Study of the embryogenesis of *Dunhevedia crassa* King, 1853 (Cladocera: Chydoridae) and a comparison of embryonic instar durations in different cladocerans

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ABSTRACT

The aim of this work is to study the embryonic development of a common tropical cladoceran *Dunhevedia crassa* King, 1853 (Anomopoda: Chydoridae) and compare the durations of embryonic instars and growth during embryogenesis in small-sized chydorids and other cladocerans. The sequence of events in the embryogenesis of Dunhevedia females was studied by means of in vitro observation in the laboratory at 28.5°C. We found that the general pattern of the sequence of events in chydorid embryogenesis is similar to that in other anomopods. However, the relative durations of the embryonic instars I and II+III are markedly short, while that of instar IV is long (delayed) when compared with other cladocerans. The observed pattern of ontogenesis in chydorids may therefore be apomorphic within the cladocerans and could reflect a neotenic evolution. Another peculiar trait of Dunhevedia is the very small increase of the size of the embryo during embryogenesis, which is correlated with a very large size of the egg relative to the mother. Therefore, our study revealed some peculiarities in the ontogenesis of small-sized chydorids, which could be a result of family-specific evolution.

Key words: Cladocera, Chydoridae, embryology, embryonic instars, ontogenesis.

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Embryogenesis of Dunhevedia (Chydoridae) using a 60 µm mesh size plankton net. The pond was dominated by Hydrilla verticillata (L.f.) Royle. The water temperature measured near the bottom, at the middle depth and near the surface of the locality was similar throughout the shallow waterbody, i.e. about 28.5°C. Females of Dunhevedia crassa were picked out from the samples in the laboratory immediately after their collection. Two series of experiments were carried out. In the first series, females with a couple of eggs were picked out, and then each individual was cultivated separately in a small Petri dish (5 cm in diameter). They were allowed to develop naturally until the neonates were released. Afterwards, each embryo was reared and monitored individually every 2 h in order to study the process until it reached maturity and laid a new egg. In the second series, females with eggs at an early stage of their development were used. A couple of eggs was removed from brood pouches and placed onto slides with a depression, containing filtered pond water. They were monitored in vitro at temperatures that were close to pond conditions and corresponded to room temperature in the lab (28.5°C) every hour, photographed under the microscope and recorded using a video camera. Some line drawings were made using a camera lucida mounted on a microscope. The terminology used for explaining events in embryogenesis (e.g. the morphological structures and the embryonic instars) is according to Kotov and Boikova (1998, 2001). Four instars are found in the development of the Ctenopoda. In the Anomopoda, the third instar is embryonised, therefore they have a first instar (I), second plus third instar (II+III), and a fourth instar (IV). Although the development was also observed in the females with eggs in the brood pouch, the relatively non-transparent valves of the females make such observations difficult. Thus the present results are focused mainly on the in vitro observations of the separated embryos. Five individuals per time were followed and the procedure was repeated five times to complete the development series. The study was carried out on parthenogenetic females, no gamogenetic/sexual stages were examined.

To compare the duration of the embryonic instars in different Cladocera we used previous papers with detailed descriptions of the embryogenesis: Kotov and Boikova (1998) (ctenopods Sida crystallina and Diaphanosoma brachyurum), Kotov and Boikova (2001) (anomopod Daphnia hyalina), and Boikova (2008) (haplopod Leptodora kindtii). We recorded the duration of the instars I, II+III (in the anomopods this is a single instar, in the non-anomopods it is a sum of duration of instars II and III) and IV, and calculated the relative duration of each instar in percentage of the complete time of embryogenesis.

