering aromatase activity and sex-steroid production. That inhibition action by high corticosteroid levels may be partly mitigated by gonadotropins but not IGF, especially at final meiotic oocyte maturation stage. The protection of aromatase activity by gonadotropins seemed lower against DOC inhibition compared to cortisol but such interaction may vary between sex-steroid production pathways.

Table 1. Experimental protocol and concentrations of the experimental pure or mixture endocrine treatments.

Stimulating factors	Dose	Corticosteroids (ng/ml)					
		Co10	Co100	Co1000	D1	D10	D100
HCG (UI/ml)	50	10	100	1000	1	10	100
IGF (nM)	100	50	50	50	50	50	50
T3-20 (ng/ml)	20	100	100	100			
T3-100 (ng/ml)	100	20	20	20			
T4-20 (ng/ml)	20	100	100	100			
T4-100 (ng/ml)	100	20	20	20			

Table 2. Changes (% control CTet) in aromatase activity and sex-steroid production of Eurasian perch ovarian tissues after 6 h of incubation with HCG, IGF-I and thyroid hormone doses (T3-T4). Values are expressed as mean of 6 replicates consisted of 6 ovarian piece tissues obtained on six maturing fish at late vitellogenic oocyte stage (LVO) or the start of the final meiotic oocyte developmental stage (FMO). Each ovarian piece tissue contained 12 late vitellogenic oocytes or final meiotic maturational oocytes.

Treatment	AA		E2		T		DHP	
	LVO	FMO	LVO	FMO	LVO	FMO	LVO	FMO
HCG	+64 ^{aB}	+267 ^{aC}	+160 ^{aC}	+149 ^C	+4 ^c	+92 ^{aC}	+103 ^{bc}	+224 ^{aC}
IGF	+24 ^{aA}	+123 ^{aC}	+86 ^{aB}	+61 ^{bb}	-17 ^c	+87 ^{aB}	+4 ^c	+81 ^{aB}
T3-20	-17 ^a	-26 ^{aA}	-6 ^a	-25 ^{ba}	-18 ^b	-37 ^{aA}	-46 ^{aA}	-17 ^b
T3-100	-22 ^{aA}	-12 ^b	-10 ^a	-13 ^a	-20 ^{aA}	-17 ^a	-32 ^{aA}	-29 ^{aA}
T4-20	-48 ^{aA}	-40 ^{aA}	-9 ^a	-23 ^{ba}	-20 ^{aA}	-25 ^{aA}	-40 ^{aA}	-28 ^{ba}
T4-100	-37 ^{aA}	-37 ^{aA}	-3 ^a	-23 ^{ba}	-24 ^{aA}	-22 ^{aA}	-53 ^{aA}	-36 ^{ba}

HCG = hCG at 50 UI/ml. IGF = IGF-1 at 100 nM. T3-20, T3-100 = triiodothyronine at 20 r A, B or C for P < 0.05, 0.01 or 0001 0.01). Significant differences related to increase or decrease are indicated by capital letters

Table 3. Decrease (% control CTet) in aromatase activity and sex-steroid production of Eurasian perch ovarian tissues by corticosteroid treatments after 6 h of incubation. Values are expressed as mean of 6 replicates consisted of 6 ovarian piece tissues obtained on six maturing fish at late vitellogenic oocyte stage (LVO) or at the start of the final meiotic oocyte developmental stage (FMO). Each ovarian piece tissue contained 12 late vitellogenic oocytes or final meiotic maturational oocytes.

Treatment	AA		E2		T		DHP	
	LVO	FMO	LVO	FMO	LVO	FMO	LVO	FMO
Co10	-85 ^{aB}	-79 ^{aB}	-24 ^{ba}	-39 ^{aA}	-39 ^{ba}	-50 ^{aA}	-60 ^{aB}	-39 ^{ba}
Co100	-92 ^{aC}	-95 ^{aC}	-54 ^{aA}	-53 ^{aA}	-36 ^{ba}	-54 ^{aA}	-87 ^{aB}	-73 ^{bb}
Co1000	-95 ^{aC}	-95 ^{aC}	-81 ^{aB}	-82 ^{aB}	-52 ^{aA}	-62 ^{aB}	-93 ^{aC}	-84 ^{aB}
D1	-83 ^{aB}	-49 ^{ba}	-13 ^b	-36 ^{aA}	-30 ^{ba}	-45 ^{aA}	-71 ^{aB}	-23 ^{ba}
D10	-98 ^{aC}	-96 ^{aC}	-52 ^{aA}	-51 ^{aA}	-34 ^{ba}	-50 ^{aA}	-89 ^{aB}	-39 ^{ba}
D100	-98 ^{aC}	-98 ^{aC}	-76 ^{aB}	-81 ^{aB}	-52 ^{aA}	-53 ^{aA}	-91 ^{aC}	-75 ^{bb}

