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Ovulation rate, latency period and ova viability after GnRH- or hCG-induced breeding in the Asian catfish *Pangasius hypophthalmus* (Siluriformes, Pangasiidae)

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Abstract − Over a 3-year period at the Sukamandi station (West Java, Indonesia), 107 *Pangasius hypophthalmus* females were selected on the basis of a modal oocyte diameter greater than 1.0 mm and treated with either Ovaprim (n = 97) or hCG (n = 10) to induce oocyte maturation and ovulation. The two hormonal treatments led to similar results in terms of ovulation rate (88 and 90 %), hatching rate (72 ± 25 and 82 ± 11 %) and relative fecundity (171 000 ± 73 000 and 128 000 ± 60 000 ova·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 and 11 h at 28–29 °C). The ovulation time was therefore difficult to predict accurately in this species. The assessment of the viability of ova retained in the ovarian cavity after ovulation showed that the process of overripening occurs rapidly in *P. hypophthalmus*. The overall quality of ova began to decline as early as 2 h after ovulation and, after 3 h, hatching rates decreased and the proportion of deformed larvae increased significantly in comparison to those observed at the time of ovulation. In some individual females this process occurred even more rapidly, with a sharp decrease in hatching rates between 1 and 2 h post-ovulation. The duration of ova survival did not appear to depend on the type of hormone treatment used to induce ovulation (Ovaprim or hCG). For optimized gamete management in hatcheries, it is therefore recommended to check carefully the females for the occurrence of ovulation (between 3 and 11 h after the last hormone injection, depending on water temperature) and to strip and fertilize the eggs less than 2 h thereafter. © 2000 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

reproduction / ovulation / ova overripening / catfish / aquaculture / Indonesia

Résumé − Taux d'ovulation, temps de latence et viabilité des ovules après induction de l'ovulation avec GnRH ou hCG chez le poisson-chat asiatique *Pangasius hypophthalmus* (Siluriformes, Pangasiidae). Au cours d'une période de 3 ans à la station de pisciculture expérimentale de Sukamandi (Java Ouest, Indonésie), 107 femelles de *Pangasius hypophthalmus* sélectionnées sur la base d'un diamètre modal de leurs ovocytes supérieur à 1 mm, ont été traitées par injection soit d'Ovaprim (n = 97), soit de gonadotropine chorionique humaine (hCG ; n = 10), pour induire la maturation ovocytaire et l'ovulation. Les deux traitements hormonaux ont conduit à des résultats similaires en termes de taux d'ovulation (88 et 90 %), de pourcentages d'éclosion (72 ± 25 et 82 ± 11 %) et de fécondité relative (171 000 ± 73 000 et 128 000 ± 60 000 ovules·kg^{-1}, avec Ovaprim et hCG respectivement). La période de latence, entre la dernière injection d'hormone et l'ovulation, est corrélée négativement à la température de l'eau, mais montre des variations importantes à une même température selon les femelles (par ex. entre 5 et 11 h à 28–29 °C). De ce fait, le moment de l'ovulation est difficile à prévoir de façon précise chez cette espèce. L'analyse du temps de survie des ovules retenus dans la cavité ovarienne après l'ovulation montre que le processus de vieillissement (surmaturation des ovules) intervient rapidement chez *P. hypophthalmus*. La qualité moyenne des ovules commence à décroître dès la deuxième heure après l'ovulation et, après 3 h, le taux d'éclosion chute et la proportion de larves déformées augmente significativement en comparaison de ceux observés au moment de l'ovulation. Chez certaines femelles, cette évolution est encore plus rapide, avec une chute brutale du taux d'éclosion entre 1 et 2 h après l'ovulation. La durée de survie des ovules apparaît indépendante du type de traitement hormonal utilisé pour induire l'ovulation (Ovaprim ou hCG). Pour une gestion optimisée des gamètes en écloserie, il est donc recommandé d'examiner régulièrement les femelles pour détecter le moment de l'ovulation (entre 3 et 11 h après la dernière injection d'hormone, en fonction de la température de l'eau) et de récolter et féconder les ovules moins de 2 h après. © 2000 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

reproduction / ovulation / qualité des ovules / poisson-chat / aquaculture / Indonésie

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1. INTRODUCTION

Pangasius hypophthalmus (Sauvage, 1878) [senior synonym of *P. sutchi* (Roberts and Vidthayanon, 1991)] is the most common cultured pangasiid catfish throughout 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 relies increasingly on artificial propagation techniques (Csavas, 1994). The species does not spawn spontaneously in captivity. It was successfully induced to breed for the first time in Thailand in 1966 after treatment with catfish pituitary gland suspension (Potaros and Sitasit, 1976). It was then introduced into Indonesia from Thailand in 1972, where its hormone-induced breeding was reported for the first time by Hardjamulia et al. (1981). Since that time, 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 can be fully optimized (Subagja et al., 1999). Poor egg quality and low hatching rates are amongst the difficulties most often reported by fish farmers.

