THYROID HORMONE-LIKE FUNCTION IN ECHINOIDS: A MODULAR SIGNALING SYSTEM COOPTED FOR LARVAL DEVELOPMENT AND CRITICAL FOR LIFE HISTORY EVOLUTION

By

ANDREAS HEYLAND

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To my teacher Larry McEdward who inspired me to think differently.

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By

Andreas Heyland

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Cooption and modularity are edifying concepts in evolutionary developmental biology. Genes function within complex networks that act as modules in development. These modules can then be coopted in various functional and evolutionary contexts. Hormonal signaling, the main focus of this thesis, has a modular character. By regulating the activities of genes, proteins and other cellular molecules, a hormonal signal can have major effects on physiological and ontogenetic processes within and across tissues over a wide spatial and temporal scale. Because of this property, we argue that hormones are frequently involved in the coordination of life history transitions (LHTs) and their evolution (LHE). Here we promote the usefulness of a comparative, non-model system approach towards understanding how hormones function and guide development and evolution, highlighting thyroid hormone function in echinoid larval development and metamorphosis as an example. We compare thyroid hormone and thyroid hormone

synthesis inhibitor effects between three species with vastly different developmental modes in order to test the hypothesis whether thyroid hormone synthesis is a pre-condition for the evolution of non-feeding development. Our findings reveal that THs are critical regulators of development to metamorphosis in echinoids. Furthermore the comparison allows us to discuss the role of THs in the evolution of non-feeding development one of the most important life history transition in echinoids and a variety of other marine invertebrates. Finally we provide evidence that LvPTO (*Lytechinus variegatus* thyroid peroxidase) is involved in TH synthesis in echinoid and discuss the role of this enzyme and TH function in larval development, metamorphosis and the evolution of alternative life histories.

CHAPTER 1
INTRODUCTION: HORMONE SIGNALING IN EVOLUTION AND DEVELOPMENT: A NON-MODEL SYSTEM APPROACH

Cooption and Modularity: The Emergence of a New EvoDevo Paradigm

There has been something of a sea of change in the past five years with respect to our general understanding of how development evolves. In the 1990s, comparative molecular and developmental data, predominantly from a fly, a rodent, a roundworm, a fish and a mustard, poured onto the pages of major journals and public sequence databases. The preponderance of raw data drove a rapid expansion in the nascent field of evolutionary developmental biology (EvoDevo), and this rapid growth was not without its growing pains. Publications purporting to synthesize data across these disparate animal (and, in some cases, plant) phyla tended to focus on their similar uses of developmental machinery, generally concluding that such similarities were evidence of shared ancestry (plesiomorphy). Favored topics included the evolution of anterior-posterior body patterning (Slack et al. 1993), homology of organs (Arendt et al. 2001; Callaerts et al. 1997) and the nature of the hypothetical protostome-deuterostome ancestor (DeRobertis 1997). These topics have been and continue to be the subject of much speculation. However, less appreciated is that inherent in the conclusion of shared ancestry is a subtle, self-justification for the model system approach. If arthropods, nematodes and mammals all use the same developmental machinery for the same processes, then any tractable model system would provide the ability to draw conclusions regarding, for example, the nature of a given human disease. While model systems have

often proven useful in this regard, the similarities among disparate animals have been frequently overemphasized (Bolker 1995).

In the closing years of the 20th century, a significantly altered paradigm began to emerge concerning two powerful concepts: cooption and modularity. Neither of these ideas was particularly new, but their application to the comparative data sets described above has had a discernible impact upon EvoDevo. The foundation for the cooption concept (the use of ancient molecules in novel developmental contexts, such as the use of the limb axis specification machinery in butterfly wing color patterns (Brunetti et al. 2001) extends at least as far back as Jacob's (Jacob 1977) notion of "bricolage." Modularity (that development is regulated by networks – i.e., modules - of interacting genes) likewise has deep roots (reviewed in Gilbert et al. 1996), and in recent years the paradigm of gene networks has largely superceded the hierarchical concept of gene "pathways". Modules have two important features: 1) the networks appear to be robust in the face of perturbation (von Dassow et al. 2000), and 2) given modules show up repeatedly in diverse developmental and evolutionary contexts (reviewed in True and Carroll 2002). Thus, the concepts of cooption and modularity are connected, and together provide an account for the aforementioned cross-phylum similarities that so energized the EvoDevo field in the 1990s. More importantly, perhaps, modularity and cooption offer a framework for understanding the nature of diversity: the hallmark of evolutionary change.

Comparative Biology: A Non-Model System Approach to Life History Evolution (LHE)

The concept of life history has been largely absent from modern developmental biology. This is surprising, especially since the holometabolous insects (including

Drosophila melanogaster) and the amphibians (including Xenopus laevis) are well-known as having complex life histories, with distinct larval and adult stages separated by a drastic metamorphosis (Fig. 1-1). Still, most of the other animal "model systems" (nematodes, mice, leeches, zebrafish) lack a distinct larval stage (i.e., have a simple life cycle; Fig. 1-1). Indeed, a simple life history may be preferable for developing a laboratory-based model system (Bolker 1995). By contrast, marine invertebrates (which, together, include 28 of the approximately 32 animal phyla) are exceedingly diverse with respect to their life histories (Strathmann 1985; Wray 1995), and this diversity can even be manifest within a single species (e.g., feeding and non-feeding larvae within a single population of the spionid polychaete (Annelida) *Streblospio benedicti* (Levin 1984). In recent years, the emerging picture of how development evolves has become decidedly more complex. Definitive conclusions concerning, for example, whether or not the ancestor of flies and mice was a segmented creature with a brain, heart, eyes and limbs, have come to seem a bit more elusive. Instead, a growing group of comparative biologists (e.g., Byrne et al. 2003; Jeffery et al. 1999; Kohn and Perron 1994; Parichy 2003; Sucena et al. 2003) are advocating a different approach: by investigating the mechanisms underlying morphological or other differences among more restricted taxonomic groups, one can begin to understand the details of the developmental mechanisms underlying evolution. Such issues are tractable, and may ultimately provide realistic hypotheses regarding larger-scale evolutionary events.

Does this approach invalidate the use of model systems? Not at all! The intensively studied model systems are crucial for identifying developmental modules and for developing techniques to study gene function. But model systems have a critical

limitation: alone, they do not allow for evolutionary conclusions. In order to understand the evolution of developmental mechanisms one should do one of the following: 1) choose a restricted taxon, whose members show substantial diversity with respect to the mechanism in question, 2) have a phylogenetic hypothesis for the taxon, and 3) be able to obtain offspring from several different members of the taxon, in order to perform experimental manipulations and descriptive observations. Other useful features are as follows: 4) taxa with a respectable fossil record; 5) taxa, or a close relative whose genome is being analyzed; 6) embryos that can be manipulated and observed while alive; and 7) short generation times, allowing genetic manipulations and selection experiments.

We will focus on hormones and their role in life history diversity in the echinoderms (sea urchins, starfish and their relatives), a group that satisfies 6 of these 7 key features (excepting feature 7, as typical echinoderms have generation times well in excess of one year; however, embryos from several different sea urchin species have proven to be amenable to modern gene knockout and knock-down techniques (Kauffman and Raff 2003; Sweet et al. 2002). Still, we advocate the application of this comparative approach to a wide diversity of organisms (not merely "satellite systems" sensu Rudel and Sommer 2003) from all of the multicellular kingdoms (Cavalier-Smith 1998) (plants, animals, fungi, brown algae and red algae), including those in which multicellularity has evolved independently on more than one occasion. It is only through such a widespread application of the comparative approach to development that we will be able to gain a fuller understanding of the mechanisms of evolution.