Co10, 100,100 = cortisol at 10,100,1000 ng/ml. D1, 10,100 = 11-deoxycorticosterone (DOC) at 1, 10,100 ng/ml. Significant differences between the two oogenesis stages are indicated by different superscript small letters (a-b or a-c for P < 0.05 or 0.01). Significant differences related to decrease are indicated by capital letters A, B or C for P < 0.05, 0.01 or 0.001.

Table 4. HCG or IGF relative mitigation* of negative corticosteroid effects on aromatase activity and sex-steroid production of Eurasian perch ovarian tissues after 6 h of incubation. Values are expressed as mean of 6 replicates consisted of 6 ovarian piece tissues obtained on six maturing fish at late vitellogenic oocyte stage (LVO) or at the start of the final meiotic oocyte developmental stage (FMO). Each ovarian piece tissue contained 12 late vitellogenic oocytes or final meiotic maturational oocytes.

Treatment	AA		E2		T		DHP	
	LVO	FMO	LVO	FMO	LVO	FMO	LVO	FMO
HCG-Co10	+85 ^{aB}	+85 ^{aB}	+33 ^{aA}	0 ^c	+100 ^{aC}	+100 ^{aC}	0 ^c	+100 ^{aC}
HCG-Co100	+84 ^{aB}	+19 ^{aA}	+6 ^a	0 ^a	0 ^c	+100 ^{aC}	0 ^c	+61 ^{aB}
HCG-Co1000	0 ^a	0 ^a	0 ^a	0 ^a	+8 ^c	+68 ^{aB}	0 ^a	+8 ^a
HCG-D1	+30 ^{aA}	+33 ^{aA}	0 ^c	+42 ^{aA}	+76 ^{bb}	+100 ^{aC}	+98 ^{aC}	+84 ^{bb}
HCG-D10	+43 ^{aA}	+25 ^{ba}	+33 ^{aA}	+31 ^{aA}	0 ^c	+100 ^{aC}	+22 ^{aA}	+100 ^{aC}
HCG-D100	0 ^a	0 ^a	+12 ^a	+7 ^a	0 ^c	+51 ^{aA}	+5 ^a	+9 ^a
IGF-Co10	+20 ^{aA}	+25 ^{aA}	0 ^c	+51 ^{aA}	+70 ^{ab}	0 ^c	0 ^c	+100 ^{aC}
IGF-Co100	+19 ^{aA}	0 ^b	+6 ^c	+51 ^{aA}	0 ^a	+4 ^a	+2 ^b	+14 ^a
IGF-Co1000	0 ^a	0 ^a	+1 ^a	0 ^a	0 ^a	+2 ^a	0 ^a	+5 ^a

Asterisk =delta % corticosteroids vs HCG/IGF + corticosteroids. HCG = hCG at 50 UI/ml. IGF = IGF-1 at 100 nM. Co10, 100,100 = cortisol at 10,100,1000 ng/ml. D1, 10,100 =11-deoxycorticosterone (DOC) at 1, 10,100 ng/ml. Significant differences between the two oogenesis stages are indicated by different superscript small letters (a-b or a-c for P < 0.05 or 0.01). Significant differences related to increase are indicated by capital letters A, B or C for P < 0.05, 0.01 or 0.001.

