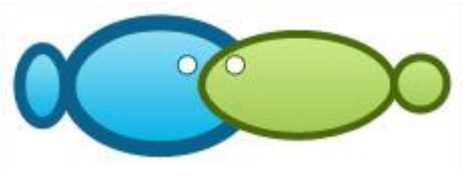

Effect of *in vivo* aging of silver carp (*Hypophthalmichthys molitrix*) ova on offspring viability rates at Beni Mellal Hatchery, Morocco

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Abstract. The quality of ova of female silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844) is of considerable importance in seed production operations. The quality influences the tolerance of ova to remain viable after ovulation and before fertilization. This tolerance significantly affects offspring viability rates (fertilization rate, embryo survival rate, larval and fry survival rates). Ideally, ova should be extracted and fertilized immediately after ovulation. However, in practice, several females reach ovulation simultaneously, which requires delaying the extraction or fertilization of ova. Each manipulation lasts about 15 min, and the time between fertilization of the ova of the first and last female varies from 60 to 90 min, leading to the aging of ova. To avoid unnecessary manipulations, an experiment was conducted at the Deroua Beni Mellal station to quantify this aging and determine the maximum duration of ova storage *in vivo* while maintaining acceptable offspring viability rates. In this study, ova from 15 females (3.2 to 9 kg live weight) were extracted at different time intervals after ovulation (0, 15, 30, 45 and 60 min). The results show that ova viability is acceptable up to 20 min after ovulation. Beyond this duration, the quality declines rapidly.
Key Words: embryos, fertilization, fry ovulation, larvae, quantification.

Introduction. In fish farming, it is crucial to understand the process of aging of eggs after ovulation, especially when spawning is triggered by hormonal treatments. This aging process occurs rapidly, particularly in common carp (*Cypinus carpio*) and grass carp (*Ctenopharyngodon idella* Vallenciennes), as demonstrated by Horvath (1978), Suzuki (1980) and Kharroubi et al (2002). To optimize reproductive success, the fish farmer must be able to accurately determine the exact time of ovulation. It is also essential to know how much time is available after ovulation to harvest and fertilize the eggs before they deteriorate. Poor management of this timing can lead to decreased ova quality and v iability, compromising offspring viability rates (fertilization rate, embryo survival rate, larval survival rate, and fry survival rate) and overall reproductive success. Knowledge of these parameters allows for optimization of interventions and ensures efficient and high-quality production in fish farming.

To date, the factors and mechanisms governing *in vivo* ova aging in cyprinids, as well as in other teleosts, remain poorly studied. The changes that occur during *in vivo* ova retention have been very little explored. However, studies have shown that prolonged stay of ova *in vivo* after ovulation results in significant biochemical changes, affecting various viability rates of the offspring (Sakai et al 1975; Suzuki 1975; Hirose et al 1979; Statova et al 1982; Billard et al 1986).

It is important to note that during storage of eggs *in vivo* under anaerobic conditions, lactic acid accumulates and pH decreases. As a result, the integrity of the vitelline envelope is compromised due to a rearrangement of lipids and structural proteins. In the ovaries, water from the ovarian fluid enters the ova, causing their

collapse (Zhukinskiy & Kim 1980). For this reason, it is recommended to fertilize ova immediately after ovulation.

In practice, during artificial reproduction operations at the Deroua station in Morocco, the injected females ovulate simultaneously, which requires delaying the stripping and fertilization of the ova of some females. Each manipulation, including the anesthesia of the fish, the extraction of the ova and the fertilization, lasts about 15 minutes. Thus, the time elapsed between the fertilization of the ova of the first female and that of the last can reach up to 90 minutes. During this interval, the ova risk aging in the abdomen of the fish. In this context, this study conducted at the Deroua station, with the main objective to evaluate the potential effects of the aging of silver carp ova subjected to prolonged retention in the ovarian cavity, in terms of the viability rate of the offspring. The study also aims to quantify the maximum duration of ova storage *in vivo* while maintaining acceptable viability rates of the offspring.

Material and Method

Place of study. The Deroua station is located in the national forest, 20 km southwest of the city of Beni Mellal, Morocco. It is located in a semi-arid region with a mild winter climate (latitude: 32°20' North, longitude: 6°45' West, altitude: 428 m). The station is based on clayey-sandy formations dating from the Mio-Plio-Quaternary (Emberger 1930).

Infrastructure and material. The nursery ponds and hatchery at the Deroua station are modern, with equipment carefully designed and installed to optimize reproductive and technical performance.

