The effect of larval age on morphology and gene expression during ascidian metamorphosis

Molly W. Jacobs,1,* Sandie M. Degnan,1 Rick Woods,3 Elizabeth Williams,1
Kathrein E. Roper,1,† Kathryn Green,1 and Bernard M. Degnan1

1Friday Harbor Laboratories, University of Washington, 620 University Road, Friday Harbor, WA 98250, USA; 2Department of Integrative Biology, University of Queensland, Brisbane 4072, Australia; 3The Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Herston, Brisbane 4029, Australia

Synopsis Metamorphosis is both an ecological and a developmental genetic transition that an organism undergoes as a normal part of ontogeny. Many organisms have the ability to delay metamorphosis when conditions are unsuitable. This strategy carries obvious benefits, but may also result in severe consequences for older larvae that run low on energy. In the marine environment, some lecithotrophic larvae that have prolonged periods in the plankton may begin forming postlarval and juvenile structures that normally do not appear until after settlement and the initiation of metamorphosis. This precocious activation of the postlarval developmental program may reflect an adaptation to increase the survival of older, energy-depleted larvae by allowing them to metamorphose more quickly. In the present study, we investigate morphological and genetic consequences of delay of metamorphosis in larvae of Herdmania momus (a solitary stolidobranch ascidian). We observe significant morphological and genetic changes during prolonged larval life, with older larvae displaying significant changes in RNA levels, precocious migration of mesenchyme cells, and changes in larval shape including shortening of the tail. While these observations suggest that the older H. momus larvae are functionally different from younger larvae and possibly becoming more predisposed to undergo metamorphosis, we did not find any significant differences in gene expression levels between postlarvae arising from larvae that metamorphosed as soon as they were competent and postlarvae developing from larvae that postponed metamorphosis. This recalibration, or convergence, of transcript levels in the early postlarva suggests that changes that occur during prolonged larval life of H. momus are not necessarily associated with early activation of adult organ differentiation. Instead, it suggests that an autonomous developmental program is activated in H. momus upon the induction of metamorphosis regardless of the history of the larva.

Introduction Metamorphosis of planktonic marine invertebrate larvae to their sessile juvenile/adult form is both a developmental and an ecological transition. Developmentally, metamorphosis occurs when the adult developmental program takes over from the larval one (Jackson and others 2002). Ecologically, metamorphosis is closely associated with the process of settlement, in which the larva abandons its pelagic habitat and joins the benthos as a juvenile (Hadfield and others 2001). The lifespan of a free-swimming larva can be divided into 2 stages: pre-competence and competence. Pre-competent larvae are obligate members of the plankton, but competent larvae are able to initiate metamorphosis whenever they encounter a suitable metamorphic cue (Hadfield and others 2001). For lecithotrophic or non-feeding larvae, the length of the pre-competent period is developmentally determined and readily measured in the laboratory. Competent larvae, however, can have variable periods in the plankton, with settlement and metamorphosis often contingent upon the availability of appropriate habitat, the sensitivity of larvae to different metamorphic cues, and the energy reserves of the larva. Jackson and Strathmann (1981) suggested that larvae with long pre-competent periods might need correspondingly long competent periods in order to find their way back to suitable habitats. In a complementary model, Pechenik (1980, 1984, 1990) proposed that both pre-competent and competent larval periods should be proportional to development rates, so that larvae with long pre-competent periods should also be capable of long competent periods.

These hypotheses have been difficult to test directly because it is almost impossible to measure the larval competent period in the field. Most field-based
Evidence of prolonged competent periods is indirect and based on comparisons of the morphology and behavior of larvae collected in the field with those reared in the laboratory (reviewed by Pechenik 1990). Such studies provide good evidence for the phenomenon of metamorphic delay but little information on the length of the delay period. Delay of metamorphosis has often been reported in laboratory studies of development, but delay periods reported in the literature are extremely variable and not obviously correlated with pre-competent periods (Pechenik 1990). Pechenik, however, correctly asserted that most of these studies were not designed to test the length of the competent period and do not realistically approximate conditions in the field.

Hadfield and colleagues (2000, 2001) pointed out that the phenomenon of competence may be peculiarly adapted to the marine environment. Metamorphosis in marine invertebrates typically occurs in response to external cues and involves extremely rapid and irreversible changes in both morphology and habitat, in contrast to the more gradual and hormonally controlled metamorphosis characteristic of amphibians and terrestrial insects. There appears to have been strong selection in marine habitats for rapid metamorphosis, so that pelagic larval performance is maximized until the last possible moment but benthic juvenile performance is maximized at the earliest possible moment. In other words, marine invertebrate larvae should become competent to metamorphose when the genetic and morphological architecture of the juvenile is formed well enough to permit a rapid transition.

