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Effect of salinity acclimatisation and administration of hormones on oocyte maturation, ovulation and spawning of goldspot mullet *Liza parsia* (Hamilton, 1822) reared in brackishwater pond

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ABSTRACT

Two separate experiments were conducted to evaluate the effect of salinity acclimatisation and administration of hormones on oocyte growth of *Liza parsia* (Hamilton, 1822). In the first experiment, broodstock fishes in the sex ratio of 2:1 (male:female) were acclimatised to 30 ppt salinity (which is the desired salinity for breeding *L. parsia*) in 24, 48 and 72 h of duration (n=6). Oocyte diameter from the experimental fishes were measured before and after salinity acclimatisation. The results showed that the acclimatisation to breeding salinity reduced oocyte diameter ($p<0.05$) in female fish and increased sperm relative mortality ($p<0.05$) in male fish. In the second experiment, males and females in the similar sex ratio (2:1) were acclimatised to the desired salinity of 30 ppt in 24 h. Subsequently, carp pituitary gland extract (PGE), human chorionic gonadotropin (HCG), combination of PGE+HCG and luteinising hormone releasing hormone analogue (LHRHa) with dopamine antagonist (DA) metoclopramide were injected intramuscularly, The dosages used were: PGE- 0.01 and 0.02 mg g^{-1}; HCG - 20 and 40 IU g^{-1}, LHRHa - 10 and 20 µg kg^{-1} as primary and secondary doses, respectively. Metoclopramide was injected at the dose of 15 mg kg^{-1} along with LHRHa. Results of the second experiment indicated that the administration of PGE or PGE+HCG is essential for final oocyte maturation and ovulation in salinity acclimatised *L. parsia.*

Keywords: Dopamine antagonist, Gonadotropin, *Liza parsia*, Luteinizing hormone, Oocytes growth, Salinity

Introduction

Goldspot mullet *Liza Parsia* (Hamilton, 1822) is a euryhaline fish that thrives well in freshwater, brackishwater and seawater (Riede, 2004). It is a migratory species that spawns in seawater (Talwar and Jhingran, 2001). Similar to other fish of commercial importance to the aquaculture sector, *L. parsia* also exhibits reproductive dysfunctions in captivity. These dysfunctions in fish are probably due to the cumulative effect of captivity induced stress (Sumpter *et al.,* 1994; Pankhurst and Van der Kraak, 1997), lack of appropriate natural spawning environment (Zohar, 1989; Yaron, 1995; Battaglene and Selosse, 1996) and inhibitory role of dopamine on release of gonadotropin (LH, Luteinising hormone) from anterior pituitary (Zohar and Mylonas, 2001). The commonly used methods to overcome this reproductive dysfunction in fish are environmental manipulation (Zohar, 1989; Yaron, 1995) and administration of exogenous hormone and a potent dopamine receptor antagonist (Zohar *et al.,* 2010). Use of pituitary gland extract (PGE), as an exogenous hormone for induced breeding of fish is a common practice. Due to uncertainty in rates of spawning success and unpredictable activity while using PGE, the use of human chorionic gonadotropin (HCG) is encouraged, because of the latter's higher chemical purity and better efficacy (Zohar and Mylonas, 2001). Use of HCG in spawning induction of thin-lipped grey mullet *Liza ramada* was reported by Mousa (1999; 2010) and Mousa and Mousa (2006). Combination of PGE and HCG was also used for induced spawning of *Liza macrolepis* (James *et al.,*1983) and *Mugil cephalus* (Shehadeh and Ellis, 1970). Moreover, a combination of HCG and ovaprim was used by Abraham *et al.* (1999) for induced breeding of *M. cephalus*. Administration of GnRHa and dopamine D2 receptor antagonist (DA) induces spawning in *M. cephalus* (Aizen *et al.,* 2005). Salinity is known to directly affect fertilisation, survival and development of fish eggs (Holliday, 1969; Alderdice, 1998). Catadromous nature of *L. parsia* indicates that salinity is an important factor which influences the final maturation and spawning (Talwar and Jhingran, 2001). It is a migratory species that spawns in seawater during December to February months (Talwar and Jhingran, 2001). With this background, the present experiment was conducted to standardise the

salinity acclimatisation process for brackishwater reared broodstock of *L. parsia* and to optimise the doses of different hormones for oocyte maturation and ovulation.

