EFFECTS OF VARYING LATENCY PERIOD ON THE *IN VIVO* SURVIVAL OF OVA AFTER OVAPRIM- AND HCG-INDUCED OVULATION IN THE ASIAN CATFISH *PANGASIUS HYPOPHTHALMUS* (SILURIFORMES, PANGASIIDAE).

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Abstract

Over a 2-years period at the Sukamandi station (West Java, Indonesia), 87 *P. hypophthalmus* females selected on the basis of a modal oocyte diameter greater than 1.0 mm were treated with Ovaprim (n=77) or hCG (n=10) to induce oocyte maturation and ovulation. The two hormonal treatment led to similar results in terms of ovulation percentage (86 and 90%), hatching rates (69 ± 28 and 82 ± 11 %) and relative fecundity (167,000 ± 18,000 and 128,000 ± 60,000 eggs.kg^{-1}, with Ovaprim and hCG respectively).

The latency period between the last hormone injection and ovulation was negatively correlated to water temperature but showed important variations at a same temperature depending on individual females (e.g. between 5-11 h at 28-29°C). The ovulation time was therefore difficult to predict accurately in this species.

The assessment of survival time of ova maintained *in ovario* after ovulation showed that the process of ageing (overripening of ova) occurs rapidly in *P. hypophthalmus*. The overall quality of ova begun to decrease as early as 2 hours after ovulation and, after 3 hours, hatching rate dropped down and fraction of deformed larvae increased significantly in comparison to those observed at the moment of ovulation. In some individual females this evolution was even more rapid, with a sharp decrease in hatching rates between 1 and 2 hours post-ovulation. The duration of ova survival did not appear to depend on the type of hormonal treatment used (Ovaprim or hCG).

For optimised gamete management in hatcheries, it is therefore recommended to check carefully the females for the occurrence of ovulation (between 3 to 11 h after the last hormone injection, depending on water temperature) and to collect and fertilise the eggs within less than two hours after this moment.

INTRODUCTION

Pangasius hypophthalmus (Sauvage, 1878) (senior synonym of *P. sutchi*; Roberts & Vidthayanon, 1991) is the most common cultured pangasiid catfish all over Southeast Asia. Its aquaculture production, reaching several thousand tons annually, is still dependent on captures of wild fry or fingerlings in some areas (e.g. in Cambodia) but rely increasingly on artificial propagation techniques (Csavas, 1994). The species was successfully induced to breed for the first time in captivity in Thailand after treatment with catfish pituitary gland suspension (Boonbrahm *et al.*, 1966; Potaros & Sitasit, 1976). It was then introduced to Indonesia from Thailand in 1972, where its hormonal induced-breeding was reported for the first time by Hardjamulia *et al.* (1981). Since that period, the culture of *P. hypophthalmus* has developed in this country both for food and ornamental purposes. However, despite the economic importance of this catfish, published data related to its biology and culture are still scarce and several problems remain to be solved before its rearing practices could be fully optimised. Poor egg quality and low hatching rates are amongst the difficulties most often reported by fish farmers.

The latency period, defined as the delay between hormonal injection and ova collection, is a key factor in the success of reproduction techniques involving hormonal induced-ovulation and artificial fertilisation in fish (Harvey & Caroldsfeld, 1993; Bromage & Roberts, 1995).

Delayed collection of gametes after ovulation leads to ageing phenomenon which can result in low fertilisation rates, increase in the number of deformed embryos, or increased mortality rates for embryos and larvae (Sakai *et al.*, 1975; Springate *et al.*, 1984). After ovulation, the *in vivo* survival of ova - estimated by the time lapse between ovulation and the moment at which the initial quality of ova begin to drop - varies according to species. This time lapse range from 6-30 days in the rainbow trout (Bry, 1981, Springate *et al.*, 1984) to a few hours in the majority of teleosts studied, including several catfish species (Woynarovitch & Horvath, 1980; Legendre *et al.*, 1996).