RESULTS

Development of a parthenogenetic egg of Dunhevedia crassa in vitro at 28.5°C

Laying of eggs

The neonates take about 47-55 h to be mature and to lay the first brood, a couple of parthenogenetic eggs. Parthenogenetic females can be easily recognised before laying eggs because, as opposed to gamogenetic females of Chydoridae, they have symmetrical, paired ovaries (Van Damme and Dumont, 2006). At that time, large as well as tiny brownish yolk granules appear inside the paired ovaries (Fig. 1A), which are continuously increasing in size during the last 2-3 h before laying the eggs. When the granules are about to generate new eggs, there are 3-4 large fat drops and plenty of small granules. A remarkable event starts when females slow their movements, which indicates that they are ready to molt. About 7-10 min after mol-
ting, the laying of new eggs starts. The egg mass with yolk granules is transferred to the sack-like brood chamber (Fig. 1B). The couple of newly released eggs are oblong-oval in shape and dull-brownish (Fig. 1B), then take on a more subspherical shape. At this stage, the 3-4 fat drops are fused together starting from 4 drops to 3 drops, 3 drops to 2 drops until there is only a single large yolk granule (Fig. 1C and 1D). This entire series of events happens in about the first 10-15 min of the egg development.

**Instar I**

By 0.5 h after laying, the egg is subspherical and filled with yolk (Figs. 1D and 2A-B).

By 4 h (Figs. 2A-C and 3B) the yellow-brownish egg is almost unchanged, the yolk granules totally fill the egg; the central area is occupied by the fat drop.

By 5 h (Figs. 2D-E and 3C) three zones are obviously recognised; the outer zone is relatively transparent, slightly

![Fig. 2. Development of embryo in Dunhevedia crassa. A) At 4 h of development; B) cleavage at 4.5 h; C) at 5 h; D-E) at 6.5 h; F-G) at 7.5 h; H) shedding of first membrane at 9 h; I) at 10 h; J) at 11 h; K) at 12.5 h; L) at 14 h; M-N) at 15 h, dorsal and ventral view, respectively; O) at 16 h; and P) at 18 h. pl-pII=thoracic limbs I-II; cp=carapace; ocl=ocellus; ep=eye pigment.](image-url)
yellow-brownish; the second zone is intensely brownish because it is filled with yolk granules, forming a yolk portion; the third or central zone is occupied by a subspherical, brown-yellowish fat drop. During this time, cleavage, blastulation, gastrulation and germ band formation take place in the outer zone. Unfortunately, some details of these events cannot be observed clearly using light microscopy, as previously noted by Kotov and Boikova (2001).

By 6.5 h (Figs. 2F and 3D), differentiation of the head appendages starts with the appearance of a transverse lateral furrow on each side of the embryo, a rudiment of antenna II. There are two membranes; the first (external) egg membrane and the second (hatching) egg membrane. During this stage embryos prepare to molt, thus the first membrane flakes off and definitely separates from the second membrane. In addition, the fat drop and yolk granules are fused to form larger granules.

By 7.5 h (Fig. 2G), the embryo is oval and the intestine

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**Fig. 3.** First 17 h of development of the embryo of *Dunhevedia crassa*. A) egg immediately after laying; B) at 1 h; C) at 4 h; D) at 6.5 h; E-H) successive stages of thoracic segment formation at 6.5 h; I-L) successive stages of carapace formation at 81 h; M) casting off the external egg membrane at 9.5 h; N) at 1 h; O) at 1 h; P) at 1 h. No scale bar was taken. yg=yolk granules; ml=first membrane (egg membrane); fd=fat drop; yp=yolk portion; mII=second membrane (hatching membrane); ant I=antenna I; ant II=antenna II; pdf=postabdomen furrow; cp=carapace; P1-P5=thoracic limbs 1-5; oc=ocellus.
becomes visible. Rudiments of antenna II are visible for the first time and shortly after their differentiation, the rudiments of antenna I, the mandible and the labrum appear. After the maxillary zone differentiation, the thoracic segments and limbs are differentiated one after another (Fig. 2G and 3E-H), as described by Kotov and Boikova (1998, 2001). The dorsal organ appears also at this time. Paired carapace rudiments appear dorsally as a posterior projection of the head (Fig. 3I-L), as suggested by Fryer (1996) and Kotov (1996), in contrast to the opinion of Walossek (1993). The embryo has a posterior furrow (Fig. 2G), forming a paired rudiment of the postabdomen.

