Biology and culture of the clown loach Chromobotia macracanthus (Cypriniformes, Cobitidae) : 1- Hormonal induced breeding, unusual latency response and egg production in two populations from Sumatra and Borneo Islands

Marc Legendre, D. Satyani, S. Subandiyah, Laurent Pouyaud, Etienne Baras, Jacques Slembrouck

HAL Id: ird-01225687
https://ird.hal.science/ird-01225687

**Aquatic
Living
Resources**

Biology and culture of the clown loach *Chromobotia macracanthus* (Cypriniformes, Cobitidae): 1- Hormonal induced breeding, unusual latency response and egg production in two populations from Sumatra and Borneo Islands

Marc Legendre[1,a], Darti Satyani[2], Siti Subandiyah[2], Sudarto[2], Laurent Pouyaud[1], Etienne Baras[1] and Jacques Slembrouck[1,2]

[1] IRD, UMR 226 – ISEM, BP 5095, 34196 Montpellier Cedex 05, France
[2] Balai Penelitian dan Pengembangan Budidaya Ikan Hias (BP2BIH), Jl. Perikanan No. 13, Depok 41152, Indonesia

Abstract – The clown loach *Chromobotia macracanthus*, endemic to Indonesia, is a major species on the international market of ornamental freshwater fish. In order to satisfy an increasing demand with a sustainable alternative to the massive capture of wild juveniles, research has been dedicated to the artificial propagation and domestication of this species. The present study, the first of a series, focused on favourable maintenance conditions for broodfish sexual maturation, criteria for identification of ripe fish, efficiency of hormone-induced breeding treatments, predictability of their latency response, and on the comparison of reproductive performances of fish from populations of Sumatra and Borneo Islands (in total, 112 females of 46 to 404 g body weight). When reared in fully controlled conditions in large water recirculation systems, broodfish originating from Sumatra had reproductive performances similar to or slightly higher than those maturing in the wild (ovulation rate of 93% vs. 82%, relative fecundity of 109 277 vs. 103 550 ova kg^{-1} and fertilization rate of 73% vs. 61%, respectively). In the same rearing conditions, captive females from Borneo ($n = 22$) showed lower ovulation rate (77%), relative fecundity (76 262 ova kg^{-1}) and fertilization rate (50%) than those originating from Sumatra ($n = 28$). By contrast, the mean individual weight of ova (around 0.8 mg) was independent from the origin or maintenance conditions of females. An initial modal follicle diameter ≥1.02 mm generally led to high ovulation success (>80%) after hormonal treatment and is recommended as the main criterion for selecting female broodfish. Two hormonal treatments for inducing oocyte maturation and ovulation (T1: two successive injections of Ovaprim at a 6 h-interval; T2: one injection of human chorionic gonadotropin (hCG)- and one of Ovaprim 24 h later), produced similar results in terms of ovulation rate, quantity and quality of ova collected. With both treatments, the latency decreased with increasing water temperature, then increased again at temperatures >28−29 °C. To our knowledge, such *U*-shaped relationship between the latency response and temperature has never been documented in teleost fishes.

Keywords: Tropical freshwater fish / Clown loach / Reproduction / Ovulation / Latency period / Egg number / Aquaculture / Ornamental fish / Indonesia

1 Introduction

Over-exploitation of aquatic species targeted by fisheries for food, recreational or ornamental purposes, have been demonstrated or suspected in many instances (FAO 2010). In Asia, the size and specific composition of most continental fisheries, as well as the abundance and life history traits of commercial aquatic species, have been affected as a result of intensive exploitation (FAO 2010). Such statements have generally fostered the development and adoption of management policies, primarily as regards fishing effort and practices (Welcomme 2001), rehabilitation strategies, essentially

for improving water quality, restoring habitats or river connectivity (Cowx and Welcomme 1998; Marmulla 2001), or remediation, for example through the use of restocking practices (Philippart 1995). However, these policies can hardly be implemented efficiently in absence of a minimal knowledge of the species biology, stock health and dynamics. One typical example of such situation comes from the clown loach *Chromobotia macracanthus* (Bleeker 1852; formerly *Botia macracanthus* Kottelat 2004). This freshwater riverine cobitid fish is highly praised by fish hobbyists because of its attractive colour pattern (three broad black bars on an orange-reddish background,) and fancy attitude. It is endemic to the rivers of Sumatra and Borneo Islands (Kottelat et al. 1995) and heavily

---

[a] Corresponding author: Marc.Legendre@ird.fr

exploited at the juvenile stage. Although information on the biology and ecology of the species remain scarce, it is generally admitted that it has carnivorous to omnivorous feeding habits, eating mostly benthic insects and aquatic snails but also plant material. Adults mostly inhabit the bottom of main river channels, often hiding under rocks, immerged woods or in mud cavities, whereas young juveniles are often found in the floodplain. In 2006, the biggest fish observed among 2 300 specimens caught in the Musi River (Sumatra) was 305 mm in total length for a body weight of 469 g (authors' unpubl. data). The harvesting techniques that are deployed by Indonesian fishermen to capture clown loach (Ng and Tan 1997; Pouyaud and Sudarto unpubl. obs.) can be destructive for other fish taxa, which are trapped as well, but merely discarded because of their low commercial value. Although highly specialized, this fishery is essential to the livelihoods of many families in these regions. Until now, there has been no direct cue that wild stocks of clown loach are critically endangered, but it is generally considered that the collection of juveniles in the wild is over-intensive (Olivier 2001). Reported around 20 million individuals fifteen years ago (Ng and Tan 1997), the number of clown loach juveniles exported annually was estimated at about 50 millions in 2009 (Satyani unpubl. data). This figure is even probably an underestimation of captures, as a significant but variable proportion of fish die rapidly from handling and transportation and is not considered in export estimations. Moreover, several Indonesian rivers, including many of those where clown loach are found, suffer from pollution associated to human activity (e.g. gold washing) and from habitat changes (e.g. substitution of the equatorial rain forest with palm or rubber tree plantations, which result in leaching of fine soil particles and increased turbidity of rivers).

From the viewpoint of conservation biology, these arguments have emphasized the need for propagating clown loach in captivity, also with the objective to meet the standards of quality and traceability, which are nowadays increasingly demanded by ornamental fish importers and retailers (Olivier 2001). Another strong incentive for the domestication of clown loach is the need of supplying continuously the international aquarium fish trade with fish of adequate commercial size (generally about 4−5 cm), knowing that the capture of juveniles in the rivers of Sumatra and Borneo is largely seasonal (high water seasons). Efforts developed during the last 15 years for breeding clown loach in captivity have largely failed, although they have been attempted by numerous hobbyists worldwide, and some fish culturists. There have been some records of spontaneous spawning of captive clown loach in aquarium (Hammond 1996; Clarke 2007). However these records were either controversial due to absence of photographic support or limited to the finding of non-fertilized or unviable eggs in the aquarium. Ng and Tan (1997) laconically reported that the breeding of clown loach had been accomplished by fish culturists in Thailand, but no reference was given. Since then, several attempts of hormonally induced reproduction have been carried out, primarily in Indonesia, but when larvae could be obtained they did not survive beyond the age of 9 days (Satyani 2004). Furthermore, broodfish were rarely observed to attain full sexual maturity in captivity. Treatments for triggering gonad development using hormone

implants or photoperiod manipulations were tested with limited success in Indonesia (Subagja et al. 1997; Satyani et al. 1999; Effendi et al. 2003). Until now, no reliable technology has been developed for the artificial propagation, i.e. induced breeding and larval rearing of the clown loach.

Hormonally induced oocyte maturation, ovulation and spermiation, followed by hand stripping of gametes and in vitro fertilization have become common procedures for controlling the breeding cycle of fish that do not reproduce spontaneously under captive conditions. A variety of hormonal treatments have been used successfully in a large number of fish species, involving either pituitary hormones acting directly on the gonads or hypothalamic hormones controlling the release of gonadotropic hormones by the pituitary (Harvey and Carolsfeld 1993; Legendre et al. 1996; Zohar and Mylonas 2001). If the basic principles of such procedures are now well established, the effectiveness of hormonal treatments is known to vary between species and local conditions (Zohar and Mylonas 2001; Mylonas et al. 2010). Therefore, it is necessary to determine the optimal maintenance and induced breeding conditions for each species.

The present paper is the first of a series dealing with the biology of *C. macracanthus* and the development of reliable methods for its artificial propagation. It is focused on the maintenance conditions for full sexual maturation of broodfish in captivity, the identification of specific criteria for selecting fish ready to respond to hormonal stimulation, the evaluation of the efficiency of two different hormonal treatments, and the characterization of latency response between injection and ova collection. In this study, reproductive characteristics (ovulation rate; number, size and quality of collected ova) of fish originating from two populations known to present marked genetic differentiation (Sudarto et al. 2008), one from Sumatra Island and the other from the Borneo Island, are compared. The reproductive characteristics of broodfish held in captivity are also examined in comparison to fish having undergone their sexual maturation under natural conditions in the wild (Musi River), which are used here as a reference.

2 Materials and methods

2.1 Fish origin and maintenance

Experiments were carried out between October 2004 and October 2008 at the Research and Development Institute for Ornamental Fish Culture (BP2BIH) at Depok (West Java, Indonesia). All broodfish were caught in the wild and originated from two distinct populations. Fish from the first population, originated from the Kapuas River in Kalimantan (Indonesian part of the Borneo Island) and were purchased from a specialized provider of ornamental fish in Jakarta. Fish from the second population came from the Musi River in Sumatra, where they were bought directly to local fishermen.