The delay between hormone injection and ova collection is a key factor in the success of reproduction techniques involving hormone-induced ovulation and artificial fertilization in fish (Harvey and Carolsfeld, 1993; Bromage and Roberts, 1995). Delayed collection of gametes after ovulation leads to overripening of ova which can result in low fertilization rates, increases in the number of deformed embryos, and increased mortality rates for embryos and larvae (Sakai et al., 1975; McEvoy, 1984; Springate et al., 1984). After ovulation, the viability of ova – estimated by the time lapse between ovulation and the moment at which the initial quality of ova begin to decline – varies according to species. This time lapse ranges from 6 to 30 days in the rainbow trout (Bry, 1981; Springate et al., 1984) to a few hours in the majority of oviparous teleosts studied, including several catfish species (Woynarovitch and Horvath, 1980; Formacion et al., 1995; Legendre and Otémé, 1995; Legendre et al., 1996).

Therefore, the aim of this study was to assess the timing of ovulation after hormonal treatment (latency period) and the viability of ova retained in the ovarian cavity 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 induction of 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 fertilization.

2. MATERIALS AND METHODS

2.1. Fish origin and maintenance

The broodfish used descended from the *P. hypophthalmus* stock initially introduced from Thailand in 1972 and were 3–5 years old and 2.3–6.4 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.

2.2. Latency period and ovulation rate

Over a 3-year period, a total of 97 *P. hypophthalmus* females were induced to breed with Ovaprim and ten others with hCG at the Sukamandi station. Although Ovaprim is generally used in Indonesian hatcheries, responses were also tested with hCG for comparison.

The mature females were chosen after intraovarian biopsy on the basis of a modal diameter of oocytes greater than 1.0 mm (1.13 ± 0.05 mm, on average). The initial position of the germinal vesicle was also examined before treatment, on a sample of about 50 oocytes cleared in Serra's fluid (60 % ethanol, 30 % formalin, 10 % acetic acid, by volume). Selected males were producing milt at stripping. Oocyte maturation and ovulation were induced with two successive Ovaprim [1 mL of Ovaprim® (Syndel Laboratories, Canada) contains 20 µg of GnRHa (D-Arg6, Trp7, Leu8, Pro9, Net) and 10 mg Domperidone intramuscular injections of 0.3 and 0.6 mL·kg^{-1} female BW given at an 8-h interval. These doses and the timing of injection were chosen because they are commonly used in Indonesian fry production units. The same procedure was applied with hCG (Organon, France) except for doses, fixed at 500 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} given at the same time as first injection of females. During the treatment the broodfish were held in hapas installed in ponds or in large concrete tanks. The mean water temperature 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 the second injection. Nevertheless, ovulation had already occurred in some females at the moment of first attempted stripping; in such cases the latency period could not be known precisely. When ovulation was observed, ova were collected by complete stripping, weighed and immediately fertilized. A sample of ova was also weighed to the nearest 0.1 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

Table I. Ovulation rate, relative fecundity and hatching rate for 97 *Pangasius hypophthalmus* females treated with Ovaprim.

Females treated	No ovulation n = 12; 12 %	Partial ovulation n = 8; 8 %	Full ovulation n = 77; 80 %
		n = 85; 88 %	
Relative fecundity (ova·kg^{-1} × 1 000)	–	6.4 ± 5.1 [1–14] n = 7	171 ± 73 [33–317] n = 66
Hatching rate* (%)	–	13 ± 17 [0–36] n = 7	72 ± 25 [3– 9] n = 55

* Only hatching rates estimated within 1 h after ovulation are considered. Mean ± sd; [range].

of 1/5) to prevent spermatozoa activation by dilution with urine, then preserved at 5 °C for subsequent fertilizations (Eeckhoutte, 1996).

The quality of ova was evaluated from hatching rates obtained with replicated batches of 200–300 eggs fertilized with 0.2 mL of diluted sperm. This corresponded approximately to 6.10^6 spermatozoa per ova. Spermatozoa activation was obtained by addition of 10 mL fresh water. After 1 min of gentle stirring, eggs were rinsed to remove excess milt and transferred for incubation to 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 fertilization.

