養殖ドジョウ雌の生殖腺発達と性ステロイドの周年変化

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Annual Changes in Ovarian Development and Sex Steroid Levels in Cultured Female Dojo Loach *Misgurnus anguillicaudatus*

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Abstract: Dojo loach *Misgurnus anguillicaudatus* is a promising species for studies into population genetics and into assessing the effects of environmental factors on reproduction. In order to document first its basic reproductive biology, we examined gonadal development and analyzed plasma sex steroid profiles during the annual cycle of captive female dojo loach. Loach sampled from May through August displayed high gonadosomatic indices (GSI) and elevated levels of plasma steroids [testosterone (T), estradiol-17β (E_2), and 17, 20β-dihydroxy-4-pregnen-3-one (DHP)]. Histologically, oocytes in advanced stages of vitellogenesis or in the migratory nucleus stage predominated in the period. Furthermore, distinct groups of oocytes in different stages of development were seen, reflecting the capacity for multiple spawns during the spawning season. The lowest GSI value (4.29±2.42) and lowest levels of T, E_2 and DHP were observed in October, coinciding with the occurrence of regressed vitellogenic oocytes. Seasonal variation in measured parameters were consistent with the reproductive cycle of this species, presenting exciting avenues for further research into environmental science and aquaculture.

Key words: Dojo loach; Reproductive cycle; Ovarian development; Sex steroid

Fishes of the family Cobitidae comprise a large group (18 genera and 110 species) of freshwater teleosts (Nelson 1994) that are widespread in different environments, both in temperate and tropical areas. One representative is the dojo loach *Misgurnus anguillicaudatus* (Cobitidae, Cypriniformes) which is a small bottom-living and facultative air-breathing freshwater fish (McMahon and Burggren 1987) widely distributed in Japan, Korea and China. These loach inhabit streams, ponds, swamps, rice fields or slow-flowing rivers with either soft muddy or sandy substrate into which they can burrow during unfavorable environmental conditions. In part of East Asia, dojo loach are utilized as aquarium pets, as fish food or in traditional medicine (Zhou et al. 2009). Because of its commercial value and widespread occurrence, several studies have been done on *M. anguillicaudatus*, mainly concerning population genetics (Oshima et al. 2005; Itono et al. 2006), activity rhythms (Naruse and Oishi 1996) and biomedical research (Zhou et al. 2009).

The dojo loach has great value as a model species for environmental research and biotechnology, since it has a moderate size that makes it easy to handle. Furthermore, mature male and female dojo loach are easily distinguished by the shape of the pectoral fins, and the animal can adapt to a wide range of freshwater conditions. In addition, they have valuable characteristics as a sentinel species in assays for environmental pollution, because dojo loach inhabit rice fields and rivers that can be exposed to high levels of pesticides and other anthropomorphic chemicals. Moreover,

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the dojo loach may be suitable as indicator of climatic and environmental changes. Besides, the wild population is decreasing because of habitat degradation (Fujimoto et al. 2008) and contamination (Zhou et al. 2009), signaling the necessity for aquaculture development for enhancement purposes. Current larval production techniques for dojo loach are dependent on the capture of mature wild breeders (Wang et al. 2010). Recently, the decrease of resources of dojo loach in wild disturbs the stable supply of larvae. The development of controlled breeding programs in indoor, tank-based, environmentally regulated systems is regarded as a solution to overcoming problems in larval production. However, notwithstanding the data on the basic reproductive biology of wild dojo loach (Fujimoto et al. 2008), information on the reproductive biology of this species within these artificial environments remains largely unknown.

In teleosts, the annual reproductive cycle is largely dependent on ovarian steroid hormones, which are produced in response to gonadotropins secreted from the pituitary gland (Rosenfeld et al. 2007). Seasonal changes in the levels of sex steroids and their value for gaining insights into mechanisms driving gametogenesis, reproductive behavior and gonadal steroidogenesis have been reported for several teleosts species (Malison et al. 1994; Pinillos et al. 2003; Sun and Pankhurst 2004). However, the endocrine control of reproductive events in captive dojo loach has received little attention. Although, dojo loach are known to be multiple spawners, our understanding of the characteristics and timing of ovarian development and on the dynamics of ovarian maturation remain deficient.