Therefore, the aim of this study was to assess the timing of ovulation (latency period) after hormonal treatment and the survival duration of ova maintained *in vivo* after ovulation in *P. hypophthalmus*. In order to test for possible difference related to the type of hormonal preparation used, survival of ova was evaluated after induced ovulation with either a mix of GnRH and Domperidone (Ovaprim®) or human chorionic gonadotropin (hCG). The ovulation rates, and the quantity and quality of ova obtained with these two treatments were also compared. The temporal evolution of ova quality was estimated by hatching rates and proportions of normal and deformed larvae obtained after artificial fertilisation.

MATERIAL AND METHODS

Fish origin and maintenance

The *P. hypophthalmus* brooders used descended from fish initially introduced from Thailand in 1972 and were 3-5-years-old and 2.4 to 5.8 kg individual body weight. They were held at a stocking density of 0.3-0.6 fish.m^{-2} in 50 m^2 ponds at the RIFF Sukamandi station (West Java, Indonesia). The broodstock was fed two times per day, 6 days a week, with a 35% crude protein pelleted feed distributed at a daily rate of 1% of fish biomass.

Latency period and ovulation rate

Over a period of two years, a total of 77 *P. hypophthalmus* females were induced to breed with Ovaprim and 10 others with hCG at the Sukamandi station. Although Ovaprim is generally used in Indonesian hatcheries (Sadili, 1999), responses were also tested with hCG for comparison.

The mature females were chosen after intra-ovarian biopsy on the basis of a modal diameter of oocytes greater than 1.0 mm (1.13 ± 0.05 mm, on average). Selected males were producing milt at stripping. Oocyte maturation and ovulation were induced with two successive Ovaprim[1] injections of 0.3 ml.kg^{-1} female BW and 0.6 ml.kg^{-1} given at 8 h interval. The same procedure was applied with hCG (Organon, France) except for doses, fixed at 500 IU.kg^{-1} and 2,000 IU.kg^{-1} for the first and second injections respectively (Campet, 1997). In all cases, males received a single Ovaprim injection of 0.3-0.4 ml.kg^{-1} applied at the moment of first injection of females. During the treatment the brooders were held in hapas installed in ponds or in large concrete tanks. The mean maintenance temperature of brooders ranged between 27.1 and 31.7°C during the latency period. Within a same trial, the amplitude of thermal variation was generally less than 2°C.

In order to detect the moment of ovulation, gentle stripping trials were generally performed every hour starting from 3 to 7 h after second injection. Nevertheless, ovulation had already occurred in some females at the moment of first stripping trial; in such cases the latency period could not be known precisely. When ovulation was observed, ova were collected by complete stripping, weighed and immediately fertilised. A sample of ova was also weighed to the nearest mg and fixed in 5% formalin for subsequent counting and total fecundity estimates.

The sperm was collected by stripping directly in a syringe containing a 0.9% NaCl solution (dilution rate of 1/5) to prevent spermatozoa activation by dilution with urine, then preserved at 5°C for subsequent fertilisations. The sperm of *P. hypophthalmus* remains generally viable for 24 h at least when preserved and used in these conditions (Eeckhoutte, 1996).

The quality of ova was evaluated from hatching rates obtained with replicated batches of 200-300 eggs fertilised with 0.2 ml of diluted sperm. This corresponded approximately to 6.10^6 spz per ova. Spermatozoa activation was obtained by addition of 10 ml freshwater. After 1min of gentle stirring, eggs were rinsed to remove excess milt and

[1] One ml of Ovaprim (Syndel Laboratories, Canada) contains 20 µg of GnRH [D-Arg6, Trp7, Leu8, Pro9, NEt] and 10 mg Domperidone.

transferred for incubation in a plastic box containing 300 ml of standing water at ambient temperature (27-30°C). Hatching ended after 26-29 h of incubation, and the hatching rates were evaluated 35-40 h after fertilisation.

In vivo *survival of ova after ovulation*

The period of *in vivo* survival of ova after ovulation was studied between May and December 1997 in 13 females treated with Ovaprim and 3 others treated with hCG. The schedule of injections and doses used were the same as those indicated above. The mean maintenance temperature of brooders ranged between 27.1 and 28.8°C during the latency period.

