Ultrastructure of the Mushroom Body: Digestion During Metamorphosis of *Utterbackia imbecillis* (Bivalvia: Unionidae)

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Introduction

Larvae of unionoid mussels encyst as parasites of fish during metamorphosis. As a result, the transition from glochidium to juvenile is not well understood. For example, larval mantle cells move ventrally and project into the mantle cavity, forming the 'mushroom body', the function of which is not clear. Blystad (1923) claimed that mushroom bodies were the site of phagocytosis of the larval adductor muscle, and Fukuhara et al. (1990) observed host tissues within vacuoles of larval mantle cells of *Anodonta woodiana*.

We examined the structure of larval mantle cells of *U. imbecillis* to ascertain their function. One advantage of using this species is its amenability to *in vitro* culture (Isom and Hudson, 1982) which provides larvae at each stage of development, and enables a comparison of *in vitro*- and fish-reared juveniles.

METHODS: Glochidia were put in culture medium or allowed to attach to bluegill sunfish (*Lepomis macrochirus*). At intervals they were removed from culture or dissected from cysts on host fish and fixed for TEM. Acid phosphatase was assayed at day 4 with Gomori lead precipitation.

Ultrastructure: days 1-3

Since larval mantle cells resemble digestive cells of adult bivalves, we adopted the terminology of Owen (1973). By day 2 mantle cells had enlarged and endocytotic pinosomes, secretory vesicles, and vesicles resembling heterophagosomes containing granular material were present in the apical portion of the cells (Fig. 1). Heterolysosomes occurred basal to heterophagosomes and contained an homogenous material (Fig. 2). By day 3 secretory vesicles were largely absent. Some heterophagosomes and hetero-lysosomes appeared to have fused (Fig. 3). Heterolysosomes were irregularly shaped and filled with granular material, but only the less electron dense vesicles contained ingested cellular material (Fig. 3).

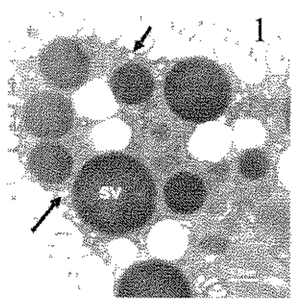

Fig 1. TEM of a larval mantle cell on day 2. Arrows indicate pinosomes SV = secretory vesicle

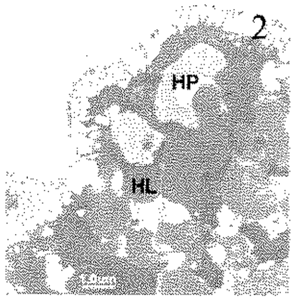

Fig 2. TEM of a larval mantle cell on day 3. HL= hetero-lysosome HP = heterophagosome

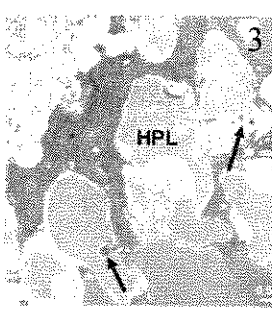

Fig.3 Mantle cell on day 3. Arrows indicate ingested material HPL = fused heterophagosome-heterolysosome

Ultrastructure: days 4-7

By day 4 there were numerous lipid and glycogen deposits in fish-reared (but not *in vitro*-) larvae (Fig. 4). Heterophagosomes of *in vitro*-reared animals were larger and less numerous, and contained granular and ingested material (Fig. 5). By day 7 all larvae had few, small heterophagosomes, with little cellular material. Heterolysosomes were smaller and less numerous, and there were fewer pinosomes or evidence of endocytosis (Fig. 6). A composite larval mantle cell is presented in Fig. 7.

