Integrative and Comparative Biology Advance Access published July 27, 2006

Signaling mechanisms underlying metamorphic transitions in animals

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Synopsis Metamorphosis in many animal groups involves a radical transition from a larval to a juvenile/adult body plan and the challenge of orchestrating 2 overlapping developmental programs simultaneously, that is, larval development and juvenile development. Metamorphic competence directly precedes this radical change in morphology and can be best described as the developmental potential of a larva to undergo the radical transition in response to internal or external signals. Several studies have employed genomic approaches (for example, microarrays or subtractive hybridization methods) to gain insights into the complexity of changes in gene expression associated with metamorphic transitions. Availability of this technology for an increasing number of organisms from diverse taxonomic groups expands the scope of species for which we can gain detailed understanding of the genetic and epigenetic architecture underlying metamorphosis. Here, we review metamorphosis in insects, amphibians, and several marine invertebrate species including the sea hare *Aplysia californica* and summarize mechanisms underlying the transition. We conclude that all metamorphoses share at least 4 components: (1) the differentiation of juvenile/adult structures, (2) the degeneration of larval structures, (3) metamorphic competence, and (4) change in habitat. While transcription levels detected by microarray or other molecular methods can vary significantly, some similarities can be observed. For example, transcripts related to stress response, immunity, and apoptosis are associated with metamorphosis in all investigated phyla. It also appears that signaling mediated by hormones and by nitric oxide can contribute to these stress-related responses and that these molecules can act as regulators of metamorphic transitions. This might indicate either that all of these distantly related organisms inherited the same basic regulatory machinery that was employed by their most recent common ancestor (RCA) in orchestrating life history transitions. Alternatively, these regulatory modules may have been used by the RCA for other purposes and have been independently co-opted to regulate metamorphic transitions in a variety of distantly related animals. We propose that such instances of independent origin or homoplasy in the evolution of metamorphosis might have resulted from specific constraints in signal transduction pathways. Modern genomic tools can help to further explore homoplastic signaling modules when used in a comparative context.

Metamorphic transitions across animal phyla

In 1978, Fu-Shiang Chia (1978) observed that the "problems of settlement and metamorphosis are diverse and complicated"; despite many studies in the interim, this remains true today. Metamorphosis among animals includes a change in habitat and the abandoning of the larva, a transitory postembryonic stage in an animal's life history that is adapted to a different environment than the adult. It possesses 2 types of structures: larval and juvenile/adult. While larval structures stop growing and differentiating directly after settlement, juvenile/adult tissues may differentiate both before and after metamorphosis. Therefore, a larva can be further characterized by the overlapping development of larval and juvenile/adult structures.

Phenotypically, there are many obvious differences between, for example, metamorphosis of flies, frogs, and sea slugs. On a more general level, Hadfield (2000) and Hadfield and others (2001) emphasized that important differences exist between metamorphic transitions in amphibians and flies as compared with the metamorphic transitions of many marine invertebrate larvae. While many marine invertebrates produce small larvae that undergo very rapid transitions from plankton to benthos, amphibian and insect larvae are generally bigger and the transition appears much more gradual (but see Hodin 2006). Settlement

From the symposium "Metamorphosis: A Multikingdom Approach" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 4–8, 2006, Orlando, Florida.

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Integrative and Comparative Biology, pp. 1–17
doi:10.1093/icb/icl023

(that is, habitat change) occurs in response to specific external cues. Data from a few selected species suggest that little *de novo* gene transcription is required during this process and that mechanisms of signal transduction appear to rely primarily on cell–cell conductance (reviewed by Hadfield 2000; Hadfield and others 2001). This contrasts with the metamorphosis of insects and amphibians where postembryonic development proceeds via hormonal coordination Nijhout (1994) and Tata (1996). Still, recent evidence indicates that hormones regulate development to metamorphosis in a variety of marine invertebrate larvae as well (Eales 1997; Heyland and Hodin 2004; Heyland and others 2004, 2005, 2006; Heyland and Moroz 2005). In the context of this evidence, signal transduction events during settlement can be viewed as a special ecological adaptation to the marine environment (Hadfield 2000).

Gene regulation is inherently modular. Networks of regulatory genes are generally robust to perturbation and can be expressed in various contexts for diverse functions (Schlosser 2002). Therefore, such networks (modules) can be co-opted for novel developmental processes. Moreover, this property also allows the testing of whether certain modules are independently co-opted for the same function owing to specific constraints.

The so-called true or primary larvae evolved several times independently among animals (Hadfield and others 2001; Wray 2000) and show a remarkable diversity of form and function. Yet from a developmental point of view, all larvae face the challenge of coordinating 2 disparate signaling networks, one that regulates the development of larval structures and one that regulates the development of juvenile/adult structures. We hypothesize that this represents a constraint at the level of the signal transduction pathways involved in this process and predict that similar modules regulating the 2 divergent developmental programs within the same organism were co-opted in disparate organisms independently multiple times. Comparing mechanisms underlying the metamorphic transition across phyla therefore has the potential to uncover some of these instances of homoplasy, that is, similarities based on convergent or parallel evolution on a mechanistic, cell-signaling level, and point to specific constraints in signaling mechanisms (see also Hodin 2000).

In order to compare metamorphic transitions between disparate species we propose to distinguish the following components of metamorphosis: (1) growth and differentiation of juvenile/adult-specific structures, (2) breakdown of larva-specific structures, (3) metamorphic competence, and (4) change in habitat. Figure 1 illustrates these general characteristics of metamorphic transitions for several species graphically. The *x*-axis represents time and the *y*-axis represents the percentage of the developmental program that was completed. The red curve shows larval development and differentiation (the larval developmental program), the green curve shows adult development and differentiation (the juvenile/adult developmental program). For example, in *Aplysia californica*, all larval structures are present after hatching and the larva grows in size while adult-specific structures continue to differentiate inside the larva. After settlement, larval structures disappear rapidly as the juvenile transforms gradually into the adult (Fig. 2).

We selected one or 2 illustrative and well-studied examples from 3 animal phyla for which we will give a brief overview of metamorphosis. These are amphibians (Chordata), ascidians (Chordata), insects (Arthropoda), and sea slugs (Mollusca). In all cases we focus on the 4 common phases of metamorphosis outlined above. We then discuss the change in habitat more specifically in the context of environmental factors influencing the regulation and timing of metamorphosis. Finally, we review selected intrinsic regulatory mechanisms underlying the metamorphic transition.

Amphibian metamorphosis

Many excellent reviews have been published on the regulation of metamorphosis in anuran amphibians (frogs) (for recent reviews see Tata 2005 and Buchholz and others 2006). We do not intend to provide a complete review here. Instead we will only discuss the 4 phases mentioned above for the metamorphosis of *Xenopus laevis* and summarize information on signaling events involved in these various parts of the metamorphic transition. A schematic representation of *X. laevis* metamorphosis is given in Figure 1.