Defining Metamorphosis

Metamorphosis is an inherently integrative concept touching different biological disciplines such as ecology, development, physiology and evolution. Considering also

that researchers working with different taxa (such as fish, amphibians, insects, and a multitude of marine invertebrates) have very different ideas of what is and what is not a metamorphosis (e.g., Callery et al. 2001; Hadfield 2000; Parichy 2003; Youson 2003), it becomes difficult if not impossible to provide a truly unifying definition that encompasses all of these examples. For the purpose of this review, we define metamorphosis as a period of dramatic ontogenetic change from a multicellular, free-living, post-embryonic stage ("larva" in animals) to a multicellular, pre-reproductive adult ("juvenile" in animals, see Wray 1995 for a list of animal phyla with metamorphic taxa with at least some members undergoing metamorphoses). This ontogenic reorganization, which can take days to months, involves major morphogenetic remodeling (e.g., loss of larval specific structures and formation of adult specific tissues) with associated cellular (e.g., apoptosis, necrosis, proliferation) and molecular events (e.g., up-regulation of tissue remodeling enzymes).

In marine invertebrates that undergo a planktonic (larval) to benthic (adult) transition, metamorphosis is also associated with settlement (the change in habitat itself). Thus, settlement is the rapid (seconds to minutes) phase of metamorphosis, which employs neurophysiological rather than transcriptional control mechanisms (Hadfield 2000). "Competence," then, is the stage at which the planktonic form can undergo settlement in response to specific settlement cues.

Interestingly, the timing of settlement relative to the longer-term ontogenetic remodeling characterizing metamorphosis varies widely among animal taxa. For example, in echinoderms and colonial sea squirts, the juvenile form has developed within the larval body, so settlement reveals the essentially fully-formed juvenile body plan. By

contrast, bryozoan and solitary ascidians, for example, settle before the major events of metamorphic remodeling have occurred, so a substantial post-settlement process of juvenile morphogenesis precedes the appearance of a recognizable juvenile body plan (Davidson et al. 2002b; Giese et al. 1991). Thus the relative timing of settlement and juvenile morphogenesis underlie important differences in life history trajectories in disparate animal taxa (Bishop and Hodin 2001). Furthermore, we feel that metamorphosis in holometabolous insects (wasps, flies, moths, etc.) follows a remarkably similar pattern to that described above for metamorphosis and settlement in marine invertebrates (c.f. Hadfield 2000). In this group of insects, pupal development (like marine invertebrate metamorphosis) is the longer-term, morphogenetic remodeling period under transcriptional control, while eclosion (like settlement) is the neurophysiologically regulated, rapid change in habitat (terrestrial to aerial).

There is an ongoing debate about whether fish as a whole are metamorphic (Balon 1999; Youson 2003). Based on our definition given above, we would not consider changes occurring between immature stages and adult life in fish to always be examples of metamorphosis. Still, substantial morphological, ecological and physiological changes do occur in some groups. The larval pattern of neural crest-derived pigment cells changes dramatically into the more complex adult pattern during zebra fish development (Parichy 2003). One of the obvious characteristics of summer flounder “metamorphosis” is the migration of the right eye to the left side of the head, as well as the transition from a primarily cartilaginous to an ossified skull, and a shift in habitat. Eel (Anguilla) and salmon life histories are characterized by major migrations of pre-adults. Such migrations are accompanied by a multitude of physiological and metabolic changes that allow the

animals to shift from fresh-water to salt water. The striking finding is that the physiological and morphological changes characterizing many such independently evolved instances of fish metamorphoses are controlled by THs, and substantial alterations in TR expression levels have also been reported (Power et al. 2001; Schreiber and Specker 2000).

Finally, we wish to point out that our definition of metamorphosis is also applicable to life stage transitions in non-animal multicellular eukaryotes, such as some fungi (the mycelium-to-fruiting body transition) and red algae (crustose-to-thallus transition). To our knowledge, no one has attempted to determine whether these metamorphic transitions in fungi and algae are similarly regulated by hormones, although a hormonal basis for other ontogenetic processes has been established for fungi (Plemenitas et al. 1999; ZakeljMavric et al. 1995). Finally, we do not consider alternations of generations in plants and algae to be examples of metamorphosis; these are, rather, reproductive LHTs which pass through an intervening zygote stage.

Nuclear Hormone Receptors: Modules and Development and Evolution

Steroids, thyroid hormones, vitamin D and retinoic acid can act as signaling molecules (ligands) for nuclear receptors (NRs) in animals. The basic structure of the more than 523 nuclear receptors genes identified from 115 or so metazoan species (Ruau et al. 2004) is well conserved, and has a remarkably modular character. NRs occupy central positions in signaling pathways relevant for metabolic functions, growth, differentiation and homeostasis (Laudet and Gronemeyer 2001). Moreover they play critical roles in life history transitions (LHTs).

An increasing number of NRs are considered orphan receptors, i.e., receptors without any known ligands. Still, several of these receptors have turned out not to be

orphans at all upon closer examination. In any case, one of the salient questions of NR evolution is whether the ancestral NR was an orphan receptor that acquired the ability to bind a specific ligand, or whether the alternative route (via a liganded receptor in the ancestral state) was taken. Although this debate is still open, arguments from structural, functional and phylogenetic studies provide evidence that the ability to bind ligands has evolved secondarily (Escriva et al. 2000). First, there is practically no correlation between the chemical composition of the ligand and its phylogenetic position. In other words, closely related receptors bind ligands that originate from vastly different biochemical synthesis pathways. Second, chemically similar ligands can bind distantly related NRs within and among taxa [e.g., the distantly-related retinoid-X receptor (RXR) and retinoic acid receptor (RAR) both bind to retinoids; steroids in vertebrates and insects bind to receptors from different NR sub-families]. This latter finding emphasizes the role of NRs as true evolutionary modules that were recruited for a broad spectrum of biological functions.

It is well known that NRs, while having extremely strong affinity for their ligands (if they have ligands at all), can be activated by a multitude of other signaling molecules, including natural and artificial compounds (reviewed in Mclachlan 2001). NR function is modulated by a battery of co-activators and repressors. Moreover, NRs can heterodimerize (build functional complex with another NR molecule), homodimerize (build functional complex with the same NR molecule) or act as monomers. NRs also have the ability to interact with other signaling pathways and their receptors through so called receptor cross-talk (Escriva et al. 2000; Laudet and Gronemeyer 2001). One aspect of this ‘open-minded’ NR behavior is their response to environmental contaminants.

Several of these components can act, for example, as estrogen mimics (or 'disruptors') by directly binding to the receptor or activating it through alternative pathways (Mclachlan 2001).

Recently it has become clear that not only NRs but also their ligands have a variety of alternative ways by which they can signal. Thyroid and steroid hormones can signal through non-genomic (also called non-nuclear or non-transcriptional) pathways. This mode of signaling is characterized by relatively fast signal transduction that does not necessarily involve de novo protein synthesis, but rather through a suite of alternative receptor molecules, resulting in changes of ion transport through membranes. Two major targets of non-genomic thyroid and steroid hormone action are the central nervous and vascular systems (Hulbert 2000; Simoncini and Genazzani 2003).

The ‘open-minded’ NR behavior, non-genomic action of several hormones, and especially the modular nature of NR ligand binding described above clearly illustrate how the effect of a signaling molecule on a given organism does not necessarily allow us to draw conclusions about how the signaling pathway evolved. This observation should also give us pause when attempting to draw conclusions about the homology of signaling pathways, as well as their functions in lesser known, non-model organisms (see also Finch and Rose 1995). The chemical compounds used by extant metazoans as hormones existed long before the origin of the animals. For example, while hormonal signaling through NR pathways is by far best investigated in vertebrates and insects, steroid signaling in plants involves a very distinct (serine/threonine kinase) receptor system (Li and Chory 1999; Wang et al. 2001). Thus, our understanding of the evolution of

hormonal signaling should not be overly biased by their mechanics in these two animal taxa, despite their seemingly similar modes of action (Bolker 1995).