Thus, the objective of the present study was to obtain information on the reproductive cycle of captive dojo loach by examining gonad development and analyzing endocrine profiles, and to correlate these in captive female dojo loach during a complete annual cycle.

Materials and Methods

Sample collection

The study was conducted between April, 2006 and April, 2007 at the facilities of the Institute for East China Sea Research, Nagasaki University. The dojo loach were obtained from a commercial supplier (Gondo Fish Farm) at Asakura, Fukuoka, Japan, twice during the expected spawning months of May, June and July, and once a month during the remainder of the survey. Accordingly, a total of 154 fish was sampled. Monthly sample sizes (9-11 fish) deviated slightly from the target of 10. The fish were kept in an outdoor wooden box pond (20 m × 2 m × 1.5 m) under ambient conditions (Fig. 1) and water temperature was recorded two times a day (in the morning and during the day time). Feeding to satiation was done once per day (commercial carp diet, P-3, Marubeni Nisshin Food, Tokyo, Japan). The average total length (TL) of adult sampled female dojo loach ranged from 140.7 to 167.4 mm and body weight (BW) ranged from 13-25 g. The fish were anesthetized by immersion in freshwater containing 2-phenoxyethanol (0.3 *ml/l*) before measuring TL and body weight (BW). The gonads and livers were removed and weighed to calculate the gonadosomatic index (GSI) and hepatosomatic index (HSI), respectively. The GSI was calculated as follows: GSI = gonad weight (GW) × 100/BW and the HSI was calculated as HSI = liver weight (LW) × 100/BW.

Histological analysis

All gonads were fixed in Bouin's solution, dehydrated in graded alcohols, embedded in paraffin, sectioned at 5 μm and stained with Mayer's Haematoxylin and Eosin Y (H-E). To identify cortical alveoli in the oocytes, some

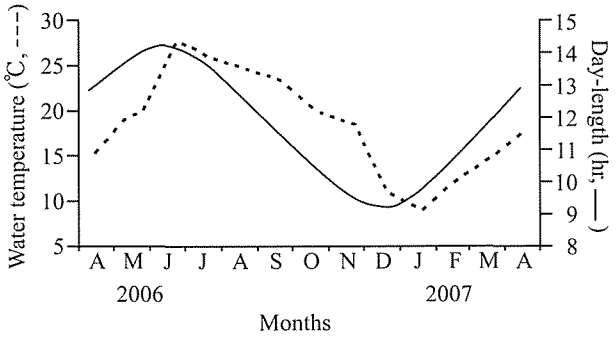

Fig. 1. Seasonal changes in water temperature and day length of the Fukuoka area.

sections were treated with Periodic Acid-Schiff (PAS) reagent.

Gonad stages and morphology

Stages of oocyte development were based on descriptions by Yamamoto and Yamazaki (1961) and Palmer et al. (1995). Ovaries were categorized into 5 stages on the basis of the most advanced type of oocytes present, according to Fujimoto et al. (2008) and Yamamoto and Yamazaki (1961) with some modifications: I- Previtellogenic; II- Early vitellogenic; III- Late vitellogenic; IV- Mature; and V- Regressed.

Oocyte size, the relative proportions of each oocyte stage, and individual ovarian stage were estimated by microscopy. Only oocytes that had been sectioned through the nucleus were counted (West 1990). The relative frequency of ovarian development was calculated as individual fish / total fish sampled.

Measurements of oocyte size

For each stage of oocyte development, the diameters of 20 oocytes from each section were measured using a computer linked to an Olympus^{TM} BX50 microscope with an Olympus FX380 CDD digital camera attachment, and using image analysis software. Since histological processing deforms the oocyte from its circle-like shape, two different types of measurement were used. For those oocytes which maintained their circular-like shape, the diameter across the nucleus was measured, whereas the diameter of non-circular oocytes was calculated from $D = P/\pi$ where P is the circumference of the oocyte.

Measurements of plasma steroid hormones

The plasma estradiol-17β (E_2) level was measured following the protocol described in the estradiol EIA kit purchased from the Cayman Chemical Company (USA). Plasma testosterone (T) and 17, 20β-dihydroxy-4-pregnen-3-one (DHP) levels were measured by enzyme linked immunosorbent assay (ELISA) according to Asahina et al. (1995) with some modifications. Rabbit anti-steroid hormone antibodies (AB) and steroid hormones labeled with horseradish peroxidase (HRP) for T and DHP were purchased from Cosmo Bio Japan. Cross-reactivities of antisera to T, E_2 and DHP have been described in the Cosmo FKA 331 and FKA 332-E kits.