2.3. Viability of ova after ovulation

The duration for which ova remain viable in the ovarian cavity after ovulation was studied between May and December 1997 in 13 females treated with Ovaprim and three others treated with hCG. The schedule of injections and doses used were the same as those indicated above. The mean water temperature ranged between 27.1 and 28.8 °C during the latency period.

From 5 h after the second injection, females were checked every hour to follow the process of oocyte maturation, on samples collected by intraovarian biopsy and fixed in Serra's fluid. 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 intraovarian biopsy was also used for fertilization trials until ovulation occurred. The moment at which the first ova could be obtained by stripping was considered as the ovulation time (0 h) 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 subsamples of 200–300 eggs, fertilized and incubated following the general procedure presented above. In each case, the sperm from two males was pooled for fertilization. Two successive strippings of the males were performed in order to prevent possible effects of a lowering in spermatozoa fertilizing ability. The sperm collected at the first stripping served to fertilize ova collected up to 3 h after ovulation, then males were stripped again for subsequent fertilization tests. During the 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.

Table II. Ovulation success, relative fecundity and hatching rate for 10 *Pangasius hypophthalmus* females treated with hCG.

Females treated	No ovulation n = 1	Full ovulation n = 9
Relative fecundity (ova·kg^{-1} × 1 000)	–	128 ± 60 [35–210]
Hatching rate* (%)	–	82 ± 11 [59–95]

* Hatching rates are estimated within 1 h after ovulation. Mean ± sd; [range].

2.4. Statistical analysis

Hatching percentages and the proportion 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 stabilize the residual variance.

3. RESULTS

3.1. Latency period and ovulation rate

In all females, the germinal vesicle of oocytes was in a non-peripheral position (central or slightly eccentric) just prior to the first hormone injection. The two hormonal treatments led to similar success in inducing oocyte maturation and ovulation of *P. hypophthalmus*. The percentages of ovulated females were 88 and 90 % with Ovaprim and hCG, respectively. It should be noted, however, that incomplete ovulation was observed in 8 % of the females treated with Ovaprim. These partial responses corresponded to females from which only a small quantity of ova could be collected, while large residual ovaries could be felt by hand in the abdomen, even after repeated stripping at intervals of a few hours. The eggs obtained from such females were of poor quality and led to low hatching rates (13 ± 17 %). In contrast, the quantity and quality of ova collected in other ovulated females were generally

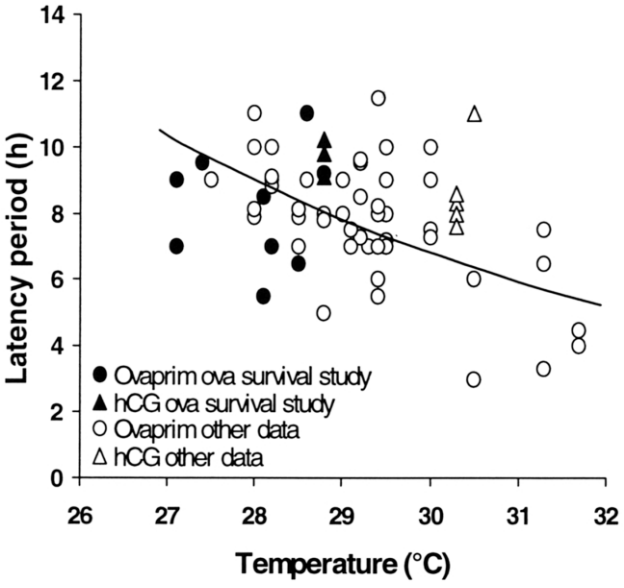

Figure 1. Latency period to ovulation, after the second Ovaprim or hCG injection, as a function of temperature in *Pangasius hypophthalmus* females. The regression line between latency period and water temperature is drawn for females treated with Ovaprim.

high, and similar for fish treated with Ovaprim or hCG (*tables I* and *II*). No relationship was found between the initial 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 59 of the 97 females treated with Ovaprim and in eight of the ten females treated with hCG. The latency period between the second hormone injection and ovulation ranged from 3 to 11 h at a mean temperature of broodfish maintenance varying between 27.1 and 31.7 °C (*figure 1*). For fish treated with Ovaprim, an inverse relationship between the latency period (LP) and water temperature (T) was found ($LP = 2582840\ T^{-3.779}$, $r = -0.505$, $F_{1,57} = 19.511$, $P < 0.0005$). Nevertheless, a high variability was observed in the latency response of the different females even at a same temperature (e.g. between 5 and 11 h at 29.4 °C for seven females in a same trial). 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 (*figure 1*). At a same temperature, the latency response was not related to the initial oocyte diameter of the different females used.