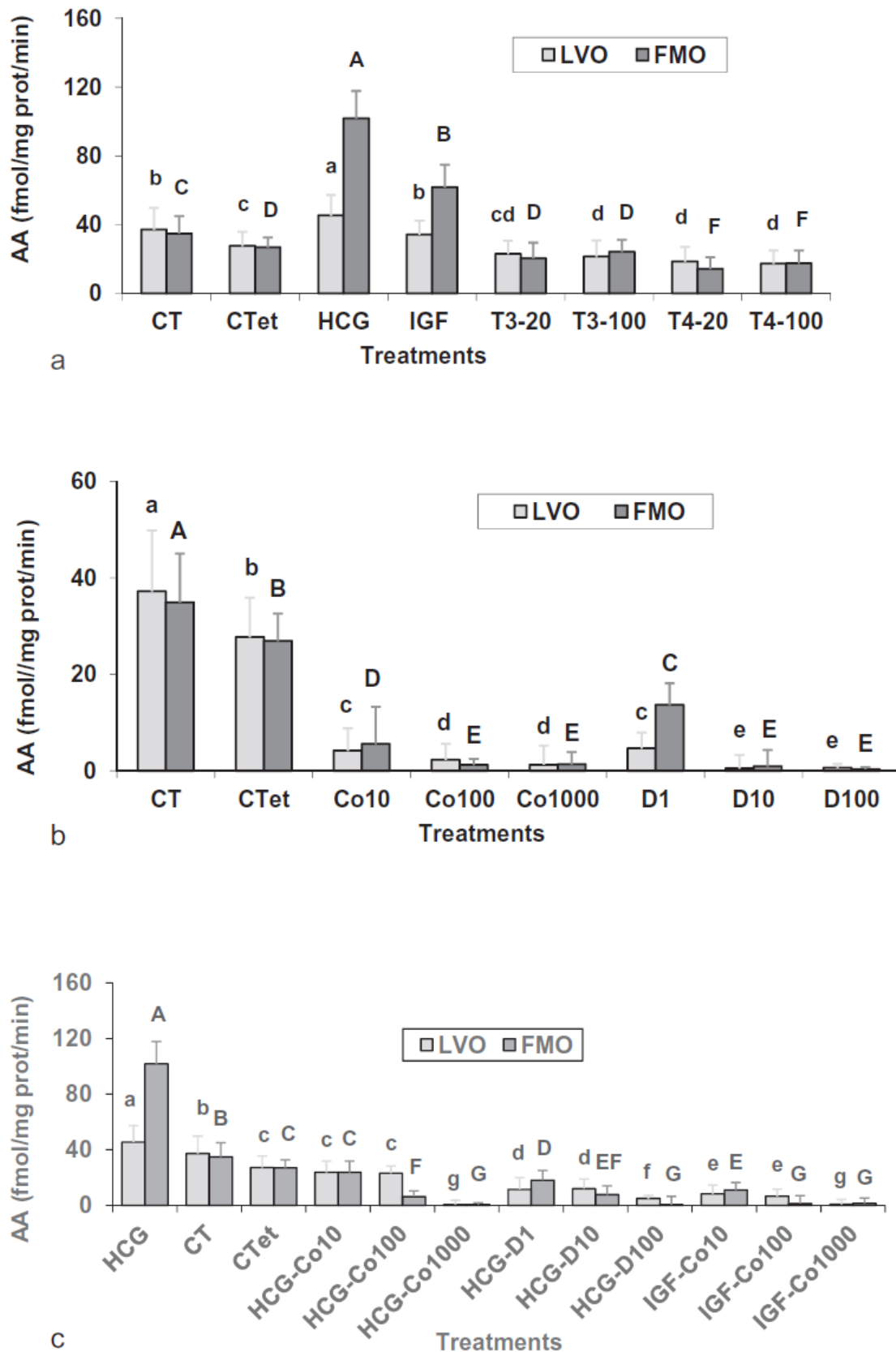


Figure 1.

Changes in *in vitro* aromatase activity (\emptyset) of Eurasian perch ovarian tissues incubated in medium containing (a) potential stimulating endocrine factors ((HCG (100UI), IGF (50 nM), T3 (20 or 100 ng/ml), T4 (20 or 100 ng/ml)), (b) corticosteroids (cortisol: 10, 100, 1000 ng/ml or DOC: 1, 10, 100 ng/ml), (c) pure HCG or mixtures composed of HCG or IGF-1 and corticosteroids. Values are expressed as mean \pm SD of 6 replicates consisted of 6 ovarian piece tissues obtained on six maturing fish at late vitellogenic oocyte stage (LVO) or at the start of the final meiotic oocyte developmental stage (FMO). Each ovarian piece tissue contained 12 late vitellogenic oocytes or final meiotic maturational oocytes. For each oogenesis stage, values with different superscript letters are significantly different ($P < 0.05-0.001$).