Materials and methods

Broodstock development

Before the start of spawning season (December to February), 250 numbers of 2 year old adult *L. parsia* (body weight range: 50 to 110 g) were stocked in a brackishwater tide-fed pond (1200 m^2) of Kakdwip Research Centre of ICAR-Central Institute of Brackishwater Aquaculture, West Bengal, India. During rearing, it was fed twice daily with a pellet diet containing 32% crude protein and 8% lipid at 3% of the fish biomass. Broodstock was reared for six months from June to November. Physicochemical parameters of the pond water salinity, temperature, dissolved oxygen and ammonia recorded during the study period were: 7±1.5 ppt, 19±2.5°C, 4.9±1.5 ppm and 0.05±0.02 ppm, respectively. During breeding season, mature females having oocyte diameter in the range of 480 to 540 µm and males with oozing milt were selected for the experiment.

Salinity acclimatisation experiment

A total of 24 females (average body weight: 78±12 g) and 48 males (average body weight: 34.35±8 g) were randomly distributed in four experimental groups. In each experimental group, six females and twelve males (Male: Female = 2:1) were stocked in FRP tanks (50 l) filled with brackishwater (salinity: 7±1.5 ppt). Thereafter, salinity was gradually increased from pond brackishwater salinity (7±1.5 ppt) to spawning salinity (30 ppt), at three different rates, namely 7.5 ppt in 6 h, 3.75 ppt in 6 h, 2.5 ppt in 6 h which took 24, 48 and 72 h, respectively. The control group was maintained in brackishwater without altering the water salinity. Before and after salinity acclimatisation, oocytes were collected by *in vivo* biopsy of ovary using a polyethylene cannula (2 mm dia) (Shehadeh *et al.,* 1973) and diameter of 30 oocytes were measured immediately under a trinocular microscope (Radical RXLr-5, India) supported with the software ProgRes Capture 2.7. Milt samples, before and after salinity acclimation, were collected and tested for vitality by Eosin-Nigrosin staining (Douglas and Kenneth, 2013). Sperm mortality was calculated as: Total number of dead sperm/Total number of sperm counted x 100.

Hormone administration experiment

The second experiment was conducted to assess the effect of administration of different hormones on oocyte growth, ovulation and spawning. From the first experiment, we found that the reduction in oocytes diameter was minimal when salinity acclimatisation from 7 ppt to 30 ppt was accomplished in 24 h. Therefore, broodstock of males and females (average body weight: females 80±8.0 g and males 38.00±4.5 g) were collected from broodstock pond and acclimatised in sex ratio of 2:1 (Male:Female) to the desired salinity of 30 ppt in 24 h. Thereafter, 30 female and 60 male fishes in sex ratio of 2:1 (Male:Female) were randomly distributed in five FRP tanks (500 l) fitted with recirculatory aquaculture system (RAS) and maintained under natural photoperiod. Different experimental groups were subjected to administration of hormones *viz*., (a) control (without hormone administration); (b) carp pituitary gland extract (PGE), (c) human chorionic gonadotropin (HCG), (d) combination of PGE+HCG and (e) luteinising hormone releasing hormone analogue (LHRHa) with dopamine antagonist (DA) metoclopramide.

PGE was prepared by homogenising carp pituitary in 0.9% physiological saline (Brzuska, 2004). HCG (5000 IU, IBSA Institut Biochimique SA, Switzerland) and LHRHa (Sigma) were dissolved separately in 0.9% physiological saline. Metoclopramide was prepared by dissolving in distilled water. PGE, HCG and LHRHa were injected intramuscularly at the rate of 0.01 and 0.02 mg g^{-1}, 20 and 40 IUg^{-1}, 10 and 20 µg kg^{-1} as primary and secondary doses, respectively. DA, metoclopramide was injected at the dose of 15 mg kg^{-1} along with LHRHa. The control group received 0.1 and 0.2 ml of 0.9% physiological saline as primary and secondary doses, respectively.

