MORPHOLOGICAL AND MOLECULAR CHANGES DURING METAMORPHOSIS IN *UTTERBACKIA IMBECILLIS* (BIVALVIA: UNIONIDAE)

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ABSTRACT

This study examines morphological and biochemical changes that occur as glochidia larvae of the freshwater mussel *Utterbackia imbecillis* (Say, 1829) metamorphose into juveniles. Metamorphosis encompasses two distinct stages. The first occurs during the first 3–4 days, and involves degeneration of the single larval adductor muscle and formation of the characteristic mushroom body by the 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 the metamorphic period and involves formation of the major anatomical structures and organ systems of the juveniles. This stage also is accompanied by an increase in DNA, RNA, and protein synthesis. The *de novo* development of the juvenile adductor muscles is described, and sites of DNA, RNA, and protein synthesis are identified.

INTRODUCTION

Freshwater unionid mussels exhibit an unusual life history in comparison to their marine counterparts. In place of a free-swimming veliger, unionids possess a larval form, the glochidium, which requires a parasitic phase during development. Glochidia develop within compartments in the gill of the parental mussel until they become competent for continued development on an appropriate host. Most species are then released into the water column where they may attach to the gills or fins of fish (Kat, 1984), or may parasitize amphibians (Watters & O'Dee, 1998); however, a few species may undergo direct development within the parental gill (Lefevre & Curtis, 1911; Howard, 1914).

After a glochidium attaches, the host responds with a proliferation of epithelial cells that results in encystment of the glochidium (Coker, Shira, Clark & Howard, 1921; Waller & Mitchell, 1989). While encysted on the host, glochidia undergo metamorphosis into juvenile mussels. This transition includes loss of the single larval adductor muscle and the larval mantle tissue. Subsequent development of the juvenile features includes formation of two adductor muscles, a foot, gill bars, stomach, crystalline style sac, and digestive glands (Tucker, 1927). When metamorphosis is complete, the juvenile mussel excysts from its host, drops to the substratum, and continues development into an adult mussel.

Although some aspects of the biology of glochidia and adult freshwater mussels have been relatively well studied, metamorphosis has been examined by only a few authors early in the last century. Blystad (1923) described metamorphosis in *Anodonta corpulenta* (Say), which possesses a hooked type of glochidium and *Lampsilis luteola* (Lamarck), which has hookless glochidia. He proposed three consecutive stages of metamorphosis: the encystment stage as the larva attaches to the fish, the mushroom body stage during which the larva digests the fish tissue between its shells, and the final stage that includes the formation of juvenile anatomical features. Both hooked and hookless glochidia undergo all three stages of development, although Blystad (1923) did report some variation between the two glochidial types. Arey (1932a,b) described metamorphosis in the same species, and his results confirmed the earlier work of Blystad (1923).

Given the lack of information concerning metamorphosis in freshwater mussels, a number of developmental questions remain unanswered. While it is agreed that the single central larval adductor muscle is absent in the juveniles, which have smaller posterior and anterior adductor muscles, the fate of the larval adductor, and the origin of the juvenile adductors are unclear. Fukuhara, Nakai & Nagata (1990) suggest that the larval adductor muscle of *Anodonta woodiana* (Lea) shifts forward and forms the anterior adult adductor muscle, while the adult posterior adductor muscle forms *de novo*. Zs.-Nagy and Lábos (1969) examined the ultrastructure of the larval and both adult adductors of *Anodonta cygnea* (Linnaeus), and found significant differences in the size and structure of myofilaments of glochidial and adult adductor muscles. Their results are consistent with the observations of Herbers (1914) that the larval and adult muscles have independent origins, and are not derived from the larval muscle.

The fate of the larval mantle cells is also unclear. These cells project into the mantle cavity of the developing juvenile and form the mushroom body (Braun, 1878). Arey (1932b) found that the mushroom body persists until the end of parasitism by the hooked glochidia of *A. corpulenta*, but that it has disappeared before excystment in the hookless *L. luteola*. This conflicts with Blystad's (1923) observations that *L. luteola* does not form a mushroom body. Because the mushroom body is assumed to facilitate the acquisition of nutrients from the mussel's host (Blystad, 1923; Arey, 1932b), Blystad (1923) predicted that *Utterbackia imbecillis* (Say, 1829), the subject of our study, should possess little or no mushroom body since this species was reported to metamorphose without a parasitic stage.