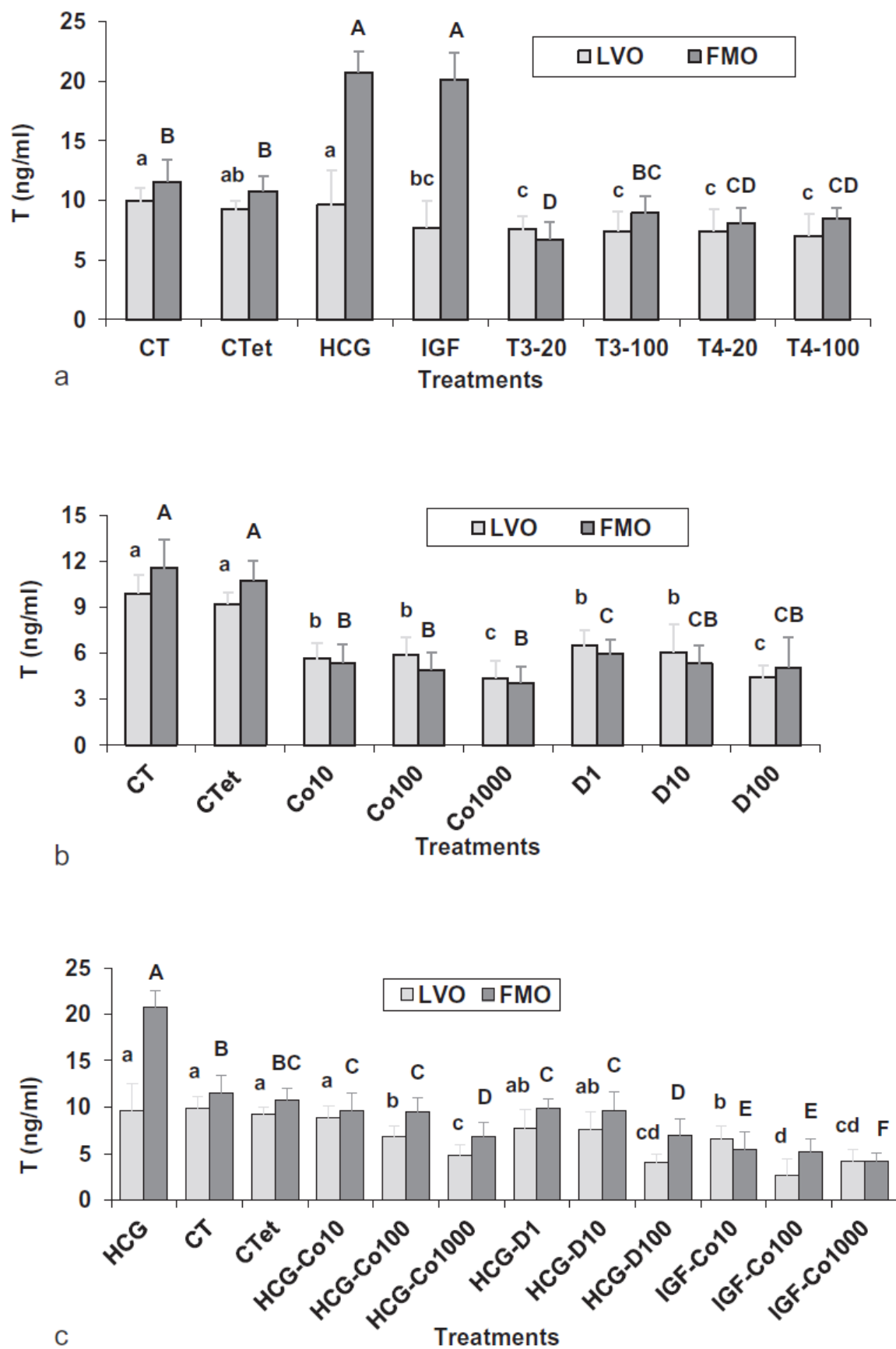


Figure 2.