Based on previous work, 3 periods can be distinguished in the metamorphic transition of *X. laevis*: premetamorphosis (stages 45–53), prometamorphosis (stages 54–58), and metamorphic climax (stages 59–66). Some of the most significant changes are the remodeling of the intestine at around stage 58, the degeneration of the larval epithelium around stages 60–62, the subsequent differentiation of the secondary epithelium of the adult frog, and finally the degeneration of the tail at the end of stage 62.

Growth and differentiation of juvenile/adult structures and breakdown of larval structures in *X. laevis*

Metamorphosis in *X. laevis* is a relatively slow and gradual process. It stretches over a period of approximately 30 days. It begins at stage 55 when the hind

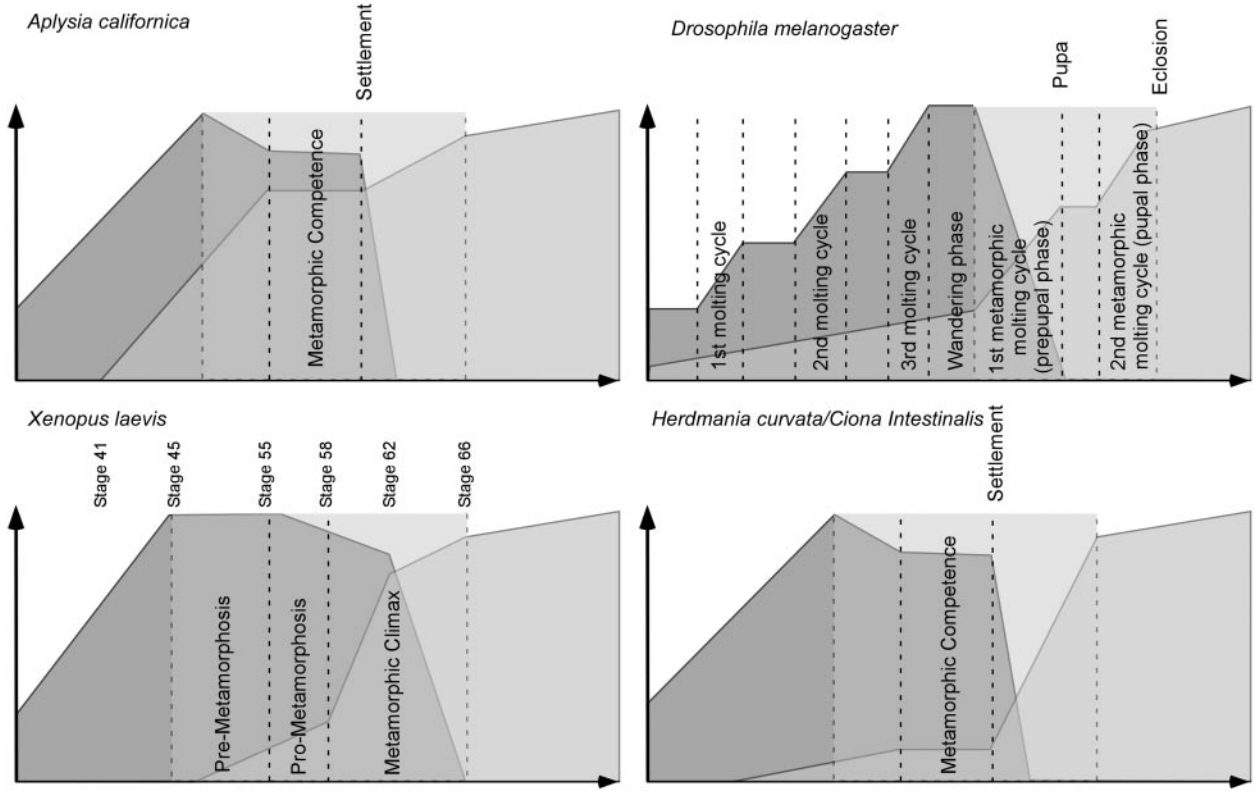

Fig. 1 Schematic representation of metamorphic transitions in four species representing three phyla: *Aplysia californica* (Lophotrochozoa: Mollusca), *Drosophila melanogaster* (Ecdysozoa: Arthropoda), *Xenopus laevis* (Chordata: Vertebrata); *Herdmania curvata* and *Ciona intestinalis* (Urochordata). The *x*-axis represents time (in relative units that have been scaled to the total length of the life history of these organisms. This means that time is not comparable between the different species in absolute values). The *y*-axis represents % morphological structures present. The red curve represents larval morphological structures. The green curve juvenile/adult morphological structures. The grey area represents the metamorphic transition. In all four cases larval structures disappear during the metamorphic transition, while juvenile/adult structures undergo differentiation, growth and development. Developmental stages are mostly based on literature data: For *Drosophila melanogaster* we referred to Nijhout (1994), *Xenopus laevis*, Nieuwkoop and Faber (1956), Shi and others (1996) and Buchholz and others (2006), *H. curvata* (Eri and others 1999), *Aplysia californica* (personal observations and literature data from Kriegstein 1977). The metamorphic transition does not include the entire adult morphogenesis. In all four cases some differentiation occurs after the metamorphic transition, such as gonadal development. In *A. californica*, the shell is present after metamorphosis. However, it is significantly reduced in the adult.

limb buds begin to grow [throughout this article we refer to the staging scheme from Nieuwkoop and Faber (1994)]. It ends at stage 66 when the tail resorption is terminated (Shi and others 1996; Buchholz and others 2006).

Throughout the metamorphic transition larval characters disappear in parallel with adult characters being formed. Relatively few structures are either formed *de novo*, such as the limbs, or resorbed, such as the tail and gills. The majority of organs are instead transformed from a larval to an adult form (Buchholz and others 2006). Both the larval and adult developmental program are under the control of various endogenous factors, 2 essential ones being the levels of thyroid hormone receptors (TRs) and the concentration of intracellular free thyroid hormone (TH). Triiodo-L-tyrosine (T3) is the active hormone in amphibian metamorphosis that binds with very high affinity to TRs. The action of TH in metamorphosis can be best described by the so-called dual-function model (reviewed in Buchholz and others 2006). Based on this model, TR without its ligand functions as a repressor of TH-regulated genes, while the conformational changes that occur upon ligand binding change TR into an activator of TH-regulated genes. Such a mechanism fulfills the very important function of repressing the metamorphic genes by TR until the larva is ready to undergo the metamorphic transition in both a developmental and ecological sense.