The present study was designed to more closely examine these questions and to provide the first detailed description of metamorphosis in *U. imbecillis*. This species is common in the Eastern USA (Johnson, 1970), and populations in North Carolina release glochidia from early spring to late fall (personal observation). Glochidia may attach to a wide range of fish hosts (Watters & O'Dee, 1998), where metamorphosis takes

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place in approximately 8–10 days. However, this species can also be reared *in vitro*. By using a modified cell culture medium (Isom & Hudson, 1982; Dimock & Wright, 1993), it is possible to effect metamorphosis of larval *U. imbecillis* in the laboratory without a host fish, thus providing large numbers of animals at the same stage of development and permitting the timing of ontogenetic events. Therefore, the sequence of developmental changes in general morphology and muscle formation was addressed. In addition, rates of DNA, RNA, and protein synthesis and sites of this activity were determined.

MATERIALS AND METHODS

Adult *Utterbackia imbecillis* were obtained from Davis' Pond (Davidson, NC) and maintained in the laboratory until mature glochidia were present in the marsupia. Glochidia were removed from the outer demibranchs of two mussels, washed in sterile moderately hard Environmental Protection Agency (EPA) water (Lewis, Klemm, Lazorchak, Norberg-King, Peltier & Heber, 1994) and pooled before use. For *in vitro* rearing, glochidia were then placed into 60 × 15-mm culture dishes in 3 ml of a standard tissue culture medium (Isom & Hudson, 1982; Dimock & Wright, 1993) and maintained in a 5% CO_2 incubator at 21°C for 7 days.

For histological examination, glochidia, metamorphosing larvae and post-metamorphic juveniles were fixed in either Bouin's fluid or formalin overnight. The shells were decalcified with Cal-Ex (Fisher Scientific, Fairlawn, NJ) for 24 h. Larvae were then dehydrated in a series of alcohols, cleared with xylene and embedded in paraffin. Sections 5–6 μm thick were stained with either haematoxylin and eosin, or Mallory's triple connective tissue stain (Presnell & Schreibman, 1997) and examined using a Zeiss Axioplan microscope. All microscopic observations are based on a minimum of six different animals for each evaluation. There were no obvious morphological differences among the relevant replicates, so the representative images shown in the results are indicative of all specimens observed.

For staining of actin filaments with the fluorochrome Alexa 488 (Molecular Probes, Eugene, OR), animals in different stages of development required different techniques to cause relaxation. Fresh glochidia were washed in EPA water and then relaxed in 0.03 M magnesium chloride for 20 min. Animals from days 1–3 of metamorphosis were removed from the culture medium, washed in phosphate buffered saline (PBS) and relaxed in 4°C carbonated water for 3–4 h. Since the larval adductor muscle has largely degenerated by day 4 of metamorphosis, animals from days 4–7 of development were relaxed by incubation in 35°C EPA water for 20 min. After relaxation, all animals were fixed in 10% neutral-buffered formalin for 15 min, washed in PBS, and then permeabilized for 1 h in 0.2% Triton X-100 in PBS. Animals were stained for 15 min with Alexa 488 in the Triton solution. Following three washes in PBS, the specimens were mounted in glycerol with *n*-propyl-galate as an anti-bleaching agent. Slides were viewed on a laser confocal microscope (Zeiss Axiovert 100 M, excitation 495 nm, emission 519 nm) and images were captured with laser scanning microscopy software (Zeiss, Germany).

For autoradiography, glochidia larvae were removed from the parental mussel, washed three times in 2 vols of sterile EPA water and then incubated in 0.5 μCi of either ^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-leucine for 24 h. Animals from each day of metamorphosis were treated the same except that they were washed in culture medium instead of EPA water. After incubation with the isotope, all animals were washed three times with 2 vols of sterile EPA water and placed in Carnoy's fixative for 30 min. They were then dehydrated, cleared in xylene, and embedded in paraffin for sectioning at 5–6 μm. After sectioning, the slides were coated with NTB-2 emulsion (Kodak, Atlanta, GA), held in the dark for 2 weeks and then developed. All sections were stained with haematoxylin and eosin.