From 5 hours after the second injection, females were checked every hour to follow the process of oocyte maturation on samples collected by intra-ovarian biopsy and fixed in Serra's solution (60% ethanol, 30% formalin, 10% acetic acid, by volume). For females treated with Ovaprim and starting when the first oocytes at a stage of germinal vesicle breakdown (GVBD) were found, part of the gametes collected by intra-ovarian biopsy was also used for fertilisation trials until ovulation has occurred. The moment at which the first ova could be obtained by stripping was considered as the ovulation time (t_0) and served as a reference. At ovulation and then every hour until 7 h post-ovulation, a partial collection of ova (approximately 10 g per stripping) was carried out for each female. For each individual stripping, ova quality was evaluated from three sub-samples of 200-300 eggs, fertilised and incubated following the general procedure presented above. In each case, the sperm from two males was pooled for fertilisation. Two stripping of the males were done in order to prevent possible effects of a lowering in spermatozoa fertilising ability. The sperm collected at the first stripping served to fertilised ova collected up to 3 hours after ovulation, then males were stripped again for subsequent fertilisation tests. During experiment, the motility of spermatozoa was regularly checked using a microscope. After hatching, the proportions of normal and deformed larvae were determined for each batch of eggs by observations with a binocular and counting over an illuminated table.

Statistical analysis

Hatching percentages and fraction of deformed larvae were compared using one way ANOVA followed by Duncan's multiple range test to determine significant differences among means at $p<0.05$. When necessary, angular transformation of data was carried out in order to stabilise the residual variance.

RESULTS

Latency period and ovulation rate

The two hormonal treatments led to similar success in inducing oocyte maturation and ovulation of *P. hypophthalmus* (Table 1 and 2). The percentages of ovulated females were of 86% and 90% with Ovaprim and hCG, respectively. It should be noted however that incomplete ovulation was observed in 10% of the females treated with Ovaprim. These partial responses corresponded to females in which only small quantity of ova could be collected, while important remaining ovarian masses in the abdomen could be felt by hand even after repeated stripping made at a few hours interval. The eggs obtained from such females were systematically of poor quality (6.4 ± 5.1%). By contrast, the quantity and quality of ova collected in other ovulated females were generally high, and similar for fish treated with Ovaprim or hCG (Table 1 and 2). No relationship was found between the initial modal oocyte diameter of the treated females (range 1.04-1.20 mm) and the quantity or quality of ova collected.

The ovulation time was assessed precisely in 43 of the 77 females treated with Ovaprim and in 8 of the 10 females treated with hCG. The latency period between the second hormone injection and ovulation ranged from 3 to 11 hours at a mean temperature of brooders maintenance varying between 27.1 and 31.7°C (Fig. 1). For fish treated with Ovaprim, a significant inverse relationship between the latency period and water temperature was found [$R = 0.486$, $F(1,41) = 12.710$, $p<0.001$]. Nevertheless, a high variability was observed in the latency response of the different females even at a similar temperature (e.g., 5-11 hours at 28.0-29.0°C). With hCG, the latency period (8-11 h) tended to be less variable than with Ovaprim and ranged amongst the highest values observed with this latter hormone (Fig. 1). At a same temperature, the latency response was not related to the initial oocyte diameter of the different females used.

In vivo *survival of ova after ovulation*

In the ova survival study, the latency period to ovulation ranged between 5 to 11 h depending on

	No ovulation	Partial ovulation	Full ovulation
N° and % of females treated	**n = 11; 14%**	n = 8; 10%	n = 58; 76%
		n = 66; 86%	
Relative fecundity ($egg.kg^{-1}$) x 1000	-	**6.4 ± 5.1** [1 – 14] (7)	**167 ± 78** [33 – 317] (49)
Hatching rate (%) *	-	**13 ± 17** [0 – 36] (7)	**69 ± 28** [3 - 99] (42)

*: Only hatching rates estimated within 1 hour after ovulation are considered.
Mean ± sd, [ ]: extreme values, ( ): N° of observations

Table 1: Ovulation percentage, mean relative fecundity and mean hatching rate for 77 *P. hypophthalmus* females treated with Ovaprim at the Sukamandi station.