Statistics

All data were presented as mean ± SD, and using SPSS for Windows software, data were analyzed by one-way ANOVA to test for differences among months. The means were subsequently compared by Tukey-Kramer test. Differences were considered statistically significant for $P < 0.05$.

Results

Water temperature and photoperiod

Changes in water temperature and photoperiod in the sampling area during the study period are shown in Fig. 1. The water temperature of the pond started to increase gradually from February and reached a peak (27.7℃) in June. Thereafter, it decreased slowly until November, dropping rapidly to its lowest level (9℃) in January. Photoperiod at Asakura-Fukuoka was shortest at the winter solstice, i.e., between December and February (10-11h L), increasing gradually from April and becoming longest during summer solstice, i.e., June and August (13-14h L).

Monthly changes in GSI and HSI

The GSI of females increased from February to May and reached its peak value (~20.0) from June to August. In September, the GSI of females started to decrease significantly, reaching its lowest value (4.3 ± 2.47 to 5.3 ± 2.24) between October and February (Fig. 2A).

The mean HSI for females increased from December and was highest (1.91 ± 0.76) in April, just prior to the onset of the reproductive season. After April, the mean HSI decreased slightly, then decreased significantly (approximately 0.9) throughout June to October (Fig. 2B).

Histological changes during an annual reproductive cycle

In the present study, a high proportion of vitellogenic oocytes at different stages were observed during spawning season (Table 1). Based on histological analysis, dojo loach ovarian development displayed characteristics that are typical of multiple spawning species (Fig. 3).

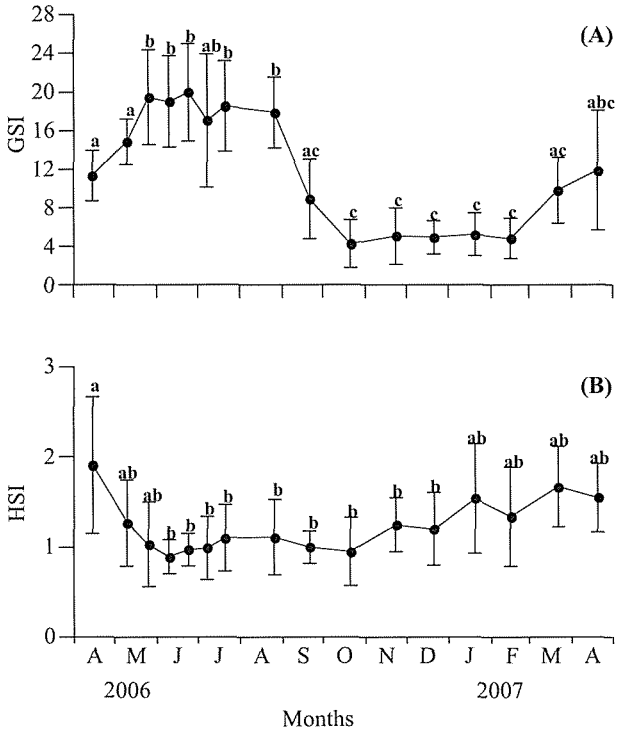

Fig. 2. Annual changes in gonadosomatic index (GSI) and hepatosomatic index (HSI) of female dojo loach (mean ± SD). Different letters indicate significantly different mean values ($P<0.05$).

Throughout the year, oocytes in perinucleolus (73-220 μm in diameter) and yolk vesicle (188-360 μm) stages were seen. During the previtellogenic stage, from October to December, perinucleolus- and yolk vesicle stage oocytes were the dominant stages observed (Figs. 3A, 4). As development progressed to the early vitellogenic stage in winter, oocytes in primary and secondary yolk stages and measuring 355-481 μm and 408-560 μm in diameter respectively, were observed at high proportions (Fig. 3B). Tertiary yolk stage oocytes with a diameter of 495-795 μm, were observed from January (Fig. 3C) and increased in abundance and size in late vitellogenic and mature ovaries from April to August; their relative abundance was maximal in June and July (Figs. 3D, 4). Accordingly, in mature gonads, the proportion of oocytes in the tertiary yolk stage and migratory nucleus stage were greatly represented. The proportion of migratory nucleus stage oocytes in individual fish ranges from 1-13% between April to August, whereas oocytes in