To measure levels of DNA, RNA, and protein synthesis during metamorphosis, a technique using radiolabelling was developed. Glochidia were removed from the parental mussel and rinsed in sterile EPA water. Forty glochidia were placed in each of nine microcentrifuge tubes and washed three times in 2 vols of sterile EPA water. The water was drawn off and 200 μl of EPA water with an antibiotic solution (5000 units penicillin, 5 mg streptomycin, 10 mg neomycin per ml, Sigma) were added to each tube. Three of the tubes then received 0.5 μCi of ^{3}H-thymidine, three received 0.5 μCi of ^{3}H-uridine, and the final three tubes received 0.5 μCi of ^{3}H-leucine. Following incubation for 24 h at 20°C in their respective solutions, the animals were washed three times in 2 vols of sterile EPA water and treated with 200 μl of 0.5% bovine serum albumin in phosphate buffered saline (PBS). The animals were then sonicated, 200 μl of 10% trichloro-acetic acid (TCA) were added and the animals were incubated for 2 h at 4°C. After incubation, the tubes were centrifuged at 660 ***g*** for 3 min and the pellet was washed three times with 400 μl of 5% TCA. The TCA was removed and the pellets were resuspended in 100 μl of 95% ethanol, which was then added to 3 ml of Scintiverse (Fisher Scientific, Fairlawn, NJ) and counted in a scintillation counter. To control for background contamination, the above procedure was carried out using animals that had been killed by exposure to 42°C for 15 min. No measurable levels of radioactivity were found in the control treatments.

Levels of DNA, RNA, and protein synthesis were also measured for animals during the metamorphic period. On each day of metamorphosis, 40 animals that were closed and appeared to be undergoing transformation to juveniles were removed, and placed into each of nine microcentrifuge tubes (total = 360). The animals were washed three times in 2 vols of culture medium and then incubated in 0.5 μCi of either ^{3}H-thymidine, ^{3}H-uridine, or ^{3}H-leucine for 24 h. Following incubation, the animals were washed three times in 2 vols of EPA water and treated as described above for the glochidia larvae.

RESULTS

Glochidia of *U. imbecillis* were approximately 300 μm long and possessed a large ventral hook on each valve. A prominent adductor muscle, encased by a thin layer of cells, occupied the majority of the cavity between the valves, and was offset slightly toward the anterior. The glochidium possessed two layers of mantle cells lining the shell. The outer layer, directly adjacent to the shell, was very thin and inconspicuous. The inner layer consisted of larger, more cuboidal cells with distinct cell boundaries and prominent nuclei (Figure 1). Located posterior to the adductor muscle and just ventral to the hinge were the ventral plate and a depression known as the lateral pits (Lillie, 1895). The cells in the ventral plate give rise to the foot and the lateral pits develop into the gills. The fluorescent stain indicated that the lateral pits have neurons, which contain actin, associated with them at this stage (Figure 2). A large neuron was also visible where the adductor muscle is inserted into each valve (Figure 3).

On the first day of metamorphosis there were few obvious changes in the structure of the developing juvenile. The adductor muscle was still prominent, although in some specimens it had begun to deteriorate slightly. Cells of the inner larval mantle had begun to cluster together and project into the mantle cavity. By day 2 of metamorphosis the larval mantle cells were enlarged and projected farther into the mantle cavity. A large number of vacuoles was now present at the base of these