Changes in *in vitro* E2 production by Eurasian perch ovarian tissues incubated in medium containing (a) potential stimulating endocrine factors ((HCG (100UI), IGF (50 nM), T3 (20 or 100 ng/ml), T4 (20 or 100 ng/ml)), (b) corticosteroids (cortisol: 10, 100,1000 ng/ml or DOC: 1,10,100 ng/ml), (c) pure HCG or mixtures composed of HCG or IGF-1 and corticosteroids. Values are expressed as mean \pm SD of 6 replicates consisted of 6 ovarian piece tissues obtained on six maturing fish at late vitellogenic oocyte stage (LVO) or at the start of the final meiotic oocyte developmental stage (FMO). Each ovarian piece tissue contained 12 late vitellogenic oocytes or final meiotic maturational oocytes. For each oogenesis stage, values with different superscript letters are significantly different ($P < 0.05$ – 0.001).

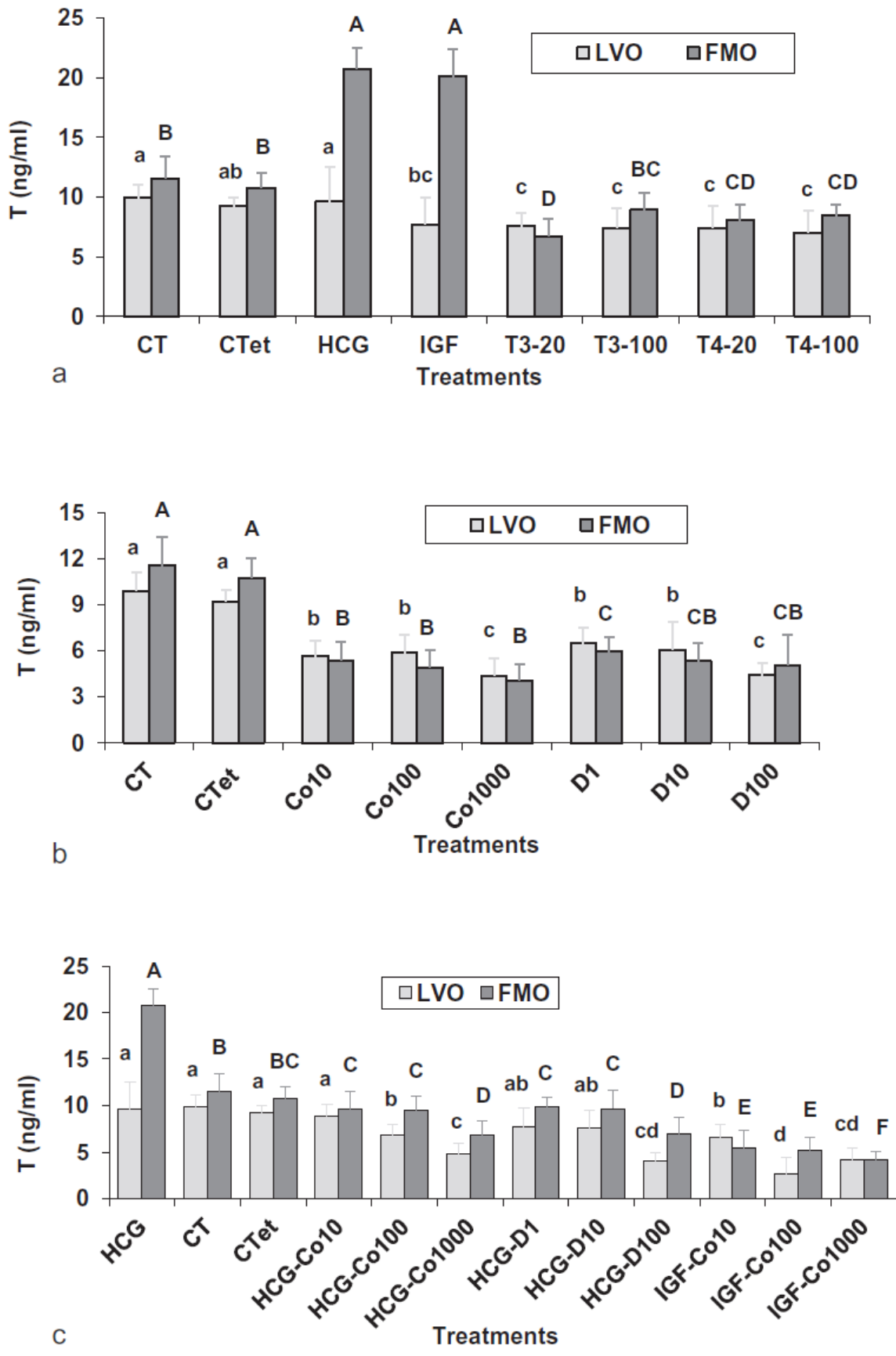
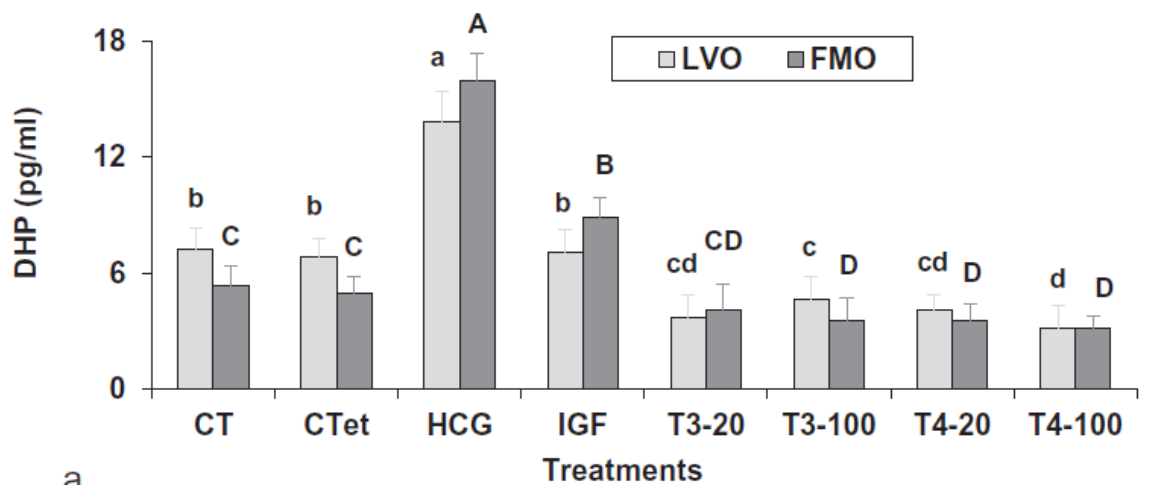
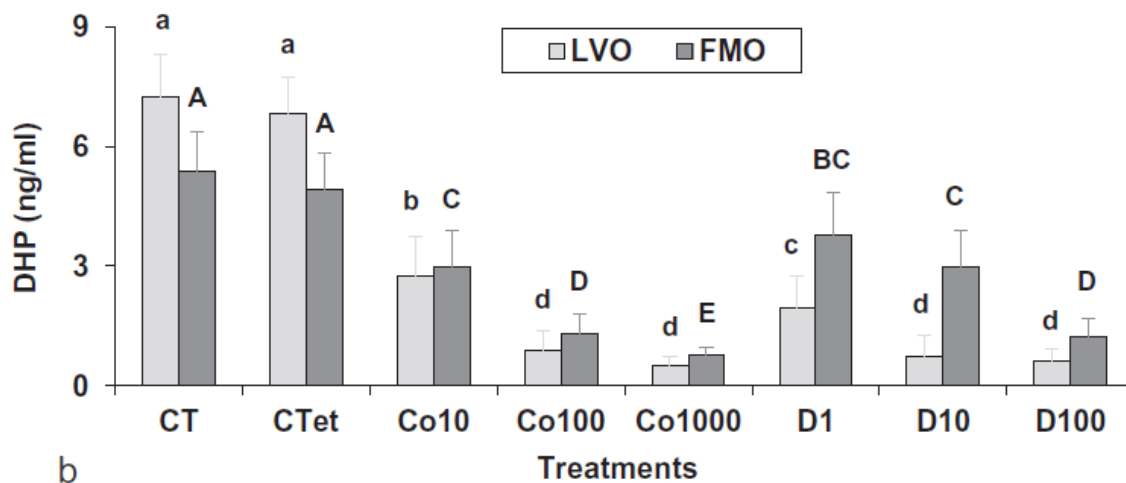


Figure 3.

