

Early Ontogenetic Development of the Amur Sleeper *Perccottus glenii* Dybowski, 1877, an Alien Invasive Fish Species Outside Its Natural Range

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Accepted April 11, 2019

Published online April 26, 2019


Issue online July 18, 2019

Original article

BŁAŻEJEWSKI M., KRÓL J., HLIWA P. 2019. Early ontogenetic development of the Amur sleeper *Perccottus glenii* Dybowski, 1877, an alien invasive fish species outside its natural range. *Folia Biologica (Kraków)* 67: 61-68.

The progressive expansion of the Amur sleeper in Europe may have serious consequences for the native ichthyofauna and results in the depletion of autochthonous biodiversity. It can be manifested by food competition with native taxa and the displacement from natural habitats or breeding grounds. Studies of the biology of reproduction of alien species reflect their adaptivity to new environmental conditions. Therefore, the aim of the study was to analyse changes during Amur sleeper embryogenesis, a representative alien invasive fish species. Mature oocytes of the Amur sleeper just after fertilisation had an elongated, symmetrically-ellipsoid shape, with a large perivitelline space. Their average total length was 3.53 ± 0.14 mm, and the total width was 1.35 ± 0.11 mm. On the vegetative pole of the egg, a sticky filiform plait with a height and width of approximately 1 mm, helpful during oviposition to the substrate, was noted. The yolk of the eggs had a homogeneous dark yellow structure with an average diameter of 1.1 ± 0.1 mm. Cleavage took place during the first day of incubation, while gastrulation was initiated around the 38thD of development and finished on the 60thD when the embryo's body was elongated and clearly divided into a head, trunk and caudal parts. The onset of the eye-colour stage correlated with the phase of the embryo moving away from the yolk sac, which occurred in the 96th hour of development (76thD). On the other hand, hatching with the head part facing forward (preceded by a 180-degree inversion of the embryo) began on the 115thD of development. The mean total length of the larvae, unable to swim freely and feed, was 2.95 ± 0.24 mm. An analysis of the specificity of the Amur sleeper's early ontogenetic development and a description of its sensitive stages may contribute to an understanding of the spread of this alien fish species.

Key words: Amur sleeper, embryogenesis, alien invasive species, eggs, larvae.

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The Amur sleeper *Perccottus glenii* Dybowski, 1877, a representative of the Odontobutidae family (Perciformes) native to eastern Asia is one of several invasive alien fish species that appeared in Polish freshwater ecosystems at the end of the 20th century (WITKOWSKI & GRABOWSKA 2012). The causes of the Amur sleeper's spread in central Europe are mainly anthropogenic and assisted by the irresponsible actions of aquarists and anglers/fishermen, who have released specimens that were kept in aquaria as ornamental fish into the wild or used them as bait for the commercial catching of predatory fish (CAMBRAY 2003; KATI *et al.* 2015). In its natural range, the Amur sleeper spawns partially from May to July at a water tem-

perature of 15-20°C (KOTTELAT & FREYHOF 2007), which results from the asynchronous maturation of the oocytes in the ovaries. The adopted reproductive strategy, increasing the overall fecundity of this species, is a specific response to unfavourable conditions, including a change in the spawning location/substrate type and water temperature fluctuations that negatively affect offspring survival (BRYLIŃSKA *et al.* 1979). With regards to invasive fish, a change in fecundity may become the first sign of the modification of their reproductive biology, triggered by a change in living conditions in the newly colonised areas (MILLS *et al.* 2004; NYESTE *et al.* 2017). The Amur sleeper reaches maturity at the age of 1-3 years when its body

length is between 5 and 6 cm (KOTTELAT & FREYHOF 2007). The largest individuals are primarily found outside their natural range, where they reach a length of 15-25 cm and a weight of 260-270 g (BOGUTSKAYA & NASEKA 2002). Amur sleeper eggs are primarily laid on substrate such as submerged rocks or roots that have been previously cleaned by a male, who takes care of the embryos during incubation by providing them with adequate oxygenation and chasing away intruders (ADAMEK & SIDDIQUI 1997). Mature oocytes of the Amur sleeper have an elongated, symmetrically-ellipsoid shape. On one of the poles, they have a special cone-shaped projection used for anchoring the eggs, which are deposited by a female, to the substrate (WAŁOWSKI & WOLNICKI 2010).