Metamorphic competence in *X. laevis*

The metamorphosing frog can synthesize THs endogenously after the development of a functional thyroid gland (stage 53). Notably, some of the most dramatic

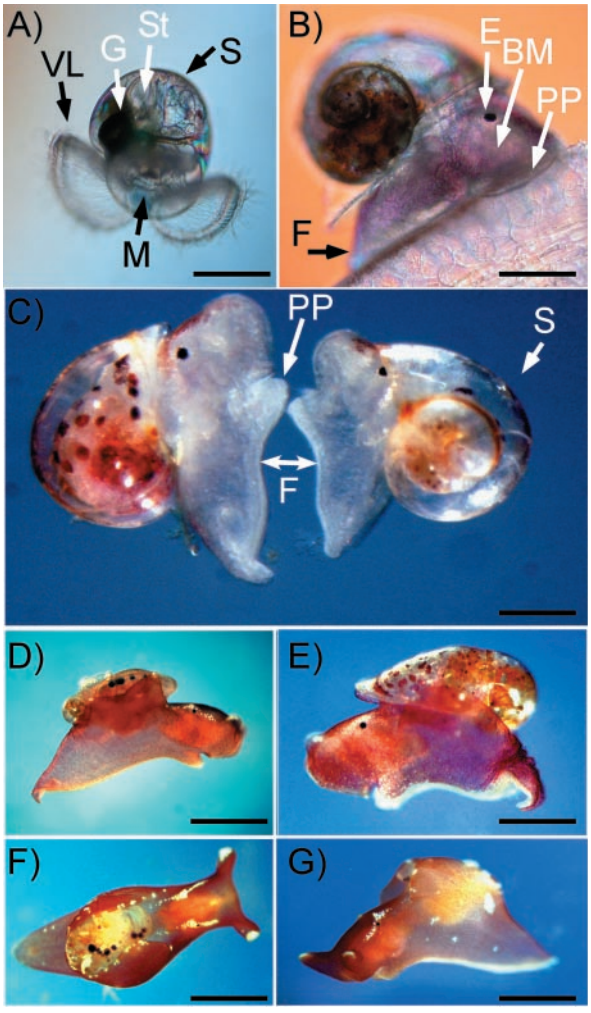

Fig. 2 Metamorphic development of *Aplysia californica* (**A**–**G**). After hatching, veliger larva (A) grow and differentiate all organ systems that can be found in the adult *Aplysia*. The propodium (PP) begins to form at stage 5. This structure is essential for settlement and crawling after settlement (stage 7, B). Although metamorphic competence (stage 6, A) correlates with several morphological characteristics, such as the formation of red spots, ink vesicles and the formation of the PP, there is no guarantee that these larvae will settle (see text). Upon settlement larvae permanently attached and shed their velar lobes (stage 7, B) after an extended period of rest that they spend retracted inside the shell. Within 24–48 h (personal observations), feeding begins (see the text). Stage 8 (C) marks the end of the metamorphic transition. It is characterized by the fusion of the two halves of velar lobe (VL) rudiments and the larval heart stops beating. This stage is characterized by adult feeding and locomotory behaviors. After juveniles start feeding a strong burst of growth sets in. Stages 9 through 13 (D–G) are characterized by the development of specific juvenile structures and reduction of the shell. VL: Velar Lobe, G: Gut, St: Stomach, S: Shell, M: Mouth, E: Eye, BM: Buccal Mass, PP: Propodium, Scale bar in A: 130 μm, in B: 120 μm, in C: 110 μm, in D: 550 μm, in E: 550 μm, in F: 800 μm, in G: 800 μm.

morphological changes happen after a thyroid gland is formed. (Buchholz and others 2006). Both T4 (L-thyroxine) and T3 (3,3′,5-triiodo-L-thyronine) levels increase exponentially until they peak at metamorphic climax (Shi and others 1996). Two forms of TR occur in *Xenopus*: TRalpha and TRbeta. Both are expressed premetamorphically. Metamorphic competence can best be described by the patterns of expression of TRalpha; late in embryogenesis, a tissue can only transform in response to TH if TRalpha is expressed (that is, at around stage 47) (Shi and others 1996; Denver and others 2002; Buchholz and others 2006). This has been confirmed by knockdown experiments using dominant negative TRs (Buchholz and others 2006). Moreover, if the larval thyroid gland is removed experimentally, tadpoles maintain the capacity to undergo metamorphosis indefinitely (Shi and others 1996; Buchholz and others 2006; Buchholz personal communication). TRalpha protein levels are ~2–3 times higher than those of TRbeta throughout larval development. However, at metamorphic climax, TRbeta expression levels increase exponentially to similar levels as TRalpha [for a description of hormone and receptor levels see Buchholz and others (2006) and Shi and others (1996)].

Environmental control of metamorphosis in *X. laevis*

The timing of metamorphosis, measured by the time from hatching until transition from the aquatic to terrestrial habitat, can vary substantially even within species. This phenotypic plasticity is triggered by various biotic and abiotic factors, which, in the majority of cases, act via the hypothalamic–pituitary axis (reviewed by Denver and others 2002). Specifically, larvae of several amphibian species accelerate development in response to desiccation and other stress-related environmental triggers (Denver and others 2002). This response involves corticosteroid stimulation of thyroid stimulating hormone, which results in TH synthesis and accelerated restructuring of the tadpole toward metamorphic climax (Denver and others 2002).

In the larval nervous system, major remodeling occurs in specific brain regions necessary for the development of adult-specific behaviors or sensory organs that will fulfill crucial functions in the adult environment. For example, the Mauthner neurons and motor neurons that innervate tail muscles are involved in the escape response of tadpoles. Another example is the transformation of the visual system from the panoramic vision of the tadpole to the binocular vision of the adult frog, involving a large part of the retina and visual projections into the diencephalon associated with it

(Denver and others 1997; Denver 1998; Gilbert 2005). Furthermore, a functional neuroendocrine system is required before the end of the metamorphic transition. Several neurosecretory structures are therefore formed during prometamorphosis and their function is under the direct control of T3 (Denver and others 1998).

Not all adult structures are formed by the time the tadpole has transformed into a frog and moved into terrestrial habitat. While sex determination and primary differentiation occur during embryonic and larval development in frogs (for review see Hayes 1998), sexual maturity is often reached long after (that is, several months to years) the metamorphic transition (Duellman and Trueb 1994).

Signaling during the metamorphic transition

We outlined above the importance of THs and TRs for the metamorphic transition in amphibians. Both subtractive hybridization and microarray approaches have been used to identify T3/TR-regulated genes. Many T3-responsive genes are differentially expressed after stage 47 (transition to competence), including TH-binding proteins, key regulators of signal transduction, transcription, and metabolism (Veldhoen and others 2002).

Tail regression is one of the most visible and dramatic morphological changes during anuran metamorphosis, and has therefore received considerable attention. Upon binding T3, TR directly triggers apoptosis and other forms of programmed cell death (PCD) in the tail at the end of the metamorphic transition (reviewed by Nakajima and others 2005). Key enzymes induced by TR are stromelysin-3, collagenase-3, and serine dipeptidyl peptidases (Ishizuya-Oka and others 2000; Ishizuya-Oka and others 2001a, 2001b).