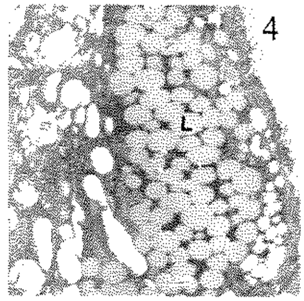

Fig 4. Mantle cell of a fish-reared larva showing lipid and glycogen L = lipid

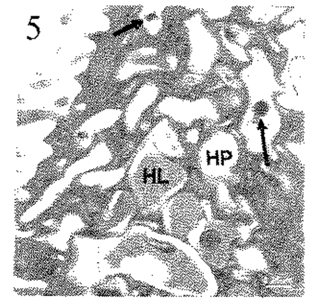

Fig 5. Mantle cell of fish-reared larva day 4. Arrows indicate ingested material HL = heterolysosome HP = heterophagosome

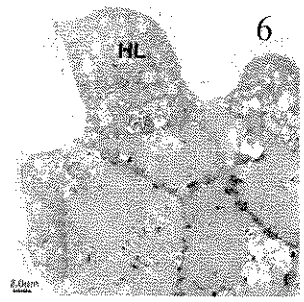

Fig 6. Mantle cells of a fish-reared larva on day 7. HL = heterolysosome

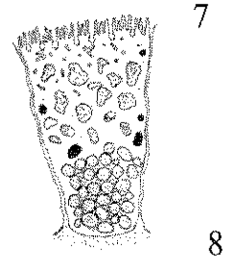

Fig 7. Mantle cell of fish-reared larva with endocytotic pits, pinosomes, heterophagosomes, heterolysosomes and basal lipid and glycogen deposits.

Histochemistry

Acid phosphatase activity occurred in hetero-phagosomes that contained ingested material (Fig. 8).

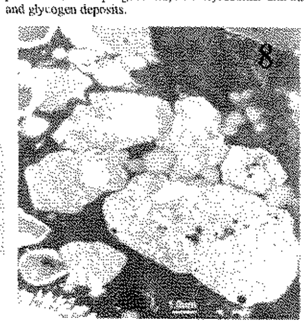

Fig 8. *In vitro*-reared larva on day 4. Acid phosphatase (black spots) is visible in the heterophagosomes.

Discussion

Larval mantle cells of *U. imbecillis* contain a system of vesicles resembling digestive cells of adult bivalves, together with microvilli and invaginations indicative of endocytosis. The numerous pinosomes, heterophagosomes and 2 types of heterolysosomes suggest that digestion begins with endocytosis of material that is passed to heterophagosomes which fuse with heterolysosomes as digestion proceeds. The presence of acid phosphatase in heterophagosomes and heterolysosomes implicates this enzyme in intracellular (and perhaps extracellular) digestion.

The nutritional condition of juveniles derived from the two rearing conditions appears to be rather different. Fish-reared larvae acquire more lipid and glycogen than do *in vitro*-reared animals. Differences in specific lipids and glycogen are quantitatively significant, and the overall physiological condition, growth, survival, and stress tolerance are greater for fish-reared animals (Fisher 2001). Since mortality of juvenile mussels is very high, animals with greater post-metamorphic energy reserves presumably have an increased probability of surviving the transition to a free-living habit. Cells of the mushroom body may serve a dual role of digestion and energy storage during metamorphosis.

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Morphological and Molecular Changes During Metamorphosis in *Utterbackia imbecillis* (Bivalvia: Unionidae)

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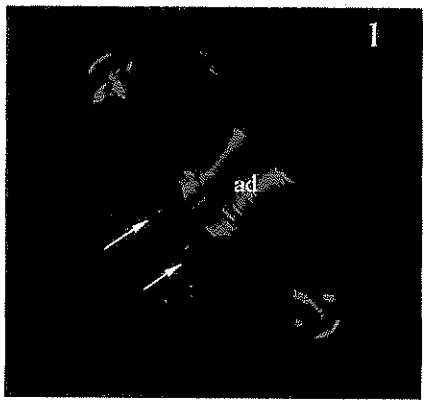

Fig. 1. Glochidium stained with Alexa 488. Arrows indicate neurons. ad = larval adductor

