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Nitrogen excretion during marsupial development in the terrestrial isopod *Armadillidium vulgare*

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ABSTRACT

Marsupial embryos of *Armadillidium vulgare* (Isopoda: Oniscidea) were collected at different stages of development and assayed for products of nitrogen excretion. Stages were classified as early stage one, late stage one (clear embryo and somite differentiation), early stage two (chorion shed, prior to blastokinesis), late stage two (following blastokinesis), and mancae (vitelline membrane shed; second embryonic molt). Stage one and stage two embryos were primarily ammonotelic. Mancae showed a significant increase in stored uric acid and decrease in ammonia production, in most cases to undetectable levels. The increased metabolic rate of mancae, and the fact that they imbibe marsupial fluid prior to exiting the marsupium, may have favored a switch from ammonotely to uricotely to avoid ammonia toxicity. Protein metabolism, estimated from ammonia production, accounted for 7% of the measured catabolic rate in Stage 2 embryos. Newly emerged juveniles showed a > 2-fold increase in metabolism relative to mancae, accompanying the transition from aquatic to aerial respiration. Following 48 h post-emergence, juveniles resumed ammonia excretion, volatilizing the base (NH_3) as in later instars. Elevated ammonia excretion in early juveniles may derive from the catabolism of remaining yolk protein. A sharp increase in whole-animal glutamine in juveniles is consistent with its role as an intermediary nitrogen store during periodic ammonia excretion. Total ammonia concentration in the marsupial fluid fluctuated but did not increase significantly over time and ammonia was not volatilized across the oostegites, indicating that embryo ammonia is transported into the maternal hemolymph for excretion.

1. Introduction

The transition from planktotrophic development in larval crustaceans to direct development *via* brooded, lecithotrophic eggs is seen in several independently derived lineages and is probably one of the more significant preadaptations enabling the broad adaptive radiations of the Amphipoda and Isopoda in the intertidal. In these two orders, uniquely, non-insect crustaceans have attained a fully terrestrial habit. The terrestrial isopods of the suborder Oniscidea represent a major radiation with over 3600 described species (Schmalfuss, 2003; Schmidt, 2008). Many of these are mesic-xeric species and a few, including *Porcellio brevicaudatus* of the Negev Desert (Kashani et al., 2011; Shachak and Yair, 1984), and *Venezillo arizonicus* of the Mojave and Sonoran-Colorado Desert (Warburg, 1965a, 1965b), exploit some of the hottest and driest habitats on Earth.

The comparative anatomy of the maternal brood pouch or marsupium in the Oniscidea has been studied by Hoese (1984) and Hoese and Janssen (1989). Following fertilization, female isopods undergo a specialized parturial molt and develop leaf-like extensions of the coxae, the *oostegites*, on the first 5 pairs of the pereopods. The oostegites overlap in an imbricate arrangement and form the floor of the marsupium. In marine isopods, the marsupium opens to the anterior and the embryos are perfused directly with seawater (Hoese, 1984). In the Oniscidea, the anterior and posterior oostegites insert into grooves on the respective sternites. Members of the basal section Ligiamorpha possess an *open* or *amphibian type* marsupium in which the marsupial fluid is provisioned externally *via* the pleural water capillary system; rows of small setae on the 6th and 7th pereopods form a capillary channel when these are appressed, serving in water uptake (Hoese, 1984, 1981). Water is sourced either from seawater (most *Ligia* species) or from freshwater (*Ligidium* spp.) supplemented with maternal ions (Yoshizawa and Wright, 2011). In the Holoverticata, comprising the

Abbreviations: ES1, early stage 1 embryos; LS1, late stage 1 embryos; ES2, early stage 2 embryos; LS2, late stage 2 embryos; EM, early mancae; LM, late mancae; VCO_2, CO_2 flux (nl min^{-1} or ml min^{-1})

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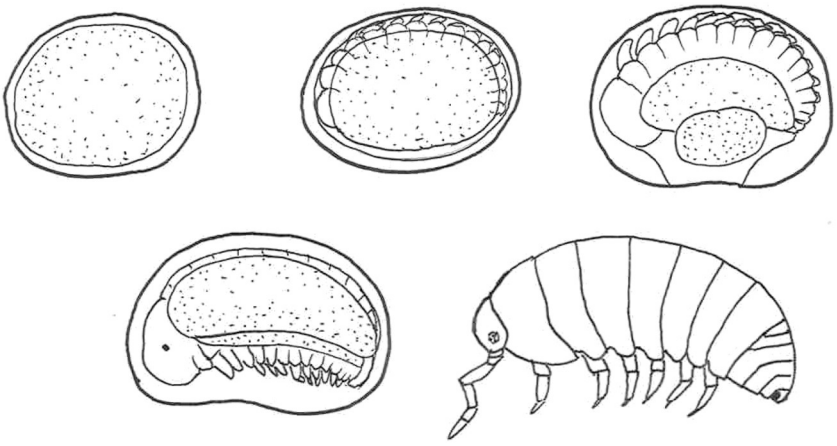

Fig. 1. Marsupial embryo stages of *A. vulgare*. Top row, left to right: early stage 1 (ES1), late stage 1 (LS1), early stage 2 (ES2). Bottom row: late stage 2 (LS2), manca. The chorion is shed as a thin, glassy envelope between LS1 and ES2. In ES2, the yolk mass is assimilated into the presumptive midgut followed by blastokinesis which marks the transition to LS2. Shedding of the vitelline membrane from LS2 yields the mancae.

sections Tylomorpha, Synocheta, Mesoniscidea and Crinocheta (see Schmidt, 2008), the marsupium is provisioned with fluid from the maternal hemolymph, and in the Crinocheta this employs modified segmental cotyledons (Akahira, 1956; Hoese and Janssen, 1989); embryo development in these sections is thus independent of external liquid water.