3.2. Viability of ova after ovulation

In the ova survival study, the latency period to ovulation ranged between 5 and 11 h depending on the females (*figure 1*). The eggs obtained at the ovulation time (0 h) 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 intraovarian biopsy were in GVBD, but had not fully achieved their maturation as indicated by very low hatching rate obtained after fertilization (*figure 2*). The changes in hatching rate and proportion 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 relationship was observed between hatching percentages and proportions of deformed larvae. The highest hatching percentages and lowest proportions of deformed larvae were observed at ovulation time (0 h) and 1 h after. As early as 2 h post-ovulation a noticeable decline in hatching rates was observed, and after 3 h, hatching rates decreased and the proportion of deformed larvae increased significantly in comparison to those observed at 0 and 1 h after ovulation. When ova were stripped 5 h or more after ovulation, mean hatching rates did not exceeded 15 % and almost all of the hatched larvae were considerably 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 h 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 a longer delay (*figure 4*). The fact that high hatching percentages were obtained for a long period of time in one female indicated that the rapid decline in egg quality observed in most females corresponded effectively to overripening of ova and not to a lowering of sperm fertilizing ability.

4. 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 was high and reached similar values with these two hormonal preparations (88 and 90 %, respectively). These results are close 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 a dopamine antagonist.

A high variability was observed in the quantity of ova collected (from 33 000 to 317 000 ova·kg^{-1}) after induced breeding with either Ovaprim or hCG. After treatment with hCG, Cacot (1999) also observed a large range of relative fecundity in *P. hypophthalmus* females cultured in ponds or floating cages in Vietnam (from 5 500 to 170 200 ova·kg^{-1}). So far, the origin of

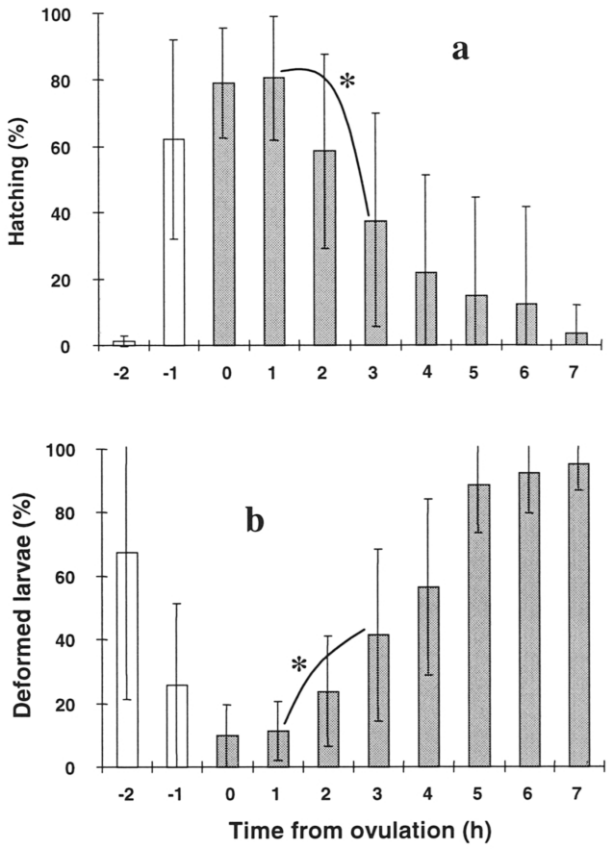

Figure 2. Changes in hatching rate (a) and proportion of deformed larvae (b) as a function of time before (bars in white) and after (bars in grey) ovulation. Means for ten *Pangasius hypophthalmus* females treated with Ovaprim (water temperature: 27.1–28.6 °C). The moment of ovulation (0 h) is considered here as the first time at which ova can be obtained by stripping. Before ovulation, the egg quality was assessed on samples taken by intraovarian biopsy. Vertical bars refer to standard deviation (*$P < 0.05$).