Water supply source. The water supply for the Deroua station hatchery comes from a well exploiting the Beni Moussa water table. According to Stickeny (1979), groundwater is highly recommended for hatcheries because it is clear and free of solid elements and aquatic predators harmful to the development of ova, larvae and fry. The ponds, for their part, are supplied by water from the irrigation canals coming from the Bine El-Ouidane dam, allowing to optimize yields and to use all the ecological niches of the water column.

Experimental protocol. To study the effect of *Hypophthalmichthys molitrix* (Valenciennes, 1844) silver carp ova aging in the ovarian cavity *in vivo* on the viability of the offspring (fertilization rate, embryonic survival rate, larval and fry survival rate), 15 females of 3.2 to 9 kg of live weight were artificially reproduced.

At the time of ovulation, a batch of ova of 100–150 g was extracted and fertilized immediately at T0. The extraction of the other batches of ova took place every 15 minutes (0, 15, 30, 45 and 60 minutes). The prolonged stay of the ova *in vivo* in the abdominal cavity was ensured by sewing the genital opening after each extraction. Each batch was incubated in a 30 L conical incubator at a temperature of 22–24°C. Dissolved oxygen was maintained at 7 mg L^{-1}, pH at 7.3, and water flow in the incubators ranged from 0.6 L min^{-1} during the first six hours of incubation to 2.5 L min^{-1} over the next 30 hours. A 25 mL pipette was immersed in the incubator, allowing ova and embryos to rise by capillary action in the pipette. This method allows the entire water column in the incubator to be crossed, thus ensuring a homogeneous and representative sample of the batch of incubated eggs. To improve reproducibility, three samples were taken from each incubator to determine each viability rate.

Determination of offspring rates. The incubation of silver carp eggs lasts between 30 to 36 hours at a temperature of 20 to 24°C. 12 hours after fertilization, mesoderm segmentation begins. At this stage, fertilized and unfertilized eggs are easily recognizable under a binocular microscope. The fertilization rate (FR) was determined 12 h after fertilization. At this stage, fertilized and unfertilized eggs are easily recognized under a binocular microscope.

FR = (Number of fertilized ova / Total number of ova) x 100

The embryonic survival rate (ESR) is determined 24 h after fertilization. At this stage, viable embryos are recognizable by their black eyes and somites, while aborted embryos have an elongated white mass on the yolk, which begins to disintegrate.

ESR = (Number of viable embryos / Total number of embryos) x 100

The larval survival rate (LSR) is determined 36 h after fertilization. At this stage, samples are separated into dishes with a specific number of embryos. The embryos, by very energetic movements, pierce the shell. Live larvae show upward swimming, while dead larvae are inert.

TSL = (Number of live larvae / Total number of larvae at the 36-h stage) x 100

The fry survival rate (FSR) is determined 56 h after fertilization. In the same boxes, viable larvae remain mobile and evolve to the fry stage, while dead larvae remain inert.

FSR = (Number of viable fry / Initial total number of larvae) x 100

Statistical analysis. One-way analysis of variance (ANOVA I) was applied to assess the effect of *in vivo* egg retention (15, 30, 45 and 60 min after ovulation) on the following parameters: fertilization rate (FR), embryonic survival rate (ESR), larval survival rate (LSR) and fry survival rate (FSR).

Correlations between the different viability indicators (FR, ESR, LSR and FSR) just after ovulation and under in vivo storage conditions (0, 15, 30, 45 and 60 min) were examined using linear regression analysis.

Results. Table 1 presents the mean viability rates of offspring (FR, ESR, LSR, and FSR).

Table 1

Viability rates of offspring (FR, ESR, LSR, and FSR)

Retention time (min)	*Viability rate of different indicators (%)*			
	FR	*ESR*	*LSR*	*FSR*
0	46.01667	50.07333	52.375	49.14833
15	40.17	38.34667	51.54167	47.35
30	36.44667	30.82667	43.146	42.52
45	29.95833	22.525	41.634	41.65333
60	23.7	12.49	39.18667	39.78333

Note: FR - fertilization rate; ESR - embryonic survival rate; LSR - larval survival rate; FSR - fry survival rate.

Figures 1, 2, 3 and 4 below present the results of offspring viability (FR, ESR, LSR and FSR) obtained in this study in 15 female silver carp at T0 and after aging of the ova in the ovarian cavity at different time intervals (15, 30, 45 and 60 minutes). In order to quantify the maximum duration of ova preservation *in vivo* while maintaining acceptable offspring viability rates, we worked with the averages of the offspring viability rates obtained for the 15 females Table 1.