Table 1. Proportional composition of various stages of oocytes in captive female dojo loach in spawning season

Sampling Date	Individual No	Stages of oocyte (%)					
		Pn	Yv	Py	Sy	Ty	Mn
Apr 20	1	40.4	13.0	16.9	16.9	11.7	1.1
	2	26.4	21.0	16.8	14.7	18.9	2.2
	3	37.1	21.6	13.2	14.2	12.9	1.0
	4	27.8	15.3	15.3	16.6	20.7	4.3
May 31	1	30.4	19.9	9.4	16.4	18.0	5.9
	2	20.0	18.0	8.0	20.0	26.0	8.0
	3	20.4	26.6	15.5	14.4	17.5	5.6
	4	21.3	23.3	11.6	5.1	27.3	11.4
Jun 29	1	33.1	22.9	2.3	8.1	26.0	7.6
	2	50.4	16.8	5.0	9.9	13.6	4.3
	3	42.8	18.6	3.3	12.4	17.5	5.4
	4	49.8	6.1	5.9	11.2	15.6	11.4
Jul 26	1	55.4	13.6	8.2	6.4	10.0	6.4
	2	25.9	20.3	14.8	6.6	17.6	14.8
	3	44.0	17.5	2.5	1.0	20.0	15.0
	4	43.8	22.2	3.7	5.2	17.1	8.0
Aug 31	1	41.6	16.9	17.9	6.1	13.3	4.2
	2	63.5	11.8	5.9	3.5	9.4	5.9
	3	64.2	12.6	3.8	4.1	11.8	3.5
	4	54.1	12.2	9.5	6.6	13.5	4.1

Pn, perinucleolus; Yv, yolk vesicle; Py, primary yolk stage; Sy, secondary yolk stage; Ty, tertiary yolk stage; Mn, migratory nucleus stage.