The progressive expansion of the Amur sleeper and its gradually increasing population numbers may have serious consequences for the native ichthyofauna and resulted in the depletion of autochthonous biodiversity. This can be manifested by food competition with native taxa, feeding on their eggs/larvae, or the displacement of native fish species from natural habitats or breeding grounds (RESHETNIKOV 2003). Another serious threat posed by the presence of invasive fish species, such as the Amur sleeper, is the possibility of transmitting exotic diseases and parasites that were previously not found in European waters, which can eliminate autochthonous fish species (MIERZEJEWSKA *et al.* 2010; RESHETNIKOV & SCHLIEWEN 2013; ANTAL *et al.* 2015; SOKOLOV *et al.* 2015). Environmental and reproductive flexibility supports the Amur sleeper in establishing numerous populations in a relatively short time (BOGUTSKAYA & NASEKA 2002). Amur sleepers can affect the food web of freshwater bodies by dramatically reducing the fish biodiversity (RESHETNIKOV & FICETOLA 2011). This is because native fish species are displaced from their natural habitats, which may lead to their complete disappearance, mainly in small, isolated water bodies (WOLNICKI & KOLEJKO 2008).

Amur sleeper succession, observed in inland waters for several years, should be monitored, according to Commission implementing regulation (EU) 2016/1141, as well as their impact on the ichthyofauna structure being inhabited. With regards to allochthonous fish, observations of their reproductive behaviour and possible changes in the biology of reproduction are of particular importance since they reflect their adaptivity to new environmental conditions. The physicochemical water parameters during reproduction and embryogenesis, differing from those occurring in their natural range, and the diversity of food resources during the post-embryonic period are factors that strongly determine offspring survival (larvae/fry). However, in the process of the expansion of alien species and

the establishment of new populations in colonised areas, embryonic development is of key importance in determining the effectiveness of natural spawning, and thus, in affecting the possibility for increasing the population size (HLIWA 2010).

The aim of this study was to indicate the critical stages in the embryogenesis process of the Amur sleeper, which determines either its normal or impaired early ontogeny and could determine its further spread in freshwaters.

Materials and Methods

This study was conducted with Amur sleeper eggs obtained from the semi-natural reproduction of matured breeders caught in the lowland Włocławek Reservoir, located in the middle course of the Vistula River, close to Brwilno Dolne (52°33'28.8''N 19°33'14.0''E) (Fig. 1) with the permission of the Local Ethics Committee (No. 17/2015 from March 25 2015). Seven males (mean body length 9.2±0.5 cm) and six females (mean body length 10.7±0.8 cm) were caught by electrofishing using an IUP-12 device (225/300 V, 6A, 50-90 Hz, RADET Ltd., Poland) in the spring of 2017 in the littoral zone of the reservoir in water temperature about 12°C. The site from which the spawners were caught is characterised by a slimy and rocky bottom, densely overgrown with macrophytes such as *Ceratophyllum demersum* L., *Elodea canadensis* Michaux and *Potamogeton* sp. After being caught, the fish were transported to laboratories of the Centre for Aquaculture and Ecological Engineering at the Faculty of Environmental Sciences, University of Warmia and Mazury in Olsztyn. They were placed in tanks with a capacity of approximately 0.5 m³ in a recirculation system with monitored physicochemical parameters of the water. After a several-day quarantine, the fish were photothermally stimulated to obtain the intended values of abiotic parameters, i.e. a photoperiod of 15L:9D and a temperature of 19.0±0.2°C (HLIWA 2010). Next, successful spawning of several breeders was noted, using PVC tubes with a length of approximately 30 cm and a diameter of 3-4 cm as an artificial substrate. After spawning, PVC tubes with eggs attached to their walls were transferred to aquaria with a capacity of approximately 30 litres and incubated at a stable water temperature of 19.0°C, pH of 7.6 and oxygen concentration close to 8.5 mg/dm³. Throughout the whole incubation period (from fertilisation to larvae hatching), egg samples were taken every 24 hours and preserved in 2.5% glutaraldehyde solution. The photographic documentation and the measurements of selected morphometric parameters were performed with an