Recent research has uncovered an interesting link between THs and NO signaling. NO is a gaseous molecule that can have both activating and repressing effects on apoptosis and various other essential cellular processes (Dimmeler and Zeiher 1997). *In vitro* and *in vivo*, NO can lead to the inhibition of catalase activity, causing oxidative stress in cells via the production of reactive oxygen species (ROS) (Brown 1995; Hanada and others 1997; Kashiwagi and others 1999; Chandra and others 2000; Inoue and others 2004). Studies on tail regression *in vitro* have confirmed that hydrogen peroxide (H_2O_2) and aminotriazole, a catalase inhibitor, enhanced markedly the apoptotic process and the activity of Cu/Zn-type superoxide dismutase (SOD) increased with a concomitant decrease in its catalase activity in the tail (Hanada and others 1997). ROS generation in the proximity of mitochondria can stimulate apoptosis and preliminary evidence suggests that this process might be involved in tail regression during amphibian metamorphosis (Inoue and others 2004). THs can enhance NO generation by stimulating the activity of inducible and constitutive NO synthase activity (NOS) (Remirez and others 2002; Fernandez and others 2005), potentially contributing to tail regression (Kashiwagi and others 1999).

Recent studies have revealed that the promoter regions of inducible peptide antibiotics are often regulated by the transcriptional control machinery of NF kappa B in the skin of amphibians. Apart from being involved in various developmental processes (Steward 1987; Kanegae and others 1998; Shimada and others 2001) NF kappa B has also been linked to inflammatory events in vertebrates including innate immune response (Belvin and Anderson 1996; Wu and Anderson 1998; Chen and others 1999; Lawrence and others 2005). Furthermore, they function as activators of cell-survival genes (Wang and others 1998). The finding of NF kappa B being involved in the immune response of the skin might have potential implications for amphibian metamorphosis as well. The skin of amphibians is one of the larval structures that is completely remodeled at metamorphosis, a process involving apoptosis (for review see Nakajima and others 2005). A direct link to this process, however, has not been established at this point.

Metamorphosis in *Drosophila melanogaster* and other insects

Insect metamorphoses are highly diverse. For the purpose of this review we will generalize a few aspects of metamorphosis as it occurs in dipterans and lepidopterans and specifically discuss mechanisms underlying the metamorphic transition for the fruit fly *Drosophila melanogaster* and related species, primarily because of the overwhelming amount of genomic data that is available compared to other species. Note, however, that some important differences exist between the metamorphic endocrinology in *D. melanogaster* and, for example, the hawkmoth *Manduca sexta*, for which there is a large amount of information on hormone levels and their role in orchestrating metamorphosis. Unless indicated otherwise, information on the metamorphosis of holometabolous insects and specifically *D. melanogaster* was derived from the studies by Bate and Martinez (1993), Nijhout (1994), Riddiford (1996b), White and others (1999), and Gilbert (2005). For a recent review on *Drosophila* endocrinology see Flatt and others (2005, 2006). A schematic representation of *D. melanogaster* metamorphic development can be found in Figure 1.

Growth and differentiation of juvenile/adult structures and breakdown of larva-specific structures in insects

In many holometabolous insects, metamorphosis represents an abrupt change in morphology, physiology, and habitat that transforms a voraciously feeding terrestrial or aquatic larva into a flying (aerial) and reproducing adult. The most dramatic part of the metamorphic transition begins after the larva has passed through several larval instars each ended by a larval ecdysis (see also Fig. 1). The rudiments of adult-specific structures begin to form in early stages of embryogenesis. These rudiments are called imaginal disks and contain the precursor cells that will give rise to all the appendages of the adult: the eyes, head appendages, legs, genitalia, and wings. Note, however, that the precursors of the abdomen and the internal organs of the adult, such as the gut, salivary glands, and brain, do not arise from discs *per se*. Instead, they differentiate from groups of histoblasts (Curtiss and Heilig 1995).

Imaginal disks form in a relatively continuous manner throughout larval development. Unlike the larval epidermis, they do not secrete any cuticle at ecdysis and their cell divisions are not coordinated with those of the molts. However, at the end of the third and final instar (for *D. melanogaster*), during the first metamorphic molting cycle (prepupal phase) which transforms the larva into a pupa, imaginal discs undergo a phase of accelerated growth and differentiate. The first metamorphic molting cycle is followed by a second one (pupal phase) during which the adult morphology differentiates. In the genus *Drosophila*, as in all of the derived cyclorrhaphous *Diptera*, adult morphogenesis occurs within the puparium, a hard case that lasts until eclosion, the hatching of the adult fly.

As in amphibians, many processes of adult morphogenesis and degeneration of larval structures occur simultaneously. Within the puparium, the larval epidermal cells, muscles, salivary glands, and prothoracic glands break down. On the other hand, adult muscles and the tracheal system are being formed. Many components of the larval nervous system degenerate while new neurons arise from groups of undifferentiated neuroblasts. These changes are essential for the development of adult-specific behaviors (Truman and others 2004).

Larval development and metamorphosis in insects is largely orchestrated by the action of 2 hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH) (Riddiford 1993, 1996a, 1996b; Nijhout 1994; Flatt and others 2005, 2006). Morphological changes during the prepupal and pupal phase occur in indirect and direct response to 20E pulses. In *Manduca*, JH acts as a *status quo* hormone: for any of the larval molts, JH has to be present when 20E is beginning to rise. For the pupal molt, on the other hand, it has to be absent at the onset of 20E rise. Both, the synthesis and secretion of 20E is controlled by another important insect hormone, PTTH (prothoracicotropic hormone, released from the neurosecretory cells in the prothoracic gland). In the 5th instar, PTTH release is inhibited by JH. This will inhibit the metamorphic molt (via 20E action) until all the JH has been cleared from the hemolymph. During a normal metamorphosis this has to happen after a critical weight has been reached by the larva which will subsequently shut off the corpora allata (JH secretion gland). If a larva is starved before it reached this critical weight, the corpora allata will remain active and pupation will be postponed.

Although the effect of 20E and JH on the development of the fruit fly *D. melanogaster* is comparable to that in *Manduca*, the degree to which JH affects metamorphosis in *D. melanogaster* is reduced, possibly due to the highly accelerated life history of the fruit fly. For example, high JH levels neither completely inhibit the larval–pupal transition nor do they inhibit subsequent differentiation of the head and thorax, although they do disrupt metamorphosis of the nervous and muscular system when applied during the prepupal period (Restifo and Wilson 1998; Zhou and others 2002). The function of another player, the transcription factor *broad*, appears to be largely conserved between *Manduca* and *D. melanogaster* where it is expressed during the larval to pupal transition (Zhou and others 2002). Generally, *broad* has the ability to suppress adult genes, while activating pupal genes. As such it fulfills a key regulatory function in the metamorphosis of holometabolous insects. Intriguingly, a recent study by Erezyilmaz and colleagues (2006) revealed that *broad* expression is maintained at each nymphal molt but disappears at the molt to the adult. Their data further suggests that evolutionary shifts in broad expression may have been involved in the evolution of metamorphosis in insects.

Metamorphic competence and the change in habitat in insects

In insects, the term competence has been used to describe the ability of tissues or imaginal disks to undergo the transition to a new stage in response to a specific hormonal trigger (that is, either larval instar to the next larval instar, larval instar to pupa, or pupa to adult) (Nagata and others 1999; Zhou and others 1998; King-Jones and others 2005; Mirth 2005).