In total, 196 sexually mature fish, of unknown age, were used in the induced breeding experiments: 112 females of 46−404 g body weight (BW) and 84 males of 15−180 g BW. No clear sexual dimorphism was apparent. Males were identified when sexually mature by the emission of sperm upon gentle abdominal hand-pressure and females when oocytes could

be collected by intraovarian biopsy. Based on fish origin and on the general conditions during fish sexual maturation, four distinct situations were considered in this study.

Fish of group 1 originated from the Kapuas River in Kalimantan and were first adapted to captive conditions at the Depok research station during at least one year before the induced breeding trials. They were maintained under natural light in indoor tanks (0.4−2.0 m^3) equipped with rudimentary mechanical and biological filters (after Satyani 2004; detailed information in Table 1). Every two days, faeces and uneaten feed were removed with a siphon, and about 10−20% of the water volume was changed with well water. The water quality parameters were as follows: dissolved oxygen concentration >5 mg L^{-1}; N-NH_3 <1.0 mg L^{-1}; NO_2 <0.01 mg L^{-1}.

Broodfish from group 2 originated from the Musi River in Sumatra and had been caught with cast nets by fishermen around the village of Beringin Teluk (upstream of Sekayu City, Province of South Sumatra, 2° 40′ S−103° 10′ E). These fish were always captured in the period between September and December, which is assumed to correspond to the main reproductive season of *C. macracanthus* in this river (authors' unpubl. data). Following capture, they were maintained in groups in wooden cages (0.5 m^3) immersed into the river and attached to a floating house. On two occasions (October and November 2004), induced breeding trials were carried out in the floating house itself. In all other instances, fish were conditioned in plastic bags with a small volume of water and oxygen atmosphere, and transported (car and plane, 10−12 h of travel) to the Depok research station, where they were transferred into 200-L quarantine aquaria. All fish (*n* = 84) survived transportation and acclimation. In all cases, breeding experiments were carried out within less than 10 days after fish capture. In the meanwhile, fish were fed exclusively with earthworms. The main difference between these fish and those from the other groups was that they had sexually matured in natural conditions in the river. Water characteristics of the Musi River were monitored continuously for temperature (Stowaway datalogger, Onset Computer Corporation, Pocasset, MA, USA) and at more infrequent intervals for water level, conductivity and pH (see Table 1).

Two additional groups of fish originating from Kalimantan (group 3, same origin as group 1) or Sumatra (group 4, same origin as group 2) were transferred to Depok research station and reared separately in two identical 10-m^3 indoor tanks in a blind room, under artificial dim red light (photographic bulb), as low light levels were reported to favour gonad development in clown loach (Satyani et al. 1999; detailed information in Table 1). Each tank was connected to a separate thermoregulated water recirculation system (water flow = 8.2 $m^3 h^{-1}$) equipped with a mechanical filter, a biological filter and a UV-sterilizing system. Fish were acclimated in this system over more than one year before being used in induced breeding experiments. The tanks were cleaned by siphoning twice a week, with a weekly renewal rate of 10% of the total water volume using well water. Water quality in the recirculation system stood as: dissolved oxygen concentration 6.7−8.1 mg L^{-1}; N-NH_3 0.0−0.1 mg L^{-1}; NO_2 0.0−0.3 mg L^{-1}. After about one year at a water temperature of 30−31 °C, the water temperature in the recirculation system was decreased progressively to 26−27 °C within two months. This change in water temperature was motivated by complementary observations showing that a higher proportion of broodfish were in advanced sexual maturity at 26−27 °C compared to 30−31 °C (J. Slembrouck unpubl. data). Broodfish held in the recirculation systems were all individually tagged soon after they were first used in breeding trials, using PIT tags (FishEagle, Lechlade, UK) inserted in the dorsal epaxial muscles.

2.2 Experimental design

The design of this study, with the use of four distinct groups, was aimed to address a series of questions. Group 2, which comprised fish maturing in the natural environment, was a control group as regards the adequacy of the environmental conditions for gametogenesis and gonad development. The comparison between groups 2 and 4 (both from Sumatra, but maturing in the wild and in captivity, respectively) precisely aimed to test for the relative adequacy of the maintenance conditions in captivity. No similar comparison could be done for fish originating from Kalimantan, for logistic reasons. The comparison between groups 3 and 4 aimed to test whether the responses differed between fish from two distinct populations, but placed in identical and presumably adequate environments (see previous comparison). The comparison between groups 1 and 3, which both comprised fish from Kalimantan, enabled testing whether induced breeding could be equally successful for fish maintained under rudimentary (group 1) or fully controlled conditions (group 3, with stable temperature, constant and low light intensity, adequate food supply and water quality, etc.). No similar comparison was done for fish originating from Sumatra, essentially as it turned out that the maintenance conditions in group 1 (which was historically the first group to be tested in captivity), were far less adequate than in group 3 (see results).

2.3 Selection of broodfish and hormonal treatments

Broodfish were anaesthetized (2-phenoxy-ethanol, 0.3 ml L^{-1}) prior to all manipulations (including hormone injections). Ripe females (i.e. ready to reproduce) were selected on the basis of three types of criteria: the degree of abdominal stoutness, the distribution of follicle diameters and the position of oocytes' germinal vesicles (GV). The degree of abdominal fullness was considered for fish from group 2 only. It was always estimated by the same observer, who ranked the fish into one of the three following categories: (1) no particular stoutness, (2) noticeable stoutness or (3) important stoutness with rebounded and soft abdomen. The follicle modal diameter was systematically determined from a sample of 40−60 follicles obtained by intraovarian biopsy using a flexible polyethylene catheter (external diameter = 2 mm). For measurements, the follicles were immersed in a 155 mM NaCl solution; otherwise they were easily damaged by manipulation. All measurements were done under a stereomicroscope (×25) by reference to a graduated eyepiece, and refer to the largest diameter of the follicles. Only females with homogenous distributions of follicle diameters (i.e. with

Table 1. Population of origin and general maintenance conditions of *Chromobotia macracanthus* broodfish used in the induced breeding experiments.

Fish population	Group	Maintenance	Stocking density (tank volume)	Sex ratio	Feeding (daily ration in % body weight)	Environment (1) Water temperature (2) Conductivity (3) pH (4) light
Kalimantan	1	Hatchery Indoor tanks in rudimentary recirculation system	10–14 m^{-3} (0.4–2.0 m^3)	±1F:1M	*Tubifex* and commercial pellets with 38% crude proteins (in slight excess)	(1) 26–30 °C (2) 99–139 μS cm^{-1} (3) 5.5–7.0 (4) Natural light, 150–500 lx (12L:12D)
	3	Specific blind room Circular tank in water recirculation system	6–7 m^{-3} (10 m^3)	±2F:1M	Morning: commercial pellets with 35–40% crude proteins (0.5–1.0%) Evening: Earthworms (>10%)	(1) 26–28 °C (2) 114–294 μS cm^{-1} (3) 7.1–7.7 (4) Artificial red light 5–10 lx (12L:12D)
Sumatra	2	Natural conditions (Musi River)	nd (open water)	nd	nd	(1) 25–31 °C (2) 25–73 μS cm^{-1} (3) 5.0–6.5 (4) Natural light, intensity nd (±12L:12D)
	4	Specific blind room Circular tank in water recirculation system	6–7 m^{-3} (10 m^3)	±2F:1M	Morning: commercial pellets with 35–40% crude proteins 0.5–10% Evening: Earthworms (>10%)	(1) 26–28 °C (2) 114–294 μS cm^{-1} (3) 7.1–7.7 (4) Artificial red light, 5–10 lx (12L:12D)

a single, clearly marked modal group) and modal diameters ⩾0.95 mm were retained for hormonal induced breeding. The initial position of the GV was examined before hormonal treatment on a sample of about 50 follicles cleared in Serra's fluid (60% ethanol, 30% formalin, 10% acetic acid, by volume). Males were selected on the basis of milt emission following gentle abdominal massage.

Two distinct hormonal treatments were applied for inducing the final maturation of oocytes and ovulation. The first treatment (T1) was modified from Satyani (2004) and consisted in two successive intramuscular injections of Ovaprim [1 ml of Ovaprim® (Syndel Laboratories, Qualicum Beach, BC, Canada) contains 20 μg of GnRHa (D-Arg6, Trp7, Leu8, Pro9, Net) and 10 mg Domperidone] of 0.4 and 0.6 ml kg^{-1} BW, at a 6-h interval. The second treatment (T2) consisted in a priming injection of hCG (human chorionic gonadotropin; Organon, France) at 500 IU kg^{-1} given 24 h before the Ovaprim ovulatory injection of 0.6 ml kg^{-1} BW. This second treatment was tested because it gave in other species more predictable latency responses than with two Ovaprim injections, the first of which might suffice to produce oocyte maturation (e.g. Pangasiid catfishes, Slembrouck et al. 2004; Legendre et al. 2008). Males were treated with a single

Ovaprim injection (0.4 ml kg^{-1}) given 16 to 24 h before sperm collection by stripping. Such treatment generally increased the volume of sperm collected and reduced its viscosity, thereby making its collect easier. Injections were always done in the dorsal musculature of the fish.