By 9 h the casting off of the external egg membrane takes place (Figs. 2H and 3M). This molt takes only 20-40 sec. The embryo quickly absorbs water, almost resembling a bubble being blown. Then, the embryo gradually takes on a swollen shape. When the elastic outer membrane is beyond the limit of turgor pressure, it splits along the embryo midline. Finally, the membrane curls up and floats off.

**Instar II+III**

By 10-15 h (Figs. 2I-N and 3N) there are five thoracic limbs, although the fifth limb is poorly discriminated (Fig. 2I and 2J). A group of yolk granules presented in the cephalic region (Fig. 2K) forms three blocks (Fig. 2L). The two rudiments of the carapace spread out and expand to the dorso-lateral areas. Thoracic limbs obviously elongate, and endopodites and exopodites become differentiated (Fig. 2M-N).

By 13-14 h the ocellus pigment appears (Figs. 2O and 3O).

By 16-18 h (Figs. 2P and 3P), rudiments of setae appear on the thoracic limbs. Postabdominal claws also appear at this time, which is evidence of their homology with setae. A cluster of pigment in the compound eye appears in the eye capsule, and then transforms into a single compound eye. Unfortunately, because of the small size of Dunhevedia embryos, the starting point of eye formation can be hardly observed. By this time, the carapace covers almost the whole body.

At 19-20 h (Fig. 4A and 4B), the yolk granules fuse into an anterior yolk block and a posterior yolk block. The second molt takes place due to the first movements of antennae II and postabdomen. However, we are not able to observe this event in detail because of the small size of the embryo of Dunhevedia; in Daphnia two membranes (second and third) are cast off at the same time (Kotov and Boikova, 2001).

**Instar IV**

At 20.5 h the embryo is somewhat compressed laterally because of the compressed carapace, which covers...
the whole body. In the Petri dish, the embryo is then situated on its side (Fig. 4C-D), slightly moving by its antennae II. The heart starts to beat. Although rudiments which generate the heart organ appear a bit earlier, the heart was defined here as organ when it started its function (by observing beating).

At 21-30 h (Fig. 4E-F), the external appearance of the embryo already resembles the juvenile female. Towards 21 h, the second molting occurs, and right after this molting, the organs are developed and embryos can actually move and swim around. The movements (wriggle and tremble) is in a way similar to an adult; however, the embryo cannot swim. It is difficult to observe further modifications due to the constant movement of the embryo at this point. We could discern the rostrum, the brood pouch and setae on the limbs of adult type (Fig. 4F). Brownish yolk blocks are present only in the dorsal portion, surrounding the digestive tract.

By 30-32 h the embryonic organs and appendages have appeared and almost completely formed, and they just need a short time to generate fine setae (Fig. 4H). The embryo moves its antennae II and other appendages, which results in strong movements of the premature female *D. crassa*.

The final molt takes place at 45-48 h. The animal pumps the water through the mouth and increases the pressure within the body, then the membrane is cast off by antennal and body movements and then the embryo strongly increases in size. Since embryos have reached the final stage at 45-48 h and can swim and feed autonomously, we can assume that the embryonic development is completed (Kotov, 2007).

**Size change in the embryo**

The three embryonic instars are well separated by the embryo size (see growth curve in Fig. 5). The length of the egg in the first h of the development is about 0.17 mm. The length almost does not change during each of the I, II, III, and IV embryonic instars, yet increases stepwise with molting events that occur at 9 h (between instars I and II-III), and at 20 h (between instars II-III and IV), and by the end of development shortly after release from the brood pouch (between embryonic instar IV and postembryonic instar I), from about 0.23 to about 0.33 mm, *i.e.* an increase of about 1.4 times.