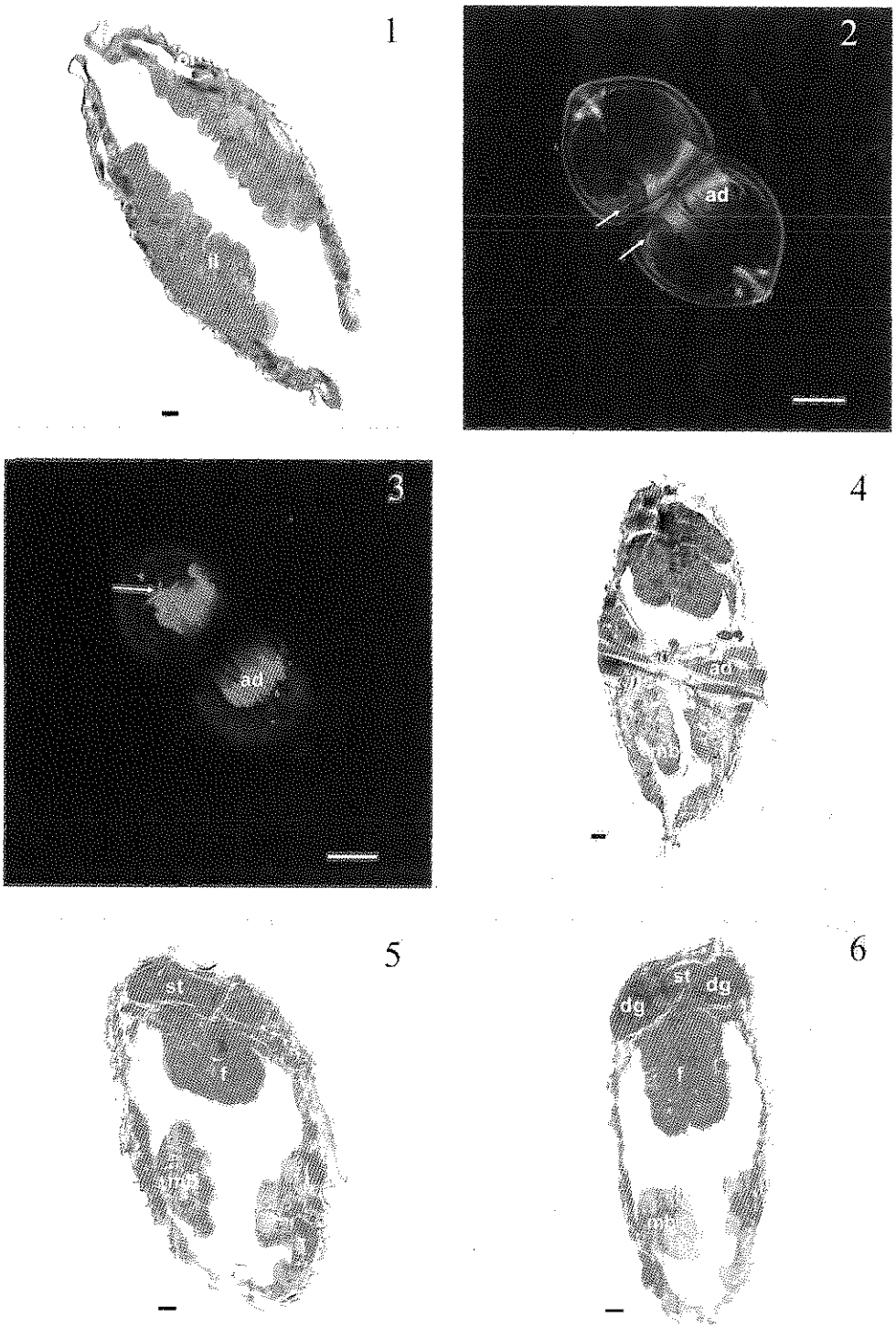


Figure 1. Frontal section through a glochidium stained with haematoxylin and eosin; scale bar = 10 μm. Abbreviations: il = inner layer of mantle cells; ol = outer layer of mantle cells.

Figure 2. Whole mount of a glochidium stained with Alexa 488 specific for actin, viewed with confocal laser microscopy; scale bar = 100 μm. Arrows indicate location of neurons in lateral pits. Abbreviation: ad = larval adductor muscle.

Figure 3. Whole mount of a glochidium stained with Alexa 488 specific for actin, viewed with confocal laser microscopy; scale bar = 100 μm. Arrow indicates neuron at insertion of adductor muscle into valve. Abbreviation: ad = larval adductor muscle.

Figure 4. Cross-section through a larva on the third day of metamorphosis. The top of the image is dorsal; scale bar = 10 μm. Abbreviations: ad = larval adductor muscle; mb = mushroom body.

Figure 5. Cross-section through a larva on the fourth day of metamorphosis. The top of the image is dorsal; scale bar = 10 μm. Abbreviations: mb = mushroom body; f = developing foot; st = stomach.

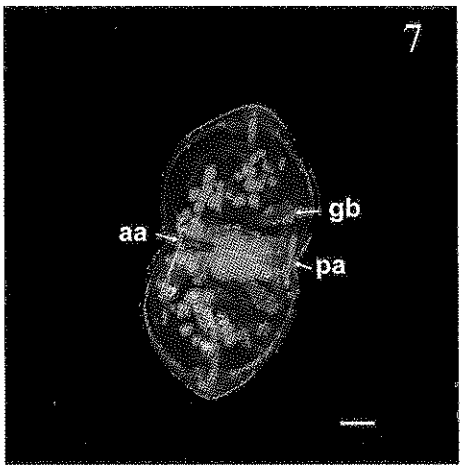
Figure 6. Cross-section through a larva on the fifth day of metamorphosis. The top of the image is dorsal; scale bar = 10 μm. Abbreviations: mb = mushroom body; f = foot; st = stomach; dg = digestive gland.