During the entire treatment period (from fish selection to gamete collection), broodfish were held in 200 or 500-L tanks (1 or 2 fish per tank for females, and groups of 3−6 individuals for males), except for trials in the field (group 2), where fish were maintained in wooden cages in the river. The mean water temperature during the latency period (i.e. between the ovulatory injection and ovulation) varied between 23.0 °C and 30.5 °C, depending on trials and fish groups. Temperatures between 25.2 and 30.5 °C corresponded to natural water temperatures on days of experiment, with thermal fluctuations never exceeding 2 °C within a same trial. Water temperatures of 23 to 25 °C corresponded exclusively to thermoregulated situations (±0.2 °C, using an aquarium chiller) and were applied only to a few fish coming from the large recirculation systems (groups 3 and 4). In this case broodfish had been progressively acclimated from 26 °C to the desired temperature (23−25 °C) over the week before the hormonal treatment.

2.4 Latency period and gamete management

In order to detect the moment of ovulation, gentle stripping trials were performed every 2−3 h, starting 4 to 8 h after the second hormonal injection (depending on water temperature). Following the observation of germinal vesicle breakdown (GVBD; verified by intraovarian biopsy and clearing oocytes in Serra's solution), the fish were examined every hour until ovulation. In four individuals (3.6% of females treated), ovulation had already occurred when stripping was first attempted. These fish were not considered in subsequent latency analyses, as the actual latency was unknown. Once ovulated oocytes were observed, the genital papilla of the female was dried with paper towel and ova were collected by stripping in a dry plastic recipient.

When GVBD were observed in females (about 2 to 4.5 h before ovulation depending on water temperature), males were caught; their papilla dried with paper towel and sperm collected by stripping directly in a syringe containing an immobilizing solution (155 mM NaCl, dilution rate 1:5). This technique, which has been used in other fish species (e.g. in Pangasiid catfishes; Cacot et al. 2003; Slembrouck et al. 2004), reduces the risk that sperm be contaminated with urine and lose its fertilizing ability. This is particularly important as the total duration of motility of *C. macracanthus* spermatozoa is relatively short (40 ± 5 s) after activation in water (authors' unpubl. obs.). Diluted sperm was kept in crushed ice or in a refrigerator (4 °C) until use. In these conditions, the sperm motility of most males could be preserved for more than 24 h. Sperm motility was evaluated prior to fertilization, using a microscope (×100) and after activation of spermatozoa in a drop of water (total dilution 1:5 × 1:100). Only sperm with over 70% motile spermatozoa were used for subsequent fertilizations.

2.5 Artificial fertilization and egg quality estimation

After complete stripping, ova were weighed (nearest 0.01 g) then rapidly fertilized. Prior to fertilization, a sample of about 50 ova was collected for diameter measurements (same method as above for follicles). Another sample of about 500 ova was also removed, weighed to the nearest 0.1 mg and preserved in 5% formalin for subsequent counting, so as to estimate the total fecundity from the mean weight of ova. On a few occasions, the mean weight of ova could not be measured due to microbalance unavailability (e.g. during field trials); in these cases fish fecundity was estimated from the mean weight of ova spawned by other females from the same fish group. Fertilization was systematically performed with sperms from two to five males pooled in equivalent volumes.

The quality of ova was estimated from fertilization percentages, which were determined in two replicate batches of about 150 ova each, and fertilized with 0.1 ml of diluted sperm. This corresponded approximately to 10^6 spermatozoa per ovum (as determined by the measurement of spermatozoa concentration with a microscope [×200] using a Thomas' cell). Spermatozoa activation was obtained by adding 10 ml of fresh water. After 1 min of gentle stirring, eggs were rinsed to remove excess milt and transferred into a plastic container filled with 300 ml of commercial spring water at room temperature (26−29 °C). The proportion of fertilized eggs was calculated from counts under the stereomicroscope (×25) in between 2 and 4 h after fertilization. At this stage, fertilized eggs have attained the morula or early gastrula stage, and could be discriminated without ambiguity from non-fertilized eggs. The hatching percentage and proportions of normal and deformed larvae are complementary indicators of egg quality. They are not documented here, but were taken into account in a specific study focusing on the requirements of *C. macracanthus* eggs and appropriate conditions (movement, temperature) for successful egg incubation in this species (Slembrouck et al. 2012).

2.6 Statistical analysis

Unless specified otherwise, values are means ± SE. One-way or two-way (populations × hormonal treatments) analyses of variance followed by Newman-Keuls's multiple range tests were used for comparisons of means. Paired *t*-tests were used for comparing the modal diameters of follicles before hormonal treatment and ova collected by stripping. Simple and stepwise multiple-regression analyses were used to test for relationships between latency response, water temperature and other variables. When necessary, analyses were performed after logarithmic or angular transformation of data in order to stabilize residual variance. Contingency table analyses (with Yates's continuity correction when individual numbers were <5) or Mann-Whitney *U*-tests were used when parametric tests could not be performed. All tests were done using Statistica (6) software. Null hypotheses were rejected at $p < 0.05$.

3 Results

3.1 Criteria for broodfish selection

The distribution of oocyte diameters in fully mature females captured during the reproductive season in the Musi River (group 2) was unimodal, both for follicles sampled in live fish by intraovarian biopsy, and for oocytes collected from the ovaries of a few females that had been killed to verify sample representativeness (Fig. 1). The initial follicle modal diameter of selected females (i.e. before hormonal treatment) ranged from 0.96 to 1.32 mm ($n = 112$ fish from groups 1−4). It did not depend on fish origin (one-way ANOVA, $p = 0.483$) or body weight (simple regression analyses, $p > 0.1$ in all fish groups). The largest modal diameter observed during this study (1.32 mm) corresponded to a female of 66 g BW.

After hormonal treatment, the ovulation success was significantly lower in females with an initial follicle modal diameter <1.02 mm (44%, $n = 9$) than in those with larger follicles (81%, $n = 103$; χ^2, $p = 0.041$). By contrast, the quality of ova, as revealed by the examination of fertilization percentages, was independent from the initial follicle diameter (simple regression analysis, $p = 0.619$). In all females examined, the oocyte germinal vesicles were systematically in central or slightly eccentric position before hormonal treatment (Fig. 2a). No difference in ovulation rate (χ^2) or egg quality (fertilization rate; *t*-test) was found as a function of the abdominal stoutness
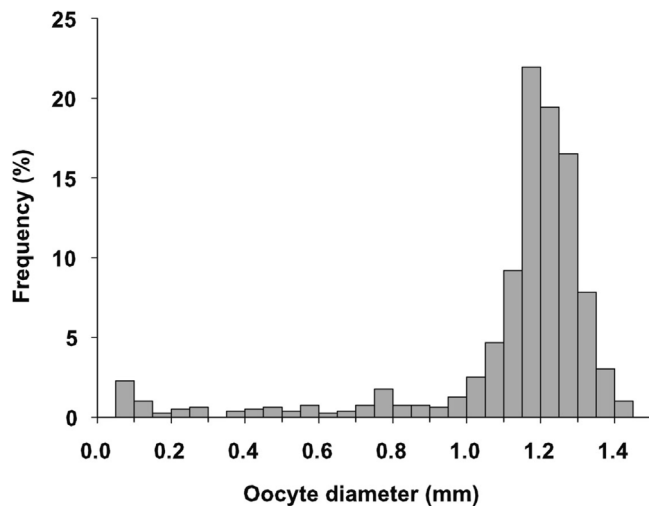
Fig. 1. Typical size-frequency distribution of oocytes from a ripe female of *Chromobotia macracanthus* caught in the Musi River (Sumatra) during the reproductive season. Oocyte diameter measurements ($n = 793$) were performed on a subsample of ovaries preserved in 5% formalin, using IMAGE J freeware (Abramoff and Magalhaes 2004).

of the females (Table 2). By contrast, the amount of ova collected after hormonal treatment was more than twice higher (one-way ANOVA, $p < 0.01$) in females displaying a medium or marked abdominal stoutness than in females with a low abdominal stoutness.

3.2 Efficiency of hormonal treatments

In total, the hormonal treatments led to the successful induction of oocyte maturation and ovulation in 87 of 112 selected females (78%, or 81% when females with initial follicle diameter <1.02 mm were excluded from calculations, see above).

The two hormonal treatments proved equally efficient in all fish groups (contingency table analyses, $p > 0.5$ in groups 1, 2 and 3. In group 4, only T2 was evaluated; Table 3). Two-way ANOVA (fish group 1, 2 and 3 × hormonal treatments) showed that the relative fecundity (number of ova collected per unit body weight) and fertilization percentages were not influenced by the hormonal treatment ($p = 0.450$ and $p = 0.150$, respectively), but varied significantly between fish groups (details given below). There was no significant effect of the interaction between factors for any of the two variables under study (fecundity: $p = 0.801$; fertilization: $p = 0.051$). Therefore, data obtained with the two hormonal treatments within a same fish group were pooled for further comparisons between fish groups.