Figure 1 presents the evolution of the average FR. Although there are fluctuations between the rates presented by each female, it is observed that, in general, they evolve in the same way. However, the longer the aging period, the more the fertilization rates decrease in a very significant way ($p<0.001$).

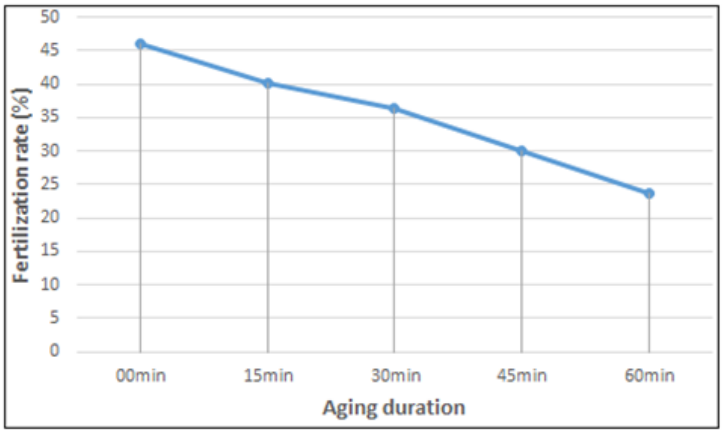

Figure 1. Evolution of means of fertilization rates after *in vivo* aging of ova.

As for Figure 2, it shows the averages of the ESR, which follow a similar evolution to that of the FR with a significant difference ($p<0.05$).

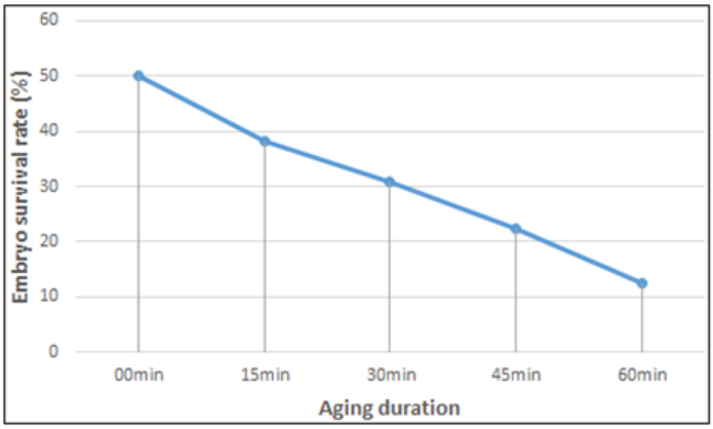

Figure 2. Evolution of means of embryo survival rates after *in vivo* aging of ova.

For Figures 3 and 4, the calculation of the LSR and FSR was carried out after separating the embryos into samples placed in boxes with a precise number. It can be seen that the effect of aging persists, leading to a decrease in the LSR and FSR as the aging period in the ovarian cavity increases significantly ($p<0.05$).

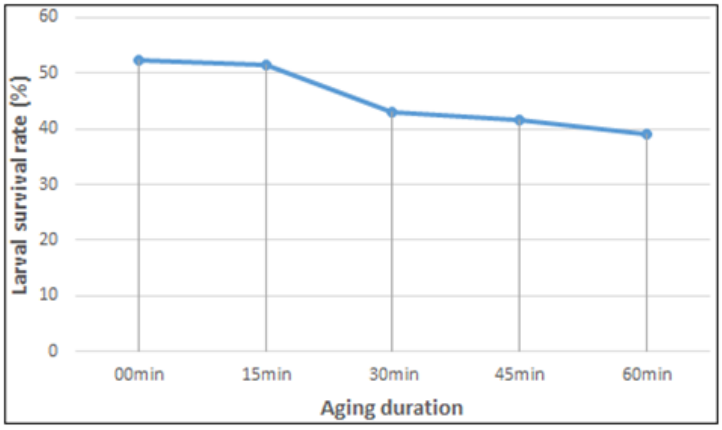

Figure 3. Evolution of means of larval survival rates after *in vivo* aging of ova.