Collectively, these diverse theoretical arguments about the relative timing of competence and metamorphosis in marine invertebrates make a compelling case for the operation of more than one independent genetic control system. Based on their study of the California red abalone _Haliotis rufescens_, Degnan and Morse (1995) proposed 2 separate developmental pathways: a “morphogenetic” pathway that is triggered by external inductive cues and an “anticipatory” pathway that is activated during larval development and controls the morphogenesis of postlarval structures. The anticipatory pathway is directly linked to the rate of metamorphosis, with prolonged activation of this pathway allowing for more rapid metamorphosis (Degnan and others 1995). The anticipatory pathway may also be closely associated with the acquisition of competence itself; Jackson and colleagues (2005) suggested that the anticipatory pathway may be responsible for building the genetic and morphological architecture needed for the rapid ecological and morphological transition highlighted by Hadfield and colleagues (2000, 2001). Interestingly, responsiveness to inductive cues has been shown to vary during the competent period for larvae of diverse taxa including abalone (Takami and others 2002), polychaetes (Pechenik and Qian 1998; Pechenik and others 2002), colonial ascidians (Marshall and Keough 2003), gastropods (Scheltema 1961; Davis 1994), and many others. Increasing sensitivity to cues or sensitivity to a wider range of cues is probably adaptive for older lecithotrophic larvae in danger of running out of energy (Doyle 1975; Pechenik 1990; Toonen and Pawlik 2001; Marshall and Keough 2003). However, it also presents a dynamic rather than a static developmental picture of competent larval life, consistent with active anticipatory development after initial competence.

Older larvae of taxa that exhibit anticipatory development may be better differentiated than are younger larvae, and time from induction to first juvenile feeding should be faster because aspects of the juvenile morphogenetic program are already engaged. Lecithotrophic larvae that delay metamorphosis past competence may thus compensate partially for the well-documented energy loss associated with prolonged pelagic life (for example, Maldonado and Young 1999; Wendt 2000; Marshall and others 2003; reviewed by Pechenik 1990; Pechenik and others 1998) by continuing to differentiate via the anticipatory pathway. Continued differentiation of postlarval structures in competent lecithotrophic larvae has been reported for abalone (Barlow and Truman 1992; Degnan and Morse 1995; Degnan and others 1995; Jackson and others 2005), hydrozoans (Plickert and others 1988; Freeman 2005), and anthozoans (Chia and Spaulding 1972), although researchers have failed to observe it in a diverse range of other taxa including limpets (Hadfield and Strathmann 1996), chitons (Barnes and Gonor 1973), and starfish (Birkeland and others 1971). Shorter time from induction to first juvenile feeding for older larvae has also been reported for abalone (Jackson and others 2005), hydrozoans (Plickert and others 1988), and polychaetes (Pechenik and Cerulli 1991).

Evidence for anticipatory development in ascidians is also mixed. Berrill (1929) reported prolonged free-swimming periods, delayed tail resorption relative to the rest of metamorphosis, and slower overall metamorphosis for larvae of _Phallusia mamillata_, _Ascidia aspersa_, and _Ciona intestinalis_ reared at high pH. It is unclear, however, whether this is evidence for anticipatory development or simply evidence that high pH inhibits tail resorption and slows development. Delay of metamorphosis for larvae of _Stylea picata_ did not significantly affect the duration of
metamorphosis or rate of development of juvenile structures (Thiyagarajan and Qian 2003). Chiba and colleagues (2004) reported for C. intestinalis that larvae that delay metamorphosis past competence exhibit some precocious differentiation of endostyle, intestinal, and heart rudiments, but do not form stigmatal perforations or stomachs. However, precociously differentiated larvae were unable to complete metamorphosis, suggesting that in this case apparent activity of the anticipatory pathway beyond competence is not adaptive.

Data from diverse taxa on metamorphosis in the presence of transcriptional or translational inhibitors suggest 2 distinct phases of metamorphosis: an early phase that does not require new gene transcription or translation and a later phase that does (Carpizo-Ituarte and Hadfield 2003). Polychaetes (Hadfield and others 2001; Carpizo-Ituarte and Hadfield 2003), nudibranchs (Hadfield and others 2001), and several species of ascidians (Davidson and Swalla 2001; Green and others 2002) initiate but cannot complete metamorphosis when transcription or translation is almost entirely blocked using 5,6-dichloro-1-B-D-ribofuranosylbemisimidazole (DRB; nudibranchs and polychaetes) or actinomycin D (ascidians). Overall transcription levels are also low during post-hatching larval life relative to postlarval and juvenile life (Hadfield and others 2001; Green and others 2002; Carpizo-Ituarte and Hadfield 2003). Although these data are not direct evidence for the operation of 2 separate developmental programs, they are consistent with the prediction that larvae respond to metamorphic cues through a pathway that is set up ahead of time (the morphogenetic pathway). However, analyses of specific genes during ascidian metamorphosis clearly demonstrate that gene expression is still occurring during early metamorphosis (Eri and others 2000; Davidson and others 2003; Woods and others 2004; Kawashima and others 2005). In some cases these genes have been shown to directly control the induction of metamorphosis and early morphogenetic events (Eri and others 2000; Woods and others 2004).

Here, we document genetic and morphological changes in competent Herdmania momus (stolidobranch solitary ascidian) larvae as they age. We relate these changes to genetic changes that occur during early metamorphosis using a microarray gene-profiling approach (Woods and others 2004). Specifically, we tested whether changes that occur in older larvae provide evidence in support of the existence of anticipatory development in this ascidian. In doing so, we explore the genetic consequences of delaying metamorphosis and relate these to the physiological and ecological realities facing larvae that initiate metamorphosis at a later age.