3.3 Variations of ovulation rate, fecundity, ova size and egg quality between fish groups

The number of female broodfish that were hormonally induced for ovulation in each of the four fish groups ranged from
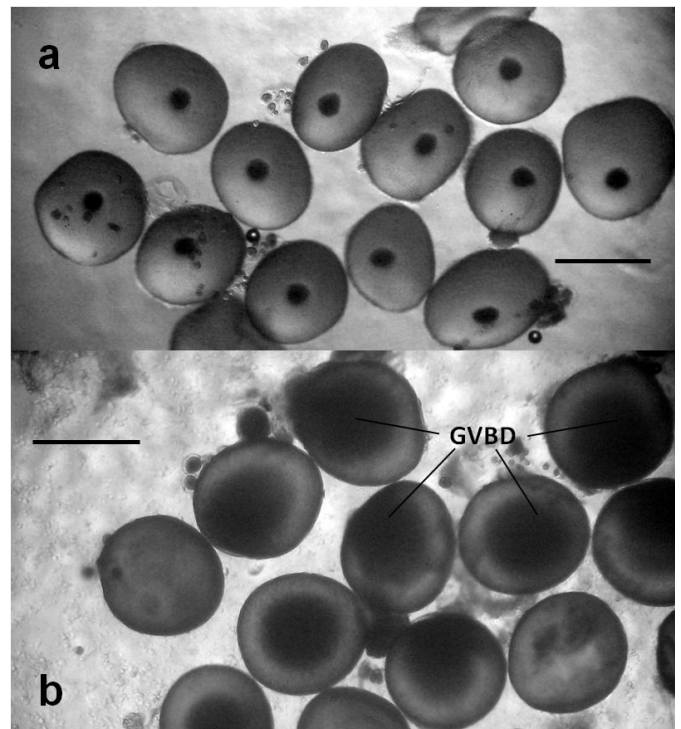

Fig. 2. Oocytes of *C. macracanthus* with germinal vesicles in central or slightly eccentric position before hormonal treatment (a) and after germinal vesicle breakdown (GVBD) 2.5 hours before ovulation (b). Horizontal bars correspond to 1 mm.

22 to 39 (Table 4). The ovulation rate was significantly lower in fish from group 1 (52%) than in those from groups 2 (82%; χ^2, $p = 0.026$) and 4 (93%; χ^2, $p = 0.003$). The ovulation rate in fish from group 3 was intermediate (77%) and did not differ significantly from any other group (χ^2, $p = 0.209, 0.650$ and 0.244 against groups 1, 2 and 4, respectively). In each of the four groups, the success of ovulation was independent from the follicle modal diameter before treatment (*t*-test; $p > 0.38$ for all groups, excluding fish with diameter <1.02 mm, see above). In groups 1 and 3 (fish from Kalimantan), the fish that ovulated were on average larger than others (105 ± 14 g versus 70 ± 5 g in group 1 and 174 ± 15 g versus 106 ± 24 g in group 3; Mann-Whitney *U*-test, $p < 0.05$ in both groups). As regards fish from Sumatra, ovulation success was independent from fish weight in group 2 (183 ± 46 g for ovulated fish versus 157 ± 83 g for others), and in group 4, the two fish that did not respond to the hormonal treatment were amongst the largest of the group (180 g and 228 g versus 143 ± 6 g for ovulated fish).

The mean diameter of ova stripped from individual females varied between 1.08 and 1.36 mm and did not differ significantly between groups (one-way ANOVA, $p = 0.072$). The mean weights of ova were close to 0.8 mg and did not differ between groups either ($p = 0.159$; Table 4).

In each female, ova were significantly larger than follicles before hormonal treatment (paired *t*-test, $p < 0.0001$, $n = 68$), irrespective of fish group or hormonal treatment. The average modal diameter increased from 1.10 ± 0.01 mm before injection to 1.20 ± 0.01 mm at ovulation, corresponding to a 9.1% relative increase on average. However, this relative increase

Table 2. Ovulation rate, quantity (relative fecundity) and quality (fertilization rate) of ova collected after hormonal treatment in *Chromobotia macracanthus* as a function of the initial abdominal stoutness of the females (mean ± SE). Only females belonging to the Sumatra population (groups 2, 4) were used in this comparison.

Abdominal stoutness	Fish n	Female body weight range (g)	Ovulation (%)	Fecundity (ova kg^{-1})	Fertilization rate (%)
Low	9	89−120	56 [a]	54 576 ± 14 622 [a]	63 ± 8 [a]
Medium to important	9	87−205	67 [a]	130 038 ± 13 094 [b]	61 ± 13 [a]

Values with same superscripts in the same column are not significantly different ($p < 0.05$).

Table 3. Ovulation rate in *C. macracanthus* as a function of hormonal treatment (T1 or T2), population of origin and environmental conditions during sexual maturation (groups 1−4, see Table 1). Contingency table analyses showed no significant difference between T1 and T2 in groups 1−3. T1 was not applied in group 4.

Fish group	Hormonal treatment	n females induced	Ovulation rate (%)
1	T1	20	50
	T2	3	67
2	T1	17	82
	T2	22	82
3	T1	7	86
	T2	15	73
4	T1	0	–
	T2	28	93

T1: two successive injections of Ovaprim (0.4 then 0.6 ml kg^{-1}, 6 h later).
T2: priming with hCG (500 IU kg^{-1}) and ovulatory injection of Ovaprim (0.6 ml kg^{-1}) after 24 h.

in diameter (RID, %) following hormonal treatment was dependent on the initial follicle diameter (FD, mm) and higher in small than in large follicles, i.e. $RID = 30.32 - 18.84 FD$ ($r^2 = 0.124$, $p = 0.0033$, $n = 68$). The smallest ovum observed in these 68 spawns was 1.04 mm in diameter. Based on the aforementioned model it can be assumed that this particular ovum might have originated from a follicle of 0.91 mm. This suggests that the cut-off diameter below which individual follicles do not respond to the hormonal treatments evaluated here is around 0.91 mm.

In all fish groups, the absolute fecundity (estimated from spawn and ova weights) was linearly and positively correlated with female body weight in all fish groups ($p < 0.05$; example in Fig. 3a). By contrast, there was no significant relationship between relative fecundity and female body weight in any of the four groups under study ($p \geq 0.6$ for all fish groups; example in Fig. 3b). Hence, the mean relative fecundity could be compared directly between groups, irrespective of female body weight.

The mean relative fecundity was significantly lower in females from Kalimantan (groups 1 and 3) than in those from Sumatra (groups 2 and 4); 61 882 ± 9 681 and 76 262 ± 9 722 ova kg^{-1} versus 103 550 ± 7 438 and 109 277 ± 6 026 ova kg^{-1}, respectively (one-way ANOVA, $p < 0.0005$; Table 4). As the mean weights of ova were similar in
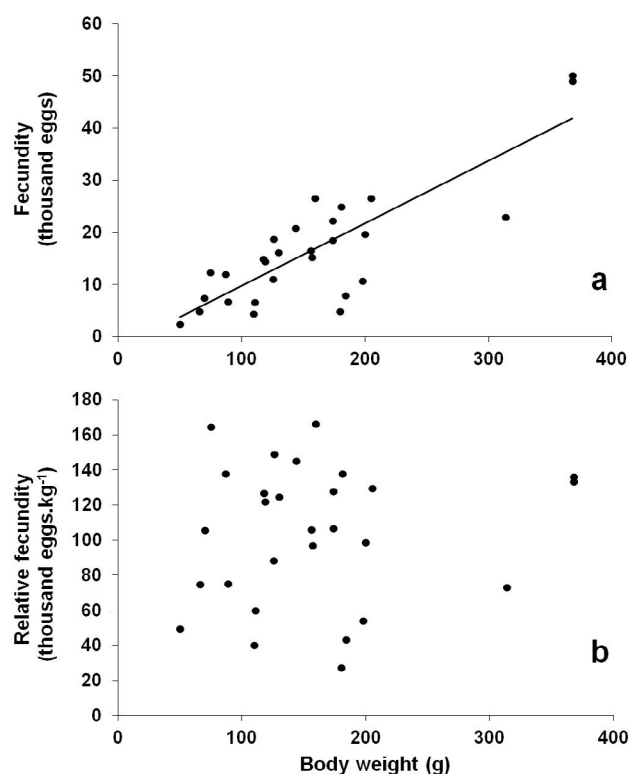

Fig. 3. Relationships (a) between absolute fecundity (quantity of ova collected by stripping) and the body weight of females [Abs. Fecundity = 119.22BW − 2160; $r^2 = 0.696$; $n = 28$; $p < 0.0001$] and (b) between relative fecundity and body weight ($p = 0.591$) in *Chromobotia macracanthus* maturing in the wild (Musi River, Sumatra, group 2).

all groups, these variations in relative fecundity reflected corresponding variations in the mean pseudo-gonado-somatic index (pGSI = weight of ova collected/fish BW, in %) between fish groups. The mean pGSI was lower in fish from Kalimantan (5.1 ± 0.8% and 6.2 ± 0.8% in groups 1 and 3) than in those from Sumatra (8.0 ± 0.6% and 9.0 ± 0.5% in groups 2 and 4). Partial ovulations were suspected on three occasions only (out of 87 ovulated females) from the presence of rather large residual ovaries that could be felt by hand through the abdominal wall, even after repeated stripping at a few hour intervals. These three fish, which originated from different groups, were not included in the fecundity analyses. No significant relationship was found between relative fecundity and initial follicle

Table 4. Comparison of ovulation rate, quantity (relative fecundity), size and quality (fertilization percentage) of ova obtained in *C. macracanthus* after hormonal treatment in fish groups sexually matured in different captive conditions or in the wild (groups 1–4, see Table 1). Fish treated with treatments T1 and T2 are pooled within each group.

Fish group	*n* females induced	Female body weight [range] (g)	Ovulation (%)	Individual weight of ova (mg)	Fecundity (ova kg^{-1})	Fertilization (%)
1	23	89 ± 14 [a] [46–226]	52 [a]	0.84 ± 0.03 [a]	61 882 ± 9 681 [a]	34 ± 6 [a]
2	39	162 ± 18[b] [50–404]	82[b]	0.79 ± 0.02 [a]	103 550 ± 7 438[b]	61 ± 4[b, c]
3	22	159 ± 16[b] [61–329]	77 [a, b]	0.82 ± 0.02 [a]	76 262 ± 9 722[a]	50 ± 8 [a, b]
4	28	147 ± 7[b] [102–228]	93 [b]	0.84 ± 0.02 [a]	109 277 ± 6 026[b]	73 ± 6[c]

Mean ± SE; values in the same column that share at least one superscript in common do not differ at *p* < 0.05.

diameter before hormonal treatment (simple regression analyses, *p* > 0.05 in all groups).