Hormonal Signaling Networks: Modules Coopted for LHTs

Hormones control and coordinate complex physiological and developmental processes in plants, animals and fungi, such as growth, differentiation reproduction and homeostasis. They are intercellular or intracellular chemical messengers, communicating over short (paracrine, autocrine) to relatively long distances (endocrine). Pheromones by contrast act as signaling molecules originating from exocrine glands and for the most part affect the behavior of organisms from the same species (but see below). A single hormone can elicit a diversity of effects, depending on the target tissue and the physiological and developmental context. In plants, hormones from five main groups (auxins, gibberellins, cytokinins, abscic aid and ethylene) control and coordinate often complex overlapping signaling systems, developmental processes and life history transitions. Examples include the role of ABA (abscisic acid) in seed dormancy and the induction of flowering by sucrose and cytokinins (reviewed in Sachs 2002). In animals, hormones signal through cell surface receptors [proteins, peptides, catecholamines and eicosanoids, which tend to activate second messenger pathways such as cyclic nucleotides (cAMP, cGMP), protein kinases and calcium and/or phosphoinositides] or signal through intracellular receptors [e.g., steroid and thyroid hormones generally act via binding to specific nuclear receptors (NRs)] (reviewed in Laudet and Gronemeyer 2001; Marchese et al. 2003). NRs are unique in that they act as transcriptional regulators that coordinate intra- and extracellular signals. This property allows them to act as nodes in complex regulatory networks that play crucial roles in development and homeostasis.

In the ontogenetic transformation from a single-celled zygote to a multicellular, reproductive adult, a tremendous number of complex processes need to be accurately timed and coordinated with one another and the environment. The result is that subsequent life cycle stages are successfully reached in the right condition and at the right place and time. In the majority of animals, such ontogenetic transformations are characterized by a metamorphic life history. Metamorphosis has evolved only a few times among terrestrial taxa, but many times independently among marine invertebrates (Hadfield 2000; Wray 1995). One uniting feature of diverse metamorphic life histories across all habitats is their control and coordination by hormones (Finch and Rose 1995; Hodin and Riddiford 2000; Matsuda 1987), a finding consistent with the hypothesis that hormonal signaling networks are modules, coopted in different lineages for evolutionarily independent cases of LHTs. This hypothesis can be tested by investigating hormonal signaling in LHTs in an evolutionary context. Such a comparative survey of the occurrence, biosynthesis, function and phylogenetic distribution of signaling molecules allows us to draw conclusions about patterns and constraints in the evolution of signaling pathways. Some illustrative cases for a diverse array of cell signaling systems are reviewed in the following articles: canabinoid signaling (Elphick and Egertova 2001), NR signaling (Laudet and Gronemeyer 2001), innate immunity signaling (Davidson and Swalla 2002) and nitric oxide signaling (Bishop and Brandhorst 2003; Moroz 1999).

Matsuda (1987) presented an immense and invaluable synthesis of comparative data concerning the function and occurrence of hormones with respect to animal LHTs. In Figure 1-2, we present Matsuda's data set (supplemented with more recent literature) in graphical form, emphasizing the role of hormones as regulatory modules in a broad

array of animal LHTs. Below, we highlight specific examples that provide strong evidence for the independent cooption of hormonal signaling in LHTs in various animal taxa.

Increasing levels of thyroid hormones (THs) (3,3',5'triiodo-L-thyronine and thyroxine promote metamorphosis in amphibians (reviewed in Shi et al. 1996). In Xenopus laevis tadpoles, TRα and TRβ [two of the four thyroid hormone receptor (TR) isoforms cloned from the frog] and RXRs (retinoid X receptors, heterodimeric partners of TR) are mediators of the THs effects during metamorphic development. Opposite to the situation in amphibians, jawless fish (lampreys) require a critical time period during which THs is absent in order to undergo the metamorphic transition (Youson 2003). This finding, in light of the substantial evolutionary distance between amphibians and lampreys, provides strong evidence for an independent acquisition of TH control of metamorphosis in these two taxa (and more transitions are probable within chordates; see below). Indeed, recent data suggests at least two additional instances of independent co-option of TH function in metamorphosis: echinoderms (see below) and urochordates. TH synthesis inhibitors block post-settlement juvenile morphogenesis in the ascidian *Boltenia villosa* (Davidson et al. 2002b), and thyroxine has been localized and measured in larvae of *Ciona intestinalis* (Patricolo et al. 2001). Also, a putative nuclear receptor for T3 in ascidians has been identified in vivo, which has a similar affinity (Kd) to those found in other chordates. However, the maximal binding capacity, one characteristic that can be compared among receptor, was low (Fredriksson et al. 1993), so the jury is still out on invertebrate TH receptors. Finally, experiments on abalone indicate a possible role for thyroid hormones in mollusk metamorphosis as well (Fukazawa et al. 2001).

TH-like function has also been reported from other invertebrate taxa (reviewed in Eales 1997), including insects, where THs may disrupt juvenile hormone (JH; which are sesquiterpenes) signaling (Davey 2000). Here we will briefly mention the cnidarians (a phylum including sea anemones, jellyfish and hydroids) due to their crucial phylogenetic position (see Figure 1-2) for testing hypotheses concerning the evolutionary mechanics of life history transitions and thyroid hormone function. Cnidarians share many patterning genes (Finnerty and Martindale 1999) with bilaterians (the protostomes, which include arthropods, mollusks, annelids, nematodes and flatworms, plus the deuterostomes), but little is known about the mechanisms underlying transitions in cnidarian life histories. Spangenberg (1974) suggested that THs can influence the benthic polyp to pelagic ephyra (pelagic life history stage) via the initiation of strobilation (reproductive strategy of polyps) in Aurelia. Complementary evidence (Silverstone et al. 1978) indicated that Aurelia may synthesize thyroid hormone precursors (mono- and dityrosine: T1 and T2), although not thyroid hormone sensu strictu (T3 and T4). Kingsley and co-workers (Kingsley et al. 2001) showed that gorgonian corals contain TH-like compounds involved in calcification. Experiments with various cnidarians have not supported a role for hormones in metamorphosis from the planula to the polyp stage, but a settlement-inducing neuropeptide in Hydractinia (a hydroid) has been identified from neurosensory cells at the anterior pole of competent larvae (Leviev et al. 1997).

Developmental transitions in insects are regulated by two classes of hormones: JH and ecdysteroids. In the completely metamorphosing insects (the Holometabola, such as bees, beetles and butterflies), ecdysteroids are further specialized as coordinators of the metamorphic transition itself. Furthermore, reproductive maturation in insects is

regulated by these same hormones (reviewed in Nijhout 1994). Still, there is good evidence for independent cooption of JH and ecdysteroids for unique roles in various insect taxa. For example, while JH promotes vitellogenesis (the synthesis and oocyte uptake of yolk proteins) in most insect taxa, it represses vitellogenesis in others such as the larch bud moth *Zeiraphera diniana* (reviewed in Ramaswamy et al. 1997, Hodin in prep.). Furthermore, in social insects (such as ants and honeybees), the adult function of JH is not in reproduction, but instead in caste determination (reviewed in Nijhout 1994). In other arthropods, ecdysteroids regulate molts as well as reproduction, and methyl farnesoate (MF; a JH precursor in insects) also plays a role in these processes in crustaceans (Laufer and Biggers 2001). While ecdysteroid regulation of molts may be a synapomorphy (shared derived feature) of the entire Ecdysozoa clade (hence the origin of the name; see Figure 1-2), there is currently no evidence to suggest whether the role of sesquiterpenes in insects and crustaceans (and possibly other ecdysozoans taxa as well) is a shared (homologous) or an independently derived (homoplasious) feature. Comparative biochemistry of insect JH and crustacean MF biosynthetic mechanisms should help resolve this issue. In either case, the comparative data from insects demonstrates multiple cooptions of JH and ecdysteroids for taxon-specific LHTs.