For example, larval tissues become sensitive to JH before the end of the final instar, and only in the absence of JH the change in commitment from larva to pupa can occur. The subsequent 20E peak (commitment peak) will induce wandering behavior, a process during which larvae stop feeding and wander around on the substrate to find a suitable pupation habitat.

On a very general level commitment followed by wandering behavior in *D. melanogaster* is comparable with competence of many marine invertebrate species (see Hodin and others 2001; Bishop and others 2006). It is even more tempting to draw a comparison between marine invertebrate larvae and aquatic larvae of mosquitoes (Brackenbury 1999). Another shared aspect between developmental transitions of marine invertebrates and insects is that they are under the strict control not only of specific exogenous factors such as temperature and photoperiod but also of specific external chemical cues, some of which remain to be characterized. Recently, Truman and others (2006) showed that a nutritional cue results in the release of a yet unknown metamorphosis initiating factor that can override the suppression of disc formation by JH.

Signaling during the metamorphic transition in insects

The transcriptional response to the main metamorphic players in *D. melanogaster*, 20E, the functional ecdysone receptor (EcR and its partner Ultraspiracle *usp*), JH, its putative receptors, and *broad* have been addressed through a variety of approaches, including the puffing pattern of salivary gland polytene chromosomes (Becker 1959; Clever 1965; Ashburner 1972, 1974), subtractive hybridizations (Hurban and Thummel 1993), and microarray analysis (White and others 1999; Arbeitman and others 2002;Rifkin and others 2003; Beckstead and others 2005; Flatt and others 2005; Tu and others 2006). Major changes in expression levels can be linked to processes that remove larval tissues and build up adult tissue, respectively. For example, the entire larval musculature is replaced in the adult, involving extensive apoptosis and myogenesis. On the level of the nervous system, adult-specific neurons establish new connections, and various factors, such as the neurotactin gene, are involved in growth cone and neuronal guidance (Delaescalera and others 1990; Truman and others 2004).

Despite many broad similarities in metamorphic regulation in different taxa, a rather sobering result came out of a recent comparison of genome-wide transcription levels during the development of several closely related *Drosophila* species. Less than 30% of all genes that change expression levels during onset of metamorphosis in one lineage show comparable changes in the other investigated lineages (Rifkin and others 2003). Genes that show consistent expression levels across lineages are primarily coding for transcription factors, while their downstream targets can be highly variable (White and others 1999; Arbeitman and others 2002; Rifkin and others 2003, 2005; Beckstead and others 2005). Outside of the *Drosophila* clade, relatively little is known about such global expression-level changes during metamorphosis in insects. However, a preliminary gene expression analysis of metamorphosis in carpenter ant (*Camponotus festinates*) partially confirms the findings by Rifkin and colleagues (Goodisman and others 2005). A large amount of variation in gene expression between species is revealed when compared with *Drosophila*, suggesting that specific molecular mechanisms involved in the regulation of metamorphosis may vary substantially among insect taxa (Goodisman and others 2005).

As in amphibians, apoptosis and other forms of PCD are crucial components of the metamorphic transition in insects, and as expected, many genes involved in this process are expressed directly in response to 20E and its binding to the nuclear hormone receptor complex ECR/USP (Beckstead and others 2005; Flatt and others 2005, 2006). Several of these genes, such as the *broad* complex, E74 and E93, regulate caspase-mediated apoptosis leading to the destruction of larval tissue during both phases of metamorphosis (Baehrecke and Thummel 1995; Yin and Thummel 2005). Intriguingly, a recent study suggested that ecdysone induces autophagy, a different form of cell death, which may allow the pupa to extract nutrients from the dying tissue, thereby supporting growth of new adult structures and tissues (Rusten and others 2004).

Using a microarray approach, Beckstead and others (2005) discovered that many immunity-related and stress-related genes are activated in response to 20E treatment at the onset of metamorphosis, a result we independently confirmed in *Drosophila* S2 cells (Heyland and Flatt, unpublished) and discuss further in this symposium (Flatt and others 2006).

NO acts as a suppressive signal of cell proliferation in several insect species. Specifically, it has been documented in *Drosophila* metamorphosis (Kuzin and others 1996) and the proliferation of neural precursors in the imaginal eye disc in *M. sexta*, a process induced by ecdysteroids (Champlin and Truman 2001). Furthermore, studies on *D. melanogaster* eye development indicate that inhibition of NOS (nitric oxide synthase, the NO synthesis enzyme) against the background of suppressed apoptosis can result in an increased number of ommatidia (Enikolopov and others 1999). In the silkworm *Bombyx mori*, an

infection signal (in this case endotoxin, a part of the outer membrane of the cell wall of gram-negative bacteria) led to the upregulation of NOS, thereby triggering apoptotic events in target cells during metamorphosis (Inoue and others 2004), a process that can also be induced by ecdysone in this species (Choi and others 1995).

Finally, a recent study by Reinking and others (2005) showed that the nuclear hormone receptor E75 has the ability to bind heme in *D. melanogaster*. The redox state of E75-heme subsequently affects the ability of this complex to bind NO and CO. Moreover, it affects the interaction between E75 and its heterodimerization partner HR3. E75 is an early 20E-responsive gene product and is, among other things, an important regulator of JH and 20E signaling during molting cycles and metamorphosis (Dubrovskaya and others 2004). Although still preliminary, these results might help explain the link between NO signaling, metamorphosis, and apoptosis in insects.

Metamorphosis in marine invertebrates

Life histories in marine invertebrate species are extremely diverse. Still, the general theme of a pelagobenthic life cycle is that a free-swimming larval form feeds and/or disperses in the plankton for a time, and then settles into the benthic habitat where it undergoes a more or less dramatic transition both morphologically and physiologically to its juvenile/adult habitat on the sea floor (benthos). Owing to the diversity of taxa with such a transition and the lack of information about the specific mechanisms underlying this process we have to focus on a few selected examples. Figure 1 shows 3 examples with such transitions: 2 solitary ascidian species and the sea hare *A. californica* (discussed below).

Growth and differentiation of juvenile structures and breakdown of larval structures

Much like the imaginal discs of insects, juvenile structures in the larvae of many species of marine invertebrates are formed during the larval period. The majority of adult tissues and organs, therefore, are often present before the larva undergoes a switch in habitat. Larvae of the sea hare *A. californica* (Mollusca: Opisthobranchia) provide a good example (Fig. 2).

Aplysia develops via a so-called veliger larva, a specialized feeding stage that can swim by means of 2 large, ciliated velar lobes (Fig. 2A). After hatching from the egg mass, it feeds on microscopic unicellular algae from the phytoplankton for approximately 3 weeks. During this time it develops all essential adult organs except the reproductive system. At stage 6 in Kriegstein's (1977b) staging scheme (see Fig. 2A), the veliger enters the competent period (see also below).

In order to illustrate differentiation of larval and juvenile structures, we will discuss the development of the nervous system of *Aplysia*. Neurogenesis in the sea hare is particularly interesting because it provides an opportunity to understand the ontogeny of individual neurons and their projections, which regulate specific behaviors such as feeding and locomotion. The mechanistic basis of these processes is well understood at the level of the adult nervous system (Carew and Sahley 1986; Bailey and Kandel 1993, 1996), but developmental information is largely lacking.