Egg quality, which was estimated from the fertilization percentages, was significantly lower (one-way ANOVA, *p* = 0.0002; Table 4), for broodfish from Kalimantan (34 ± 6% and 50 ± 8%, in groups 1 and 3) than for those from Sumatra (61 ± 4% and 73 ± 6%, in groups 2 and 4). Egg quality was independent from female BW, mean weight of ova or pGSI in the ranges observed in this study (simple regression analyses; *p* > 0.05 in all groups).

3.4 Latency period

The ovulation time could be determined in 29 of the 30 females that ovulated after hormonal treatment T1 (Ovaprim + Ovaprim), and in 54 of 57 of those ovulating after treatment T2 (hCG + Ovaprim). The latency period between the second hormone injection and ovulation (hereafter, *LP*) ranged between 4.5 h and 18.2 h with hormonal treatment T1 (at a mean temperature of fish maintenance varying between 23.0 °C and 30.5 °C), and between 9.0 h and 21.5 h with treatment T2 (at a mean water temperature of fish maintenance between 25.0 °C and 30.5 °C). The dynamics of latency response as a function of water temperature were almost identical in the groups with Sumatra and Kalimantan fish, which suggested that data from both types of groups could be analysed altogether. Nevertheless, for the sake of completeness and information to the reader, the origin of data points is shown in Figure 4.

For both treatments, stepwise multiple-regression analyses of *LP* against several explicative variables (water temperature, fish body weight and initial follicle diameter) only retained the significant influence of water temperature (*WT*). Likewise, *LP* was seemingly independent from the initial stoutness of females, as estimated from 10 females, for which this criterion was taken into account and which ovulated after treatment T2. For both treatments, the relationships between *LP* and *WT* were best modelled with second order polynomials (Fig. 4). These models indicate that 1) *LP* is always shorter with T1 than with T2, 2) the shortest *LP* with T1 and T2 are obtained at 27.6 and 28.3 °C, respectively; and 3) the gap between the

LP obtained with the two treatments decreases when *WT* increases.

Nevertheless, with both treatments the latency period at any particular temperature of maintenance varied substantially between individuals (Fig. 4). To test whether this variation was temperature-dependent and variable between treatments, the thermal range under study was categorized, using 1 °C classes, and for each class, the variation of *LP* (*VLP*, %) was calculated as $VLP = 100 (LP_{max} − LP_{min}) LP_{min}^{-1}$, where LP_{max} and LP_{min} are the longest and shortest *LP* for the 1 °C class. This method was preferred to the use of coefficients of variation, which are strongly influenced by the number of observations for small numbers. *VLPs* were independent from temperature for both treatments (simple regression analyses, *p* > 0.05), and on average, they were similar with both treatments over the thermal range that was common to the two treatments (25–29 °C, *VLP* of 72 and 70%, for T1 and T2, respectively).

The possible influence of *LP* on the quality of ova was tested by examining the variation in fertilization rates as a function of residue values of individual *LP* from predictions of the *LP*-to-*WT* models for T1 and T2. No significant relationship was found between residues and fertilization percentages (*p* > 0.6 for T1 and *p* > 0.3 for T2, either when considering all fish together or taking fish origin into account). This indicated that egg quality was independent from the latency response to ovulation.

When *LP* was expressed in terms of degrees × hours (d-h), significant second order polynomial relationships were still found between latency and water temperature ($r^2 = 0.548$; *n* = 25 with T1, and $r^2 = 0.329$; *n* = 54; with T2, *p* < 0.001 for all coefficients). However, when the thermal range under consideration was restricted to 26.0–29.0 °C, these relationships were no longer significant. In this thermal range, the mean *LP* were 241 ± 12 d-h (*n* = 9) and 359 ± 9 d-h (*n* = 37), for treatments T1 and T2, respectively.

Throughout the trials, no female attained the GVBD stage after the first injection of hormonal treatments, except during one particular trial, which referred to fish from group 2 (Sumatra, maturation in the river) in November 2004. In this trial (treatment T1), 31 to 46% of the oocytes of the four
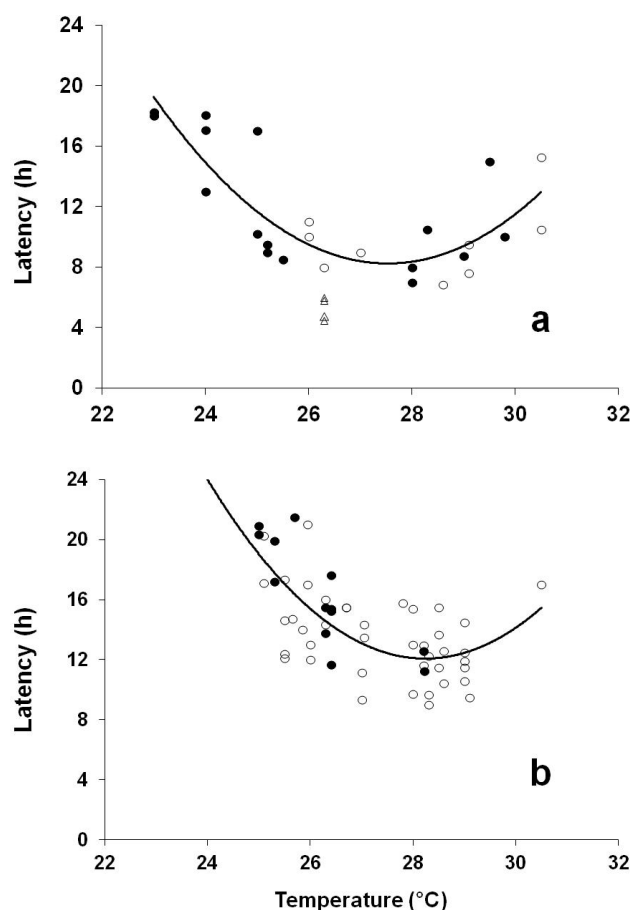
Fig. 4. Latency period (*LP*) since the second Ovaprim injection as a function of water temperature (*WT*) in females of *Chromobotia macracanthus*. **a)** females treated with two successive injections of Ovaprim (0.4 then 0.6 ml kg^{-1} at 6-h interval; treatment T1). $LP =$ 413.97 − 29.4723 WT + 0.5352 WT^2; $r^2 = 0.670$; $n = 25$; $p < 0.0001$ for all coefficients. **b)** females treated with a priming injection of hCG (500 IU kg^{-1}) followed 24 h later by an ovulatory injection of Ovaprim (0.6 ml kg^{-1}; treatment T2). $LP = 545.34 − 37.7630WT +$ 0.6685WT^2; $r^2 = 0.479$; $n = 54$; $p < 0.0005$ for all coefficients. Open circles: females originating from Sumatra; closed circles: females originating from Kalimantan; open triangles: four females from Sumatra for which the process of oocyte maturation was already engaged after the first Ovaprim injection (presence of GVBD; data not included in the "T1" model; see also text, Figs. 2b and 5).

females under study had already attained the GVBD stage at the moment of the second injection. Their *LP* was exceptionally short (4.5–6 h at 26.3 °C) and the corresponding data were not included in the model of latency period to temperature presented above; as they departed from all other data (see the triangle symbols in Fig. 4a). Such fast oocyte maturation following the first hormonal injection was never observed in any other trial, neither for fish maturing in the wild nor for those maturing in captivity. A close examination of the environmental factors in the Musi River showed that this exceptionally rapid oocyte maturation took place after a period of heavy rains, with a subsequent rise in water level and a rapid drop in water temperature, from 28.5 °C to less than 26.0 °C

(Fig. 5). By contrast, identical field trials carried out in October 2004 with wild fish resulted paradoxically in long latency response (Figs. 4a,b). This time of the year corresponded to a period of warm water temperature (31.5 °C), with no rainfalls (Fig. 5). It is worth pointing out that the broodfish that exhibited these contrasting latency responses in October and November 2004, showed similar characteristics in terms of initial position of GV (central or slightly eccentric in all cases), initial follicle diameter (1.22 ± 0.03 versus 1.25 ± 0.01 mm) and egg quality (fertilization rates of 62 ± 10 vs. 75 ± 3%).

4 Discussion

This study is the first ever to provide a reliable procedure for the induced breeding of *Chromobotia macracanthus* in captivity, although it has been attempted for decades. It also provides key information as regards the general conditions for adequate broodfish maintenance, criteria for the selection of ripe individuals, effective hormonal treatments and their latency responses. In addition to these applied aspects, the study highlights reproductive traits of the species, in particular as regards size-dependent aspects, relative fecundity and egg quality, for two fish populations originating from Sumatra and Borneo Islands to which *C. macracanthus* is endemic.

4.1 Criteria for assessment of female sexual maturity

The capacity of assessing the reproductive state of broodfish is crucial to successful induced breeding. Criteria used as indicators for ripeness can be external (abdominal distension and softness) or internal, referring mostly to the aspect and diameter of follicles sampled by biopsy and to the position of the oocyte germinal vesicle (Lam 1982; Harvey and Carolsfeld 1993; Legendre et al. 1996; Mylonas et al. 2010).