	No ovulation	Full ovulation
N° and % of females treated	**n = 1; 10%**	**n = 9; 90%**
Relative fecundity ($egg.kg^{-1}$) x 1000	-	**128 ± 60** [35 – 210] (9)
Hatching rate (%) *	-	**82 ± 11** [59 - 95] (9)

*: Hatching rates are estimated within 1 hour after ovulation.
Mean ± sd, [ ]: extreme values, ( ): N° of observations

Table 2: Ovulation percentage, mean relative fecundity and mean hatching rate for 10 *P. hypophthalmus* females treated with hCG at the Sukamandi station.

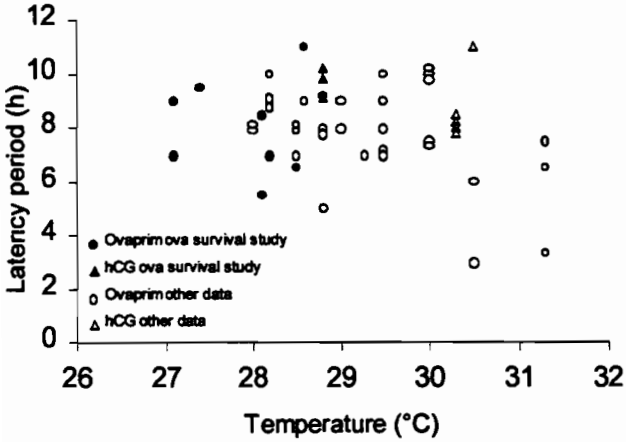

Figure 1: Latency period to ovulation, after the second Ovaprim or hCG injection, as a function of temperature in *P. hypophthalmus* females.

the females (at 27.1-28.8°C). The eggs obtained at the ovulation time (t_o) were generally of good quality with hatching rates ranging between 64 and 96% for all females, except for three individuals induced with Ovaprim (below 40%). Data from these three latter fish were withdrawn from the analysis.

Two hours before ova could be collected by stripping, the majority of oocytes obtained by intra-ovarian biopsy were GVBD, but did not fully achieved their maturation as indicated by very low hatching rate obtained after fertilisation (Fig. 2).

The evolution of hatching rates and fraction of deformed larvae obtained after partial stripping as a function of time from ovulation are given in Figures 2 and 3 for females treated with Ovaprim and hCG, respectively. Responses observed with the two hormonal treatments were very similar. In both cases, a clear inverse evolution was observed between hatching percentages and fraction of deformed larvae. The highest hatching percentages and lowest proportions of deformed larvae were observed at the ovulation time (t_0) and one hour after. As early as 2 hours after ovulation a noticeable decrease in hatching rates was observed, and after 3 hours hatching rates dropped down and fraction of deformed larvae increased significantly in comparison to those observed at t_0 and t_0+1h. When ova were stripped 5 hours or more after ovulation, mean hatching rates did not exceeded 15% and almost all of hatched larvae were strongly deformed.

Nevertheless, individual variations were observed. In the two extreme situations with Ovaprim, high hatching rates (superior to 80 %) were maintained for more than 4 hours in one female while, in another one, hatching percentages dropped from 74% to 9% between 1 and 2 h post ovulation and no hatching occurred at longer latency periods (Fig. 4). The fact that high hatching percentages were obtained for a long period of