**Methods**

**General collection and culture**

H. momus (formerly H. curvata, Kott 2002) is a tropical solitary ascidian that free-spawns its gametes. Adult H. momus were either collected from the under-sides of coral rubble on the reef crest at Heron Island, Australia (23°27′S, 151°55′E) and maintained in flow-through seawater tables at Heron Island Research Station or collected from among crevices and boulders on the north shore of North Stradbroke Island, Australia (27°34′S, 153°28′E) and maintained in recirculating aquaria at the University of Queensland, Brisbane. Adults were under constant light to prevent spawning (Degnan and others 1996) for not more than 1 week. Gametes were obtained by dissection of gonads from ripe adults. Eggs from several adults were mixed with sperm in large beakers of 0.22 μm filtered seawater (FSW) and washed several times after fertilization as described by Degnan and colleagues (1996). Approximately 8 h after fertilization at 24°C, developing embryos were sorted and unfertilized eggs discarded. Development is similar to that of other solitary ascidians (Satoh 1994; Degnan and others 1996). Tadpole larvae hatched at ~10 h after fertilization. Hereafter we use “larva” to refer to the stage from hatching to initiation of tail resorption, “postlarva” to refer to the stage from tail resorption to the opening of siphons and the initiation of filter feeding (~5 days at 24°C, Degnan, Souter, and others 1997), and “juvenile” to refer to the stage from the initiation of feeding to reproductive maturity.

**Competence assay**

In order to measure the length of the competent period, larvae were maintained undisturbed in sterile 6-well plates at 24°C. We added 40 mM KCl-elevated FSW to 6 replicate wells containing 30 larvae at 2, 3, 4, 9, 24, 36, 50, 75, and 100 h after hatching. One hour after the addition of KCl, larvae were scored for initiation of metamorphosis on the basis of complete tail resorption (see Degnan, Degnan, and others 1997 for methodological details).

**Morphometrics**

Metamorphosis in ascidians is normally accompanied by dramatic morphological rearrangements and extensive migration of mesenchyme cells across the mesenchyme into the tunic, and so changes in these characters could be indicative of early initiation of metamorphic events. We assessed morphological
changes during the competent period by measuring changes in the size and shape of larvae and counting the number of cells present in the space between the larval epidermis and the tunic. Larvae were maintained undisturbed in sterile 6-well plates at 24°C as above. At 4 h after hatching, 12 h after hatching, and every 6 h thereafter, 10 larvae were removed from dishes, placed on a microscope slide, and photographed at 10× and 20× using a trinocular compound microscope and digital camera. At 18 and 48 h after hatching, larvae were transferred after photography to a separate dish and induced to metamorphose using 40 mM KCl-elevated FSW (Degnan, Degnan, and others 1997) to ensure they were still competent. We imported photographs into Image J 1.32 and measured tail length from the base of the neck to the tip of the tail, head length from the base of the neck to the base of the papillae, head width at the widest part of the head, and papilla length (Fig. 1A) for each larva. We also counted the number of cells visible in the photos in the space between the larval head epidermis and the tunic. JMP 5.1.2 was used to measure pairwise Pearson product–moment correlations between larval age and morphological characters.

RNA extraction and microarray analyses

We assessed genetic changes within the competent period by comparing gene expression profiles at competence and at several intervals during the first half of the competent period, based on the competence assay. Based on both the competence and morphometrics assays, we chose to sample in the first half of the competent period when an extremely high percentage of larvae are still competent and developmentally normal in appearance (see below). RNA was extracted from batches of 200 tadpoles at 4, 18, and 30 h after hatching and from batches of 100 postlarvae 14 h after induction of either 4- or 30-h larvae (Fig. 1) using TRI-reagent (Sigma) as described by the manufacturer. Quantity and quality of RNA were assessed using gel electrophoresis and UV spectrophotometer readings at A260 and A280.

From each of these 5 samples, 2 µg total RNA was used to prepare labeled cDNA, coupled with either Cy3 or Cy5 fluorescent dye (Amersham), and purified as described by Woods and colleagues (2004). Microarray chips containing 4836 clones created from a partial cDNA library of *H. momus* developmental genes (Woods and others 2004) were hybridized with Cy3-labeled and Cy5-labeled cDNA. Each of the 5 samples was assayed 3 times, at least once with each dye (dye-swapping), following a loop design (Churchill 2002; Oleksiak and others 2002). In addition, because each

Fig. 1 Morphology of *Herdmania momus* larvae at 4 (A), 18 (B), 30 (C), 42 (D), and 60 h (E) after hatching. We harvested RNA from batches of larvae at 4 (A), 18 (B), 30 (C), 42 (D), and 60 h (E) after hatching. Four-hour (A) and 30-h (C) larvae were induced to metamorphose, and RNA was harvested from 14-h postlarvae (not shown) in each case. t = test cells; p = papilla (labeled in “A” only).
microarray slide contained 2 replicate printed arrays, each replicate assay itself contained 2 replicates, which were used to detect hybridization errors. Chips were washed 1 time in 0.2× SSC, 0.05% SDS and 2 times in 0.2× SSC. A GMS 418 Array Scanner (Genetic Microsystems) was used to detect Cy3 and Cy5 fluorescence hybridized to the cDNA elements on the microarray chips. The .tiff files created by the GMS scanner were imported into ImaGene 4.2 software (Biodiscovery Inc.) to quantify the intensity of labeling on each of the 4836 clones (spots) on each array. Spot signals were collected as fluorescence intensities for each dye excitation wavelength and were subsequently imported as 1-color data into GeneSpring version 7.0 (Silicon Genetics) for further analysis.