Follicle diameter proved a reliable criterion for evaluating female ripeness in *C. macracanthus*. It was also the main or even exclusive criterion used for successfully selecting mature females responsive to hormonal treatments in several other fish species (Richter and Van Den Hurk 1982; Lee et al. 1986; Legendre and Otémé 1995; Legendre et al. 2000). In *C. macracanthus*, a modal follicle diameter ⩾1.02 mm generally led to high ovulation success (>80%) after hormonal treatment. No significant relationship was found between follicle diameter and female body weight in *C. macracanthus*, so this cut-off diameter can be used confidently for females of all sizes. In females already selected on the basis of their initial follicle diameter, the presence of a rebounded abdomen was generally associated to an abundant egg production. However, high ovulation rates and good egg quality after hormonal treatment were independent from abdominal stoutness (Table 2). Hence, the exclusive use of abdominal stoutness for identifying ripe female broodfish in *C. macracanthus* can be misleading, as it would dismiss fish that can be responsive to the hormonal treatment, but have smaller gonads (and thus a lower fecundity) than others. Moreover, complementary observations from the dissection of fish raised in tanks showed that females with a rounded abdomen could also correspond
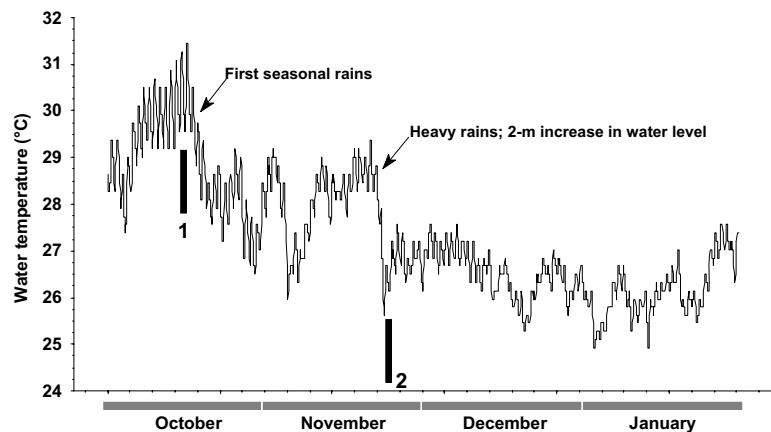
Fig. 5. Variation of water temperature in the Musi River (Sumatra) from October 2004 to January 2005 (hourly records with a Stowaway recorder in the main stream, at mid of the water column). Black vertical bars indicate the days of two induced breeding trials in a field laboratory (see methods).

to fish with abundant perivisceral fat, but in situation of reproductive rest (authors' unpubl. data). In many other fish species, the position of oocyte's germinal vesicle (GV) has been used as the main criterion for selecting mature females (e.g. Bry 1981; Pouvreau et al. 1983; Rothbard and Yaron 1995). However in the present study, GV was systematically in a central or slightly eccentric position in all follicles collected before hormonal treatment, even when oocyte maturation occurred very rapidly thereafter (e.g. field trial in November 2004). Hence, the position of GV was not a discriminating criterion for evaluating ripeness in female *C. macracanthus*. In some species (e.g. pikeperch, *Sander lucioperca*; Zarski et al. 2011), the presence and coalescence of oil droplets were also used as criteria to determine pre-ovulatory oocyte maturation stages. However oil droplets were absent at any development stage in *C. macracanthus* oocytes (e.g. Fig. 2), as in other Cypriniformes (Kamler 1992).

The ovaries of ripe females of *C. macracanthus* contain oocytes at all developmental stages, with the presence of a large unimodal group of postvitellogenic oocytes (Fig. 1). These characteristics correspond to the group-synchronous ovarian type defined by Wallace and Selman (1981). So far it remains uncertain whether *C. macracanthus* belongs to the subcategory of annual multiple spawners (Nuñez and Duponchelle 2009) or spawns just once during a same reproductive season in the wild. The capacity of *C. macracanthus* to undergo annual multiple spawning has been verified for fish held in the large water recirculation systems (groups 3 and 4), in which females (identified by PIT-tags) were responsive to a second hormonal treatment within 2−3 months, without any significant reduction in the quantity and quality of collected ova (authors' unpubl. data). However, this observation is no univocal demonstration that wild broodfish might be capable of doing so, by analogy with observations in other fish species. For example, the common European barbel *Barbus barbus*, a European cyprinid, can spawn repeatedly under controlled conditions (up to 15 spawns per individual female per year; Poncin 1988), but exhibits a single seasonal spawning in the wild (Baras and Philippart 1999).

4.2 Efficiency of hormonal treatments

GnRHa, alone or in combination with dopamine antagonist, has been used to successfully induce final maturation and ovulation in many cultured fishes, including several species of Cypriniformes (Harvey and Carolsfeld 1993; Zohar and Mylonas 2001). The two hormonal treatments tested in the present study, led to similar efficiency in terms of ovulation rate, and quality and amount of ova collected. Except for fish from group 1 in which low ovulation rates were noticed, the percentage of ovulated females was high with both treatments (82−86% and 73−93%, for T1 and T2, respectively; Table 3). Although not maximal, these scores fall into a range considered as successful (70−100% depending on fish species and hormonal treatments; Harvey and Carolsfeld 1993; Legendre et al. 1996) and suffice for starting the artificial propagation of *C. macracanthus* at a commercial scale.

The hormone dose applied for the ovulatory injection in this study ($12 \, \mu g \, kg^{-1}$ GnRHa + $6 \, mg \, kg^{-1}$ DOM) compares with those successfully applied in other Cypriniformes (10 to $20 \, \mu g \, kg^{-1}$ LHRHa + 5 to $10 \, mg \, kg^{-1}$ DOM; Arabaci et al. 2001; Dasgupta et al. 2009; Wang et al. 2009). The injection of hCG at a dose of $500 \, IU \, kg^{-1}$ (or lower) has been reported successful for inducement of oocyte maturation and ovulation in several fish species (see Zohar and Mylonas 2001, for review), but not in several others (e.g. Pangasiids; Legendre et al. 2000; Cacot et al. 2002). In *C. macracanthus*, this hCG dose acted as a primer only. It resulted in GV migration to sub-peripheral position in part (0−57%) of the postvitellogenic oocytes, as well as in a slight increase in oocyte diameter within 24 h (data not shown), but never induced full GV migration nor GVBD.

4.3 Latency period

The predictability of the latency period (LP) is a key factor in the success of hormonally induced reproduction in fish (Harvey and Carolsfeld 1993; Legendre et al. 2000). The duration of LP is species- and treatment-specific, and it also depends on environmental conditions (Mylonas et al. 2010).

Here, LP was shorter with T1 than with T2 (mean latency of 241 versus 359 d-h at 26−29 °C, respectively). As the second injection was identical in both treatments, this indicates that the first injection of Ovaprim in T1 was more potent to engage oocytes in the process of final maturation than the priming with hCG in T2. In *P. hypophthalmus*, Legendre et al. (2000, 2008) also reported a shorter LP with a treatment similar to T1 than with T2. They also found that in comparison to T1, T2 strongly increased the predictability of the latency response at a given temperature in this catfish species. In *C. macracanthus*, there was no gain in predictability, as both treatments were accompanied with highly variable LP at any particular temperature. This variability suggests that the gonads of the different females selected on the basis their oocyte size distributions were not exactly at the same physiological state. Therefore more accurate or complementary indicators of ripeness should be sought for reducing LP variability. In the devil stinger *Inimicus japonicus*, Takushima et al. (2003) reported that after treatment with LHRHa the LP was related to the initial oocyte diameter. This was not the case in *C. macracanthus*.

Many studies reported that the process of oocyte maturation is temperature-dependent, with LP linearly (or curvilinearly) and negatively correlated to water temperature (Hogendoorn and Vismans 1980; Lam 1983; Harvey and Carolsfeld 1993; Drori et al. 1994; Legendre and Otémé 1995; Rothbard and Yaron 1995; Legendre et al. 2000). In *C. macracanthus*, the relationships between LP and water temperature depart from conventional models, as the latency responses observed both with T1 and T2 rebound for water temperature above 28−29 °C (Fig. 4). To our knowledge, it is the first time that such *U*-shaped pattern is reported in teleost fishes. From a physiological perspective, this pattern is largely paradoxical in poikilothermic organisms, as warmer temperatures normally result in higher metabolism and shorter LP. However, from an ecological perspective, it might have an adaptive value, as fertilized eggs of *C. macracanthus* incubated at temperatures >28−29 °C do not hatch or produce high rates of deformed fish (Slembrouck et al. 2012), so it is most unlikely that females would spawn at these thermal regimes in natural conditions. By contrast, a particularly short LP was noticed for the four females induced to ovulate in our field laboratory (Musi River) in November 2004. In this particular situation, it had been observed that the first Ovaprim injection (treatment T1) had sufficed to induce GVBD in a large proportion of oocytes, which was exceptional by reference to other trials with the same treatment, and indicated a particularly high sensitivity to the hormonal stimulation. This observation, particularly when compared to the situation prevailing in October 2004, suggests that the rapid drop in water temperature taking place just before the hormonal treatment (from 28.5 to 26 °C in 2−3 days; Fig. 5), either alone or associated to heavy rains and rise in water level, might have acted as an environmental cue preparing fish for spawning. This hypothesis is supported by the fact that the thermal optimum for *C. macracanthus* eggs is close to 26 °C (Slembrouck et al. 2012). Conversely, the increase in LP at water temperatures above 28−29 °C might be related to the inadequacy of such temperature for successful embryo development in *C. macracanthus*. It should be noticed however that the latter increase in LP was not associated to

a loss in egg quality, as the corresponding fertilization percentages were still high (see results). Mylonas et al. (2010) stated that outside the range of "physiological temperatures" for spawning induction, a higher temperature is unfavourable and may affect spawning success and progeny quality. This was particularly the case in the rainbow trout, *Oncorhynchus mykiss*, in which the responsiveness to hormonal treatment was lower at 18 °C than at 12 °C. This effect of high temperature was interpreted as being due to either an initial lack of pituitary responsiveness to LRHRa or the incapacity of ovaries to synthesize 17,20βP (Pankurst and King 2010). In the grass carp *Ctenopharyngodon idella*, a warm-water species, the ovulation rate was lower at 28 °C than at 24 °C after injection of a combination of pimozide and GnRH (Glasser et al. 2004). This negative effect of high temperature was observed acting at various levels of the brain-pituitary-gonad endocrine axis. However in the latter study, the effects of water temperature on the latency response or egg quality were not documented. In *C. macracanthus* from group 2, there was also a tendency for a lower ovulation rate at 30.5 °C (50%, three of six females injected with T1 or T2) than at lower temperatures (88%, 33 fish). So far the physiological mechanisms behind the unexpected temperature-dependent pattern for LP in *C. macracanthus* remain to be elucidated.