Some cestode (Platyhelminthes) and arthropod parasites utilize the hormones of their host to regulate their own LHTs (reviewed in Matsuda 1987; Nijhout 1994). Since such hormones are not produced endogenously by these parasite taxa, this phenomenon can be considered a unique class of cooption of hormonal mechanisms for LHTs. For example, the release of the rabbit's hormones (estrogen and corticosteroids) and pheromones stimulate ovarian maturation in the rabbit flea via up-regulation of JH in the

parasite (reviewed in Nijhout 1994). Other aspects of the flea's physiology and life history, such as the feeding rate, defecation rate and mating behavior, are also coordinated with the release of their host's hormones and pheromones.

Epitoky is a life history transition that occurs during sexual maturation in some benthic annelids. In an extreme version of epitoky (called “schizogamy,” found for example in psyllid polychaetes), segments in the posterior of the animal become specialized for dispersal, gonads in this region mature, and a second head develops with elaborate sensory structures. This posterior reproductive entity buds off and disperses to spawn and degenerate, while the benthic form regenerates the lost segments. Other annelid taxa (including nereid polychaetes) undergo a single transformation from the benthic form to a dispersive reproductive form (this is known as “epigamy”), which likewise dies in a massive, coordinated spawning event. Careful experimental manipulations done by Durchon and later Hauenschild revealed that an unidentified head hormonal factor is involved in this unique metamorphic process (reviewed in Fischer 1999).

Hormones and Life History Evolution (LHE)

Matsuda (1987) hypothesized that hormones are also major players in LHE in a broad array of animal groups. This proposal seems eminently sensible: evolutionary alterations in organismal life histories must involve a radical reorganization of the mechanisms underlying the LHTs themselves (see also Finch and Rose 1995). As we have reviewed above, hormones coordinate and orchestrate LHTs in a wide variety of taxa; therefore, LHE must involve modifications in the production, regulation and/or tissue-specific response of these very same hormones. Comparative studies have

uncovered examples of each of these LHE mechanisms, and we will review several such cases here.

Evolutionary alterations in organismal life histories have been profitably described in terms of heterochronies: changes in the relative timing of developmental events (Alberch et al. 1979; Gould 1977). For example, neoteny, which has evolved mutiple times independently in various salamanders from metamorphic ancestors, can be described in terms of alterations in the relative timing of sexual and somatic differentiation (Alberch et al. 1979). Specifically, metamorphosis is blocked, and sexual maturation proceeds within an otherwise larval morphology. This metamorphic block has different physiological causes in different taxa (reviewed in Denver et al. 2002). In one case (e.g., *Ambystoma tigrinum*), a hypothalamic neurohormone in the thyroid axis is non-functional, in another (e.g., the axolotl *A. mexicanum*) TH is not produced, while a third neotenic taxon (*Necturus maculosus*) has apparently lost a functional TH receptor (TR). These examples clearly support the notion that LHE, of necessity, involves modifications in hormonal signaling, albeit distinct (i.e., convergent) modifications in different taxa (Hodin 2000).

The second major heterochrony underlying LHE in amphibians is the evolution of direct development, where metamorphosis is skipped (but see Callery et al. 2001), and little froglets hatch directly from their egg masses. In this case, the heterochrony involves early activation of adult development within the embryo. This precocious adult development is correlated with precocious TH synthesis as well as early up-regulation of TRs in the Puerto Rican tree frog Eleutherodactylus coqui (Callery et al. 2001; Hanken et al. 1997; Jennings and Hanken 1998).

Alterations in the timing of hormone receptor expression also underlie the independent evolution of larval reproduction (paedogenesis: ovarian maturation in the larval stage) in two species of fungus-eating cecidomyiid gall midges (Insecta: Diptera). Here the heterochrony is facultative. Under plentiful food conditions ovarian development is activated early, correlated both with a rise in ecdysteroids (Went 1978) and early appearance, specifically in the ovarian cells, of the two proteins [Ecdysone Receptor (EcR) and Ultraspiracle (USP)] that constitute the functional ecdysteroid receptor (Hodin and Riddiford 2000). When the food quality is poor, the ovarian expression of these proteins is down-regulated, ovarian development is delayed, and the midges proceed through metamorphosis, hatch and disperse to find a new fungal patch.

Metamorphosis in fish, as we (see above; defining metamorphosis) and others (Youson 2003) have defined it, is relatively rare. Thus, the presence of a distinct larval stage in the life history of vertebrates in general is most likely secondarily derived from an ancestral direct life history (i.e., Hadfield 2000; Nielsen 1998). Well known examples for metamorphoses in fish are lampreys, eels and flounder (Youson 2003). In each of these taxa, thyroid hormones are involved in metamorphosis, though in distinct ways in different taxa (as described above for lampreys). Thyroid hormones also regulate other taxon-specific LHTs in fish, such as smoltification in salmon (Kulczykowska et al. 2004). So if we are to accept the proposal that direct development is ancestral for the vertebrates, then each of these examples would represent instances of independent cooption of TH control of fish LHTs.

Life history evolution in some plants is associated with similar hormonal alterations. For example, mangroves are a polyphyletic assemblage of coastal plants that

come from sixteen distinct plant families and are often more closely related to upland, non-mangrove taxa than they are to other mangroves. Of these sixteen distinct experiments in mangrove evolution, vivipary (where the embryos lack a seed dormancy stage) has evolved six times independently. The plant hormone abcissic acid is known to regulate seed dormancy in a wide variety of plants. In independently evolved mangroves from four of the six viviparous families, embryonic ABA levels are reduced relative to both non-mangrove outgroups and one non-viviparous mangrove species (Sonneratia alba) (reviewed in Farnsworth 2000). Other viviparous plants that live in predictably moist environments (the amazonian cocoa *Theobroma cacao*, the english oak *Quercus robur*, the southeast Asian heavy hopea tree *Hopea odorata* and the east Asian red machilus *Machilus thunbergii*) are also characterized by low embryonic ABA levels (reviewed in Farnsworth 2000). Thus, reduced embryonic ABA levels have evolved in parallel with vivipary in a wide variety of dessication-intolerant plants.

In summary, a careful interspecific comparison of reasonably closely related species allowed the conclusion that shifts in timing of hormonal release and/or the cellular response to hormones in the target tissues were intimately associated with the evolution of derived life history strategies, a hypothesis we will further test taking advantage of the immense life history diversity in echinoderms and specifically echinoids.

There are five extant classes within the phylum Echinodermata: Echinoidea (sea urchins and sand dollars), Asteroidea (starfish), Ophiuroidea (brittle stars and basket stars), Holothuroidea (sea cucumbers), and Crinoidea (feather stars and sea lilies). The majority of taxa have a free swimming larval stage that disperses from the parental

location, undergoes metamorphosis and settles to the benthos as a pre-reproductive juvenile. A minority of echinoderms brood their offspring. Those echinoderms with dispersing larvae can be further subdivided into those that feed as larvae and those that do not (reviewed in McEdward and Miner 2001). Similarities in morphology and feeding biology among the disparate larvae of echinoderms (and even their sister group the hemichordates) suggest that the ancestral mode (plesiomorphy) of development in the echinoderms is development via a feeding larva, and that non-feeding development has evolved independently on many occasions (Strathmann 1985, see Figure 1-3 for echinoids; Wray 1995). Much has been written regarding the ecological and evolutionary consequences of feeding versus non-feeding larval development (Hart 2002; Strathmann 1985; Wray 1995), including trade offs between egg size and egg number, differences in survival in the plankton, limitations to dispersal, as well as differences in juvenile growth and mortality in feeding and non-feeding taxa. Still, the developmental mechanisms involved in the multiple evolutionary transitions from feeding to non-feeding have remained obscure.