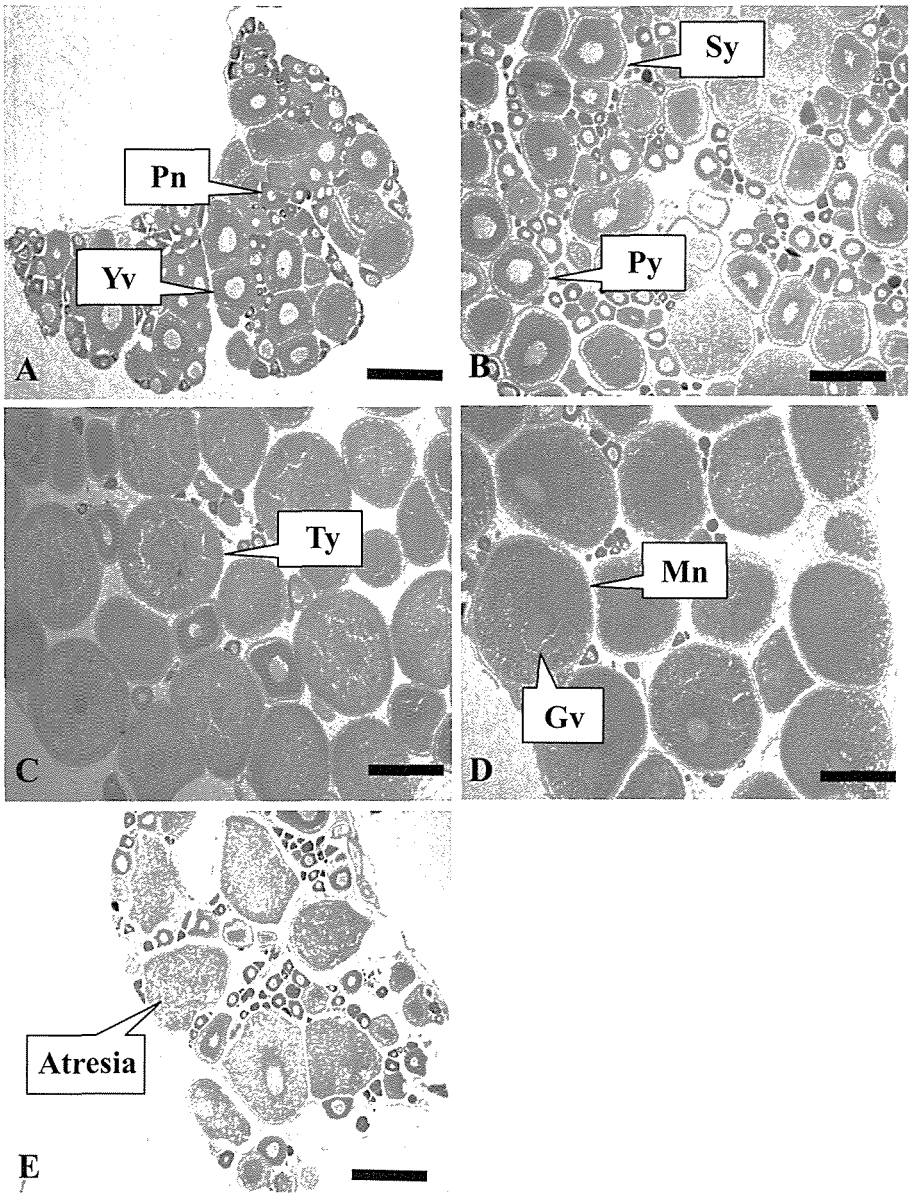

Fig. 3. Photomicrographs of female dojo loach ovaries showing various stages of ovarian development. A, pre-vitellogenic stage; B, early vitellogenic stage; C, late vitellogenic stage; D, matured stage; E, regressed stage. Pn, perinucleolus stage; Yv, yolk vesicle stage; Py, primary yolk stage; Sy, secondary yolk stage; Ty, tertiary yolk stage; Mn, migratory nucleus stage; Gv, germinal vesicle. Scale bar = 100 μm.

the tertiary yolk stage had a relative frequency of 9-26% (Table 1). Throughout the sampling period, we only observed one post-ovulatory follicle in one fish. This postovulatory follicle was found surrounded by oocytes at secondary and tertiary yolk stages. Atretic oocytes were found in most ovaries throughout the year, and constituted 8-11% of vitellogenic oocytes during September to November, when most regressed ovaries were encountered.

Plasma sex steroid levels

Low levels of T (<0.66 ± 0.28 ng/*ml*) were detected in September and these were maintained until January (Fig. 5A). T started to increase in March (1.13 ± 0.44 ng/*ml*) and peaked from May to August (~1.5 ng/*ml*). Mean plasma E_2 levels increased with ovarian development, from lows between October (0.22 ± 0.12 ng/*ml*) and February (0.25 ± 0.09 ng/*ml*). E_2 tended to increase slowly from March (0.36 ± 0.15 ng/*ml*) to a peak level (~0.9 ng/*ml*) in June and August during which oocytes in vitellogenic and migratory nucleus stages were observed (Fig. 5B). In September, E_2 levels started to decline, followed by a further significant decrease in October (P<0.05). Plasma DHP levels increased from April to May and peaked in July