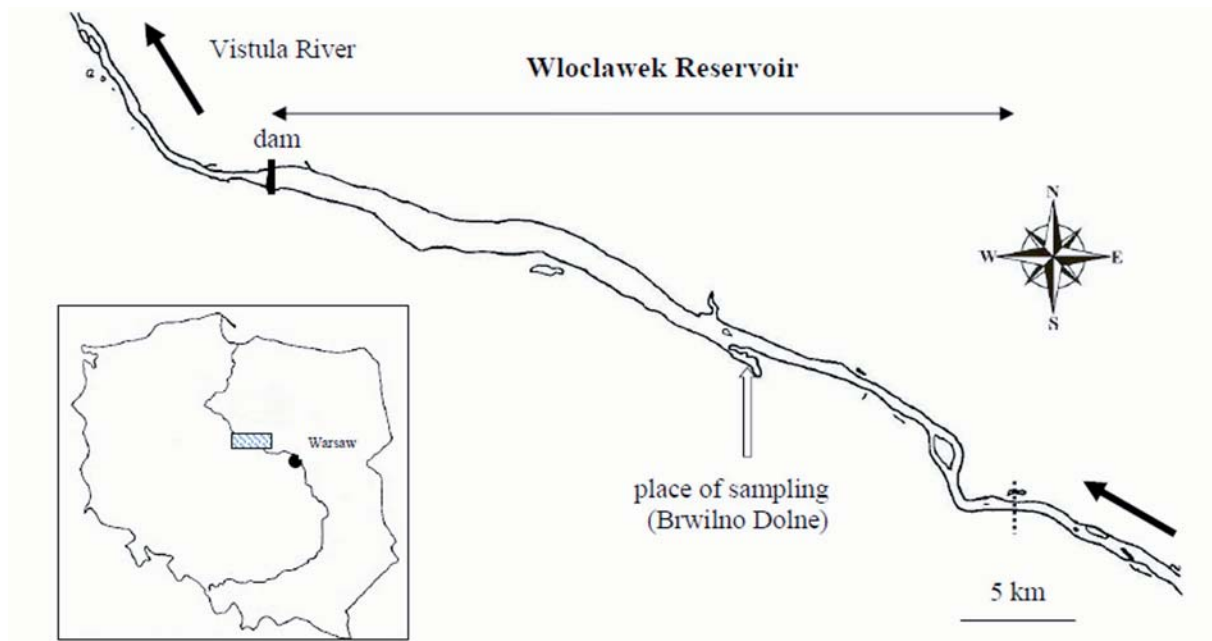


Fig. 1. Catching site of Amur sleeper spawners within the Vistula River (Poland).

accuracy of 0.01 mm using a LEICA MDG33 stereoscopic microscope (LEICA Microsystems Ltd, Switzerland) coupled with image analysis software JENOPTIK ProgRes C3 (JENOPTIK Optical System, Germany). For morphometric investigations, 30 eggs or freshly hatched larvae were taken from each sample. They were transferred to a Petri dish and photographed in the lateral position. Based on the digitised micrographs, the following characteristics were measured:

- total diameter of unfertilised egg – TDue (mm),
- volume of unfertilised egg – TVue (mm³), calculated as: $\frac{4}{3}\pi r^3$, where: r – egg radius,
- total length of fertilised egg – TLe (mm),
- total width of fertilised egg – TWe (mm),
- total volume of fertilised egg – TVe (mm³), calculated as: $\frac{4}{3}\pi nk^2$, where: k – short axis of egg radius; n – long axis of egg radius,
- diameter of fertilised egg yolk – DYe (mm),
- total length of freshly-hatched larvae – TLI (mm),
- total height of larvae head – THl (mm),
- diameter of larvae eye – DEl (mm),
- diameter of larvae yolk sac – DYl (mm),
- total volume of larvae yolk sac – TVl (mm³), calculated as: $\frac{4}{3}\pi r^3$, where: r – yolk sac radius (Fig. 2a, b, c).

The parameters TVue, TVe, TVl were calculated using the formulas presented by BLAXTER and HEMPLE (1963). All measured values were expressed as means \pm SD.