**Microarray data analysis**

The total raw dataset comprised 30 measurements (2 replicate measurements on each chip, nested within 3 replicate chips assayed for each of 5 treatments, or developmental stages) for each of 4836 array elements. Raw data were normalized per chip to the 50th percentile and per gene to the median using the 2 replicate measurements on each chip, and measurements <0.01 were set to 0.01. These normalizations controlled for variation in strength of hybridizations between chips and removed negative and extremely low values that could interfere with analysis.

To increase the reliability of measurements, data were then filtered to retain only those spots with a flag value of “present” (meaning a spot that conformed to a minimum intensity above background in addition to an expected shape and size across a spot; this was assessed as part of image processing using ImaGene 4.2) and a normalized expression level of at least 0.5 in all 3 replicates of at least 1 developmental stage. This eliminated 1544 clones that were not present in any of the developmental stages or present only at extremely low levels. The remaining data were filtered to eliminate clones that exhibited highly variable intensities between replicates within developmental stages. We set the cross-gene error model in Genespring to “replicates” and eliminated 486 clones for which the standard deviation was >0.5 between the 3 replicates in one or more of the developmental stages. After these filtration steps, 1058 reliable clones remained in the analysis.

We applied ANOVA (parametric, variances assumed equal) to the normalized, filtered data, with a P-value cutoff of 0.05 and a Benjamini-Hochberg multiple testing correction to identify genes that varied significantly between treatments (larvae and postlarvae of different ages). Treatments were compared using Tukey’s HSD post-hoc test. This methodology allows for the detection of differential expression well below the nominal 2-fold rate. We explored the relationships between treatments by using Genespring to perform cluster analysis (with Spearman’s correlation coefficient). We also used Primer 6.0 (Clarke and Warwick 2001) to perform a principal components analysis of significantly variable clones.

We used ANOVA post-hoc tests and cluster analysis results to assign all clones that varied significantly between treatments to 1 of 4 profiles based on gene-expression patterns during prolonged larval life. These profiles were then used as starting points to compare gene expression profiles during postponed metamorphosis and immediate metamorphosis. In an earlier microarray study using the same organism and the same cDNA library, Woods and colleagues (2004) assigned profiles based on a 2-fold change in gene expression after induction of (immediate) metamorphosis. The 2 datasets are not directly comparable because we used ANOVA instead of arbitrary fold change to generate our profiles, but the comparison is useful because the timepoints studied by Woods and colleagues are much earlier (0.5 and 4 h after induction of immediate metamorphosis) than our timepoints (14 h after induction of immediate or postponed metamorphosis). We compared our results to this earlier dataset in order to infer expression patterns earlier in metamorphosis and overall variability of expression across the metamorphic period.

We did not attempt to ground-truth our microarray data using *in situ* hybridization or quantitative PCR. However, Woods and colleagues (2004) found good correspondence between expression patterns revealed by the microarrays and expression patterns revealed by quantitative PCR despite a less stringent approach to clone selection.

**Results**

**Effect of delay of metamorphosis on larval morphology and competence**

By 4 h after hatching at 25°C, almost 100% of larvae were competent to respond to a metamorphic cue (Fig. 2). Percentage competency remained high until 50 h after hatching. After this point, percentage metamorphosis declined sharply. The same result was clearly observed during the morphological assay, albeit from fewer timepoints: 10 of 10 larvae metamorphosed readily 18 h after hatching, but by 48 h after hatching only 6 of 10 larvae had completely resorbed their tails 1 h after induction (although 2 additional larvae successfully resorbed their tails by 12 h after induction). After 50 h metamorphosis was noticeably slower
and more erratic and tail resorption was often incomplete (M. Jacobs, personal observations).

Several significant changes in morphology were observed during prolonged larval life. Larval tail length was significantly negatively correlated with larval age, and most of this reduction in tail length occurred abruptly between 30 and 54 h after hatching (Fig. 3A). Conversely, larval head width and papillae length (Fig. 1A) increased gradually, but significantly, with larval age. There was no significant relationship between larval head length and larval age. These changes were associated with a change in larval head shape over larval life; as larvae aged, heads became trumpet-shaped (Fig. 1C–E) rather than ovoid (Fig. 1A and B). We also observed a significant increase in the number of test cells over time. As cell density increased (Fig. 1), cells became increasingly difficult to count using our photographic method, and so cell counts late in larval life are probably underestimates.

Effect of delay of metamorphosis on gene expression profiles of larvae and postlarvae

We assayed RNA from larvae at 4, 18, and 30 h after hatching and from postlarvae 14 h after the induction of either 4- or 30-h larvae (Fig. 1). Thus, the 2 postlarval stages were the same age post-induction (14 h), but different ages post-hatching (18 and 44 h). ANOVA with Benjamini-Hochberg multiple testing correction revealed 235 clones that vary significantly (P-value cutoff 0.05) between these developmental stages. Of these, 87 (37%) have been sequenced and 64 (27%) matched to known genes and are listed in Table 1.