One commonly used marker of selected larval neurons is serotonin (5HT) (Croll and Chiasson 1989; Goldberg and Kater 1989; Barlow and Truman 1992; Marois and Croll 1992; Kempf and Page 2005). The first serotonergic cells appear on day 5 in embryogenesis (Marois and Carew 1990) and are located in the apical organ. This organ is critical in mediating sensory input from the velar lobes and, possibly, is involved in response to specific settlement cues (Byrne and others 2001; Byrne and Cisternas 2002; Leise and others 2004; Kempf and Page 2005). Adult ganglia are starting to form within the first 10 days of embryonic development in parallel with the larval nervous system. At the moment of hatching both the cerebral and pedal ganglia are present (Kriegstein 1977a). Other ganglia are formed subsequently resulting in a complete adult nervous system by the time competence is reached.

The role of the adult nervous system during larval development is not well understood. It is, however, likely that the larval and adult nervous systems interact during larval development. Possible functions of such interaction could be related directly to metamorphic competence and the integration of development with environmental cues. Behavioral changes developing during the metamorphic period are likely based on the re-organizations and rewiring of the ganglionic adult nervous system since the velar lobes and other parts of the larval nervous system disappear at metamorphosis.

Aplysia represents just one extreme in the spectrum of metamorphic patterns of marine invertebrates. Some larvae such as those of cnidarians and bryozoans form adult structures after transition to the adult habitat and not before (Fig. 1). Larvae of solitary ascidians generally spend a very short time (few hours to few days) in the plankton before they become sensitive to settlement cues (Cloney 1982).

In *Herdmania curvata* (a species we will discuss below in terms of mechanisms underlying metamorphic transition) differentiation of juvenile organs primarily occurs after settlement (Fig. 1). While this is true for many ascidian species, considerable variation exists within this group (Davidson and others 2002). Postsettlement morphological changes involve the resorption of the tail, large parts of the larval nervous system, and several transitory larval organs (Cloney 1982). At the same time adult structures such as the gut, muscles, heart, and adult nervous system differentiate.

Metamorphic competence

Several contributions to this symposium discuss metamorphic competence in marine invertebrate larvae (Bishop and others 2006; Hodin 2006; Jacobs and others 2006) and other papers have significantly expanded on this issue (Pechenik 1987; Pechenik and others 1998; Hadfield 2000; Bishop and Brandhorst 2001, 2003; Hadfield and others 2001).

Competence can be best described as the potential of larvae to undergo metamorphic transition in response to appropriate settlement cues. As such, it fulfills several important functions in an animal's life cycle. From an ecological standpoint it gives the larvae an opportunity to find suitable settlement sites. Developmentally, it appears that some larvae undergo little to no differentiation at this stage (Hadfield 2000; Del Carmen 2003), indicating that all necessary structures required for the juvenile stage are either fully developed or will develop after settlement. From the point of view of costs and benefits, a larva needs to weigh the costs of staying in the plankton (high mortality in the plankton, loss of energy with eventual burn out, loss of the capacity to settle at all) against the benefits of staying in the plankton (high mortality in the benthos, increased probability to find suitable habitat) (but see Pechenik 1987).

Competent larvae of *Aplysia,* for example, can remain at stage 6 for weeks and will only settle if they encounter appropriate cues (Fig. 2) (Kriegstein 1977b). While competence correlates with several morphological characteristics, such as the formation of red spots, ink vesicles, and the formation of the propodium (Fig 2B and C), there is no guarantee that these larvae will settle, indicating that internal factors such as hormones or neurotransmitters determine this stage as well. Note, however, that these morphological characters can be viewed as a minimal requirement in that larvae will not settle if these structures or characters are absent. Still, if competent larvae are exposed to the red alga *Laurencia*, they will try to attach to it using a mucous-like compound secreted by the metapodial glands (Kandel 1979).

The larvae of *H. curvata* are the opposite of *Aplysia* in that they will undergo spontaneous settlement, that is, they will settle in the absence of specific cues. In fact, the timing of acquiring competence is rather predictable. Larvae can usually be induced to settle a few hours after hatching (Degnan and Johnson 1999). It is possible that postsettlement morphogenesis and low specificity to settlement cues (see below) are both direct consequences of the dramatically shortened larval period in many solitary ascidians.

Environmental control of metamorphosis and settlement

Aplysia larvae that have encountered appropriate settlement cues usually explore the substrate (a particular kind of seaweed) from several minutes to several hours. No permanent settlement takes place unless they are metamorphically competent. After an extended period of rest which they spend retracted inside the shell, the larvae settle and shed their velar lobe (stage 7; Fig. 2B). Within 24–48 h (personal observations), larvae start to feed on the seaweed using their radular apparatus (adult mode of feeding). The buccal mass moves constantly, indicating that the settled larva is actively feeding. Stage 8 marks the end of the metamorphic transition (Fig. 2C). It is characterized by the fusion of the 2 rudiments of the velar lobes and by cessation of the larval heart beating. At this point, all adult feeding and locomotory behaviors have developed. Stages 9 through 13 (Fig. 2D–G) are characterized by the development of specific adult structures such as the rhinophores and the reproductive system. Note that we do not consider this differentiation as part of the metamorphic transition since it is comparable with the growth and differentiation that occurs in many organisms without metamorphosis.

As discussed above for amphibians, insects, and *Aplysia*, the larval nervous system plays an essential role in coordinating metamorphic events and integrating them with the environment in marine invertebrate species (Burke 1983; Marois and Carew 1990; Degnan and others 1997a; Byrne and Cisternas 2002; Page 2002a, 2002b; Kempf and Page 2005). Chemosensory pathways, which frequently can be activated by various neurotransmitters and other nonspecific compounds are mediators between settlement cues and subsequent metamorphic changes (reviewed in Degnan and Morse 1995).

Still, for some species, such as the ascidian *H. curvata*, a functional central nervous system is not required to propagate signals that induce

morphological changes during the metamorphic transition (Degnan and others 1997a). While separate, competent anterior fragments can be induced to 'metamorphose'; posterior fragments can only be induced to do so in the presence of papillae from the anterior fragment. It appears that specific cells from these papillae have the ability to employ the post-larval morphogenetic program directly via the stimulation by a secreted factor (Degnan and others 1997a).

A recent mutagenesis screen on the ascidian species *Ciona savignyi* identified a mutant called *vagabond* (*vag*). The *vag* mutant fails to form the 3 palps on the anterior trunk, has defects in the morphology of the sensory organs in the sensory vesicle, and is unable to settle on a substrate and/or to complete metamorphosis. While mutants do have palp neurons, they form ectopically in the trunk, rather than in the normal triangular field (Tresser, personal communication).