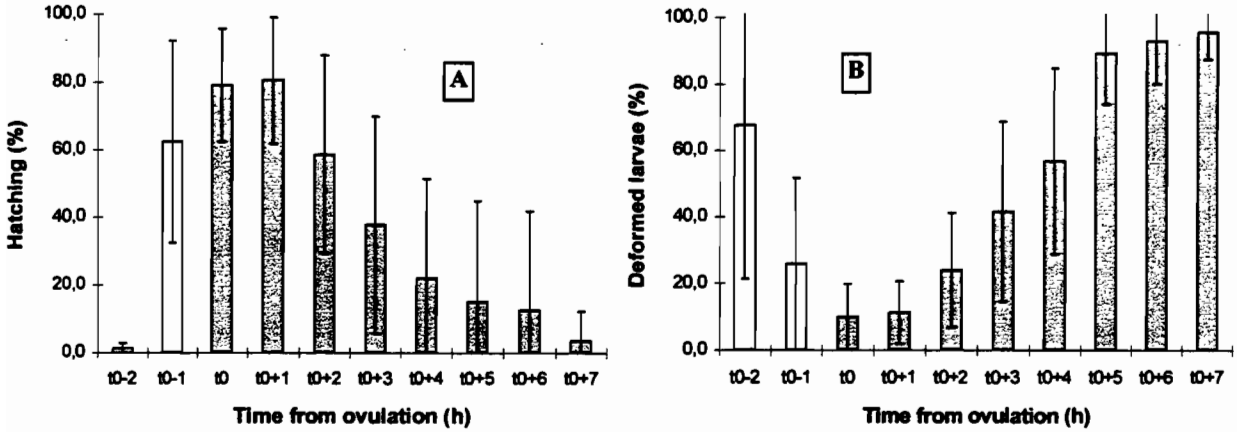

Figure 2: Evolution of mean hatching rate (A) and fraction of deformed larvae (B) as a function of time before (bars in white) and after (bars in grey) ovulation. Means for 10 *P. hypophthalmus* females treated with Ovaprim (water temperature: 27.1-28.6 °C). The moment of ovulation (t_0) is considered here as the first time at which ova can be obtained by stripping. Before ovulation, the egg quality was assessed on samples taken by intra-ovarian biopsy. Vertical bars refer to standard deviation.

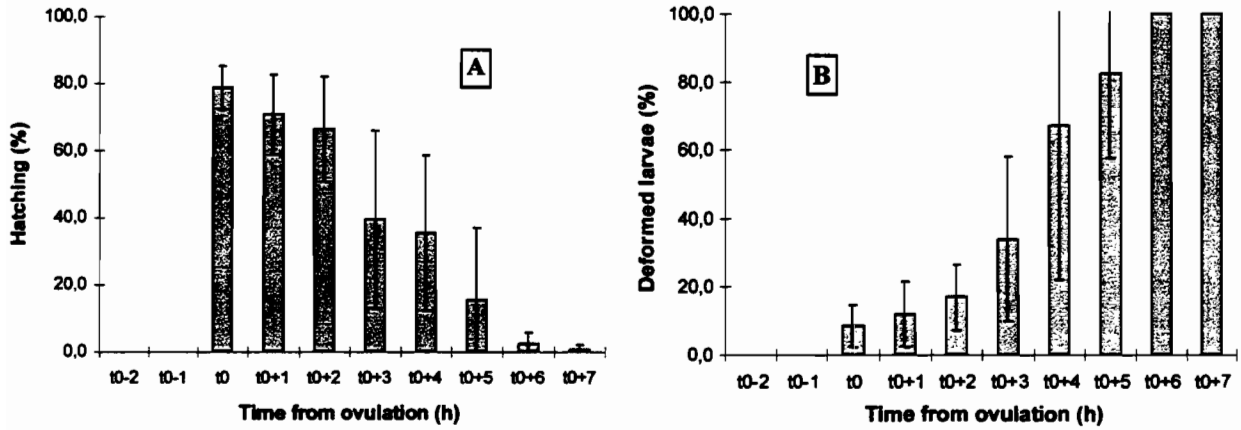

Figure 3: Evolution of mean hatching rate (A) and fraction of deformed larvae (B) as a function of time after ovulation. Means for 3 *P. hypophthalmus* females treated with hCG (water temperature: 28.8 °C). The moment of ovulation (t_0) is considered here as the first time at which ova can be obtained by stripping. Vertical bars refer to standard deviation.

time in one female attested that the rapid drop in egg quality observed in most females corresponded effectively to ageing of ova and not to a lowering of sperm fertilising ability.

DISCUSSION

In this study, both Ovaprim and hCG proved to be efficient in inducing oocyte maturation and ovulation in *P. hypophthalmus*, and led to the collection of ova of an overall good quality. The percentage of ovulated females were high and reached similar values with these two hormonal preparations (86 and 90 %, respectively). These results are equivalent to those of Cacot (1999) who reported ovulation in 88 % of 67 *P. hypophthalmus* females treated with hCG in Vietnam. Saidin *et al.* (1988) obtained 33-100% ovulation after treatment with LHRHa administered alone, i.e. not combined with anti-dopamine antagonists.