In the next 4 chapters I will provide data in support of the hypothesis that THs are critical players in echinoid development and life history evolution. This comparative approach allows us to draw conclusions beyond simply the mechanisms of TH function. I hypothesize that THs were critically involved in the evolution of non-feeding development in echinoids and that endogenous hormone synthesis is a pre-adaptation for this important evolutionary step. Finally I explore the mechanistic basis for TH signaling in a sea urchin species and provide evidence for a thyroid peroxidase homologue in the

sea urchin *Lytechinus variegatus* that appears to be involved in TH synthesis during larval development.

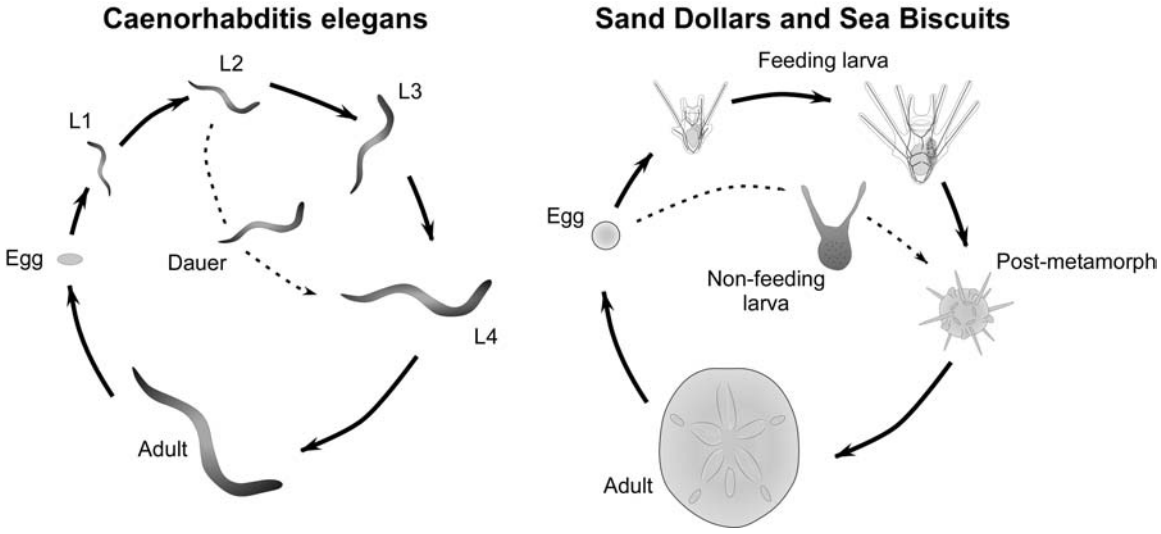


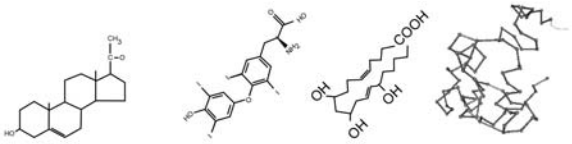
Figure 1-1. Simple (the roundworm *Caenorhabditis elegans*, phylum Nematoda) and complex (sand dollar and sea biscuit; Echinodermata, Echinoida see Figure 1-3) development. These life cycles - often called direct and indirect, respectively - are two extreme examples of life history strategies found among the Metazoa. Sand dollar and sea biscuit larvae undergo a radical transformation after their larval life into a benthic juvenile (post-metamorph), which then develops into the reproductive adult. In *C. elegans*, the embryo develops through a sequence of pre-adult stage (L1-L4; also called larval stages) into the adult form. But, unlike the larval stage in sand dollars and sea biscuits, *C. elegans* pre-adult stages are quite similar morphologically to the adult. The dotted line in the *C. elegans* life history diagram represents dauer stage production of a diapause-like, alternative developmental pathway within the same genotype (phenotypic plasticity). The life cycle depicted for sand dollars and sea biscuits is development through metamorphosis via a feeding (pluteus) larva. The dotted line in the sand dollar and sea biscuit life history diagram represents an alternative, non-feeding developmental pathway characterizing some sand dollar species (here exemplified by the larva of the sand dollar Peronella japonica).

Figure 1-2. Wide spread involvement of hormones in life history transitions (LHTs). "+" indicates that this class of hormones has been demonstrated to be involved in a LHT [hatching, larval transitions, resting stages (such as dauer and diapause), metamorphosis, settlement, migration, attainment of reproductive maturity and ovulation/oviposition]. "?" indicates that such a role has not been demonstrated for that phylum (note that this category also includes those cases where the phenomenon has been examined but no effect was found). "+?" indicates preliminary evidence for such a role. Note that the four large hormone families considered here do not encompass all animal hormones involved in LHTs; certain hormones were omitted for simplicity. Also, hormones belonging to the same family can be synthesized by vastly different biochemical pathways. Furthermore, the original sources of animal hormones might have been plants/algae in many cases, and could well have been independently derived from plants/algae in distinct lineages. Finally, different taxa use similar hormones to regulate totally different LHTs (such as steroids in molluscan reproduction and arthropod molting), and in some cases the same hormone has been coopted multiple times to regulate independently evolved metamorphoses (TH is the prime example; see the text). For these reasons, the reader should not interpret the presence of a similar class of hormones in related animal taxa to be sufficient evidence for common ancestry of particular LHT functions and their hormonal control. (data for phylogeny from (Peterson and Eernisse 2001); numbers in table refer to references listed below). 1.(Spangenberg 1974); 2. (Howard and Stanley 1999) 3. (Leviev et al. 1997) 4. (Kuervers et al. 2003) 5. (Daugschies and Ruttkowski 1998) 6. (Gerisch et al. 2001) 7. (Nijhout 1994) 8. (Davey 2000) 9. (Clare 1999) 10. (Pertseva and Shpakov 2002) 11. (Schallig et al. 1991) 12. (Matsuda 1987) 13. (Laufer and Biggers 2001) 14. (Fischer 1999) 15. (Takeda 2000) 16. (Fukazawa et al. 2001) 17. (Giese et al. 1991) 18. (Eales 1997) 19. (Pestarino 2000) 20. (Jackson et al. 2002) 21. (Sugimoto et al. 1997) 22. (Shi et al. 1996) 23. (Crews 1996)

Ecdysozoans

Lophotrochozoans

Deuterostomes



	Steroids	Amino Acid Derivatives	Eicosanoids	Peptides & Proteins
Porifera	?	?	?	?
Cnidaria	?	+(1)	?	+(3)
Ctenophora	?	?	?	?
Nematoda	+(4)	?	+(5)	+(6)
Arthropoda	+(7)	+(8)	+(9)	+(10)
Platyhelminthes	+(11)	?	+(2)	?
Nemertini	?	?	?	+(12)
Annelida	?	?	+(14)	+(13)
Echiura	?	?	?	?
Mollusca	+(15)	+?(16)	+(15)	+(15)
Bryozoa	?	?	?	?
Echinodermata	+(17)	+(18)	+(2)	+(17)
Hemichordata	?	?	?	?
Urochordata	+?(19)	+(19)	?	+(20)
Cephalochordata	?	?	?	+?(19)
Vertebrata	+(23)	+(22)	+(21)	+(35)