Maturity assessment

The stages of maturity were examined based on oocyte diameter and the germinal vesicle (GV) position and classified as: Stage I (central GV), Stage II (migrating/ eccentric GV), Stage III (peripheral GV), Stage IV (germinal vesicle breakdown, GVBD) and Stage V (ovulated oocytes), as per Levavi and Yron (1986). To locate the GV position, a sample of 30 eggs from each female was cleaned with cleaning solution (ethanol: formalin: acetic acid=6:3:1 v/v) and observed under a compound microscope.

Ovulation and spawning

Females were tested for ovulation by hand stripping of the abdomen and ovulation rate was calculated as: Ovulation rate (%) = (No. of ovulated females/No. of injected females) x 100 (Richter *et al.,* 1987). After noticing the spawned eggs in tank, spawning rate was calculated as: Spawning rates (%) = (No. of spawned fish/ Total No. of injected fish) x 100. Latency period (h) *i.e.,* the time period from injection to spawning was also calculated.

Statistical analysis

In the first experiment, mean values of initial and final oocyte diameter were compared by paired t-test and percentage change in oocyte diameter among different

acclimatisation times were subjected to one-way ANOVA. In the second experiment, mean value of final oocyte diameter, ovulation and spawning were subjected to one way ANOVA. All statistical analyses were performed with SPSS v. 20.0.

Results and discussion

Environmental factors such as salinity are known to directly affect fertilisation, survival and normal development of fish eggs (Holliday, 1969; Alderdice, 1998). Salinity does not affect oocyte maturation in European seabass *Dicentrarchus labrax* (Zanuy and Carrillo, 1984); striped mullet *M. cephalus* (Lee and Weber, 1986; Tamaru *et al.,* 1994) and black bream *Acanthopagrus butcheri* (Haddy and Pankhurst, 2000). In the present study, we recorded maturation of male and female *L. parsia* in brackishwater pond system, where salinity ranged from 5 to 8 ppt (mean: 7±1.5 ppt). Similar to this, maturation of *L. parsia* in brackishwater pond having water salinity of 7 ppt was reported earlier by Alam *et al*. (2008).

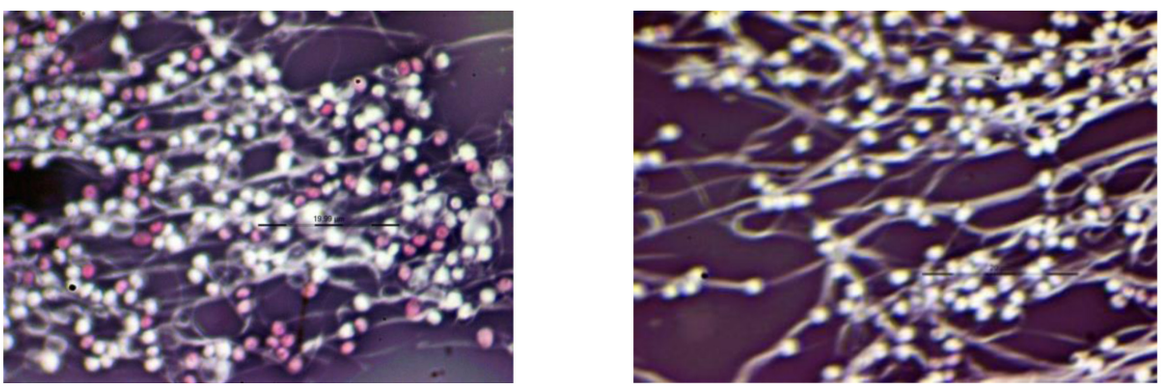
Results of the first experiment showed that the acclimatisation of brackishwater reared brood fish to 30 ppt salinity significantly ($p<0.05$) reduced oocyte diameter; however the reduction in oocyte diameter was minimal in 24 h of acclimation (Table 1). This reduction in oocyte diameter might be due to salinity stress and poor feeding. However there is no published literature to support this finding. However, Yashouv (1969) in *M. cephalus* and Mousa (2010) in *L. ramda* reported 24 h as an ideal time period for salinity acclimatisation. Contradictory to this, Alam *et al.* (2008) acclimatised brackishwater reared *L. parsia* to 20 ppt salinity in 72 h and achieved successful spawning through hormonal administration. Acclimatisation of male broodstock from pond rearing salinity (7 ppt) to breeding salinity (30 ppt) in 24, 48 and 72 h significantly increased ($p<0.05$) sperm relative mortality by 15±4.7, 28±2.8 and 33.25±2.6%, respectively (Fig. 1a; b). This inverse relationship between acclimatisation time and viability of sperm in *L. parsia*, might be due to osmotic imbalance of seminal plasma caused by salinity stress. Lee and Weber (1986) reported that sperm of *M. cephalus* loses its motility when reared at 10 ppt or below. However, in the present study, males of *L. parsia* reared at 7 ppt had viable sperms, which is similar to the finding of Hu and Liao (1981) in *M. cephalus,* who reared the brooders at 7 ppt.