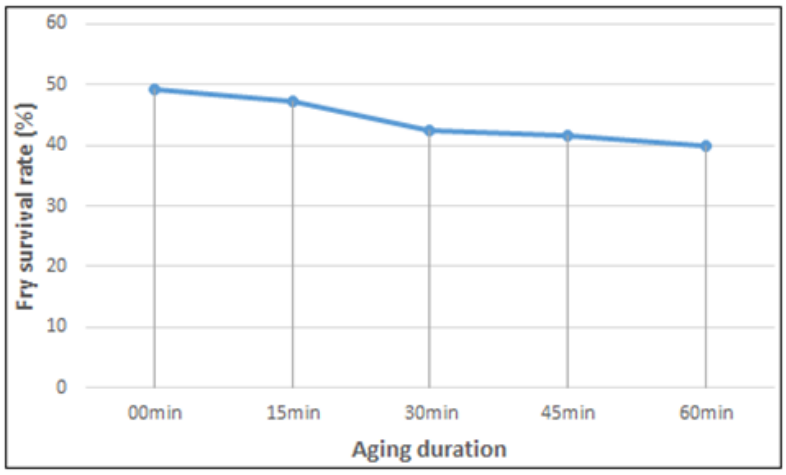

Figure 4. Evolution of means of fry survival rates after *in vivo* aging of ova.

Table 2 presents the results of the analysis of variance (ANOVA I) for each parameter (FR, ESR, LSR, FSR) to assess the effect of *in vivo* egg retention (15, 30, 45, and 60 min after ovulation). These results show that for FR and ESR, the p-values are very low, indicating that retention time strongly influences these parameters. For LSR and FSR, the effect is significant, although less pronounced than for FR and ESR. Overall, the retention time of eggs has a significant effect on all viability indicators.

Table 2

Analysis of variance (ANOVA I) for each parameter (FR, ESR, LSR, FSR)

Parameter	*p-value*	*Conclusion*
FR	0.000257	Significant effect of time.
ESR	0.000155	Very significant effect of time.
LSR	0.011044	Significant effect of time.
FSR	0.005625	Significant effect of time.

Correlations between indicators. Table 3 presents the correlation coefficients (r) and their statistical significance (p-values) between the viability parameters (FR-ESR, FR-LSR, FR-FSR, ESR-LSR, ESR-FSR, and LSR-FSR). These results indicate that all viability indicators are strongly correlated with each other, with highly significant positive relationships. The strongest correlation is observed between LSR and FSR (r=0.9935), suggesting that these two parameters evolve almost identically.

Table 3

Correlations between offspring viability parameters (FR, ESR, LSR, and FSR)

Parameters	*Coefficient (r)*	*p-value*	*Relationship*
FR-ESR	0.9957	0.0003	Very strong and positive
FR-LSR	0.9300	0.0220	Strong and positive
FR-FSR	0.9488	0.0138	Very strong and positive
ESR-LSR	0.9403	0.0173	Very strong and positive
ESR-FSR	0.9643	0.0081	Very strong and positive
LSR-FSR	0.9935	0.0006	Very strong and positive

Linear regressions (time vs indicators). Table 4 presents the linear regression equations calculated for each parameter as a function of time. FR and ESR show rapid decreases with time (slopes of -0.37 and -0.61, respectively). LSR and FSR decrease more slowly, but their trends remain significant. The high R^2 values (close to 1) indicate

that time well explains the variation of the indicators. In conclusion, the linear regressions show progressive decreases in parameters over time.

Table 4

Linear regressions (time vs indicators)

Parameter	*Equation*	*p-value (Time)*	*R^2*
FR	FR = 46.23 - 0.37 · Time	0.00026	0.993
ESR	ESR = 49.05 - 0.61· Time	0.00015	0.995
LSR	LSR = 52.83 - 0.24 · Time	0.01104	0.914
FSR	FSR = 48.98 - 0.16 · Time	0.00563	0.945

Discussion. The results obtained in this study highlight the impact of *in vivo* egg retention time in the ovarian cavity of silver carp on offspring viability rates (FR, ESR, LSR, and FSR). The analysis of variance (ANOVA I) showed a significant effect of storage time on all the studied indicators, with a particularly marked influence on FR and ESR ($p<0.001$), confirming that prolonged retention of eggs *in vivo* leads to a rapid degradation of their ability to be fertilized and develop normally. These results corroborate the findings of Zarski et al (2017), who reported a decline in egg quality with post-ovulation time in several cyprinid species.

The rapid decrease in FR and ESR observed in our study also suggests a rapid deterioration in egg quality after ovulation. This deterioration can be attributed to biochemical and hormonal changes in the follicular fluid and alterations in the egg membrane (Bobe & Labbé 2010). Furthermore, the reduction in ESR after 30 minutes of retention is consistent with the work of Linhart et al (2016), who showed that prolonged egg retention in cyprinids increased the rate of embryonic malformations.