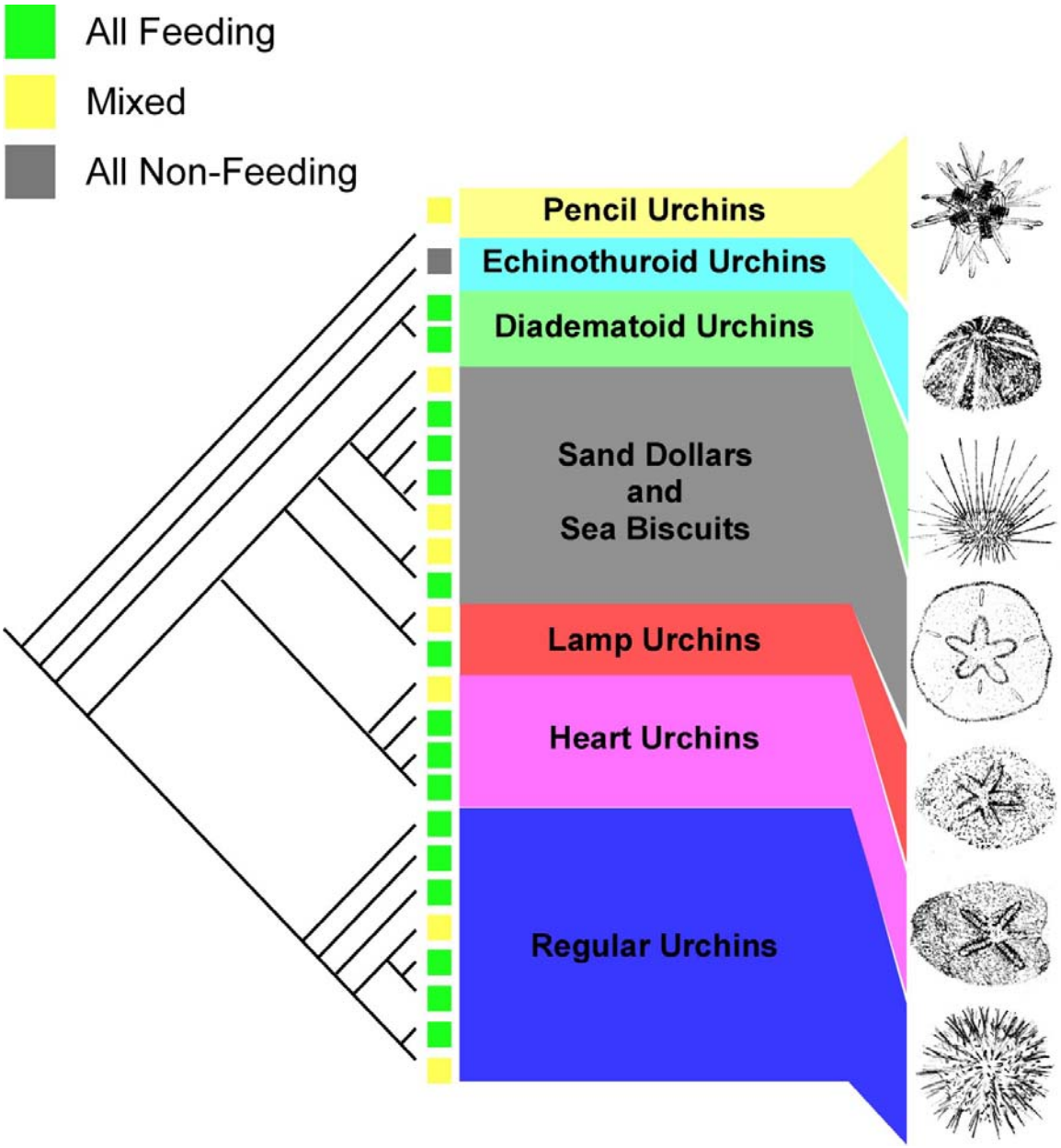


Figure 1-3. Life history strategies in echinoids (Echinodermata). It is generally accepted that a feeding mode of development is ancestral for the echinoids (Strathmann 1985; Wray 1995), but non-feeding development has certainly evolved many times independently in the group (Wray and Bely 1994). These multiple independent evolutionary transitions provide fertile ground for studying the mechanisms underlying LHTs and LHE. Phylogeny modified from Littlewood and Smith (1995). Life history characters compiled from McEdward and Miner (McEdward and Miner 2001).

CHAPTER 2
HETEROCHRONIC DEVELOPMENTAL SHIFT CAUSED BY THYROID HORMONE IN LARVAL SAND DOLLARS AND ITS IMPLICATIONS FOR PHENOTYPIC PLASTICITY AND THE EVOLUTION OF NON-FEEDING DEVELOPMENT

Introduction

A range of developmental strategies characterizes life history patterns in echinoderms (sea urchins, sand dollars, starfish and their kin) and other marine invertebrates (terminology sensu McEdward and Janies 1997; reviewed in Wray 1995). Planktotrophic development, as exemplified by the pluteus larva of sand dollars and the veliger larva of snails, represents one extreme in this spectrum where larvae develop from relatively small, energy poor eggs. The elaborate and highly efficient feeding structures in these larvae compensate for the fact that the eggs of planktotrophs do not contain sufficient maternally derived resources to complete development in the absence of food. Lecithotrophic development proceeds from much larger eggs. Since sufficient maternally derived reserves are available for lecithotrophic larvae, feeding structures are reduced or even absent. Most planktotrophic and lecithotrophic larvae then undergo a radical metamorphosis into a settled juvenile. Direct development is a special case of lecithotrophy in which the larval stage is completely lost and the embryo develops directly into the juvenile with no intervening metamorphosis. The presumed ancestral mode of development in echinoderms is planktotrophic, and it is generally accepted that lecithotrophy has evolved multiple times independently from planktotrophic ancestors through the loss of larval feeding (e.g., Strathmann 1978; Strathmann 1985; Wray 1996)

but see Lacalli (1993). Traditional life-history models and their modifications (Christiansen and Fenchel 1979; Havenhand 1995; McEdward 1997; Roughgarden 1989; Vance 1973a; Vance 1973b) have assumed that egg size is the prime determinant of developmental mode, and that ecological factors explaining selection for increased egg size can account for the evolution of lecithotrophy. Mechanistic (ontogenetic) factors have received little attention in these models.

Planktotrophic echinoderm larvae, most famously those of echinoids (sea urchins and sand dollars, the main focus of this study), respond in a phenotypically plastic way to the abundance of food under laboratory and field conditions (e.g., Boidron-Metairon 1988; Fenaux et al. 1985; Fenaux et al. 1994; George 1999; Hart and Strathmann 1994; Strathmann et al. 1992). Planktotrophic echinoids develop via a feeding pluteus larva, which captures algae using the convoluted ciliary band that runs along its several pairs of long, skeletal arms (Fig. 2-1A). The juvenile rudiment (Fig. 2-1B) develops as an essentially separate entity from the larval body. When the larvae of several echinoid species are reared under high food conditions, a differential increase in the growth of juvenile structures relative to the growth of larval structures occurs. Under low food conditions, by contrast, juvenile growth is delayed, and the larvae instead invest in longer larval arms (Boidron-Metairon 1988; Fenaux et al. 1994; Hart and Scheibling 1988; Hart and Strathmann 1994; Strathmann et al. 1992). This differential allocation of energy to larval versus juvenile structures under low food conditions has been shown to result in an increased ability to capture food items (Hart and Strathmann 1994), and is thus considered an example of adaptive phenotypic plasticity.