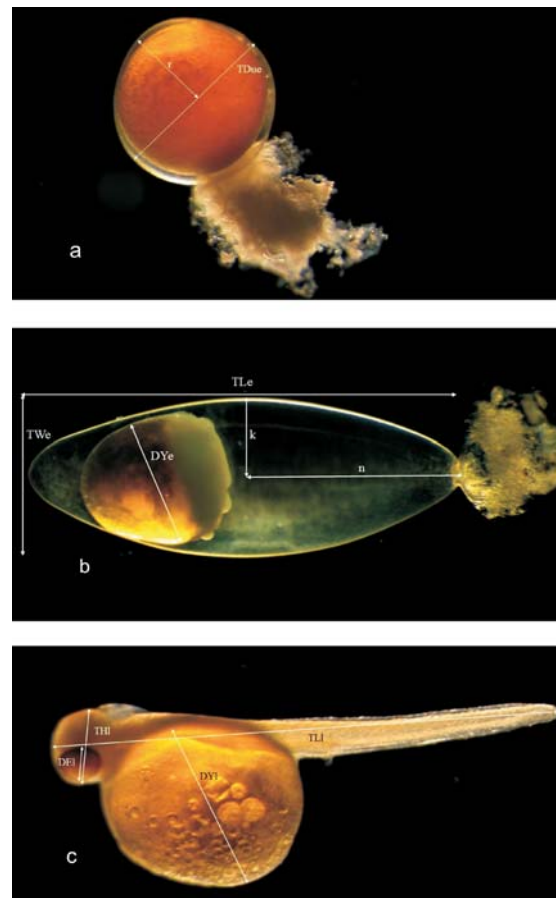


Fig. 2a-c. Morphometric landmarks measured in the egg and yolk sac larvae of the Amur sleeper.

Table 1

Characteristics of eggs and freshly hatched Amur sleeper larvae (n = 30).

Parameter	Range	Mean ± SD
total diameter of unfertilized egg (mm)	0.89-1.30	1.2±0.02
total length of egg (mm)	3.15-3.82	3.53±0.14
total width of egg (mm)	1.20-1.69	1.35±0.11
total volume of unfertilized egg (mm ³)	0.83-1.12	0.91±0.03
total volume of egg (mm ³)	7.93-10.70	8.79±0.72
diameter of egg yolk (mm)	0.66-1.51	1.1±0.1
total length of larvae (mm)	2.34-3.35	2.95±0.24
total height of larvae head (mm)	0.40-0.65	0.5±0.04
diameter of larvae eye (mm)	0.19-0.30	0.24±0.04
diameter of larvae yolk sac (mm)	0.70-1.16	1.07±0.07
total volume of larvae yolk sac (mm ³)	0.41-0.74	0.64±0.09

SD – standard deviation.

Results

Mature, unfertilised Amur sleeper eggs were spherical in shape and coloured from yellow to light orange. Their mean diameter was 1.2 ± 0.02 mm and the mean volume was estimated to be 0.91 ± 0.03 mm³ (Table 1). During spawning, the eggs were attached by a female to the substrate and the anchoring element was a characteristic cone-shaped projection comprised of several sticky threads about 1 mm in length (Fig. 3). After fertilisation and swelling, the eggs changed to become symmetrically-ellipsoid in shape, with a mean length of 3.53 ± 0.14 mm, width of 1.35 ± 0.11 mm and volume of 8.79 ± 0.72 mm³ (Table 1). The yolk, with a mean diameter of approximately 1.1 ± 0.1 mm, had a homogeneous, compact structure, and the perivitelline space, due to the unusual and varied shape, was relatively spacious. The ratio of the size of unfertilised eggs and eggs after fertilisation and swelling was approx. 1:10, with the volume of the yolk 10-15% of its total size (Fig. 3, Table 1).

The cleavage process in the Amur sleeper was initiated immediately after fertilisation, when the embryo shifted to the animal pole opposite to the substrate attachment. After several hours, numerous blastomeres were clearly visible as a result of serial mitotic divisions. Finally, about 15°D of the blastodisc that is typical of most Teleostei fishes was noted (Figs 3, 4a). In the next stage of embryonic development, i.e. gastrulation, which takes place between 20°D and 38°D, a gradual slide of the blastopore over the yolk was observed. At this stage, the embryonic disc started to elongate to