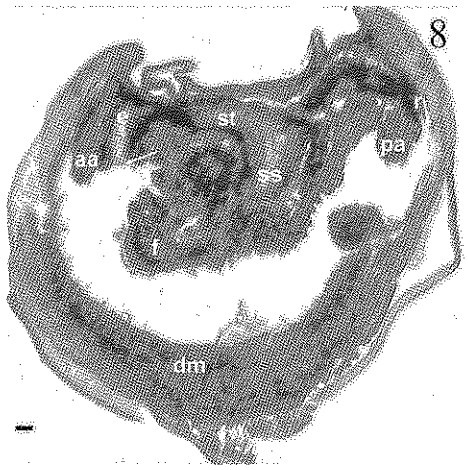
cells. The larval adductor muscle had begun to degenerate and some of the individual fibres were torn in the centre and no longer connected to both valves. On day 3 of metamorphosis it was clear that the adductor muscle was disintegrating with many fewer fibres connected across the body of the animal. The larval mantle cells became constricted at their bases and formed a mass of cells with indistinct cell boundaries (Figure 4).

By the fourth day of metamorphosis, most of the larval adductor muscle had degenerated and the very few remaining fibres were located toward the anterior of the animal. The larval mantle cells underwent substantial alterations during the first 4 days of metamorphosis, forming the mushroom body (Figures 4 and 5). The mantle cells were constricted at the base, the cell boundaries were indistinct, and the cells projected far into the mantle cavity. The beginnings of the definitive mantle were also present immediately adjacent to the larval shells. These cells were small and cuboidal, and appeared between days 3 and 4. The cells of the ventral plate had become more numerous and began to form the juvenile foot. A rudimentary stomach was discernible in some specimens (Figure 5).

By day 5 the muscle fibres of the larval adductor were largely absent and the juvenile adductor muscles had begun to develop. The stomach and digestive glands had formed and the foot had become enlarged (Figure 6). The lateral pits had given rise to gill buds, with one on each side of the animal at this stage. Days 6 and 7 of metamorphosis were characterized by continued development of the juvenile structures. The stomach and style sac were present, and the intestine appeared to be complete. The two adductor muscles were located anteriorly and posteriorly, the positions they will occupy in the adult. Three gill buds were seen in the posterior and ventral aspect of the animal, where they have arisen from the lateral pits (Figure 7). The mushroom body persisted throughout the end of metamorphosis.

Upon completion of metamorphosis the juvenile possessed a ciliated foot with pedal retractor muscles and pedal ganglia. The mouth opened into a ciliated oesophagus leading to the stomach with its crystalline style sac and to the intestine, which terminated in the rectum (Figure 8). The larval mantle cells had degenerated and the definitive mantle cells took their





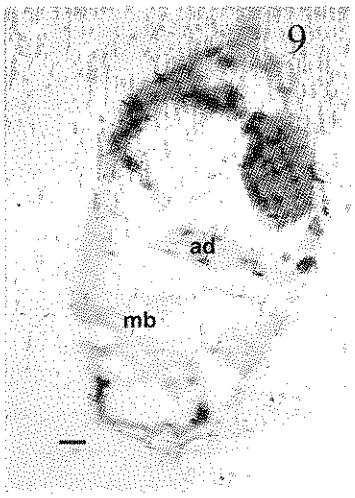


Figure 7. Whole mount of a larva on the sixth day of metamorphosis stained with Alexa 488 specific for actin; scale bar = 100 μm. Abbreviations: aa = anterior adductor muscle; gb = gill bud; pa = posterior adductor muscle.

Figure 8. Saggital section of a juvenile mussel one day after completion of metamorphosis. The top of the image is dorsal; scale bar = 10 μm. Arrow indicates the location of the mouth. Abbreviations: aa = anterior adductor muscle; dm = definitive mantle; e = oesophagus; pa = posterior adductor muscle; r = rectum; ss = style sac; st = stomach.

Figure 9. Cross-section through a larva on the third day of metamorphosis, with the dorsal hinge at the top of the image; scale bar =10 μm. Dark areas indicate sites of DNA synthesis. Abbreviations: ad = larval adductor muscle; mb = mushroom body.