When they initially pass from the ovary into the marsupium, oniscidean eggs possess two extra-embryonic membranes, an outer chorion and inner vitelline membrane (Strömberg, 1965, 1972), and are filled with vitellocytes. As the embryo develops, the yolk mass decreases in volume and energy reserves are assimilated into the presumptive midgut (Fig. 1). After 10–12 days, eggs of *Armadillidium vulgare* shed the chorion at the first embryonic molt and transition from Stage 1 to Stage 2 (Surbida and Wright, 2001). After a further 5–6 days, the yolk mass disappears and the embryo undergoes blastokinesis, rotating about the longitudinal axis, so the ventral surface and pereopods now orient to the concave face of the egg. Blastokinesis coincides with the atrophy of the embryonic dorsal organ (Goodrich, 1939; Wright and O'Donnell, 2010). After the subsequent shedding of the vitelline membrane, Late Stage 2 embryos become the first instars or mancae which remain in the marsupium for a further 5–6 days. During this period, they imbibe the marsupial fluid (Hoese and Janssen, 1989; Surbida and Wright, 2001) before emerging as free-living juveniles.

Energy reserves in the yolk include both vitellin proteins and lipids (Okuno et al., 2000). The yolk proteins provide an important catabolic substrate as well as serving for anabolic tissue growth (Rønnestad et al., 1999). Very little is known, however, about waste nitrogen excretion by the embryos of crustaceans with lecithotrophic development. Crustaceans generally eliminate deaminated nitrogen from amino acid catabolism as ammonia, either *via* the antennal gland (Binns, 1969; Greenway, 1991) or the gill epithelia (Weirauch et al., 2004). The one notable exception is the coconut crab *Birgus latro* which is purinotelic, synthesizing a mixture of guanine and urate in the fat body (Greenaway and Morris, 1989; Linton et al., 2016); purines are transported across the midgut and voided as a white pellet. Among other groups with lecithotrophic eggs, precipitated purines comprise about 50% of the accumulated waste nitrogen stored in the allantoic sac of the American alligator *Alligator mississippiensis* (Clark et al., 1957), while other reptile and bird eggs mostly accumulate urea (Fisher and Eakin, 1957; Packard and Packard, 1983, 1987; Sartori et al., 2012). By contrast, the aquatic, yolky eggs of fish (Wright and Fyhn, 2001), amphibians (Munro, 1953), and freshwater snails (Sloan, 1964), are primarily ammonotelic.

The present study sought to examine nitrogen excretion during and immediately following marsupial development in *Armadillidium vulgare* (Oniscidea, Crinocheta, Armadillidiidae). Embryo excretion in the Oniscidea represents an interesting case. Being aquatic, embryos could release ammonia directly into the marsupial fluid, but the limited fluid volume of the marsupium imposes the potential for toxic ammonia accumulation. Gravid females may possess a mechanism for excreting marsupial fluid ammonia, for example volatilizing NH_3 across the oostegites or transporting ammonia into the maternal hemolymph and volatilizing NH_3 from the pleopodal fluid in the usual manner (Wieser et al., 1969; Wieser and Schweizer, 1970; Wright and O'Donnell, 1994). Alternatively, embryos may switch to storage excretion during all or part of marsupial development, as in the cleidoic eggs of reptiles and birds. Possible candidates for storage excretion include urea, purines and nitrogen-rich amino acids such as glutamine. Uric acid is present in small amounts (typically 1–3 μmol g^{-1}) in probably all Oniscidea (Linton et al., 2016), and all crustaceans appear to possess xanthine oxidoreductase and other critical enzymes for urate synthesis (Hartenstein, 1968; Linton et al., 2016). Glutamine is known to serve as a temporary nitrogen store in terrestrial (Wieser and Schweizer, 1972; Wright et al., 1994; Wright and Peña-Peralta, 2005) as well as littoral (Nakamura and Wright, 2013) Oniscidea.

2. Materials and methods

2.1. Collection and culture

Armadillidium vulgare were collected from the campus of Pomona College and in the local San Gabriel Mountains and maintained in lab terraria with oak litter and occasional potato and carrot as supplementary food. Gravid females were identified by the tumid ventral marsupium, and it was typically possible to identify the brood stage using x40–80 magnification. Suitable embryos were sampled by lifting the anterior edges of the oostegites gently using fine forceps and transferred to marsupial fluid saline solution. The saline was based on previously measured electrolyte concentrations (Yoshizawa and Wright, 2011): 280 mM NaCl, 23 mM KCl, 13 mM $CaCl_2$, 5 mM $MgSO_4$, and 5 mM HEPES buffer, adjusted to pH 7.8. Although prior work (Ban, 1950; Surbida and Wright, 2001) has shown that embryos can be reared from Stage 1 through to the manca stage in a similar saline solution, all experiments here were initiated within 1 h of isolating embryos from the female.

2.2. Staging embryos

Embryos were categorized into five developmental stages following Surbida and Wright (2001), (Fig. 1): Early Stage 1 (ES1), Late Stage 1 (LS1), Early Stage 2 (ES2), Late Stage 2 (LS2), and mancae. ES1 possess minimal embryo differentiation while in LS1 the embryo somites are clearly visible. Following shedding of the chorion, the embryos are classified as Stage 2. ES2 and LS2 are pre- and post-blastokinesis respectively.