A high variability was observed in the quantity of egg collected (from 33,000 to 317,000 egg.kg^{-1}) after induced breeding, either with Ovaprim or hCG. After treatment with hCG, Cacot (1999) observed a similar range of relative fecundity in *P. hypophthalmus* females cultured in ponds or floating cages in Vietnam (from 10,100 to 297,500 egg.kg^{-1}). So far, the origin of such high individual variability, which could not be related to fish size or age, remains poorly understood and requests further investigations.

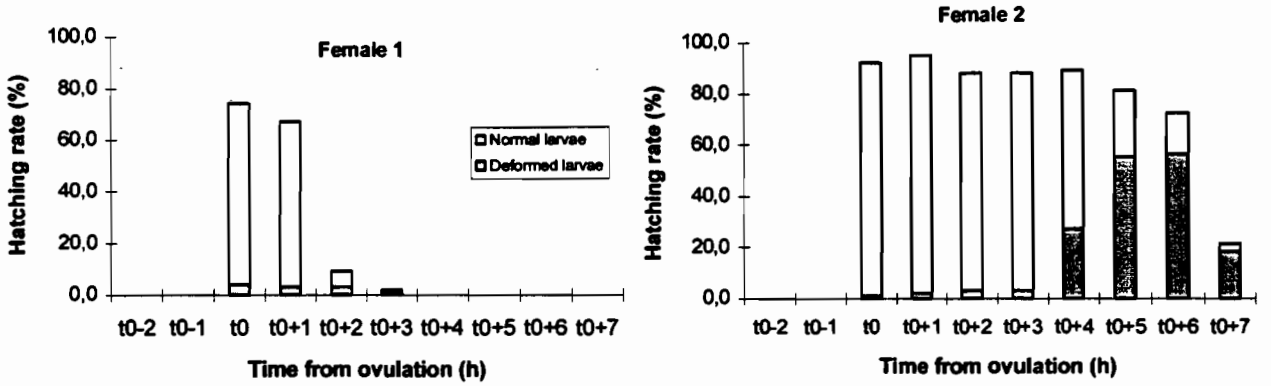

Figure 4: Extreme situations observed in the evolution of hatching rate and proportion of deformed larvae as a function of time after ovulation in two *P. hypophthalmus* females treated with Ovaprim (water temperature: 28.0-28.5°C).

As in other fish species (Woynarovitch & Horvath, 1980; Bromage & Roberts, 1995), a negative relationship was observed in *P. hypophthalmus* between the latency period and water temperature. However, at a same water temperature, the latency period between the second Ovaprim injection and ovulation showed a relatively high range of variation between individuals (5-11 h at 28-29°C). This suggested that gonads of the different females selected on the basis of their oocyte size, were not exactly at a same physiological state. Therefore, more accurate indicators than oocyte diameter alone should be identified to evaluate sexual stage before hormonal treatment in order to reduce variance in latency period.

The assessment of survival time of ova maintained *in ovario* after ovulation showed that the process of ageing (overripening of ova) occurs rapidly in *P. hypophthalmus*. The overall quality of ova begun to decrease as early as 2 hours after ovulation and, after 3 hours, hatching rates dropped down and fraction of deformed larvae increased significantly in comparison to those observed at the moment of ovulation. In some individual females this evolution was even more rapid, with a sharp decrease in hatching rates between 1 and 2 hours post-ovulation (Fig. 4). The evolution of hatching rates and fraction of deformed larvae as a function of time after ovulation were very similar with Ovaprim or hCG and did not appear to depend on the hormonal treatment used to induced ovulation. After hCG-induced ovulation in four *P. hypophthalmus* females in Vietnam, Campet (1997) observed that the initial mean quality of ova drop significantly after 3 hours from ovulation, but the ageing process occurred earlier (less than 2 hours after ovulation) in one individual female. In other catfish species studied, the reported duration of ova survival is generally longer, varying between 2-4 h in *Heterobranchus longifilis* and 10-12 h in *Clarias macrocephalus* (Legendre *et al.*, 1996). Therefore the correct timing of ovulation and moment of ova collection are particularly crucial for further egg development in *P. hypophthalmus* and insufficient checking may explain to a large extent the poor egg quality often reported on fish farms.