In the second experiment, salinity acclimatised females with oocyte diameter in range of 480-500 µm (mean: 470±5.30 µm), having central GV (Fig. 2a; b) were selected for hormonal administration. In this study, dose of PGE (Lee *et al.,*1987; Vazirzadeh and Ezhdehakoshpour, 2014), HCG (Mousa, 2010) and LhRHa (Aizen *et al.,* 2005)

Table 1. Effect of salinity acclimation from 7 to 30 ppt in 24, 48 and 72 h of duration on oocyte development in *L. parsia*

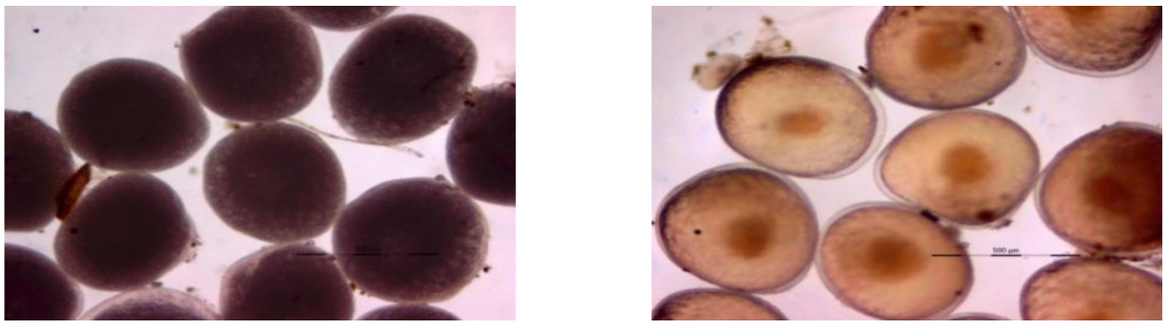
Time (h)	Initial oocyte diameter (µm)	Final oocyte diameter (µm)	% change in diameter
24	513.50±5.63	504.83±7.14	1.63^{c}±0.02
48	547.83±10.63	530.16±7.03	3.09^{b}±0.50
72	521.00±21.15	482.66±14.77	6.26^{a}±0.03
Control	519.33±3.77	518.83±4.18	0.086^{d}±0.01

Mean values in the same row (Initial and final oocyte diameter) does not differ significantly ($p>0.005$).
Mean values of percentage change in oocytes diameter in a column under each salinity acclimation having different superscripts differ significantly ($p<0.05$). Data are shown as mean±SE.



(a) (b)

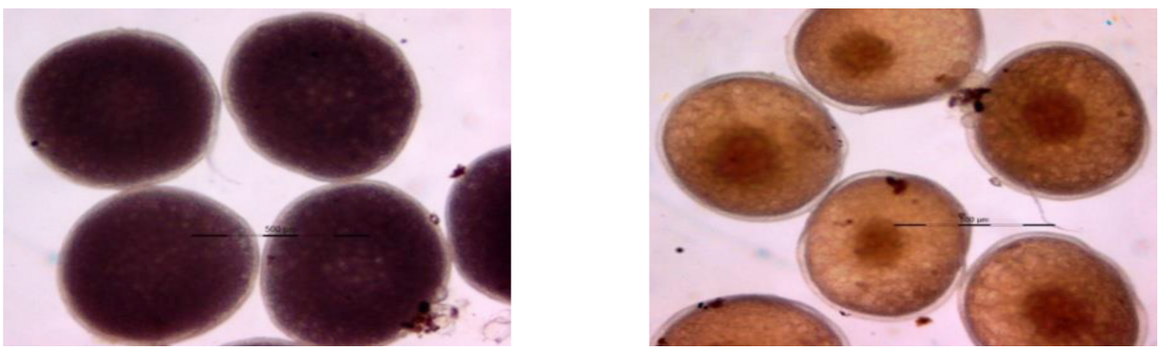
Fig.1. (a) Effect of salinity acclimation from 7 to 30 ppt on relative mortality of *L. parsia* spermatozoa (x100). (b) Spermatozoa of *L. parsia* reared in brackishwater pond at salinity of 7 ppt. Spermatozoa were stained with Eosin-Nigrosin stain. Live spermatozoa remained unstained and dead spermatozoa stained pink or red