Principal components analysis of the 235 variable clones revealed that 4-, 18-, and 30-h larvae formed separate clusters in multivariate space, but postlarvae clustered together regardless of age post-hatching (Fig. 4). The first 3 principal components combined to explain 82.3% of the variation observed across treatments (axis 1 = 44.3%, eigenvalue (λ) = 23.48; axis 2 = 22.6%, λ = 12.00; axis 3 = 15.3%, λ = 8.14). Older larvae (18 and 30 h) clearly segregated from early competent (4 h) larvae and all postlarvae along principal component 1 (Fig. 4A and B). Principal component 2 separates 18-h larvae from all other treatment groups (Fig. 4A), and similarly principal component 3 separates newly competent (4 h) larvae from all other treatment groups (Fig. 4B). The 2 postlarval treatments could not be distinguished from one another along any principal components axis.

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Figure 5 shows a heatmap (a graphic in which intensity of gene-expression is represented by color intensity, with black representing high intensity) for the 235 variable clones. Each column is 1 sample, and each individual bar in the column is 1 clone. The color of the bar indicates the expression level of that clone for that sample. Variation in gene-expression for an individual clone or group of clones can be observed by reading horizontally across the figure, and variation in gene-expression level between clones in a sample can be observed by reading vertically down the columns. Cluster analysis of genes grouped clones with similar expression patterns together, revealing blocs of genes that vary in similar ways between treatments. Cluster analysis on samples grouped samples that contained similar global expression patterns according to Spearman’s correlation coefficient. This analysis is shown by the tree at the top of Figure 5 and revealed that larvae of different ages formed separate clusters, but postlarvae clustered together regardless of age (Fig. 5), as indicated by the PCA analysis.

Several gene-expression profiles are immediately evident and are marked by brackets and numbers that refer to principal-components axes that best explain variation across samples for that bloc of genes. One large bloc of genes was downregulated (light colors) in older tadpoles only (“1” in Fig. 5). A second bloc of genes (“2” in Fig. 5) was distinguished by low expression levels for 18-h larvae. Expression level of a third bloc of genes rose steadily over tadpole life and was also relatively high in postlarvae (“3” in Fig. 5).

Gene expression profiles during prolonged larval life

Profile 1 (Fig. 6A) is approximately analogous to bloc 1 in Figure 5 and includes 65 clones that decreased
significantly during extended larval life (Fig. 6A). Of these, 53 (82%) increased again during postponed metamorphosis while 12 (18%) remained constant. Interestingly, 97% of all clones in profile 1 did not change in expression level over the first 14 h after induction of immediate metamorphosis. However, Woods and colleagues (2004) found that more than half of these clones (58%) varied by at least 2-fold (13 up, 25 down) during the first 4 h after induction of immediate metamorphosis (Table 1). Sequenced clones in profile 1 included several copies of a post-like protein (HrPost-1), 1 myosin gene, 1 sequence-specific DNA binding protein (Hec-T2AG), and a number of genes involved in basic cellular processes such as metabolism and RNA processing (Table 1).

Profile 2 (~bloc 2; Fig. 5) includes 51 clones that decrease temporarily during extended larval life but are upregulated again prior to postponed metamorphosis (Fig. 6B). Of these, the vast majority exhibited no change over either postponed (49 clones; 96%) or immediate (48 clones; 94%) metamorphosis in our study. Again, fold change over the first 4 h of immediate metamorphosis (Woods and others 2004) was more variable: 8 clones (16%) increased 2-fold and 14 clones (27%) decreased 2-fold. Sequenced clones falling into profile 2 included 1 myosin gene, 1 acyl carrier protein, several cytochromes, and a number of ribosomal proteins (Table 1).

Profile 3 (~bloc 3; Fig. 5) includes 49 clones that increased significantly during extended larval life (Fig. 6C). Of these, 35 (71%) remained upregulated...
### Table 1  
Gene identities and expression profiles for significantly variable genes

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during postponed metamorphosis while 14 (29%) decreased again (Fig. 6C). Clones in profile 3 increased significantly (24 clones) or remained constant (25 clones) but did not decrease during immediate metamorphosis in our study (Fig. 6C). During the first 4 h of immediate metamorphosis, 10 clones (20%) increased by at least 2-fold, 4 clones (8%) decreased, and 35 clones (72%) remained constant (Woods and others 2004) (Table 1). Sequenced clones in profile 3 included **Hec-cip2A** and **Hec-cip2B** (solid black lines, Fig. 7), several copies of mannose-binding lectin (dashed gray lines, Fig. 7), 1 copy of profilin, a transport ATPase (**Hec-SPCA1d**), and a number of ribosomal proteins (Table 1).

Profile 4 includes the remaining 70 significantly variable clones. These clones exhibited no significant change in expression level during extended larval life (Fig. 6D), although many exhibit trends similar to profiles 1–3. Expression profiles during metamorphosis were extremely variable (Fig. 6D); 15 clones (22%) varied significantly during immediate metamorphosis and 33 clones (47%) during late metamorphosis in the study, and 25 clones (36%) varied by at least 2-fold during the first 4 h of immediate metamorphosis (Woods and others 2004). Sequenced clones in profile 4 included several mannose-binding lectins, several signaling receptors including **hikaru genki** (solid gray line, Fig. 7) and a complement receptor-like protein, 1 myosin, several ribosomal proteins, and a number of genes involved in intermediary metabolism and catabolism (Table 1).