INTRODUCTION

The glochidium larva of unionid bivalves develops within the gill of a parental mussel until it is competent to attach to a host fish. Once encysted on a host, these parasitic glochidia metamorphose to the juvenile mussel, excyst, and grow into benthic adults. Although glochidia and adults have been rather well studied, little is known about metamorphosis. For example, the single larval adductor muscle is absent in juveniles, but its fate and the origin of paired juvenile adductors are unclear. Fukuhara et al. (1990) suggest that in *Anodonta woodiana*, the larval adductor shifts forward, giving rise to the anterior adductor of the adult. In contrast, Zs.-Nagy and Labos (1969) noted significant differences in ultra-structure of larval and adult adductor muscle of *A. cygnea*, indicative of an independent origin. The fate of larval mantle cells that project into the mantle cavity forming the 'mushroom body', also is unknown. Arey (1932) and Bivstad (1923) reached conflicting conclusions about the presence or absence of the mushroom body and its persistence during metamorphosis of *A. corpulenta* and *Lampsilis luteola*. Since the mushroom body may facilitate acquisition of nutrients from the host Bivstad (1923) predicted that *Utterbackia imbecillis* (the subject of this study) should possess little or no mushroom body since this species was thought at that time to undergo direct development within parental gills.

The present study describes metamorphosis in *U. imbecillis*, together with an analysis of DNA, RNA and protein synthesis. Since this species can be cultured *in vitro* (Isom & Hudson, 1982), numbers of animals are available at each stage of development, facilitating sequencing of ontogenetic changes.

Fig. 2. Glochidium stained with Alexa 488. Arrow indicates neuron at insertion of adductor muscle

METHODS

Mature glochidia were removed from the gills and placed in culture medium at 20°C under 5% CO_2. Under these conditions metamorphosis typically occurs in 8 days. For histological examination, animals were fixed in Bouin's and embedded and sectioned in paraffin. Actin filaments of relaxed formalin-fixed animals were imaged with the fluorochrome Alexa 488 viewed with laser confocal microscopy. For autoradiography animals were incubated in 0.5 µCi of ^{3}H-thymidine, ^{3}H-Uridine, or ^{3}H-Leucine for 24 h, fixed in Carnoy's and embedded in paraffin. After sectioning, slides were coated with NTB-2 emulsion, developed, and stained with hematoxylin and eosin. To quantify DNA, RNA, and protein synthesis animals were incubated with isotope as above and then were treated with 0.5% BSA, sonicated, precipitated in 10% TCA, centrifuged, and the pellets were resuspended in 95% ETOH prior to scintillation counting.

Fig 3. Cross section on day 3
a = adductor muscle mb = mushroom body
Scale =10 µm

Fig 4. Cross section on day 4
mb = mushroom body f = foot
st = stomach Scale bar = 10µm

RESULTS

Glochidia had a prominent adductor muscle and lateral pits, both of which are innervated (Figs. 1 & 2). By day 2 of metamorphosis larval mantle cells had enlarged and projected into the mantle cavity, and the adductor muscle had begun to degenerate. By days 3 & 4 mantle cells had formed the mushroom body, and the larval adductor muscle had degenerated (Figs. 3 & 4). By day 5 the two juvenile adductor muscles had begun to develop, the stomach and digestive glands were present, and the foot had enlarged (Fig. 5). By days 6 and 7 the style sac and intestine were present, as were gill buds in the posterior/ventral aspect of the animal, having arisen from the lateral pits (Fig. 6).

The mushroom body persisted throughout development, but had disappeared by final metamorphosis to the juvenile. Juveniles had a ciliated foot with pedal retractor muscles and pedal ganglia, and the gut was fully developed (Fig. 7).

RNA and DNA synthesis occurred in different cells at different points in development. Cells of the ventral plate and lateral pits were active sites during the first 4 days but after day 4, activity was broadly distributed. Synthesis of DNA, RNA and protein was bimodal, with peaks between days 1 & 4 and 5 & 8 (Fig. 9).