(a) (b)

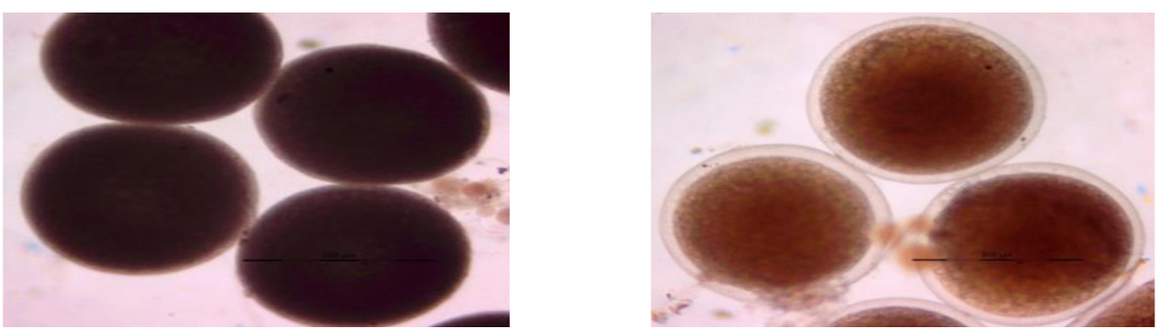
Fig. 2. Photomicrograph of *L. parsia* oocytes before administration of primary dose of hormone. (a) Oocytes immediately observed after ovarian biopsy (oocyte diameter: 470±5.30 µm); (b) Oocytes with centrally located germinal vesicle (x40)

were calculated based on commonly used dose for final oocytes maturation (FOM), ovulation and spawning of *M. cephalus* and *L. ramda.* The effects of different exogenous hormone treatments on growth and maturation of *L. parsia* oocytes is shown in Table 2. In all the experimental groups, 24 h after primary dose of hormones (PGE, HCG and PGE+HCG) administration, oocyte growth, migration of GV and fusion of lipid droplets was seen (Fig. 3a; b). Twelve hours after the second dose of PGE, HCG and PGE+HCG administration, oocyte growth, GVBD, homogenisation of yolk protein and thickening of chorion were noticed (Fig. 4a; b). Zaki *et al*. (1998) have also observed lipid droplet coalescence after administration of double dose of HCG in *M. cephalus*. Recently Vazirzadeh and Ezhdehakoshpour (2014), reported lipid droplet fusion and oocyte growth



(a) (b)

Fig. 3. Photomicrograph of *L. parsia* oocytes 24 h after administration of primary dose of hormone (PGE, HCG and PGE+HCG). (a) Oocytes immediately observed after ovarian biopsy; (b) Oocytes with migrating or eccentric germinal vesicle and fusion of lipid droplets (x40)



(a) (b)

Fig. 4. Photomicrograph of *L. parsia* oocytes 12 h after administration of secondary dose of hormone (PGE, HCG and PGE+HCG) (a) Oocytes immediately observed after ovarian biopsy; (b) Oocyte with germinal vesicle break down and yolk homogenisation (x40).