4.4 General maintenance conditions of broodfish and reproductive traits

The design of the present study, which examined fish from different populations in different conditions of maintenance and maturation, enabled three kinds of global comparisons of reproductive traits: (1) comparison between fish that matured sexually in the wild or in fully controlled condition (fish from Sumatra population; groups 2 and 4), (2) comparison between fish held in rudimentary or fully controlled culture conditions (fish from Kalimantan population; groups 1 and 3), and (3) comparison between fish from Kalimantan and Sumatra, held in the same controlled culture conditions (groups 3 and 4).

All reproductive traits of fish from Sumatra that sexually matured in captivity (group 4) did not differ significantly from those of fish having undergone their sexual maturation in the wild (Musi River, group 2). The ovulation rate, mean pseudo-gonadosomatic index (pGSI), relative fecundity and fertilization rate were even slightly higher in fish from group 4 than in those from group 2. Conversely, all four indicators were slightly lower in group 1 than in group 3. Yet, in view of the positive correlation between ovulation rate and female body weight found for Kalimantan fishes, it cannot be excluded that the comparison between groups 1 and 3 for this particular indicator was skewed by the smaller size of females in group 1. All three other indicators were independent from fish size, which suggests that their lower scores in group 1 originated from less adequate environmental conditions. These two comparisons indicate the overall adequacy of the environmental conditions in the large recirculation systems (groups 3 and 4) for sustaining a complete sexual maturation of *C. macracanthus*. In such systems the proportion of mature fish upon monthly checks could reach over 80−90%, either for females or males (data not presented). So far, the poor success in obtaining full

gonad development of *C. macracanthus* in captivity has generally been considered as a major obstacle to its artificial propagation, and the proportion of fish able to mature sexually at a same time in stocks cultivated in more rudimentary conditions did generally not exceed about 40% (Satyani 2004).

Rearing conditions in groups 1 and 3 differed in terms of food supply, water quality, tank size, fish density, temperature and lighting, so it is not permitted to determine which factor or combination of factors was decisive. Nevertheless, some inferences can be drawn from the comparisons herein. The fact that reproductive characteristics of fish from groups 2 and 4 were similar although maturation took place in contrasting situations for pH (5.5 to 7.7) and conductivity (25 to 294 μS cm^{-1}; Table 1), suggests that these two parameters do not have an overwhelming influence on gametogenesis in *C. macracanthus*, at least within the ranges evaluated here. As regards other environmental factors, Satyani et al. (1999) reported a positive effect of low light levels on gonad development in *C. macracanthus*. A dim red light was used in the present study; however it is not known yet whether lights of different wavelength could influence physiological or behavioural responses in the clown loach. In another Cypriniforme, the tench, *Tinca tinca*, Owen et al. (2010) reported that red light increased the activity level of juveniles and might reduce their intrinsic stress level in comparison to white light. An additional factor that might be of importance in explaining the observed differences between groups 1 and 3 is the greater degree of fish disturbance in group 1, which were reared in a hatchery used for several species, with more frequent passage of workers and higher noise level than in the blind, quiet room specifically dedicated to the clown loach hatchery for groups 3 and 4. Stress-induced impairments of gamete quality and quantity (fecundity) are paramount, although stress can affect many other aspects of fish biology associated with reproduction (see Schreck 2010, for review).

In identical and suitable rearing conditions, the mean relative fecundity was significantly higher in females originating from the Musi River in Sumatra than in those from the Kapuas River in Kalimantan (about 109 000 vs. 76 000 ova kg^{-1}, Table 4). This difference can hardly be attributed to a differential sensitivity to the hormonal stimulation, especially in view of the very low occurrence of partial ovulation in both groups. By contrast, it might be related to the strong genetic differentiation between the two populations that was reported on the basis of mitochondrial and nuclear DNA analyses (Sudarto et al. 2008). Besides fecundity, the ovulation rate and quality of ova obtained after hormonal induced ovulation also tended to be lower in fish from Kalimantan. This may also indicate a lower acclimation of the Kalimantan fish to the rearing conditions in this study, resulting in an overall poorer reproductive performance. It is currently uncertain whether these differences are of genotypic or phenotypic nature, as the fish probably experienced different life histories before being captured in their respective rivers.

In contrast to all other variables, the mean individual weight of ova was remarkably stable whatever the origin or maintenance conditions of *C. macracanthus* females. As a matter of fact, the balance between fecundity and egg size that has been documented in many fish species (e.g. tilapias;

Duponchelle et al. 2000) was not evidenced in *C. macracanthus*, as variations of fecundity were not accompanied with opposite variations in egg size.

5 Conclusion

This study provides a reliable procedure for broodfish maintenance and induced breeding of *Chromobotia macracanthus* in captivity. It includes criteria for the identification of ripe fish, efficient hormonal treatments and description of corresponding models of latency period to water temperature. The results showed that a set of conditions implemented in large water recirculation systems was fully satisfactory for the sexual maturation of *C. macracanthus*. Nevertheless further research will be necessary to identify the respective importance and role of the environmental factors involved in the control of gametogenesis in this species. The two hormonal treatments under evaluation were equally successful in terms of ovulation rate, and quantity and quality of ova collected, but treatment T2, involving two injections at a 24 h-interval (instead of 6 h in T1), can facilitate work organization in the hatchery. As regards the thermal biology of clown loach, the present study highlighted an unusual pattern in the latency response to water temperature, which echoes well to the thermal requirements of embryos in this species (Slembrouck et al. 2012). This study provided further evidence that fish from contrasting origins within the distributional area of clown loach (Sumatra and Kalimantan) exhibited similar responses, which is an encouraging perspective for transposing the techniques for the artificial propagation of this species. Yet, between-population differences were reported, but their extent is unlikely to compromise the artificial propagation of fish from any of these rivers at least as regards the possibility of obtaining gametes, which is just the first step in a domestication process. The next crucial steps refer to techniques of egg incubation and larval rearing, which have also largely compromised the artificial propagation of this species until now. These investigations are prolonged in the other articles of this series.

References

Abramoff M.D., Magalhaes P.J., 2004, Image processing with image. J. Biophoton. Internat. 11, 36−42.

Arabaci M., Cagirgan H., Sari M., 2001, Induction of spawning in common carp (*Cyprinus carpio*) using LHRHa ([D-Ser (tBu)6, Pro9-NEt]-LHRH) combined with haloperidol: effects of different treatment time and determination of latency period dependence on temperature. Turk. J. Fish. Aquat Sci. 1, 1−5.

Baras E., Philippart J.-C., 1999, Adaptive and evolutionary significance of a reproductive thermal threshold in *Barbus barbus* (L.). J. Fish Biol. 55, 354−375.

Bry C., 1981, Temporal aspects of macroscopic changes in rainbow trout (*Salmo gairdneri*) oocytes before ovulation and of ova fertility during the post-ovulation period. Effect of treatment with 17α-hydroxy-20βdihydroprogesterone. Aquaculture 2, 153−160.

Cacot P., Legendre M., Dan T.Q., Hung L.T., Liem P.T., Mariojouls C., Lazard J., 2002, Induced ovulation of *Pangasius bocourti* (Sauvage 1880) with a progressive hCG treatment. Aquaculture 213, 199−206.

Cacot P., Eeckhoutte P., Muon D.T., Trieu T.T., Legendre M., Lazard J., 2003, Induced spermiation and milt management in *Pangasius bocourti* (Sauvage 1880). Aquaculture 215, 67−77.

Clarke M., 2007, British fishkeeper spawns Clown loach. Practical Fishkeeping,
http://www.practicalfishkeeping.co.uk/content.php?sid=1381

Clarke M., 2008, Fishkeeper successfully breeds Clown loach. Practical Fishkeeping, http://www.practicalfishkeeping.co.uk/content.php?sid=1584

Cowx I.G., Welcomme R.L. (eds.), 1998, Rehabilitation of Rivers for Fish. Fishing News Books, Blackwell Science Ltd., Oxford, UK.

Dasgupta S., Sarkar S.K., Sarangi N., Bhattacharya S., 2009, Variation in spawning responses, egg and larvae productions from induced rohu (*Labeo rohita*) during pre-monsoon and monsoon seasons: Relationship with hormonal changes and oocyte responsiveness during final maturation. Aquaculture 290, 320−326.