form the initially weakly-outlined body of the embryo (Figs 3, 4b). At 39°D, when the blastopore was closed, until 56°D, organogenesis was observed. During the formation of the embryo, the presence of the dorsal notochord and the primordia of the central nervous system, i.e. the brain, were visible (Figs 3, 4c). On the fourth day of development, which corresponds to 75°D, the embryo's body was divided into the cephalic and thoracic parts, and the caudal part was clearly separated from the yolk. In the cephalic part, the brain case was also visible, along with the optic cups and the formation of the otic vesicles (Fig. 4c). On the next day, the total length of the embryo's body increased by approximately one third. Between 75°D and 94°D, the embryonic finfold appeared and the primordia of the pectoral fins was observed. The head and the tail had already clearly separated from the yolk. At this stage, the division of somites occurred in the dorsal and abdominal parts of the embryo's body (Figs 3, 4c). The eyed stage, i.e. the appearance of pigment in the eyes, was noted on the sixth day of embryonic development, which corresponds to approximately 110°D. At this time, internal organs, such as the beating heart, nostrils and developing jaws, were visible in the cephalic part, and the anus was visible in the thoracic part (Figs 3, 4d). The hatching phase was preceded by violent embryo movements, with a 180-degree rotation of their bodies inside the egg chorion. Properly formed Amur sleeper embryos hatched with the cephalic part facing forward.

The mean length of newly-hatched Amur sleeper larvae was 2.95 ± 0.24 mm, and their relatively spacious

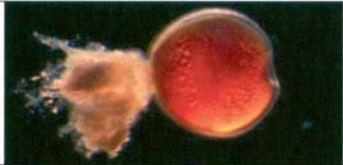

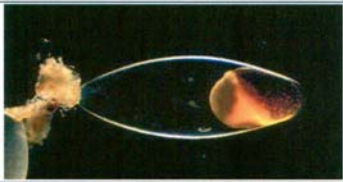
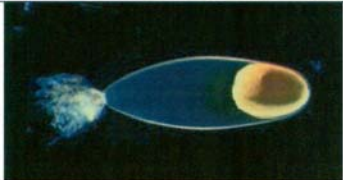
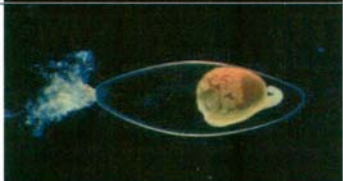



Day of embryonic development/ degree-day (°D)	Photos	Description
0 / 0		a mature, spherical oocyte after fertilisation changes its shape to ellipsoidal with a visible, characteristic cone-shaped projection on the vegetative pole, used to anchor the egg to the substrate
1 / 1 – 19		the cleavage stage, the appearance of the blastodisc
2 / 20 – 38		gastrulation through covering of the blastopore (epiboly)
3 / 39 – 56		blastopore closure, the beginning of the formation of an embryo's body, the appearance of the dorsal notochord and the primordia of the brain
4 / 57 – 75		a clear division of the embryo's body into the cephalic, thoracic and caudal parts, deflection of the caudal part from the yolk; a distinctly formed brain cased with developed optic vesicles
5 / 76 – 94		formation of a fin-fold, a division of somites in the dorsal and abdominal parts of body; the emergence of the pectoral fins primordia
6 / 95 – 114		the eyed stage (visible a pigment cells in eyes); clearly visible beating heart, nostrils at the top of the head and an isolated lower jaw (mandible)
7 / 115 – hatching		the embryo makes violent movements with a 180-degree rotation of their bodies inside the egg chorion; due to the effects of hatching glands, the egg chorion broken and the embryo leaves outside with the cephalic part facing forward

Fig. 3. Characteristics of the Amur sleeper's embryonic development stages.

yolk sac had a mean diameter of 1.07 ± 0.07 mm and a total volume of 0.64 ± 0.09 mm³ (Table 1). The whole lower side of the head was separated from the yolk sac, and the mouth opening in the form of a hole was

perforated (Fig. 3). On the next day, at 19°C, the larvae resorbed the contents of the yolk sacs, while lying mostly at the aquarium bottom, moved over short distances and started to feed using exogenous food.