The strong correlations observed between the various viability indicators, notably between FR and ESR ($r=0.9957$) and between LSR and FSR ($r=0.9935$), suggest that fertilization and embryonic development processes are interdependent and influenced by similar environmental factors. This close relationship between embryonic survival and larval survival was also described by Kucharczyk et al (2019), who observed similar results in common carp (*Cyprinus carpio*).

The linear regression analysis indicates a progressive and significant decrease in the studied parameters with increasing retention time. The high R^2 values (>0.91) for all the studied variables confirm that egg aging time is a major explanatory factor for the observed variations. In particular, the steeper slopes of the FR and ESR regressions (-0.37 and -0.61, respectively) indicate an increased sensitivity of these parameters to egg aging. These trends suggest that egg retention primarily affects the early stages of development. These results reinforce the conclusions of Migaud et al (2013), who suggested that egg viability is compromised after 30 minutes of retention in freshwater fish with external fertilization.

The results of this study reveal a progressive decrease in the viability rates of the offspring (FR, ESR, LSR, and FSR). Whenever we increase the aging time of the ova in the ovarian cavity (15, 30, 45, and 60 minutes), ANOVA reveals a significant effect of storage time on all indicators, with a marked influence on FR and ESR ($p<0.001$). This could be explained by the introduction of aging in this cavity, where a set of biochemical reactions occur that are at the origin of the changes that affect the biochemical constituents of the ova, which causes the degradation of their quality. As shown in other studies (Hirose et al 1977; Cerda et al 1995; Evans et al 1996), these biochemical changes are due to the storage environment of the ova, which, when inadequate, leads to their degradation. It follows that the quality of the ova is closely linked to the duration of their retention in the ovarian cavity. Therefore, it is recommended in hatcheries to fertilize the ova immediately after ovulation to minimize the effect of aging.

A significant variation ($p<0.001$) was also observed between different batches of ova from females who received the same hormonal treatment and incubated under identical conditions, although they were extracted and fertilized at the same time intervals. This variation could be explained by intrinsic differences in the quality of ova

from each female, which affects their tolerance to aging. Indeed, poor quality ova give poor viability rates even when fertilized immediately after ovulation. Craik (1985) and Billard et al (1986) suggest that variability in egg quality is influenced by both hereditary and non-hereditary factors. The former are intrinsic and non-modifiable, while the latter are related to environment and nutrition, and therefore modifiable. In this study, modifiable factors included prolonged *in vivo* aging and storage conditions. Furthermore, viability rates can also be affected by hygienic conditions in aquaria and nursery tanks (Folch et al 1957; Cerda et al 1994). Poor hygiene can result in total mortality of larvae or fry. Furthermore, the care of broodstock, as well as the expertise of personnel, are essential for successful silver carp reproduction.

The results show that ova fertility remains acceptable when their stay in the ovarian cavity does not exceed 20 minutes. Beyond this time, they begin to deteriorate, leading to a drop in the viability rates of the offspring. It is therefore crucial to collect and fertilize ova before they deteriorate.

These observations confirm our ongoing research on optimizing the reproductive performance of silver carp, which showed that ova tolerate *in vitro* storage conditions better than *in vivo*, as biochemical changes are less rapid in the *in vitro* environment.

Thus, to optimize the reproductive performance of silver carp, we recommend collecting and fertilizing ova within 20 minutes of ovulation, taking care to maintain good hygienic conditions. It is also advisable to schedule hormonal injections in a staggered manner on small groups of females, in order to avoid synchronization of spawning, which helps to reduce the aging time of the ova and improve the viability of the offspring.

Conclusions. This study has provided a better understanding of the phenomenon of *in vivo* aging of silver carp ova. It appears that the ova of this species must be collected and fertilized within 20 minutes after ovulation. Beyond this time, their prolonged stay in the ovarian cavity considerably reduces their ability to be fertilized, leading to very low viability rates of the offspring (fertilization rate, survival rate of embryos, larvae and fry). In addition, the operations become laborious and very costly. These results highlight the importance of careful planning of artificial reproduction operations. In practice, it is crucial not to handle several females simultaneously to avoid synchronized spawning. It is recommended to stagger hormonal injections in order to be able to collect the ova from each female immediately after ovulation. Any delay of more than 20 minutes results in a deterioration in their quality, making them unsuitable for fertilization. The objective of artificial reproduction operations in hatcheries is to produce a sufficient number of fry, with optimal viability rates, to stock the nursery ponds. To achieve this objective, it is essential to space out hormonal injections of females in order to avoid synchronization of spawning and to prevent over maturation of ova *in vivo*.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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