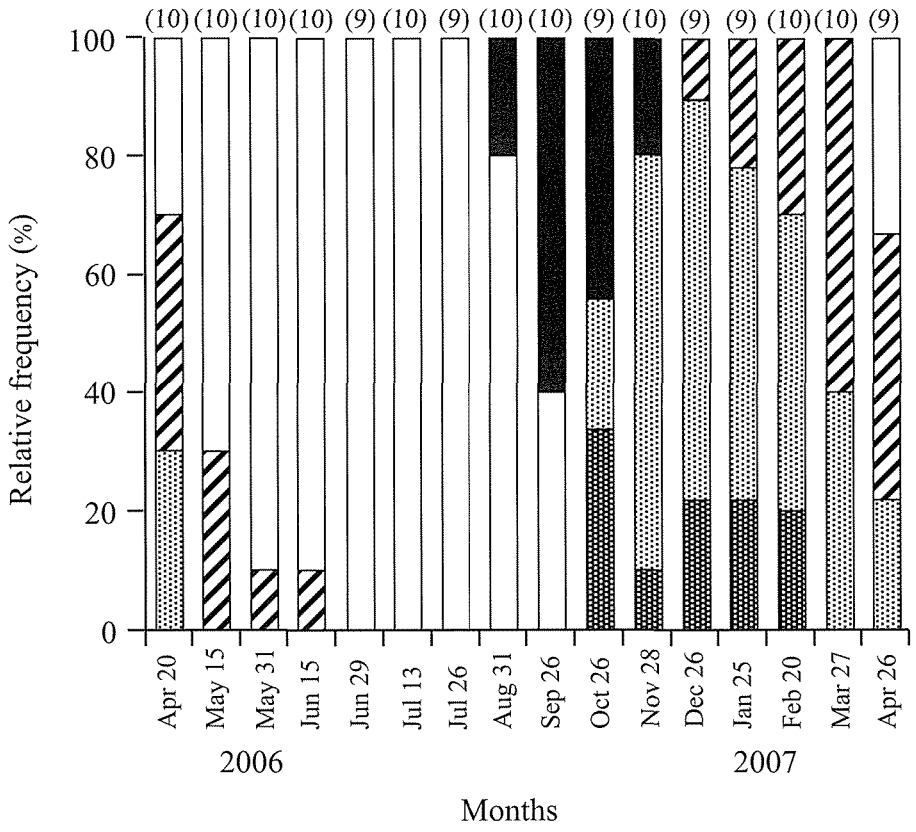

Fig. 4. Monthly changes in relative frequencies of the ovarian developmental stages in cultured female dojo loach. ▦, pre-vitellogenic stage; ▨, early vitellogenic stage; ▨, late vitellogenic stage; □, matured stage; ■, regressed stage.

and August (1.36 ± 0.44 and 1.22 ± 0.28 ng/*ml*, respectively); concentrations fell significantly, to 0.53 ± 0.2 ng/*ml*, in September and remained low until January (<0.36 ± 0.1 ng/*ml*) (Fig. 5C). The T level was slightly higher than E_2 throughout the year.

Discussion

Results of our histological studies indicate that the active spawning period of dojo loach extends from May to August, and that ovarian development of dojo loach had characteristics comparable to those of other multiple spawning fish (Wallace and Selman 1981; West 1990; Fujimoto et al. 2008). GSI values, similarly, peaked between May and August. During these months, most ovaries were in the mature stage containing oocytes in the migratory nucleus stage (1-15%), or in vitellogenesis. Oocytes in the yolk vesicle stage were present throughout the reproductive cycle of the female dojo loach. This pattern of development was also reported by Babiker and Ibrahim (1979) for *Oreochromis niloticus* and Junchno et al. (2007) for spined loach *Cobitis taenia*, suggesting that pools of these stages were constantly available for recruitment by multiple spawning fishes (Rinchard and Kestemont 1996).

In the present study, it was not possible to determine the interval of spawning or the number of eggs released during a spawning event. However, the ovaries of dojo loach contained oocytes at all stages of development whilst mature, implying that dojo loach can spawn more than once during the course of a prolonged breeding season. This is in keeping with previously published studies (Rinchard and Kestemont 1996; Shimizu 1997; Sun and Pankhurst 2004). Indeed, in one fish, advanced developmental stages were found together with a postovulatory follicle (POF) in the ovary, reinforcing that this species is a multiple spawner.

The spawning season of captive dojo loach in the Kyushu area is approximately one month longer than that in Iwate, Northern Japan (Fujimoto et al. 2008), although the peak spawning season is similar. This may be related to the