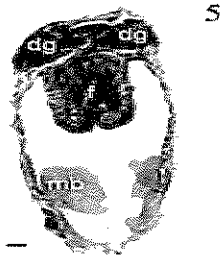

Fig 5. Cross section on day 5 mb = mushroom body f = foot dg = digestive gland
Scale = 10 µm

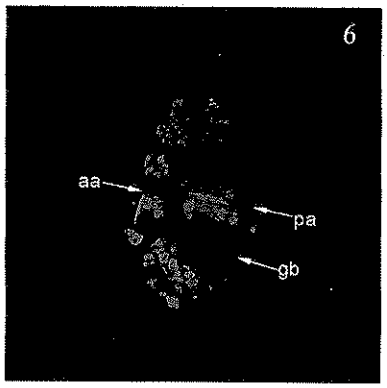

Fig 6. Larva on day 6 stained with Alexa 488 aa = anterior adductor gb = gill bud pa = posterior adductor

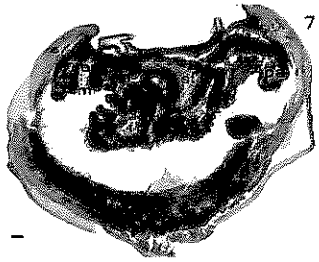

Fig 7. Saggital section of a juvenile aa = anterior adductor
e = esophagus f = foot m = mouth pa = posterior adductor
st = stomach Scale = 10µm

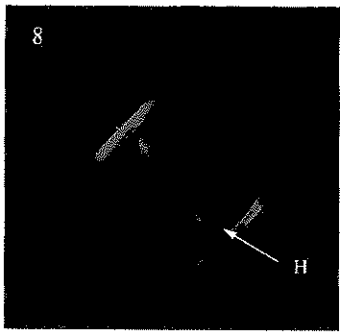

Fig. 8 Ventral view of post-metamorphic juvenile at day 10. Arrow indicates heart in pericardial sinus, near posterior adductor. Also visible are the foot and pedal muscles.

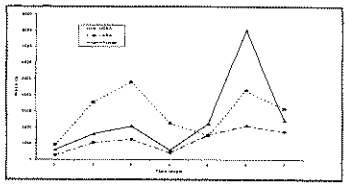

Fig 9. Transcription, translation and protein synthesis during metamorphosis. Points are mean counts/min ± SE N = 40

DISCUSSION

During *in vitro* metamorphosis of *U. imbecillis*, the larval adductor muscle contracts and the valves remain closed until development to the juvenile is complete. Histochemical and actin-specific staining show complete degradation of the larval adductor muscle during the first few days, and *de novo* synthesis of juvenile muscles. This observation is consistent with that of Zs.-Nagy and Labos (1969) indicating that larval and adult adductor muscles are distinctly different structures. Larval mantle cells form the characteristic mushroom body which persists throughout metamorphosis. Autoradiography and DNA, RNA and protein synthesis indicate that metamorphosis in occurs in two stages. The first (days 1-4) includes degeneration of the larval adductor muscle, formation of the mushroom body, and high levels of DNA, RNA, and protein synthesis. The second stage occurs during the final 4 to 8 days as the larva completes its transition to a free-living juvenile, with formation of the remaining organ systems (Fisher, 2001).

ABSTRACT

This study examines morphological and biochemical changes during metamorphosis of the freshwater mussel *Utterbackia imbecillis*. Progression from glochidium larva to juvenile encompasses two distinct stages. The first occurs during the first 3-4 days of metamorphosis and involves degeneration of the single larval adductor muscle and formation of the characteristic mushroom body by larval mantle cells. These morphological changes are accompanied by an increase in DNA, RNA, and protein synthesis. The second stage occurs during the final 4 days of metamorphosis and involves formation of major anatomical structures and organ systems of the juvenile, and also is accompanied by increased DNA, RNA, and protein synthesis.

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