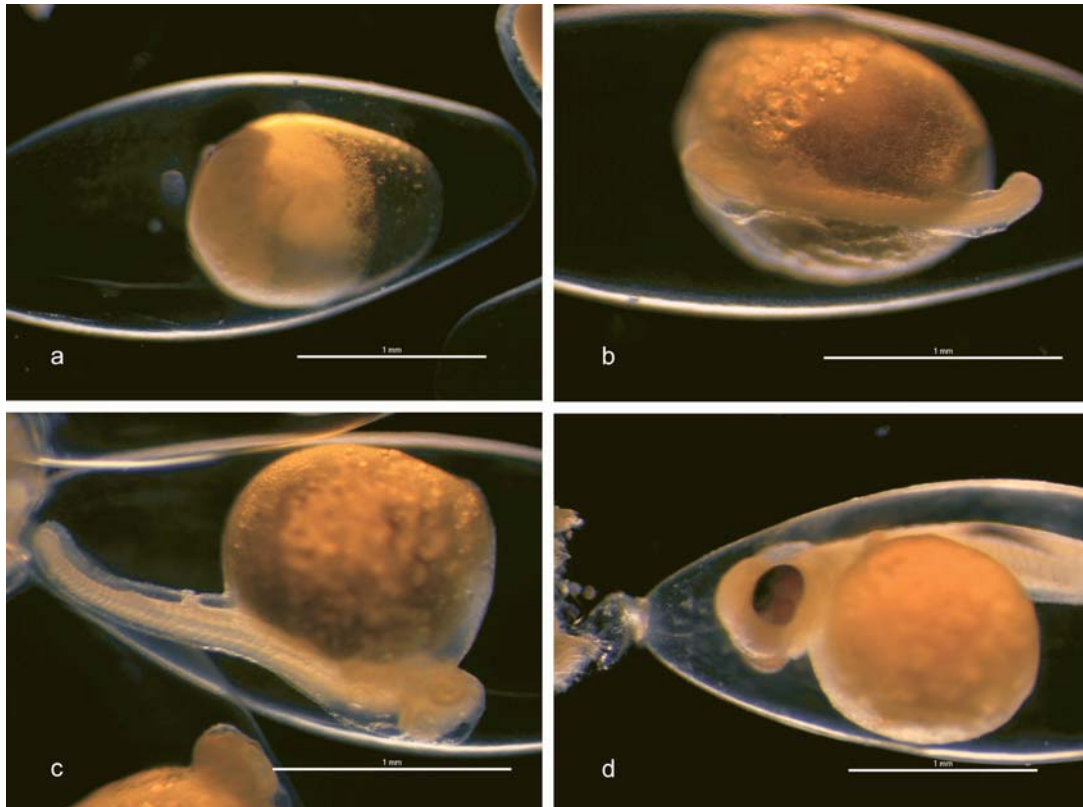


Fig. 4a-d. Development of the Amur sleeper: a – late stage blastoderm, high cell count stage imaged 15°D, greater cell accumulation on the yolk surface (arrowhead) and individual cells indistinguishable; b – embryo 75°D (4 dpf) at the blastopore, optic cup stage, with a visible double-layered optic cup (arrowhead); c – appearance of a finfold and the primordia of the pectoral fins, head and tail clearly separated from the yolk, the division of somites has occurred in dorsal and abdominal parts of the embryo, d – eyed stage embryo 110°D with completion of retinal pigmentation (arrowhead). Scale bar = 1mm.

Discussion

The succession of the Amur sleeper in new habitats primarily includes the specific reproductive determinants associated with the prolonged multi-spawning period, and the active care of the eggs by males (KOTTELAT & FREYHOF 2007; GRABOWSKA *et al.* 2011; NYESTE *et al.* 2017). The diversity of spawning substrates preferred by the Amur sleeper during reproduction, i.e. rocks, roots, macrophytes, and even anthropogenic waste (bottles, pipes, tires), is another element that supports the spread of this alien invasive fish species. This ability and opportunism were confirmed during our experiment, in which pieces of PVC pipes were used as the spawning substrate for breeders.

The results of the present study indicate that the mean length and width of eggs deposited by Amur sleeper females, characterised by an elongated, symmetrically-ellipsoid shape, were similar to the values described by KOSTRZEWA *et al.* (1999). On the vegetative pole, a filiform projection was used to anchor the eggs to the substrate during spawning; it was placed similarly to the element observed in round goby (*Neogobius melanostomus*)

eggs, another representative alien invasive fish species occurring in Poland (BONISŁAWSKA *et al.* 2014), and in the endemic Korean spotted sleeper, *Odontobutis interrupta*, which is phylogenetically closely related to the Amur sleeper (PARK *et al.* 2014). The yolk of the eggs accounted for only part of the egg volume, with a homogeneous structure, a dark yellow colour and a mean diameter of 1.1 ± 0.1 mm. This is in contrast with for example whitefish, in which the yolk occupies c. 95% of the total volume of the germ cell and the small perivitelline space separating the yolk and the chorion (SREETHARAN *et al.* 2015).