Strathmann et al. (1992) noted that planktotrophic echinoid larvae that develop from relatively large eggs exhibit a developmental trajectory superficially similar to that of larvae with smaller eggs that are raised under high food conditions: both tend to exhibit increased relative allocation to juvenile rather than to larval structures. These authors hypothesized that endogenous (maternally-derived) and exogenous (derived from the plankton) food resources are essentially equivalent, and that the evolution of lecithotrophy, and the accompanying loss of larval feeding structures, could be explained simply by an evolutionary increase in maternal investment in the egg. Later experiments with egg size manipulations (Bertram and Strathmann 1998, Strathmann pers. comm. 2001), however, showed that alterations of exogenous and endogenous resources do not produce equivalent effects on larval morphology, contrary to the prediction of the aforementioned hypothesis.

An alternative hypothesis is that exogenous food is fundamentally different from endogenous food, or, more specifically, that planktotrophic larvae need to obtain specific compounds from planktonic algae that are not provided in the egg. A corollary to this hypothesis is that lecithotrophic larvae either synthesize these compounds themselves, or that these compounds are supplied to the larvae maternally. Recent work on the mechanisms of echinoderm metamorphosis (Chino et al. 1994; Hodin et al. 2001; Johnson 1998; Johnson and Cartwright 1996; Saito et al. 1998; Suyemitsu et al. 1997) suggests that thyroid hormone may be one such compound.

Thyroid hormones [T4 (thyroxine) and T3 (3, 3', 5- triiodothyronine) and others] are best known for orchestrating metamorphosis in amphibians: several thyroid hormone synthesis inhibitors (such as $KClO_4$ and thiourea) prevent metamorphosis, while

exogenous hormonal applications cause precocious metamorphosis (reviewed in Rose 1999). Here we investigate the role of thyroid hormones in larval development and metamorphosis of the sand dollar *Dendraster excentricus*. We demonstrate that *D. excentricus* larvae do, indeed, respond to exogenous thyroid hormone (thyroxine) and a thyroid hormone synthesis inhibitor (thiourea) in a similar manner to the adaptive, plastic response of *D. excentricus* larvae to increased and decreased larval nutrition, respectively. We also present data suggesting that *D. excentricus* larvae can produce some endogenous thyroid hormone (or a thyroid hormone-like compound; but see Materials and Methods), but not enough to support development to metamorphic competence in the absence of food. We discuss our results in the context of phenotypic plasticity and life history evolution in echinoderm larvae.

Materials and Methods

We report on four experiments with larvae of *Dendraster excentricus* (Eschscholtz, 1831) from spring and summer 2000, 2001 and 2002: the Morphometrics Experiment, the Phenotypic Plasticity Experiment, the Metamorphosis Experiment and the Inhibitor Experiment. Sand dollars (*D. excentricus*) were collected at Crescent Beach, Orcas Island, Washington, USA at low tide in the spring and summer, held in flow through sea tables with pre-conditioned sand, and spawned later the same year.

We do not isolate or measure thyroid hormones in this study. Chino et al. (1994), measured thyroid hormone [T3 (3, 3', 5- triiodothyronine) and thyroxine (T4)] concentrations in larvae of two sea urchin species and one unicellular algal species that sea urchin larvae feed on (this was the apparent source for the thyroid hormones in the larvae) using radioimmunoassays after separation of individual thyroid hormones by HPLC. Suyemitsu et al. (1997) and Saito et al. (1998) showed that inhibitor (thiourea)

treatment of non-feeding larvae of the sand dollar *Peronella japonica* resulted in lower T3 and T4 levels. Based upon these studies, we refer here to the active compounds, inhibited in our experiments by thiourea and rescuable by exogenous thyroxine, as thyroid hormone (TH).

We prepared thyroxine (Sigma-Aldrich: T-1775) as described in Chino et al. (1994), and thiourea (a thyroxine synthesis inhibitor which acts by blocking iodine peroxidase activity; Sigma-Aldrich) in MFSW (millipore-filtered seawater; 0.45μm) at appropriate concentrations.

Experimental Designs

D. excentricus larvae can be reared from 10°C to 24°C (Strathmann 1987) without affecting their relative developmental trajectories (McEdward 1985). Temperatures for the four experiments were as follows: Morphometrics Experiment 11.5-14°C [30/6/00-12/8/00 (42 days)]; Plasticity Experiment 21-22°C [14/6/02-26/6/02 (12 days)]; Metamorphosis Experiment 21-23°C [8/4/00-12/8/00 (37 days)] and the Inhibitor Experiment 18.8 + 1.3°C std. dev. [24/7/01-14/8/01(34 days)].

In order to obtain gametes, we injected one male and one female (different ones in each experiment) with 0.55M KCl and set larval cultures up after hatching (as described in Strathmann 1987). The cultures were gently stirred using a motor-driven stirring apparatus (Strathmann 1987) or a shaker table, and we changed the water every two to three days. Unless stated otherwise, we set up larval cultures (at a maximal initial larval density of 1 larva/5ml MFSW) at the stage when the invaginating echinus rudiment contacts the left hydrocoel, and fed larvae 6000 cells/ml *Dunaliella tertiolecta*. Larvae in the Inhibitor Experiment were starved when they had reached rudiment stage 3 (see Table 2-1).

Experimental Treatments

Morphometrics Experiment: HIGH TH (10^{-9}M thyroxine); LOW TH (10^{-11}M thyroxine); CONTROL; LOW INHIBITOR (10^{-4}M thiourea); HIGH INHIBITOR (10^{-2}M thiourea); Plasticity Experiment: HFHT (6000cells/ml *D. tertiolecta*; 5 x 10^{-10}M thyroxine); LFHT (2000cells/ml *D. tertiolecta*; 5 x 10^{-10}M thyroxine); HFLT (6000cells/ml *D. tertiolecta*; 5 x 10^{-11}M thyroxine); LFLT (2000cells/ml *D. tertiolecta*; 5 x 10^{-11}M thyroxine); HF (6000cells/ml *D. tertiolecta*), LF (6000cells/ml *D. tertiolecta*). Metamorphosis Experiment: (HIGH TH (10^{-9}M thyroxine); LOW TH (10^{-11}M thyroxine); CONTROL; INHIBITOR (1.67 x 10^{-3}M thiourea); MIXED (1.67 x 10^{-3}M thiourea plus 10^{-9}M thyroxine); Inhibitor Experiment (no food): TH (10^{-9}M thyroxine); CONTROL; INHIBITOR (10^{-3}M thiourea) and MIXED (10^{-3}M thiourea plus 10^{-9}M thyroxine).

Morphometric Measurements and Rudiment Stages

In the morphometrics experiment, we took measurements on five randomly-chosen larvae (see Results for details) per independent replicate (three replicates in all experiments) after each water change. From captured images (using a Nikon compound microscope with an attached Polaroid^{TM} video camera) we took the following morphometric measurements (as indicated in Figure 2-1A): post-oral (PO) arm length, postdorsal (PD) arm length, body-midline length (BM), stomach length (SL), stomach width (SW), rudiment length (RL) and rudiment width (RW) using NIH Image (public domain) software and our own macros written for NIH Image. We calculated stomach size (SS) and rudiment size (RS) as the square root of the cross sectional area of an

ellipsoid, using SW or RW and SL or RL as the ellipsoid axes respectively. For relative morphological character measurements we divided each absolute morphological character measurement by rudiment size. We consider PO, PD and BM as larval characters, the stomach as both a larval and juvenile character since it is retained in the juvenile (Chia and Burke 1978) and the juvenile rudiment as a juvenile character. We also assigned each larva to developmental stages based on adult rudiment characters (Table 2-1; Fig. 2-1B), using images taken with DIC optics and/or polarized light.