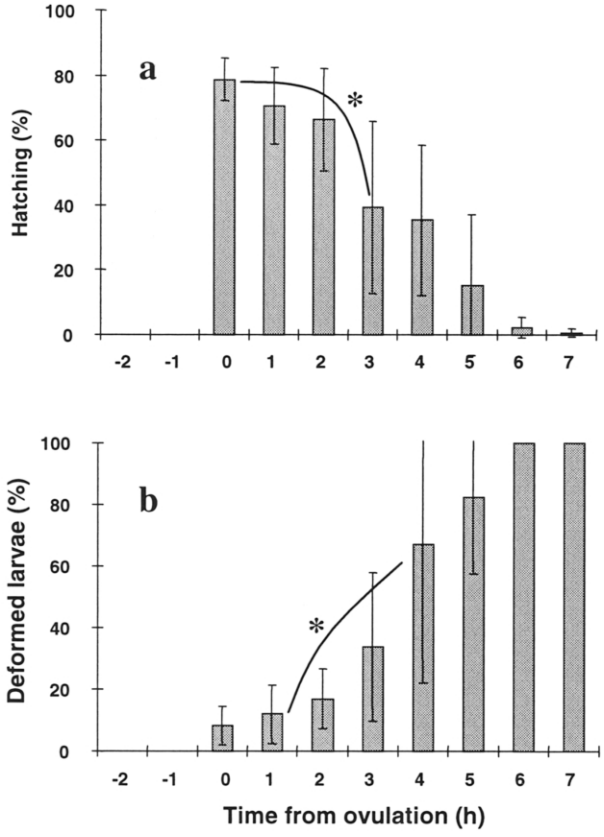

Figure 3. Changes in hatching rate (a) and proportion of deformed larvae (b) as a function of time after ovulation. Means for three *Pangasius hypophthalmus* females treated with hCG (water temperature: 28.8 °C). The moment of ovulation (0 h) is considered here as the first time at which ova can be obtained by stripping. Vertical bars refer to standard deviation (*$P < 0.05$).

such a high individual variability, which could not be related to fish size or age, remains poorly understood and requires further investigations.

As in other fish species (Lam, 1982; Bromage and Springate, 1995), an inverse relationship between the latency period and water temperature was observed in *P. hypophthalmus*. 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. This suggested that the 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 the sexual stage before hormonal treatment, in order to reduce the variance in latency period.

The assessment of the viability of ova retained in the ovarian cavity after ovulation showed that the process of overripening occurs rapidly in *P. hypophthalmus*. The overall quality of ova begun to decline as early as 2 h after ovulation and, after 3 h, hatching rates decreased and the proportion of deformed larvae increased significantly in comparison to those observed at the moment of ovulation. In some individual females, this process occurred even more rapidly, with a sharp decrease in hatching rates between 1 and 2 h post-ovulation. The temporal changes in ova quality after ovulation were very similar in fish treated with Ovaprim or hCG, and did not appear to depend on the hormonal treatment used. After hCG-induced ovulation in four *P. hypophthalmus* females in Vietnam, Campet (1997) observed that the initial mean quality of ova decreased significantly 3 h after ovulation, but the overripening process occurred earlier (less than 2 h 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 the moment of ova

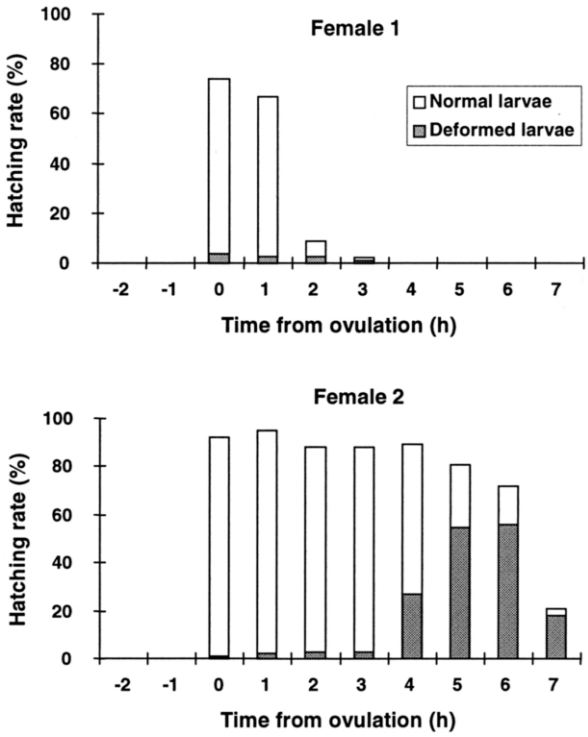

Figure 4. Extreme situations observed in hatching rate and proportion of deformed larvae as a function of time after ovulation (0 h) in two *Pangasius hypophthalmus* females treated with Ovaprim (water temperature: 28.0–28.5 °C).

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 h after the last hormone injection; ovulated fish are then stripped and ova fertilized while non-ovulated females are returned directly to the ponds (unpublished inquiries on fish farms). From the present results showing the variability of latency period and the short viability of ova, it appears clearly that such a procedure may lead either to discarding of fish still in the course of oocyte maturation or to collection of ova already engaged in the process of overripening.

In practice, it is therefore recommended to inspect the females carefully for the occurrence of ovulation (between 3 and 11 h after the last hormone injection, depending on water temperature) and to strip and fertilize the eggs lessα than 2 h thereafter.

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