Significant differences in the time between particular embryonic development stages, presented previously by SIKORSKA *et al.* (2007), were noted. For example, the separation of the caudal part of the embryos' bodies from the yolk sac was noted at 41°D post-fertilisation, while similar changes were observed in the experiment between 65°D and 75°D of development. Another incoherent element of both experiments is the total duration of embryonic development which, according to SIKORSKA *et al.* (2007), lasts for 12-13 days at a temperature of 18°C, and for approximately 10 days at a tem-

perature of 20°C. On the other hand, in our study, embryogenesis lasted about 120°D, resulting in the incubation of eggs at a constant water temperature of 19°C after only seven days; this is convergent with the data from YANG *et al.* (2012). However, our results of Amur sleeper embryogenesis indicate few significant differences to the early ontogenesis of another member of the Odonotobutidae family – *Odontobutis interrupta*. The main difference is the much shorter time for larvae hatching, which occurred at the stage when the jaws were opening in the developing embryos, whereas in *O. interrupta*, it is about 326°D until the complete metamorphosis of larvae and the acquisition of the ability to actively swim and engage in exogenic feeding (PARK *et al.* 2014).

During the first 3-4 days of development, due to the ellipsoidal shape and extensive perivitelline space of the eggs, embryos had laxity and were lying on the pole opposite to the threads anchoring the eggs to the substrate. In the next stages, the body length and weight of embryos gradually increased and began to change their position and move towards the central part. The specific element distinguishing the final phase of the Amur sleeper's embryogenesis was the 180-degree turn of the embryos within the chorion, which occurred immediately prior to hatching, with the cephalic part facing forward. This is an important, critical moment of the embryogenesis process, which is likely to determine the effectiveness of natural reproduction, and thus, the spread into new habitats.

In our study, the recorded size of newly-hatched larvae, with an average total length of 2.95±0.24 mm, was significantly different from the results presented by SIKORSKA *et al.* (2007), in which the larvae reached a length of approx. 4.47-4.75 mm immediately after hatching. These data are comparable with the size of freshly-hatched round goby larvae (average 5.00±0.32 mm). Despite the larger size and more advanced development of larvae during the hatching time, the mean volume of the yolk sac of round goby larvae is larger than that of the Amur sleeper, measuring 0.81±0.22 mm³ and 0.64±0.09 mm³, respectively (BONISŁAWSKA *et al.* 2014). Additionally, they were characterised by well-developed jaws and a relatively small yolk sac, which was already resorbed on the second day after hatching. These differences between the appearance of particular stages of embryonic and larval development may be due to different experimental conditions, and document the environmental flexibility of the Amur sleeper. The observed changes may have been due to the different physicochemical parameters of the water, such as the temperature, hardness, pH value or oxygen concentration. Furthermore, the fast and simultaneous hatching of larvae in our study, contrary to

SIKORSKA *et al.* (2007), could be due to the artificially-forced water flow; it was caused by the aeration in the PVC tubes, which was required to avoid hypoxia and death of the incubated eggs. In natural conditions, these activities are carried out by the male.

The presented data of the early ontogenetic development of the Amur sleeper confirmed the predispositions for its mass spread in new ecosystems. Multi-spawning, an extension of the spawning season, males guarding the eggs and the plasticity of the embryo/larvae reaction to environmental factors may be the special adaptations that promote the progressive success of invasions.

Acknowledgments

This study was financed by state funds appropriated to University of Warmia and Mazury in Olsztyn under the code no. 18.610.011-110 and project financially co-supported by Minister of Science and Higher Education in the range of the program entitled “Regional Initiative of Excellence” for the years 2019-2022, Project No. 010/RID/2018/19, amount of funding 12.000.000 PLN”.

Author Contributions

Research concept and design: M.B., P.H.; Collection and/or assembly of data: M.B., J.K., P.H.; Data analysis and interpretation: M.B.; Writing the article: M.B., P.H.; Critical revision of the article: M.B., P.H.; Final approval of article: P.H.

Conflict of Interest

The authors declare no conflict of interest.

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