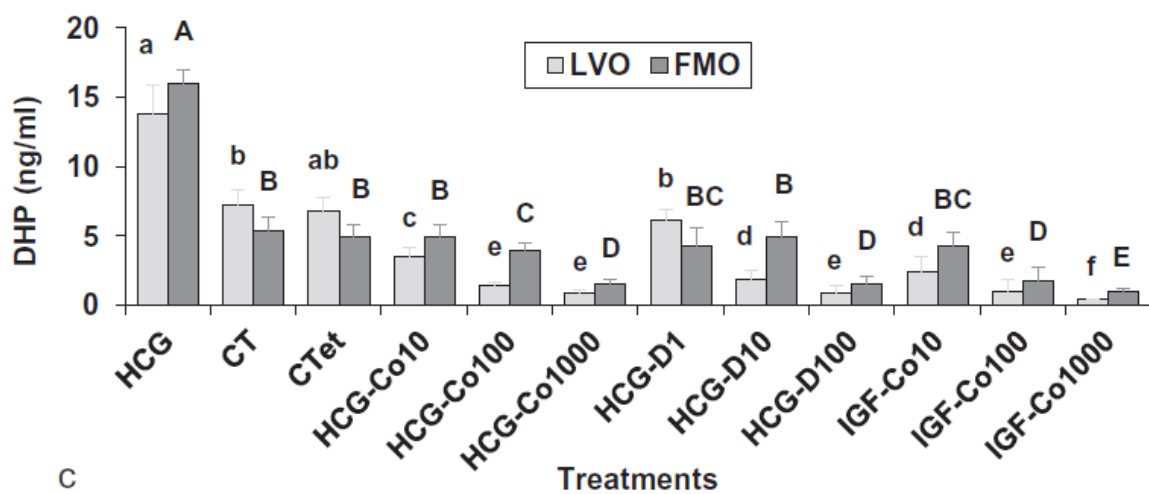
Changes in vitro T production by Eurasian perch ovarian tissues incubated in medium containing (a) potential stimulating endocrine factors (HCG (100UI), IGF (50 nM), T3 (20 or 100 ng/ml), T4 (20 or 100 ng/ml)), (b) corticosteroids (cortisol: 10, 100, 1000 ng/ml or DOC: 1,10,100 ng/ml), (c) pure HCG or mixtures composed of HCG or IGF-1 and corticosteroids. Values are expressed as mean \pm SD of 6 replicates consisted of 6 ovarian piece tissues obtained on six maturing fish at late vitellogenic oocyte stage (LVO) or at the start of the final meiotic oocyte developmental stage (FMO). Each ovarian piece tissue contained 12 late vitellogenic oocytes or final meiotic oocytes. For each oogenesis stage, values with different superscript letters are significantly different ($P < 0.05-0.001$).



a



b



c

Figure 4.

Changes in *in vitro* DHP production by Eurasian perch ovarian tissues incubated in medium containing (a) potential stimulating endocrine factors ((HCG (100UI), IGF (50 nM), T3 (20 or 100 ng/ml), T4 (20 or 100 ng/ml)), (b) corticosteroids (cortisol: 10, 100,1000 ng/ml or DOC: 1,10,100 ng/ml), (c) pure HCG or mixtures composed of HCG or IGF-1 and corticosteroids. Values are expressed as mean \pm SD of 6 replicates consisted of 6 ovarian piece tissues obtained on six maturing fish at late vitellogenic oocyte stage (LVO) or at the start of the final meiotic oocyte developmental stage (FMO). Each ovarian piece tissue contained 12 late vitellogenic oocytes or final meiotic oocytes. For each oogenesis, values with different superscript letters are significantly different ($P < 0.05$ – 0.001).

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