Figure 10. Cross-section through a larva on the sixth day of metamorphosis. The top of the image is dorsal. Dark areas indicate sites of RNA synthesis; scale bar = 10 μm. Abbreviations: f = foot; st = stomach.

place. The three gill bars were ciliated and vigorous ciliary action was observed in live specimens.

The autoradiographic data indicated that synthesis of RNA, DNA, and protein was more extensive in the glochidia larvae and juvenile mussels than in animals undergoing metamophosis. However, during metamorphosis, RNA and DNA synthesis occurred in distinctly different populations of cells at different times during development. The cell populations dorsal to the larval adductor muscle, where the ventral plate and lateral pits were located, were sites of synthesis during the first four days (Figure 9). From day 4 to the end of metamorphosis, RNA, and DNA synthesis appeared to occur more or less equally throughout the body of the animals (Figure 10). Protein synthetic activity showed no such pattern and took place throughout all cells for the entire metamorphic period. The rates of DNA, RNA, and protein synthesis were bimodal, with one peak occurring between days 1 and 4 of metamorphosis, and a second occurring between days 5 and 8 (Figure 11).

DISCUSSION

Our histological data are consistent with previous descriptions of glochidia larval morphology. The presence of two layers of mantle cells has been reported for a variety of species including *Anodonta corpulenta, Lampsilis luteola* (Blystad, 1923; Arey, 1932b), and *Margaritifera margaritifera* (Linnaeus) (Karna & Millemann, 1978). Pekkarinen (1996) observed three pairs of ganglia in the glochidia of *Anodonta anatina* (Linnaeus) and *Pseudanondonta complanata* (Rossmässler), which she referred to as the cerebral, pedal, and visceral ganglia, all located near the lateral pits. The glochidia of *U. imbecillis* possess two obvious pairs of ganglia before metamorphosis, one of which can be seen near the lateral pits. The other pair innervate the larval adductor muscle, and are presumably involved in contraction of the muscle and shell closure. The response of larvae of *U. imbecillis* to mechanical and chemical stimuli has been documented previously (Shadoan & Dimock, 2000), and probably involves these neurons.

For *U. imbecillis* in tissue culture medium and when attached to a host fish (Fisher, 2001), the adductor muscle contracts and the valves remain closed until development to the juvenile is complete. During this time the larval muscle degenerates and the two juvenile adductor muscles develop *de novo*. In fact, the histochemical and actin-specific staining of *U. imbecillis* show the complete degradation of the larval adductor muscle during the first few days of metamorphosis. This observation is consistent with that of Zs.-Nagy and Lábos (1969) indicating that the larval and adult adductor muscles are two distinctly different structures. The adult muscles are initially quite small and presumably enlarge as development continues. While Fukuhara *et al.* (1990) suggest that the adult anterior adductor muscle is derived in part from the larval muscle in *Anodonta woodiana*, such an origin for the adductor muscles of *U. imbecillis* is not supported by this study.

Another aspect of freshwater mussel metamorphosis that has been the subject of some debate is the fate of the larval mantle cells. Braun (1878) described a process by which the cells enlarge and project into the mantle cavity, forming the mushroom body. Although Blystad (1923) did not find this structure in *L. luteola*, Arey (1932b) described it in detail for both *L. luteola* and *A. corpulenta*. The results of the present study indicate that