Signaling during metamorphic transitions of marine invertebrates

As discussed for amphibians and insects, metamorphosis involves increased amounts of cell proliferation and degradation via various forms of PCD. The successful coordination of these 2 systems in the same organism during the metamorphic transition presumably requires the expression of genes in a specific spatial and temporal pattern. What factors might regulate such a coordination?

Tail regression is one of the 2 main apoptotic events during development of *Ciona intestinalis*, which evokes a comparison with the similar, but independently evolved, process of amphibian tail resorption, described above. Ascidian tail regression, as in amphibians, has been shown to be regulated via caspase-dependent apoptosis (Chambon and others 2002). The second main apoptotic event in ascidian development is test cell regression during embryogenesis. Despite occurring well before settlement, test cell death is indirectly metamorphic in nature, since removal of test cells results in perturbation of the metamorphic transition (Sato and others 1997). Maury and others (2006) recently established that test cells undergo caspase-dependent apoptosis, which is also true for tail resorption. Test cell death can be repressed by the transcription factor NF kappa B upon activation by the TH thyroxine (T4) which *Ciona* larvae can synthesize endogenously (Patricolo and others 2001) and which also might be maternally provisioned.

EGF signaling has been implicated in coordinating differentiation and cell death during adult eye development in *Drosophila* (Bangs and White 2000). Recently, an EGF-like molecule (HEMPS) in the ascidian *H. curvata* has been shown to be involved in the regulation of cell differentiation and proliferation. The levels of HEMPS increase during development to competence suggesting that this factor may be involved in metamorphic coordination in this species (Eri and others 1999; Woods and others 2004). Furthermore, HEMPS appears to be acting as a modulator of gene expression rather than transcriptional initiator. A macroarray analysis of genes affected by HEMPS reveals that it affects several signal transduction pathways including those involved in innate immunity (Woods and others 2004). Note, however, that no direct link between HEMPS and apoptosis has been established and Eri and others (1999) emphasized, that no expression of HEMPS could be detected in the proximity of the resorbed tail. This suggests that other signals must be involved in PCD of the tail in this species. Still, an indirect link is considered likely (Degnan personal communication).

Previous to the study by Woods and others (2004), subtractive hybridizations in the ascidian species *Boltenia villosa* identified transcripts involved in invertebrate innate immunity that are differentially expressed during metamorphic competence (Davidson and Swalla 2002; Davidson and others 2002). Some of these factors may be associated with the ability of larvae to respond to bacterial films in the benthos and might be used as receptors for settlement cues (see also Maki and Mitchell 1985). Alternatively, this response could be a result of cellular stress associated with morphological changes during and after metamorphosis (Davidson and Swalla 2002). Finally, it could simply be a general mechanism protecting metamorphosing juveniles from infectious microbes encountered in their new benthic habitat.

Hormones have remarkably pleiotropic effects on development (see also Nijhout 1994; Denver and others 2002; Heyland and others 2005; Flatt and others 2006) and are ideal candidates for differential regulation of larval and juvenile morphogenesis and apoptosis. THs have been discussed as regulators of life history transition in many organisms (Eales 1997; Heyland and others 2005; Flatt and others 2006) and new data particularly emphasize the role of THs as regulators of larval development to competence in echinoderms (Heyland and Hodin 2004; Heyland and others 2004, 2005; Heyland and Moroz 2005; Bishop and others 2006; Hodin 2006). Intriguingly, in all experiments THs promote differentiation of juvenile structures while simultaneously leading to the early degeneration of larval structures.

TH action has also been investigated in ascidian species. In *B. villosa* TH synthesis inhibitors dramatically inhibited adult differentiation after

settlement (Davidson and others 2002). Interestingly, thyroxine appears to accelerate development to metamorphosis in another ascidian species (*C. intestinalis*), that is, it exerts effects presettlement (Patricolo and others 2001; D'Agati and Cammarata 2006). This example demonstrates that the action of specific signaling molecules has to be viewed in the context of an animal's life history. THs clearly affect the differentiation of juvenile structures in both species. The timing of this event, however, is shifted relative to settlement, leading to the different effects. Moreover, retinoic acid mediates patterning of the adult endoderm after settlement in *H. curvata*. As in *B. villosa*, this species also undergoes the majority of juvenile differentiation after settlement (Hinman and Degnan 1998, 2001; Hinman and others 2000).

Still, the signaling pathway activated by TH and other hormones in ascidians and other marine invertebrates remains elusive. Ectopic application of hormones in *B. villosa* did not rescue the effects of inhibitors of TH synthesis when applied to settled juveniles (Davidson and others 2002). Carosa and others (1998) identified a TR ortholog (CiNR1) in the ascidian species *C. intestinalis*. However, structural and functional analysis of this nuclear hormone receptor revealed that this form does not bind THs and expression was never investigated postsettlement.

The barnacle species *Balanus galeatus* responds to JH-1 and a JH analog by undergoing the cyprid to adult transition (metamorphosis) precociously (Gomez and others 1973; Ramenofsky and others 1974). An unepoxidated form of JH, methyl farnesoate (MF), has also been shown to have identical effects in other barnacle species (Yamamoto and others 1997a, 1997b) and MF has now been identified in over 30 crustacean species (reviewed in Laufer and Biggers 2001). These effects can be considered rather unusual compared to the inhibitory effects JHs have on the development and differentiation of many dipterans and lepidopterans (see above) and also on several marine arthropods (but see Laufer and Biggers 2001; Erezyilmaz 2006). While many of the functions of this hormone still remain to be elucidated, there is little doubt that MF acts as a signal for both reproduction and morphogenesis in many crustacean species (Laufer and Biggers 2001).

Recent evidence led to the hypothesis that NO acts as a repressive signal in the metamorphic transition of mollusks, echinoderms, and ascidians (reviewed by Bishop and Brandhorst 2003). Experiments with different species from these groups (one mollusk, 2 ascidians, and an echinoid) suggests that NO is required to maintain the larval state at metamorphic competence. Moreover, Bishop and Brandhorst (2003) discussed NO signaling in the context of the stress response based on the dependence of NOS (nitric oxide synthase) on HSP90. As pointed out earlier, TH signaling has now been proposed as a signal regulating development in a variety of echinoderm larvae and has been further discussed as a regulator of the metamorphic transition in these groups (Heyland and others 2005; Hodin 2006). Future research will be necessary to establish the link between NO and TH-signaling within the metamorphic signaling architecture (Bishop and others 2006; Hodin 2006).

Finally, muscle degeneration and differentiation in the abalone *Haliotis rufescens* during metamorphic transition is regulated by divergent forms of tropomyosin simultaneously in the larvae and postlarvae (Degnan and others 1997b). In *Haliotis asinia* competence may be influenced by genes that determine the developmental or physiological state of chemosensory cells or their targets (Jackson and others 2005). Moreover, several studies suggested that threshold concentration of chemoreceptors inside specific larval tissues can be linked to the development of metamorphic competence (Trapidorosenthal and Morse 1986). Such chemoreceptors could be involved in the detection of settlement cues and modulate the rapid expression of metabolic and developmental genes necessary to bring about radical morphological changes.