**Comparison of gene expression changes during metamorphosis**

The expression of 44 clones increased or decreased significantly during immediate metamorphosis (30 upregulated and 14 downregulated, Fig. 8A).
In contrast, 102 clones increased or decreased significantly during postponed metamorphosis (69 upregulated and 33 downregulated, Fig. 8B). There was almost no overlap between clones that changed during immediate metamorphosis and clones that changed during postponed metamorphosis. In general, genes that were upregulated during immediate metamorphosis (black solid lines, Fig. 8A) were also upregulated during extended larval life. During postponed metamorphosis, the expression levels of these clones did not markedly change, or they decreased, but they never increased (gray solid lines, Fig. 8B). For example, Hec-cip 2A and 2B follow this pattern (Fig. 7). Two clones were upregulated in both immediate and postponed metamorphosis: 1 clone of mannose-binding lectin (mbl) and 1 unsequenced clone (thick black lines, Fig. 8). Genes downregulated during immediate metamorphosis (black dashed lines, Fig. 8A) either remained constant (gray dashed lines, Fig. 8B) or were also downregulated (black dashed lines, Fig. 8B) during postponed metamorphosis.

Most genetic changes during postponed metamorphosis reflected reversals of genetic trends during extended tadpole life. Clones that decreased in expression level during extended larval life were significantly upregulated during postponed metamorphosis (black solid lines, Fig. 8D; see also Fig. 6A), so that upregulation during metamorphosis represented a return to the genetic status quo at competence. With the exception of 1 clone of mbl and 1 unsequenced clone (see above), these clones did not change in expression level over immediate metamorphosis (gray solid lines, Fig. 8C). Conversely, many clones that increased in expression over extended larval life were significantly downregulated during postponed metamorphosis (black dashed lines, Fig. 8D; see also Fig. 6C). These clones remained constant (gray dashed lines, Fig. 8C) or were downregulated (black dashed lines, Fig. 8C) during immediate metamorphosis.

**Discussion**

Delay of metamorphosis for *H. momus* larvae was accompanied by several morphological changes, including a decrease in tail length, a change in head shape from roughly elliptical to trumpet-shaped, a ballooning of the tunic, and an increase of cells within the tunic. The change in head shape could be caused by cells migrating from the anterior part of the head to the posterior part of the head, resulting in a wider posterior, by addition of material from the tail to the posterior of the head as the tail shortens and is partially resorbed, by changes in epidermal cell shape, by differentiation of endodermal structures, or by a combination of these.

Older larvae also had longer papillae (Figs 1 and 3), possibly as a consequence of the general reduction observed in the anterior end of the head or localized restriction of head tissue. It is unknown whether papillae length affects initial attachment strength or whether the stickiness of papillae changes with larval age, but it would be interesting to measure dislodgement force of newly attached larvae of different ages. The larval tunic expands around the head in older larvae. The increase in tunic cells may underlie this expansion, with these possibly synthesizing additional...
material for tunic deposition. During metamorphosis, trunk mesenchyme cells undergo extensive migration across the epidermis into the tunic (Cloney 1982, 1990; Eri and others 2000; Davidson and Swalla 2002). While our data are not direct evidence that these same cells were migrating during prolonged larval life, Woods and colleagues (2004) have shown that mesenchyme cells expressing \textit{Hec-Cip2B} migrate into the tunic during early metamorphosis and Eri and colleagues (2000) demonstrated that PAT (papillae-associated tissue) cells may originate from this larval region; the movements of mesenchymal cells during prolonged larval life merit further closer study. Movement of mesenchyme cells across the epidermis and tunic expansion also may be associated with differentiation of the tunic as an immunocompetent organ and may be associated with upregulation of immunity-related genes (Davidson and Swalla 2002). Important to this study, cell migration within and out of the head as well as tail shortening are typical of morphological changes associated with early metamorphosis (Degnan and others 1996; Degnan, Degnan, and others 1997; Degnan, Souter, and others 1997).

Morphological changes were accompanied by genetic changes. Principal components analysis of gene-expression data clearly segregates older larvae, younger larvae, and postlarvae. These differences can be explained by several major changes in steady-state transcript levels during development. First, the abundance of many transcripts decreases during prolonged larval life. Most of these increase again when these larvae ultimately metamorphose. Second, some transcripts accumulate during prolonged larval life. Levels of these transcripts remain high or decrease only slightly in postlarvae. Most remaining clones that vary significantly between treatment groups are upregulated in 4- and 30-h larvae relative to 18-h larvae and (to some degree) both sets of postlarvae.

Clones that decrease in levels of expression over prolonged larval life may do so because transcription has been halted and RNA degradation has increased. There is a low level of gene transcription during the first phase of larval development in \textit{H. momus} (that is, acquisition of competence, Green and others 2002), although the expression of some of these genes is crucial for the induction of metamorphosis (Eri and others 2000; Woods and others 2004). Despite changes in expression profiles of many genes during the first 4 h of metamorphosis (Woods and others 2004), marked increases in global gene expression do not begin until a few hours later (Green and others 2002). Our results are consistent with the continuation of limited general gene activity during larval life, with transcript levels remaining the same or decreasing in a majority of genes. As in other studies that have looked at specific genes, some genes display opposite patterns, with transcript levels higher in older larvae (for example, Profile 3).