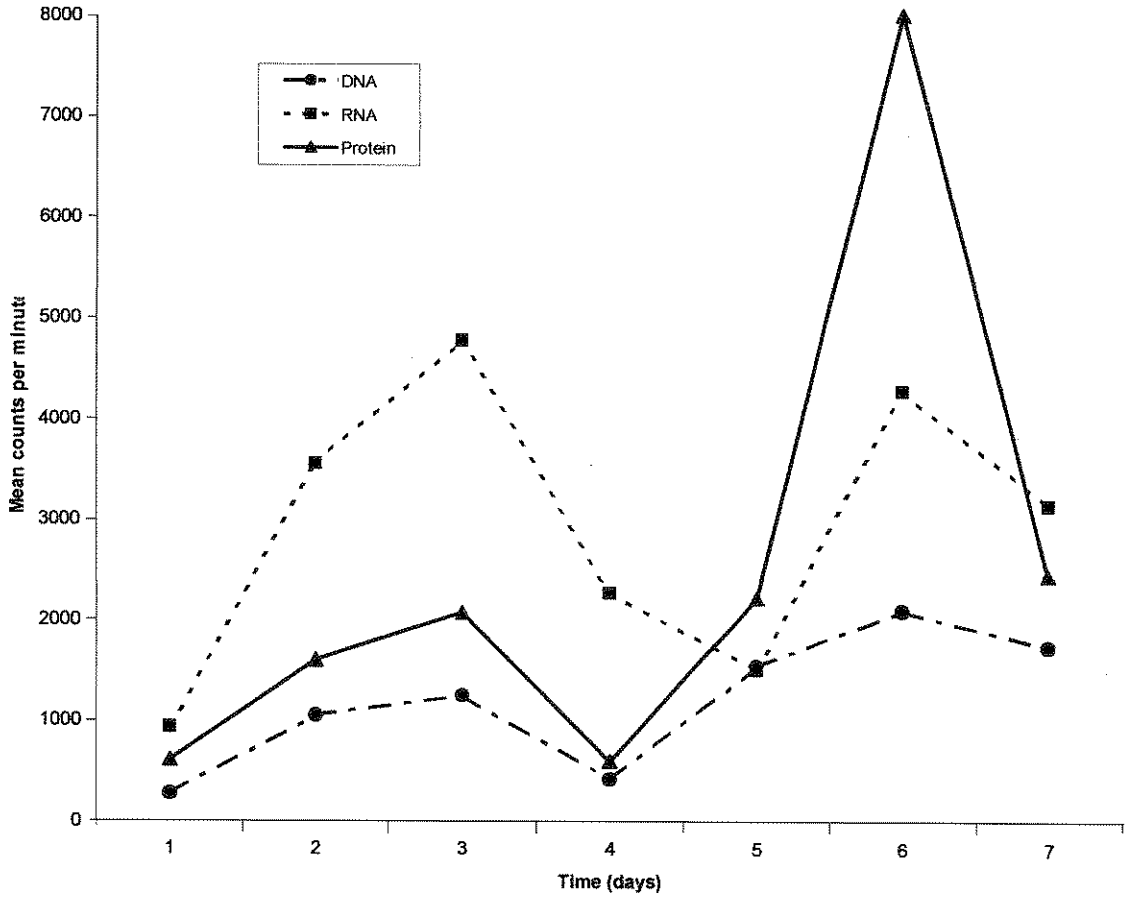


Figure 11. Transcription, translation and protein synthesis throughout metamorphosis. Points are mean ± SE. $n = 40$.

in *U. imbecillis* the larval mantle cells do form the mushroom body and this structure is maintained until the end of metamorphosis. On the final day of metamorphosis, the mushroom body is clearly visible, but by the next day it has disappeared. It is unclear if the cells are digested by the animal itself or if they are sloughed off during the final transition to a juvenile.

The roles of the ventral plate and lateral pits were first described by Lillie (1895) in the glochidia of *Unio* and *Anodonta*. The ventral plate is located in the posterior region of the animal, immediately ventral to the hinge, whereas the lateral pits are posterior to the ventral plate. The ventral plate gives rise to the foot (Lillie, 1895), while the lateral pits give rise to the gills (Blystad, 1923). The autoradiographic data indicate that these structures are areas of high rates of RNA and DNA synthesis during the first few days of metamorphosis. Transcription and translation are confined primarily to the lateral pits and the ventral plate, which is not surprising since they form many of the juvenile structures. Our observations also confirm that the lateral pits are the sites for the origin of the gill bars.

Based on the results from autoradiography and the macromolecular synthesis studies, there appear to be two stages of metamorphosis from glochidium to juvenile mussel in *U. imbecillis* reared *in vitro*. The first stage occurs within the first 4 days and is characterized by high levels of DNA, RNA, and protein synthesis. This is also the period when the larval adductor muscle is degenerating and the larval mantle cells form the mushroom body.

The second stage of metamorphosis occurs during the final few days of metamorphosis before the animals develop into free-living juveniles. This stage is also accompanied by high levels of DNA, RNA, and protein synthesis, and it is during this time that juvenile structures including the stomach, intestine, digestive glands, foot, gill bars, and nerve cords develop. There is no longer a concentration of cellular activity in the lateral pit cells, rather there appears to be normal cell division throughout the animal. This pattern of two stages of metamorphosis appears clear for *in vitro*-reared *U. imbecillis* and has also been observed in *U. imbecillis* reared on host fish; however, other species need to be examined to determine if it is species-specific or a general trend among unionids.

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