Table 2. Effect of different hormonal (pituitary gland extract, PGE; human chorionic gonadotropin, HCG; PGE+HCG and luteinizing hormone-releasing hormone analogue, LHRHa + dopamine antagonist (DA) treatments on growth, germinal vesicle position and lipid droplet coalescence in oocytes of *L. parsia*

Treatments	Initial oocyte diameter in µm (Beforeprimary dose/after salinity acclimation)	Oocyte diameter in µm (24 h after primary dose/ during secondary dose)	Oocyte diameter in µm (24 h after secondary dose)	Germinal vesicle position after 48 h of primary dose	Lipid droplet coalescence	% Mortality of females
Control	458.15±11.20	521.17^{c}±9.20	520.00^{c}±7.35	Central	Not seen	Nil
PGE	486.13±15.28	558.63^{a}±12.72	604.38^{a}±16.48	Eccentric and GVBD	Observed	27±2.00
HCG	463.88±10.27	576.63^{a}±11.66	586.25^{a}±14.27	Eccentric and GVBD	Observed	38±5.00
PGE+HCG	457.13±12.43	564.88^{a}±11.74	603.63^{a}±7.88	Eccentric and GVBD	Observed	35±3.00
LHRHa+DA	535.00±3.78	547.50^{b}±11.49	551.75^{b}±3.47	Central	Not seen	80±5.00

Mean values in a column under hormonal treatments having different superscripts differ significantly ($p<0.05$). Data are shown as mean±

with primary and secondary doses of PGE and LHRHa, respectively in *M. cephalus*. Percentage increase in oocyte diameter, 24 h after second dose of hormonal administration was significantly ($p<0.05$) higher in PGE group (19.2±4.73%) followed by HCG (11.98±2.19%), PGE+HCG (8.74±2.97%) and LHRHa+DA groups (3.14±0.52%) (Fig. 5). Compared to other groups, PGE group showed significantly ($p<0.05$) higher percentage of ovulation (75%), which does not vary significantly ($p>0.05$) from HCG and PGE+HCG groups (Fig. 6a). Compared to other two groups, significantly higher spawning percentage was noticed in PGE and PGE+HCG groups (Fig. 6b). In the present experiment, ovulation and spawning were noticed at 18±2 h and 24±3 h after second dose of PGE and HCG, respectively. Ovulated oocytes were in size range of 580-615 µm, which were characterised by single yolk sac (Fig. 7a). Spawned oocytes (diameter range: 680-715 µm; mean: 710±1.0 µm) were characterised by 6-7 oil globules (Fig. 7b). We did not observe spawning after second dose of LHRHa+DA, however ovulation (Fig. 6a) and atretic oocytes were noticed (Fig. 7c).

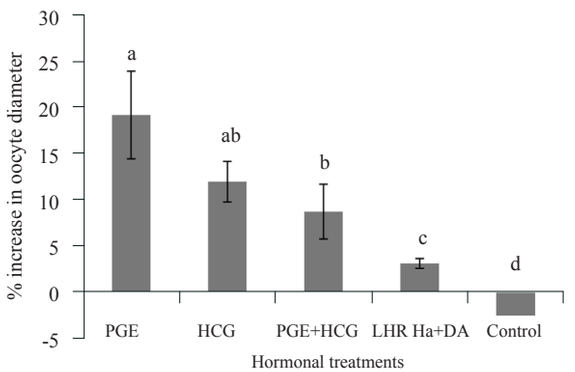


Fig. 5. Effect of different hormonal treatments on percentage increase in oocyte diameter after 48 h of injection in *L. parsia.* Different superscripts indicate significant differences between hormonal treatments ($p<0.05$).

Results of the present study demonstrated that primary dose of PGE significantly improved oocyte diameter and maturation in salinity acclimatised *L. parsia*. This might be due to the presence of many other hormones, such as growth hormone (GH) and thyroid stimulating hormone (TSH), which also have implication in fish maturation (Rocha *et al.,* 2007; Moussavi *et al.,* 2009) in cooperation with gonadotropin. Further, lowest mortality of broodfish was noticed in PGE group, which showed that administration on PGE causes comparatively less stress. In case of group administered with LHRHa+DA, significantly poor ovulation and spawning were noticed, which might be due to sub- and supra-optimal doses of exogenous hormones (Sahoo *et al.,* 2007). In this study, the latency period of ovulation and spawning recorded were 18±2 h and 24±3 h, respectively. These differences could be attributed to the type and dose of hormonal treatment and environmental conditions. The results of this study demonstrated that primary injection of PGE is required for oocyte growth, and a secondary dose of either PGE or HCG is essential for final oocyte maturation and ovulation in *L. parsia*. Present findings are in agreement with the studies by Tamaru *et al.* (1989); Samira *et al.* (2008) and Yousif *et al.* (2010), who reported that the prime injection of gonadotropin (PGE or HCG) is essential for successful spawning in *M. cephalus*.