Changes in gene expression during prolonged larval life disappear after metamorphosis

Despite significant changes in transcript levels over prolonged larval life, we find only moderate evidence that the genetic consequences of delaying metamorphosis carry over into postlarval life. Most changes in gene expression that occur in older larvae appear to be reversed when these larvae eventually metamorphose.
That is, despite clear differences in abundances of a large number of transcripts in newly competent (4 h after hatching) and older (18 and 30 h after hatching) larvae, the gene expression profiles of postlarvae 14 h after induction could not be distinguished regardless of the age at which induction occurred. These data suggest that to a large extent transcript levels are recalibrated during early metamorphosis and postlarval developmental genetic patterns are not influenced by variation in larval age. For example, *Hec-cip2A* and *Hec-cip2B*
genes are activated during the acquisition of competence and during early metamorphosis in mesenchyme cells fated to be localized in the tunic (Woods and others 2004). Five clones in our study matched to either Hec-cip2A or Hec-Cip2B, and transcript levels of all of these increased with larval age in a manner reminiscent of an anticipatory pattern. Although transcript levels in 30-h larvae were much higher than transcript levels observed in younger larvae or postlarvae, after metamorphosis transcript levels in postlarvae from younger and older larvae were indistinguishable, indicating a marked decrease in transcript abundance during the metamorphosis of older larvae (Fig. 7). Thus, some genetic changes in older tadpoles were reminiscent of changes observed during immediate metamorphosis in the present study and in previous studies (Woods and others 2004). However, those differences disappeared after metamorphosis and it is unclear whether this putative anticipatory development carries over into postlarval life or helps to mitigate some of the negative physiological consequences of depleting maternally provided nutritional reserves during prolonged larval life.

While the bulk of gene change that occurs during aging appears to have no effect on gene expression profiles in postlarvae, anticipatory development could result in faster overall metamorphosis for older tadpoles. Our study did not test this question directly, but differences in metamorphic timing could explain some of the discrepancies we observed in gene change between immediate and postponed metamorphosis. For example, several genes involved in innate immunity are known to play a critical role in the metamorphic response pathway coordinated by Hemps, an EGF-like protein secreted in the anterior papillary region of the larva and known to be a key regulator of early metamorphic events (Eri and others 2000; Davidson and others 2003; Woods and others 2004). We identified 4 significantly variable genes in this category: 3 distinct clones of mannose-binding lectin (mbl) and 1 of Hikaru genki. Expression patterns of these clones were extremely variable: Hec-mbl1a and Hec-mbl1b increased over immediate metamorphosis in accordance with previous studies, but did not consistently increase over postponed metamorphosis. Hec-mbl2B and Hikaru genki decreased or remained constant during metamorphosis (Fig. 7).

This discrepancy between immediate and postponed metamorphosis could have several explanations. First, mbl1a and mbl1b may be involved in the Hemps-coordinated morphogenetic response pathway in immediate metamorphosis but not postponed metamorphosis, which suggests that the genetic mechanism for responding to inductive cues varies with larval age (see Pechenik and others 2002). Second, the timing of the response pathway may be different for larvae of different ages. Woods and colleagues (2004) recorded a peak in mbl expression between 30 min and 4 h after induction. It is possible that during immediate metamorphosis expression levels were still high enough at our 14-h timepoint for us to detect a significant increase, but that we did not detect a change during postponed metamorphosis because the genetic changes associated with metamorphosis were accelerated and expression levels had already dropped to pre-induction levels. This hypothesis—based on these few genes—is consistent with the premise of an anticipatory developmental pathway in ascidians that results in a faster metamorphic transition for older larvae (Jackson and others 2005).
Anticipatory development in ascidians?

The induction of metamorphosis in *H. momus* is characterized by rapid retraction of the papillae, resorption of the tail, and initiation of body axis rotation. About 4 h after induction, major morphogenetic changes begin that lead to the formation of the filter-feeding juvenile body plan (Degnan and others 1996; Degnan, Degnan, and others 1997). This phase of metamorphosis takes ~5 days and includes continued body axis rotation, differentiation of the endodermal primordium, and synthesis of the juvenile tunic. These changes are typical of many marine invertebrate larvae, for whom metamorphosis consists of a series of rapid morphogenetic movements coupled with a slower period of differentiation (Hadfield 2000; Hadfield and others 2001). Differentiation can take place ahead of the rapid morphogenetic movements associated with settlement (for example, colonial ascidians) or afterwards (for example, solitary ascidians). For solitary ascidians, anticipatory development can be viewed as precocious activation during the competent period of gene expression pathways associated with the slower differentiation phase at metamorphosis or as the response pathway to inductive cues (for example, the Hemps pathway).

The dramatic morphological changes that occur during the initiation of metamorphosis (that is, the first 4 h) are accompanied by dramatic changes in gene expression. About 50% of the *H. momus* genome is activated or repressed by at least 2-fold during this stage of immediate metamorphosis (Woods and others 2004). While some of these genes are only transiently activated or repressed, a large proportion of these genetic changes last well into metamorphosis and presumably contribute to subsequent morphogenetic events. Based on observations of other marine invertebrates such as the abalone *H. rufescens* (for example, Jackson and others 2005) we predicted that activation of the anticipatory pathway (Degnan and Morse 1995; Degnan and others 1995) in *H. momus* would result in changes in gene expression in older larvae reminiscent of changes observed during immediate metamorphosis. We also predicted that some of these genetic changes would carry over into the postlarval stage, so that postlarvae derived from older larvae would display accelerated development and significantly different gene expression profiles than postlarvae derived from younger larvae.