Drori S., Ok M., Levavi-Sivan B., Yaron S., 1994, Spawning induction in common carp (*Cyprinus carpio*) using pituitary extract or GnRH superactive analogue combined with metoclopramide: analysis of hormone profile, progress of oocyte maturation and dependence on temperature. Aquaculture 119, 393−407.

Duponchelle F., Cecchi P., Corbin D., Nunez J., Legendre M., 2000, Variations in fecundity and egg size of female Nile tilapia, *Oreochromis niloticus*, populations from man-made lakes of Côte d'Ivoire. Environ. Biol. Fish. 57, 155−170.

Effendi I., Prasetya T., Sudrajat A.O., Suhenda N., Sumawidjaja K., 2003, Pematangan gonad induk ikan botia (*Botia macracanthus*) dalam kolam. Gonad maturation of clown loach (*Botia macracanthus*) in pond. Jurnal Akuakultur Indonesia 2, 51−54 (in Indonesian with English abstract).

FAO, 2010, La situation mondiale des pêches et de l'aquaculture. FAO, Rome.

Glasser F., Mikolajczyk T., Jalabert B., Baroiller J.-F., Breton B., 2004, Temperature effects along the reproductive axis during spawning induction of grass carp (*Ctenopharyngodon idella*). Gen. Comp. Endocrinol. 136, 171−179.

Hammond G., 1996, You can breed clown loaches. Practical Fishkeeping February, 29−31.

Harvey B., Carolsfeld J., 1993, Induced breeding in tropical fish culture. Int. Development Research Center (IDRC), Ottawa, Ont., Canada, 144 p.

Kamler E., 1992, Early life history of fish. Chapman & Hall, London.

Kottelat M., Whitten A.J., Kartikasari S.N., Wirjoatmodjo S., 1995, Freshwater Fishes of Western Indonesia and Sulawesi. Periplus edn. Ltd., Jakarta.

Kottelat M., 2004, *Botia kubotai*, a new species of loach (Teleostei: Cobitidae) from the Ataran River basin (Myanmar), with comments on botiine nomenclature and diagnosis of a new genus. Zootaxa 401, 1−18.

Lam T.J., 1982, Applications of endocrinology to fish culture. Can. J. Fish. Aquat. Sci. 39, 111−137.

Lee C.S., Gordon M., Watanabe W. (eds.), 1986, Aquaculture of milkfish: state of the art. Oceanic Institute, Waimanalo, HI.

Legendre M., Otémé Z., 1995, Effect of varying latency period on the quantity and quality of eggs after hCG-induced ovulation in the African catfish *Heterobranchus longifilis* (Teleostei, Clariidae). Aquat. Living Resour. 8, 309−316.

Legendre M., Linhart O., Billard R., 1996, Spawning and management of gametes, fertilized eggs and embryos in Siluroidei. In: Legendre M., Proteau J.-P. (eds.), The biology and culture of catfishes. Aquat. Living Resour., Special Issue, 9, 59−80.

Legendre M., Slembrouck J., Subagja J., Kristanto A.H., 2000, Ovulation rate, latency period and in vivo survival of ova after GnRH- or hCG-induced breeding of the Asian catfish *Pangasius hypophthalmus* (Siluriformes, Pangasiidae). Aquat. Living Resour. 13, 145−151.

Legendre M., Subagja J., Kristanto A.H., Baras E., Bardon A., Slembrouck J., 2008, Priming with hCG increases the predictability of latency period in hormonal induced ovulation of the catfish *Pangasianodon hypophthalmus*. Abstracts of World Aquaculture 2008, May 2008, Busan, Korea. (https://www.was.org/Meetings/SessionsByDay.asp?Meetingcode=WA2008).

Marmulla G., 2001, Dams, fish and fisheries. Opportunities, challenges and conflict resolution. FAO Techn. Rep. 419, FAO, Rome.

Mylonas C., Fostier A., Zanuy S., 2010, Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516−534.

Ng P.K.L., Tan H.H., 1997, Freshwater fishes of Southeast Asia: potential for the aquarium fish trade and conservation issues. Aquat. Sci. Conserv. 1, 79−90.

Nuñez J., Duponchelle F., 2009, Towards a universal scale to assess sexual maturation and related life history traits in oviparous teleost fishes. Fish Physiol. Biochem. 35, 167−180.

Olivier K., 2001, The ornamental fish market. FAO/GLOBEFISH Research Programme 67. FAO, Rome.

Owen M.A.G., Davies S.J., Sloman K.A., 2010, Light colour influences the behaviour and stress physiology of captive tench (*Tinca tinca*). Rev. Fish Biol. Fish. 20, 375−380.

Pankhurst N.W., King H.R., 2010, Temperature and salmonid reproduction: implications for aquaculture. J. Fish Biol. 76, 69−85.

Philippart J.C., 1995, Is captive breeding an effective solution for the preservation of endemic species? Biol. Conserv. 72, 281−295.

Poncin P., 1988, Le contrôle environnemental et hormonal de la reproduction du barbeau, *Barbus barbus* (L.), et du chevaine, *Leuciscus cephalus* (L.) (Pisces Cyprinidae), en captivité. Cah. Ethol. Appl. 8, 173−330.

Pouvreau M., Maisse G., Jalabert B., Breton B., 1983, Efficacité du contrôle de l'ovulation par traitement gonadotrope dans une population hétérogène de brochet (*Esox lucius*) : relation avec le stade ovocytaire et comparaison avec les résultats d'ovulation spontanées. In : Billard R. (ed.), Le brochet: gestion dans le milieu naturel et élevage. INRA Publ., Paris, pp. 85−95.

Richter C.J.J., Van den Hurk R., 1982, Effects of 11-desoxycorticosterone-acetate and carp pituitary suspension on follicle maturation in the ovaries of the African catfish, *Clarias lazera* (C. & V.). Aquaculture 29, 53−66.

Rothbard S., Yaron Z., 1995, Carps (Cyprinidae). In: Bromage N.R., Roberts R.J. (eds.), Broodstock management and egg and larval quality. Blackwell Science Ltd., Oxford, UK, pp. 321−352.

Satyani D., 2004, Percobaan pemijahan ikan Botia (*Botia macracantha* Blkr.) di laboratorium. Spawning experiment of Botia broodstocks (*Botia macracantha* Blkr.) in laboratory. Jurnal Penelitian

Perikanan Indonesia 10, 55−59 (in Indonesian with English abstract).

Satyani D., Kadarini T., Komarudin O., 1999, Perkembangan gonad berbagai ukuran induk botia (*Botia macracantha* Blkr) pada pemeliharaan degan fotoperiod 0 jam/hari. Gonad development of botia (*Botia macracantha*) broodstock reared with photoperiod of 0 h/day. Jurnal Penelitian Perikanan Indonesia 2, 37−42 (in Indonesian with English abstract).

Schreck C.B., 2010, Stress and fish reproduction: the roles of allostasis and hormesis. Gen. Comp. Endocrinol. 165, 549−556.

Slembrouck J., Komarudin O., Maskur, Legendre M. (eds.), 2004, Technical manual for the artificial propagation of the Indonesian catfish, *Pangasius djambal*. IRD-BKP edn., ISBN 979-8186-92-3.

Slembrouck J., Priyadi A., Permana A., Ginanjar R., Baras E., Satyani D., Sudarto, Pouyaud L., Legendre M., 2012, Biology and culture of the clown loach *Chromobotia macracanthus* (Cypriniformes, Cobitidae). 2. Importance of water movement and temperature during egg incubation. Aquat. Living Resour. 25, 109−118.

Subagja J., Komarudin D., Effendi J., 1997, Effek implantasi hormone LHRH-a pada ikan botia (*Botia macracantha* Bleeker) terhadap keragaan pematangan gonadnya. The effect of LHRH-a implantation on the gonadal development of clown loach (*Botia macracantha*). Jurnal Penelitian Perikanan Indonesia 3, 10−17 (in Indonesian with English abstract).

Sudarto, Hidonis K., Paradis E., 2008, Genetic differentiation among populations of *Chromobotia macracanthus* Bleeker from Sumatra and Kalimantan based on sequencing gene of MtDNA cytochrome B and nucleus DNA RAG2. Indones. Aquac. J. 3, 107−117.

Takushima M., Nozaki R., Kadomura K., Yasumoto S., Soyano K., 2003, Induced-ovulation using LHRHa and artificial fertilization in devil stinger, *Inimicus japonicus*. Fish Physiol. Biochem. 28, 521−522.

Wang Y., Hu M., Wang W., Liu X., Cheung S.G., Shin P.K.S., Song L., 2009, Effects of GnRHa (D-Ala6, Pro9-NEt) combined with domperidone on ovulation induction in wild loach *Misgurnus anguillicaudatus*. Aquaculture 291, 136−139.

Welcomme R.L., 2001, Inland fisheries – Ecology and management. Fishing News Books, Blackwell Science Ltd., Oxford, UK.

Zarski D., Kucharczyk D., Targonska K., Palinska K., Kupren K., Fontaine P., Kestemont P., 2011, A new classification of preovulatory oocyte maturation stages in pikeperch, *Sander lucioperca* (L.), and its application during artificial reproduction. Aquac. Res. 27, 1155−1158.

Zohar Y., Mylonas C.C., 2001, Endocrine manipulations of spawning inductions in cultured fish: from hormones to genes. Aquaculture 197, 99−136.