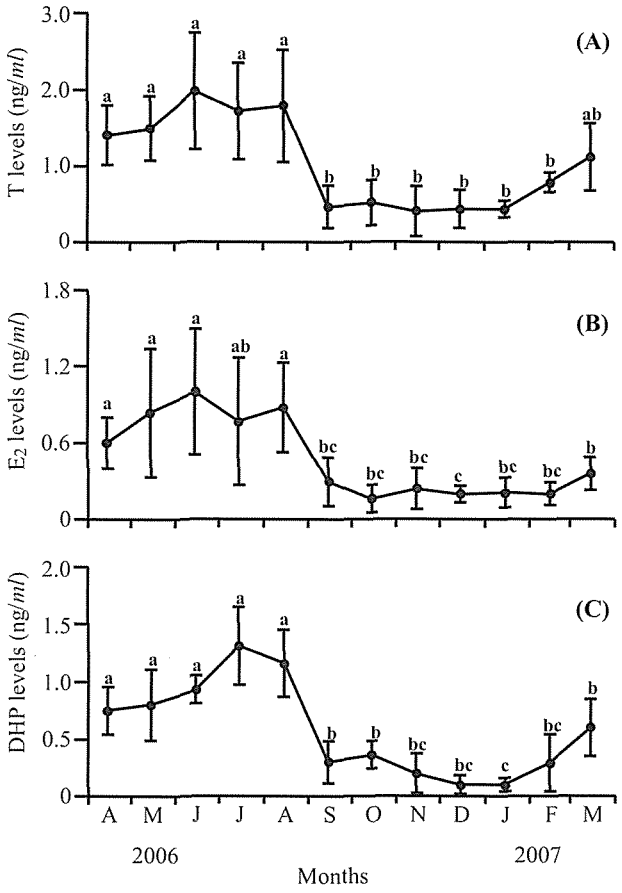

Fig. 5. Monthly changes in serum hormone levels of Testosterone (T), estradiol-17β (E_2), and 17,20β-dihydroxy-4-pregnen-3-one (DHP) in female dojo loach. Solid bar represents SD of 5-10 fish. Different letters indicate significantly different mean at $P<0.05$.

availability of feed and intensive management in artificial environments. Moreover, the rise and fall in the seasonal water temperature is obviously different in both areas and are obvious factors that could impact on the timing of initiation of gonad development and spawning.

In the dojo loach, the GSI started to decrease significantly from August to September and the lowest value was observed in October during which most of the ovaries had regressed. This coincided with a high rate of atresia, which was maximal between September and November, heralding the end of spawning period (Sivakumaran et al. 2003). The gradual increase in GSI during late autumn and winter corresponded to slow growth of yolk vesicle and early yolk stage oocytes. This implies that following spawning there is a very short period of gonadal regression followed by gonadal recrudescence during October. Similarly, the appearance of vitellogenic oocytes from October onwards was reported in wild dojo loach collected from Hokkaido (Teranishi et al. 1981). The GSI value increased markedly from February to April when ovaries of dojo loach were in vitellogenesis, reflected by rapid increases in oocyte diameters. These results indicate that the dojo loach have the ability to develop and maintain vitellogenic oocytes for long periods.

In dojo loach, oocyte size within a given developmental stage varied widely compared to reports from other teleosts. Particularly during March to August, there was considerable variation in the size of vitellogenic oocytes between the ovaries of different females. Suzuki (1983) reported this phenomenon previously and further described that there is a positive correlation between mean oocyte diameter and the body weight of female dojo loach. This may also be the case in the present study. Such differences in mean oocyte diameters within the same developmental stage was also reported for rainbow trout *Oncorhynchus mykiss* (Tyler et al. 1990) and Murray cod *Maccullochella peelii* (Newman et al. 2007).

Female dojo loach tended to have higher HSI values before and at the start of the spawning season (February to April) than during the spawning season (May to August). A similar pattern has been reported for other teleosts such as white bream *Blicca bjoerkna* (Rinchard and Kestemont 2003). The high HSI during March and April is probably related to the beginning of feeding activity (Naruse and Oishi 1996). This indicates that before the spawning season, females must have high energy reserves and thus have more resources to divert toward reproduction. During the spawning season, females had a reduced HSI and many vitellogenic oocytes, suggesting that the energy reserve, particularly hepatic substances, are transferred from liver to ovary.

We observed high levels of T during late vitellogenic and mature stages, which could be related to a role of T as a substrate for aromatization to estrogen (Nagahama et al. 1995). Our results clearly showed that the rise in levels of T and E_2 matched the increased incorporation of yolk globules in the oocyte, and thus,