**DISCUSSION**

Only very few illustrations of some stages were previously made for chydorid embryogenesis (Shan, 1969; Smirnov, 1971). An approximate sequence of embryonic stages was superficially described only for a single chydorid species, *Leydigia acanthocercoides* (Leydig, 1860) (Murugan and Job, 1982) and for the closest relative of...
the chydorids, the eury cercid Eury cercus lam ellatus (O.F. Müller, 1776) (Kotov, 1996), but without determination of the stage durations. For other Chydoridae, Santos-Wis newski et al. (2006) gave an overview of embryonic development times of C. sphae ricus, C. dentifer, C. pubescens, Pneuroxus uncinatus and A. harpae, based on Bottrell (1975a, 1975b), Melão (1997, 1999) and their own observations. From our data, we can say that the general patterns of the sequence of events in chydorid embryogenesis is similar to that in other anomopods, such as the daphniids Daphnia magna (Obreshkove and Frazer, 1940), D. hyalina and D. galeata (Kotov and Boikova, 2001), and Ceriodaphnia reticulata (Shuba and De Costa, 1972). Our unpublished observations of Karaulona sp. and Chydorus sp. suggest that the development of other chydorids also had similar patterns of stage sequence, and that it is not only limited to the subfamily Chydorinae, to which Dunhevedia belongs.

Ontogeny of specific structures was not investigated in detail, but an Anlage of a sixth thoracic limb could be noticed (Fig. 3L). The sixth limb is absent in adult Dunhevedia, in fact, it is so in nearly all Chydorinae, but it is present in many Aloninae (e.g. the so-called Hexalona-clade in Alona). Even though no appendage is formed that is later resorbed, the Anlage is present, though very small. In daphniid embryos, where mature females also have only five limbs, the sixth segment is not so clear. This confirms that, like the Aloninae, chydorines derive from a sixlimbed ancestor. Other structures still need to be investigated in detail, but in comparison to daphniids the eye development seems also different. As in most crustaceans, the structure is paired in daphniid embryos as the two eyes that fuse later (Kotov and Boikova, 2001). Also, the fat drop seems relatively larger in Daphnia embryos. In Dunhevedia, we did not observe this, but more studies should be carried out on chydorids in order to provide comparable data on these aspects.

Important differences of the chydorid parthenogenetic females from that in daphniids-sidids are: i) small body size; ii) only one-two eggs in the brood pouch; and iii) very large size of the eggs relative to the mother (Smirnov, 1971).

It is well-known that the duration of the embryonic development in the Cladocera depends strongly on the temperature (Bottrell, 1975a, 1975b). For example, Bottrell observed an embryonic development of 76 h for A. harpae under 20°C, while Melão (1999) found that the development is about twice as fast (37 h) for the same species – though for a population from a different region – under 25°C. When comparing within the Chydoridae, our observed length of embryonic development of Dunhevedia at 28.5°C is within a realistic range, yet indeed rather on the short side of what Santos-Wisnewski (2006) listed as the embryonic development times for temperatures between 20-25°C (ca. 37-76 h, for different taxa). In general, a fast development in the tropics, in function of increased temperature, would have an advantage. Strong predation pressure favours small-bodied cladocerans in the tropics (Dumont, 1994) and high temperatures allow for a fast development of chydorids.

However, the observed duration of embryogenesis of Dunhevedia is not too short. Shuba and De Costa (1972) observed 38 h for Ceriodaphnia in the tropics. Indeed, the duration of the embryogenesis in a cladoceran may depend also on other factors, i.e. volume of the egg and of yolk in it (Kotov and Boikova, 2001). In general, the duration of the embryogenesis at a particular temperature in small-sized chydorids is shorter than in daphniids (Bottrell, 1975a, 1975b). We believe that in our case of Dunhevedia, the short embryogenesis is a reflection of high temperature (in the tropics – tropical conditions for a tropical population), small body size of these animals and, presumably, its phylogenetic position.