In Indonesia, after induced breeding of *P. hypophthalmus*, fish farmers generally apply a standard procedure consisting in the checking of females for ovulation 8-9 hours after the last hormonal injection; ovulated fish are then stripped and ova fertilised while non ovulated females are returned directly to the ponds (unpublished inquiries on fish farms). From the present results showing the effects of water temperature and individual variability on latency period, on one hand, and the short survival duration of ova, on the other hand, it appears clearly that such practice may lead either to discard fish still in the course of oocyte maturation or to collect ova already engaged in the process of overrippening.

In practice, it is therefore recommended to check carefully the females for the occurrence of ovulation (between 3 to 11 h after the last hormone injection, depending on water temperature) and to collect and fertilise the eggs within less than two hours after this moment.

REFERENCES

Boonbrahm M., Tarnchalanukit W. & Chuapoehuk W. (1966) Induced spawning by pituitary

injection of Pla-Sawai *Pangasius Pangasius* (Hamilton) in captivity. *Kasetsart J.*, **6**, 97-110.

Bromage N.R. & Roberts R.J. (eds) (1995) *Broodstock management and egg and larval quality*. Blackwell Science, 424 p.

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 17 -hydroxy-20 dihydro-progesterone. *Aquaculture*, **2**, 153-160.

Cacot P. (1999) Description of the sexual cycle related to the environment and set up of the artificial propagation in *Pangasius bocourti* (Sauvage, 1880) and *Pangasius hypophthalmus* (Sauvage, 1878), reared in floating cages and in ponds in the Mekong delta. *Proceedings of the mid-term workshop of the Catfish Asia project*, this volume.

Campet M. (1997) *Qualité des ovules d'un poisson chat élevé en cages flottantes dans le delta du Mekong (*Pangasius hypophthalmus*) durant le processus de maturation ovocytaire*. Mémoire DAA, ENSA-Rennes, France, 31 p + annexes.

Csavas I. (1994) Status and perspectives of culturing catfishes in East and Southeast Asia. *FAO Aquaculture Newsletter*, **8**, 2-10.

Eeckhoutte P. (1996) *Maîtrise de la reproduction de deux poissons-chats (*Pangasius bocourti *et* Pangasius hypophthalmus*) élévés en cages flottantes dans le Delta du Mékong (Vietnam)*. Institut National Agronomique Paris-Grignon, Paris, France, 66 p.

Hardjamulia A., Djajadiredja R., Atmawinata S. & Idris D. (1981) Pembenihan jambal siam (*Pangasius sutchi*) dengan suntikan ekstraks kelenjar hipofise ikan mas (*cyprinus carpio*). *Bull. Pen. Perik Darat.* **1**, 183-190.

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

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

Potaros M. & Sitasit P. (1976) Induced spawning of *Pangasius sutchi* (Fowler). *FAO, IPFC/76/SYM/36*, **17**, 349-353.

Roberts T.R. & Vidthayanon C. (1991) Systematic revision of the Asian catfish family Pangasiidae, with biological observations and descriptions of three new species. *Proceedings of the Academy of Natural Sciences of Philadelphia*, **143**, 97-144.

Sadili D. (1999) Marketing of pangasiid catfishes in Java and Sumatra, Indonesia. *Proceedings of the mid-term workshop of the Catfish Asia project*, this volume.

Sakai K., Nomura M., Takashima F. & Oto H. (1975) The over-ripening phenomenon of rainbow trout: II. Changes in the percentage of eyed eggs, hatching rate and incidence of abnormal alevins during the process of over-ripening. *Bull. Jpn. Soc. Sci. Fish.*, **41**, 855-860.

Saidin T., Othman A.A. & Sulaiman M.Z. (1988) Induced spawning techniques practised at Batu Berendam, Melaka, Malaysia. *Aquaculture*, **74**, 23-33.

Springate J.R.C., Bromage N.R., Elliott J.A.K. & Hudson D.L. (1984) The timing of ovulation and stripping and their effects on the rates of fertilization and survival to eying, hatch and swim-up in the rainbow trout (*Salmo gairdneri* R.). *Aquaculture*, **43**, 313-322.

Woynarovich E. & Horvath L. (1980) The artificial propagation of warm-water finfishes - A manuel for extension. *FAO Fish. Techni. Pap.*, **201**, 183 p.

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