increased GSI and oocyte diameter starting from March. Such a correlation between plasma T levels with gonad developmental stage and oocyte growth has also been reported for female jundia *Rhamdia quelen* (Barcellos et al. 2001). It is well known that gonadal steroids exert both positive and negative feedback on GtH synthesis and secretions depending on the sex and developmental stage (Huang et al. 1997; Kobayashi and Stacey, 1990). Therefore, apart from being a precursor for the synthesis of E_2, T seems to play an important role in stimulating LH in the pituitary, as was demonstrated for goldfish *Carassius auratus* (Kobayashi et al. 2000) and catfish *Heteropneustes fossilis* (Tiwary et al. 2002). This feedback, in turn, stimulates the production of steroids involved in induction of final oocyte maturation. This is consistent with the high concentration of T observed in mature female dojo loach during the spawning period. However, the potential significance of T in feedback to influence LH during the ovarian development and its specific functional role in dojo loach is yet to be determined. Interestingly, Tosaka et al. (2010) found that levels of mRNA encoding androgen receptors were high in Japanese eel *Anguilla japonica* oocytes in the late oil droplet to the mid vitellogenic stage, which indicates that androgens may play a key role during early stages of oogenesis. This is reinforced by the ability of 11-KT, the predominating androgen in female eels (Lockman et al. 2002), to stimulate lipid accumulation in the oil droplet stage (Lockman et al. 2007; Endo et al. 2008). Whereas the androgen receptors are likely to predominantly bind 11-KT in eel, the higher levels of T in most other female fishes (Lockman et al. 2002) together with the high affinity of androgen receptors for T, for instance in red seabream *Pagrus major* (Touhata et al. 1999), make it likely that T is an important ligand for the androgen receptors in most female teleosts. Collectively, it appeared that T plays an important role in oocyte development of dojo loach, and that research on androgen action during gonadal development of female fish be pursued.

Profiles of E_2 were similar to changes in GSI and oocyte diameter from April to August. In individuals with atretic ovaries, E_2 levels sharply declined and GSI decreased from September to November, remaining low until February. These results are consistent with the role of estrogen in promoting hepatic synthesis of the yolk precursor vitellogenin (Tyler and Sumpter 1996). The presence of a wide range of vitellogenic oocytes in individual ovaries together with migratory nucleus stage confirm that dojo loach displays asynchronous ovarian development, and oocytes are probably recruited into vitellogenic growth throughout the spawning period. High levels of E_2 during spawning were indeed observed in other multiple spawning fishes such as goldfish (Kagawa et al., 1983), sea bream *Sparus aurata* (Kadmon et al. 1985) and sea bass *Dicentrarchus labrax* (Prat et al. 1990). E_2 synthesis may be continued in multiple spawners, including dojo loach, until the fish complete vitellogenesis, which is controlled by environmental stimuli.

In dojo loach, DHP levels were elevated during the spawning season at a time that relatively high proportions of migratory nucleus stage oocytes were observed. DHP, which controls final oocyte maturation and ovulation in most teleosts (Suwa and Yamashita, 2007), may be the oocyte maturation inducing hormone (MIH) in dojo loach. The annual profiles of steroid hormones were comparable to those shown for wild female dojo loach by Fujimoto et al. (2008). However, the peak level of our study is different from that of wild dojo loach. These differences may be attributed to the difference in sources or area in which the specimens were collected and differences in the ovarian developmental stage.

In conclusion, information on GSI, oocyte diameter, histology and sex steroid levels indicated that dojo loach has the characteristics of a multiple spawner that spawns from May to August. The sex steroid hormone profiles suggest an involvement of these hormones in regulating the reproductive cycle. Nonetheless, more information is required at higher levels of the endocrine cascade to better understand the mechanisms controlling ovarian development in dojo loach. These data have importance

for environmental monitoring, for the control and management of wild dojo loach populations, and for the establishment of techniques for artificial seed production. Furthermore, our findings contribute to the basic knowledge of the reproductive cycle and ovarian development of a teleost that inhabits small rivers with muddy substrate.

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養殖ドジョウ雌の生殖腺発達と性ステロイドの周年変化

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九州北部で飼育された養殖ドジョウ雌の生殖腺発達を組織学的に観察するとともに，テストステロン（T），エストラジオール17β（E_2）及び17,20βジヒドロキシ4プレグネン3オン（DHP）の血中濃度を周年に亘り測定した。その結果，本種の卵巣には発達段階の異なる卵母細胞が同時に存在すること，また産卵期には成熟期の卵母細胞に加えて異なる卵黄形成過程にある卵母細胞が常に観察されることから，比較的産卵間隔の短い多回産卵魚であることが組織学的に明らかとなった。本種は産卵後わずかの期間をおいて卵黄形成を開始し，4月以降成熟することが分かった。特に6月および7月には観察した全ての雌成魚が成熟した卵母細胞を有したことから，この時期が成熟盛期と考えられる。またこの時期に何れの性ステロイドも高値を示したことから，継続して発達を続ける卵母細胞の卵黄形成及び最終成熟にこれらのホルモンが関わっていると推測された。