Our data provide limited support for the existence of anticipatory development in *H. momus*. Over the competent period, larvae that delayed metamorphosis underwent several consistent and predictable morphological changes that were clearly reminiscent of changes that occur during metamorphosis, such as tail shortening and migration of cells into the tunic. Similarly, many genetic changes observed in older larvae were similar to changes observed during immediate metamorphosis. These differences were not, however, reflected in postlarvae derived from young (newly competent) and older larvae. Despite large genetic changes during larval life, we did not observe any genetic carryover effects, and it is consequently unclear.

**Fig. 8** Gene expression profiles over metamorphosis. Black solid lines: significant upregulation; black dashed lines: significant downregulation; gray lines: no significant change. Lines in (A) and (B) represent clones that either decreased or increased significantly in mean expression level during immediate metamorphosis. Lines in (C) and (D) represent a different set of clones that either decreased or increased significantly in mean expression level during postponed metamorphosis. Five clones decreased significantly during both immediate and postponed metamorphosis [black dashed lines in (B) and (C)]; 2 clones increased significantly during both immediate and postponed metamorphosis [thick black lines in (A–D)], and 2 clones increased significantly during immediate metamorphosis but decreased significantly during postponed metamorphosis (gray lines). The sets of clones in A+B and C+D are otherwise entirely non-overlapping.
whether the observed morphological and genetic changes in older larvae represent compensation for the reduction in energy associated with prolonged planktonic periods.

We propose 3 explanations for the genetic and morphological changes observed in older larvae. First, genetic and morphological changes observed during extended larval life in the current experiment may be unrelated to metamorphosis. Under this scenario, inherent in the *H. momus* early-metamorphosis program is a mechanism that recalibrates transcript levels so that postlarvae are morphogenetically identical regardless of past history. Perturbation of gene expression pathways associated with metamorphosis, for example by the inhibition of Hemps signaling (Eri and others 2000), should have the same impact on postlarvae (Woods and others 2004) regardless of larval age at induction.

Second, observed genetic and morphological changes may represent anticipatory activation of adult developmental pathways. While postlarval transcript levels and anecdotal observation of rates of postlarval development do not support this hypothesis, more detailed morphometric analyses may reveal evidence of anticipatory development. For example, the timing of adult organ differentiation relative to induction of metamorphosis in larvae of different ages could be mapped using a combination of detailed morphological observations and analysis of organ-specific gene expression patterns.

Finally, observed genetic and morphological changes may represent anticipatory activation of pathways associated with the response of larvae to metamorphic cues, but not differentiation of adult organs. Early activation of these pathways could result in a faster pelagic-to-benthic transition for larvae that delay metamorphosis (that is, Hadfield 2000; Hadfield and others 2001) or in a change in responsiveness to metamorphic cues (for example, Marshall and Keough 2003). We did not, however, find any evidence of upregulation of Hemps in older larvae, which would be compatible with increased ability to initiate metamorphosis rapidly (Eri and others 2000; Woods and others 2004). In addition, *H. momus* is known to be induced by a wide range of substrata when competence first develops (Degnan 2001), making an increase in sensitivity to cues during prolonged larval life less likely.

Anticipatory development (or the lack thereof) may be linked to the phenomenon of “spontaneous” metamorphosis. In the laboratory, a large percentage of competent larvae of *H. momus* and many other species of ascidians will initiate spontaneous metamorphosis in sterile culture conditions (Degnan, Souter, and others 1997) without an apparent inductive cue. Development of these ascidians is normal and identical to development of those individuals that metamorphose in response to naturally occurring metamorphic cues (Degnan 2001). Evidence of such spontaneous metamorphosis in nature has been observed, with the collection of unattached, metamorphosing *H. momus* in the plankton (B. Degnan, unpublished data). Although the probability of survival for these pelagic postlarvae is extremely low, it is still higher than the probability of survival for a larva that has waited too long and no longer has the energy to complete metamorphosis (that is, older than 75 h; Fig. 2). Larvae of solitary ascidians are often retained near parents (Havenhand and Svane 1991), increasing the probability of passive transport to suitable habitat for pelagic postlarvae. High rates of spontaneous metamorphosis may provide an alternative mechanism to increase survival of species that lack the ability to genetically anticipate metamorphosis by activating metamorphic developmental pathways early. In contrast to *H. momus*, the abalone *Haliotis* exhibits extensive anticipatory development (Degnan and Morse 1995; Degnan and others 1995; Jackson and others 2005; E. Williams and B. Degnan, unpublished data) but has little or no spontaneous metamorphosis and has relatively specific settlement requirements. Prolonged time in the plankton may trigger activation of the anticipatory pathway for lecithotrophs such as *Haliotis*, but species such as *H. momus*, which display only limited or no anticipatory development, may compensate by allowing high rates of spontaneous metamorphosis.

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References