Synthesis

Based on the discussion of metamorphosis in frogs, flies, and diverse marine invertebrate species we highlight some of the commonalities and differences between these species.

Generalization of metamorphic competence and the metamorphic transition

We proposed to distinguish between the following 4 phases in the metamorphic transition: (1) growth and differentiation of juvenile/adult-specific structures, (2) breakdown of larva-specific structures, (3) metamorphic competence, and (4) change in habitat. These phases appear to be present in all metamorphoses discussed. Since the metamorphic transition of animals evolved under various selective pressures, however, some phases appear temporally shifted relative to others. For example, in solitary ascidians and some other phyla (not discussed here) such as bryozoans, cnidarians, and sponges, settlement precedes the differentiation of adult structures while in many other groups such as amphibians, insects, colonial ascidians, and mollusks, adult morphogenesis precedes settlement (Hodin 2006). It is therefore critical to define these

phases of the metamorphic transition in an organism in order to make meaningful comparisons across phyla.

Metamorphic competence among many invertebrate species can be viewed as a unique adaptation to the marine environment in that it allows organisms to quickly transform the planktonic larva into a benthic juvenile (Hadfield 2000; Hadfield and others 2001). Mechanistically, competence always requires larval and possibly also juvenile tissues to become sensitive to inductive cues. This clearly parallels competence in amphibians and insects in the ability of tissues to respond to THs and ecdysteroids, respectively. Still, there are important differences. In late-stage embryos of amphibians morphological changes can be induced precautiously as soon as TRalpha is present by applying ectopic TH. Such a precocious metamorphosis often leads to abnormal development (Buchholz and others personal communication), indicating that the TH response is, probably, not well coordinated in this case, possibly, because some of the tissues have not acquired the ability to respond appropriately to the hormone.

Comparison of signaling mechanisms underlying metamorphosis

In the majority of metamorphoses we reviewed, PCD of larval tissues occurs in parallel with the differentiation of adult tissues during the metamorphic transition. Hormones, specifically THs and steroid hormones, as well as several other compounds such as nitric oxide (NO), and epidermal growth factors can initiate and/or regulate PCD. We hypothesize that the interaction between hormonal signals and NO with specific components of cell death pathways orchestrates these 2 divergent developmental programs, thereby leading to the successful formation of adult tissues and organs (see Hodin 2006). We hypothesize that in many organisms, NO can suppress differentiation of adult tissues and at the same time activate apoptosis of larval tissues. Increasing hormone levels can subsequently lower NO levels leading to reduced apoptosis and increased cell proliferation in adult tissues.

Injury, stress, or exposure to pathogens can initiate cell proliferation and/or various forms of PCD (Cohen and others 1992; Chandra and others 2000; Eldadah and Faden 2000; Creagh and others 2003). In many of these pathways hormones or NO are mediators of cell proliferation and apoptosis, respectively, and we emphasize the putative role of the transcription factor NF kappa B, which plays a central role in immunity and anti-inflammatory responses and has the ability to link hormonal signaling with NO signaling and apoptosis (Ruff and others 1994; Natori and others 1999; Bates 2004; Yamada-Okabe and others 2004; Maury and others 2006). Moreover, innate immunity genes, such as components of the *toll* signaling pathways, are frequently represented as components in signaling networks underlying metamorphic transitions. At this point it is impossible to predict what role innate immunity played in the evolution of metamorphosis. Increased cellular stress and exposure to pathogens might have led to the integration of immunity pathways into the metamorphic signaling network during the evolution of metamorphosis. Still, it could be simply a proximate mechanism of the juvenile that protects it from pathogens during the transition (see also Davidson and others 2002).

Conclusions and future directions

The somewhat surprising difference of transcriptional levels preceding metamorphosis in closely related *Drosophila* species (Rifkin and others 2003) is in stark contrast with the idea that similar signaling modules were independently co-opted for the regulation of metamorphosis across phyla. If we assume that metamorphoses in amphibians, insects, ascidians, and mollusks evolved independently, we have to admit to a remarkable convergence in regulatory mechanisms implying specific constraints on the evolution of the metamorphic signaling architecture. Such constraints could originate from the marine environment in which these transitions take place (for ascidians and mollusks), as we and others have previously discussed for the time course and the predominance of cell–cell signaling events during the settlement process. Moreover, the use of THs and NO as signaling molecules during metamorphosis in amphibians, insects, and marine invertebrates suggests that these compounds are particularly suitable to coordinate the complex morphological and physiological changes occurring during the metamorphic transition. Finally, the association of apoptosis and innate immunity responses with metamorphosis provide a good example for the importance of homoplasy rather than homology as a process shaping the evolution of complex life history transitions such as metamorphosis.

Another field that has not received sufficient attention in this context is the characterization and function of nuclear hormone receptor signaling among marine invertebrate species. These transcription factors can be activated by hormones but are also critically involved in receptor cross talk and can activate other signaling pathways during the metamorphic transition. For example, in *D. melanogaster* 17 out of the 18 classical characterized NHR have no known ligands (reviewed in Thummel 1995). The ecdysone receptor (EcR)

requires USP (ortholog to vertebrate RXR) to build a functional complex that initiates transcription upon binding 20E. Still more co-activators and repressors which are involved in integrating other metamorphic signals with EcR are required. Seven-up (*svp*), an ortholog to the chicken ovalbumin upstream promoter transcription factor (COUP-TF) (Zelhof and others 1995; Gates and others 2004) interacts with EcR. Orthologs of this gene exist in many marine invertebrate species (see also Heyland and others 2006) and could, potentially, be involved in signaling events during the metamorphic transition.

We propose a scheme that compares the metamorphic transition in different phyla and emphasize both similarities and differences (Fig. 1). The similarities lie in the temporal pattern of morphogenesis of larval and juvenile/adult structures, while important differences between species primarily lie in the temporal shifts of specific phases relative to each other. We hope that this scheme will serve as a useful illustration for comparisons of metamorphic transitions of a variety of organisms. As we have discussed for ascidians, it is important to consider the life history of an organism as a whole in order to make meaningful comparisons between species. Moreover, the proposed categorization might turn out to be useful for future genomic studies of metamorphosis since it illustrates which phases of metamorphic transition should, or should not, be compared. We are currently using microarray studies on *A. californica* to specifically test some of the hypotheses discussed in this review.

Acknowledgments

We thank Drs. Jason Hodin, Lynn Riddiford, Cory Bishop, Svetlana Maslakova, Bernie Degnan, James Tresser, Hal Heatwole, Dan Buchholz, and two anonymous reviewers for discussions and suggestions on earlier versions of the manuscript. We also thank SICB for promoting and partially funding this symposium. Furthermore, we would like to thank the following organizations for generous financial support of the symposium: the University of Florida, the Whitney Laboratory for Marine Biosciences, the Division of Evolutionary Developmental Biology through SICB, and the American Microscopic Society. This research was supported by the Swiss National Science Foundation (to A.H.) and McKnight Brain Research Foundation (to L.L.M.).

Conflict of interest: None declared.

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