At this point it is useless to discuss the absolute durations of stages in the embryogenesis of different cladocerans, because we cannot standardise the temperature in different species, especially keeping in mind that the species from tropical and temperate zones and perhaps even different populations of the same species from different regions have presumably different thermal developmental preferences.

However, relative durations could be compared. Although absolute time of the embryonic development as well as of its stages is very different in different cladocerans, relative durations of instars in Diaphanosoma, Daphnia and Leptodora are surprisingly similar (Fig. 6). Only in Sida, the duration of instar IV is somewhat more prolonged when compared with other non-chydorid cladocerans. In contrast, in the chydorid Dunhevedia, relative durations of instars I and II-III are very short, while that of instar IV is strikingly long in comparison with other cladocerans. Such a chydorid pattern seems therefore apomorphic, while the pattern of daphniids-sidids is plesiomorphic.

Why are the instars I and II+II so short and instar IV so long in Dunhevedia? We could interpret the pattern as a sign of paedomorphic evolution in chydorids. All the morphological structures are differentiated and then further develop in the embryo during instars I and II+III, while during instar IV the embryo is already similar to a released animal and no significant morphological changes happen. Duration of instars I-III is shorter in Dunhevedia, which means that the morphogenesis in chydorids stops at earlier stages in comparison with other cladocerans. Therefore, at the beginning of embryogenesis, the development of the chydorid embryo is fast, but then stops, which is characteristic of neoteny as a variant of paedomorphic evolution. Typically, in neoteny, the development of an animal is slowed, resulting in juvenile characters in mature stages. The chydorids’
small body size (in mature animals) could be seen as a characteristic of neoteny. By altering the development time relative to other cladocerans, sexual maturity is reached relatively faster as well. This is another characteristic of neoteny, the significance of which for chydorid evolution should be further explored.

A neotenic origin of the Cladocera was discussed by many authors (Kotov, 2007; Boikova, 2010), although some recent investigators doubted this hypothesis (Olesen, 2004). We think that the possibility of a neotenic evolution of a group within the cladocerans, namely the small-sized chydorids, should be particularly investigated and discussed in the future. We should take into account that these observations have been done at high temperatures on a tropical species and therefore relative speeds of development in the embryo and the relative duration of stages could be different in other chydorids.

Another peculiar trait of Dunhevedia is a very small increase in the size of the embryo during embryogenesis (from the laying of the egg to release of the embryo from the brood pouch, but before the final molt): about 35% increase from the initial size of the egg. In Daphnia this increase is 80%, in Sida it reaches 115%, and in Leptodora it is even 322% (Kotov and Boikova, 1998, 2001; Boikova, 2008). In general, the relative increase of the embryo in the anomopods is smaller than in the members of other orders. In both Daphnia and Dunhevedia, the size of the embryo during instar I is almost unchanged. But during instars II+III and IV, the embryo of Daphnia increases significantly in size, while in Dunhevedia the change is minimal. In addition, instar II+III – i.e. the stage when the maximum relative growth happens in Daphnia – is particularly small in Dunhevedia, which also contributes to differences between the former and the latter.

A small relative growth of the embryo during the whole period of development in small-sized chydorids is correlated with very large size of the egg relative to the mother. It seems that such large eggs in the chydorid brood pouch even have no chance to grow, already occupying all the volume of the brood pouch (Fig. 1). Such relative growth is not necessary, because the chydorid egg is already too large compared to the mother. We can also speculate that the covers of the chydorid embryos are much thicker (Fryer, 1968) and, as a result, less elastic when compared to daphniids, preventing the increase of the embryo size.

**CONCLUSIONS**

Our study revealed some peculiarities of the embryogenesis in small-sized chydorids which could be the result of a family-specific evolution of their ontogenesis. More species need to be studied for firm conclusions on the phylogenetic and evolutionary significance of these observations.

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