In conclusion, from this study we were able to infer that acclimatisation to the breeding salinity of 30 ppt in 24 h is ideal for *L. parsia*. PGE as priming dose (0.01 mg g^{-1}) is essential for oocyte growth and the secondary dose of either PGE (0.02 mg g^{-1}) or HCG (40 IU g^{-1}) is required for final oocyte maturation and ovulation . This understanding will be useful in the development of captive breeding and seed production technology for *L. parsia.*

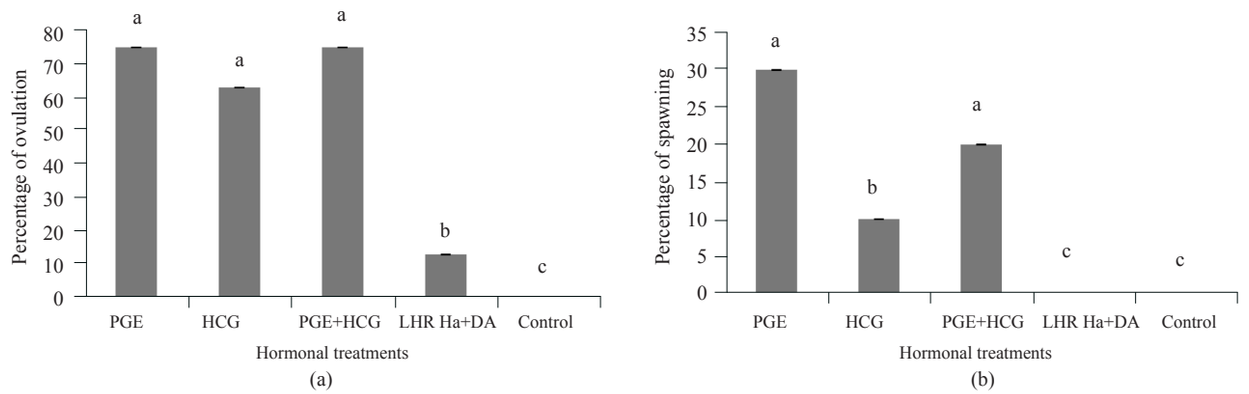
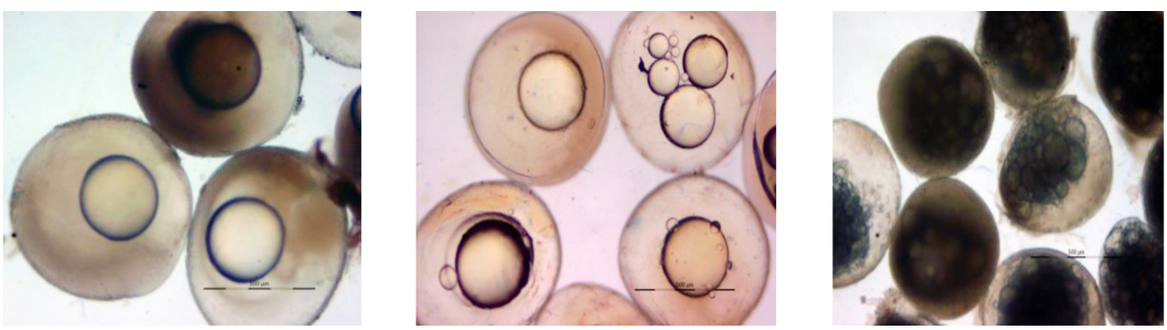


Fig. 6. Effect of different hormonal treatments on (a) ovulation (%) and (b) spawning (%) in *L. parsia.* Different superscripts indicate significant differences between hormonal treatments ($p<0.05$).



(a) (b) (c)

Fig. 7. Photomicrograph of *L. parsia* oocytes, (a) Ovulated/hydrated oocytes showing single yolk sac (oocyte diameter: 590±2.0 µm); (b) Spawned oocytes with 6 to 7 oil globules (oocyte diameter: 710±1.0 µm); (c) Atretic oocytes (x40).

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