For the Plasticity Experiment, we used a Nikon microscope E600 with a Nikon Coolpix 990 digital camera attached to it, which did not give clear views of skeleton using polarized light. Therefore, we did measurements and staging as described above, except that we ultimately flattened each larva underneath a cover slide for rudiment stage analysis (see Table 2-1). We have since demonstrated that both techniques (polarized light in living larvae versus flattening larvae) yield nearly identical results for rudiment staging in our hands (data not shown).

Metamorphic Competence and Settlement

We define settlement as the moment when tube feet stick out of the larva and it attaches firmly to the bottom of the culture dish. In the Morphometrics Experiment and the Metamorphosis Experiment, settlement occurred naturally (i.e., we added no settlement-inducing chemical). In the Inhibitor and Plasticity Experiment, we induced settlement with 40mM excess KCl in MFSW. We define metamorphic competence as the time at which more than 30% of the larvae in a given replicate settled spontaneously or upon induction of settlement with KCl. Because the KCl response was so robust in the Plasticity Experiment, we did not use the 30% threshold but compared percent settlement

in response to KCl directly. All juvenile measurements are test diameters (without spines).

Many observers have noted (e.g., Cameron and Hinegardner 1974) that echinoid larvae often have emergent tube feet well before settlement, and that such larvae can form non-permanent attachments to the substrate. Our experience, though, is that a suction challenge to attached larvae distinguishes settled larvae from those merely sampling the substrate.

Different echinoid species differ in the extent to which they require a specific settlement cue or inducer. *D. excentricus* larvae will readily settle onto clean plastic or glass. Therefore, we feel justified in defining metamorphic competence here as either 30% spontaneous or 30% KCl-induced settlement. We recognize that such a broad definition for competence would not be applicable to those echinoid (or other) species that fail to settle so readily in the absence of a cue or inducer.

Statistics

We compared time to metamorphic competence, juvenile sizes and larval growth among treatments as a function of rudiment stage using general linear model commands in SPSS^{TM}. We used a nested design to test for homogeneity of measurements among replicates. For the rudiment stage specific analysis and juvenile size, we used estimated marginal means for the comparison among treatments due to unequal number of replicate measurements (we present Standard Deviation instead of Standard Error in the relevant graphs). If necessary we used Bonferroni corrections for multiple comparisons. We analyzed larval mortality using Kolmogorov-Smirnov statistics. All p-values are from null hypotheses testing that the mean difference equals 0.

Results

Analysis of Thyroxine Effects on Larval and Juvenile Morphology

Comparison of age and rudiment stage specific analysis

We used the Morphometrics Experiment to compare measurements of larval and juvenile characters (Fig. 2-1) at age versus juvenile rudiment stage in the sand dollar *D. excentricus* (Fig. 2-2; see Table 2-1). In Figure 2-2 we plot the age at which given rudiment stages were reached for the CONTROL (x-axis) against the age at which the comparable rudiment stages were reached (y-axis) in the presence of exogenous thyroid hormone [HIGH TH (10^{-9}M thyroxine)] and a thyroid hormone synthesis inhibitor [HIGH INHIBITOR (10^{-2}M thiourea)]. Thus, larvae measured at the same time were not necessarily at the same developmental stage. Specifically, larvae in HIGH TH developed rudiment structures early; the reverse was true (though to a lesser degree) in HIGH INHIBITOR.

In order to undertake a meaningful comparison of relative investments into larval and juvenile structures by larvae reared under divergent food and chemical treatment conditions, we henceforth will focus on comparisons among treatments grouped by juvenile rudiment stage (Table 2-2).

Analysis of larval, juvenile and stomach growth and development

Morphometrics Experiment.- Figure 2-3 shows growth trajectories for each morphological character measurement (see Fig. 2-1) as a function of rudiment stage (see Table 2-1) for all experimental treatments [HIGH TH (10^{-9}M thyroxine), LOW TH (10^{-11}M thyroxine), CONTROL , LOW INHIBITOR (10^{-4}M thiourea) and HIGH INHIBITOR (10^{-2}M thiourea)]. A nested ANOVA revealed homogeneity for all

measurements of morphological characters among replicates in each treatment ($p > 0.40$). Therefore, we pooled measurements of all replicates from the same treatment (for reference see Underwood 1997, p. 268). Table 2-2 summarizes the ANOVA results; we present the details of the ANOVA in Table 2-4.

All three larval characters were significantly smaller in HIGH TH than in the CONTROL after rudiment stage 2 (Fig. 2-3A-C; Table 2-2; Table 2-4). For LOW TH, we detected no consistent trend (Fig. 2-3A, B; Table 2-2; Table 2-4). HIGH INHIBITOR larvae had significantly smaller PO and PD arm-lengths at rudiment stages 1 and 2 (Fig. 2-3A, B; Table 2-2; Table 2-4). For LOW INHIBITOR, PO and PD arms were significantly shorter at rudiment stage 4 (Fig. 2-3A,B; Table 2-2; Table 2-4). Stomach size was significantly smaller in HIGH TH after rudiment stage 2 (Fig. 2-3D, Table 2-2; Table 2-4). For HIGH INIHIBITOR and LOW INHIBITOR, stomach size was increased compared to the control (Fig. 2-3D, Table 2-2; Table 2-4). We detected no divergence in rudiment size between the control and any of the treatments after rudiment stage 1 (Fig. 2-3D; Table 2-2; Table 2-4).

We analyzed growth trajectories using a correlation analysis (Table 2-3) between each larval characteristic (PO, PD, BM; see Fig. 2-1A and below) or stomach size (SS) against rudiment size (RS) (see Material and Methods). We used absolute rudiment size because it was the character least influenced by the experimental treatments and followed comparable growth trajectories in all treatments (Fig. 2-3E, Table 2-2; Table 2-4). For each independent replicate (beaker) we compared the Pearson correlation coefficients among treatments using an ANOVA with simple contrast. HIGH TH growth trajectories all had negative correlations, whereas HIGH INHIBITOR and the CONTROL had

positive correlations, indicating that HIGH TH larvae allocated relatively more energy into juvenile rather than larval structures.

Phenotypic plasticity experiment and comparison with morphometrics experiment.- In Figure 2-4, we present the ratios of a given morphological character measurement over juvenile rudiment size for the Morphometrics Experiment and the Phenotypic Plasticity Experiment. These ratios indicate the relative allocation of energy into growth and development of larval structures (PO, PD, BM) or the stomach versus juvenile rudiment structures (see also Strathmann et al. 1992). We only present data from rudiment stages 3 and 4 (see Table 2-1). Larvae allocate relatively more energy into growth and development of juvenile structures than into larval structures, when they are reared with either excess thyroid hormone (thyroxine) or with high food (Fig. 2-4). The reverse is true when larvae are reared either with high levels of the thyroid hormone synthesis inhibitor thiourea (Fig. 2-4A) or with low food (Fig. 2-4B). The combined treatment of low levels of excess thyroid hormone plus low food (LFLT) resulted in a similar allocation ratio to high food (HF) (Fig. 2-4B; see also Fig. 2-8).

In summary, exposure either to high levels of exogenous thyroid hormone or to high food levels resulted in an increased relative allocation of energy into the growth of juvenile structures and a reduced relative allocation into the growth of larval structures (see Table 2-3). The investment profile in low food-reared larvae (increased investment into larval rather than juvenile structures) was reversed with the addition of low levels of exogenous thyroid hormone. Together, these results strongly suggest a connection between the morphometric effects of food and thyroid hormone on larval and juvenile growth in echinoid larvae.

Metamorphic Competence and Juvenile Size

We present the timing of settlement (see Materials and Methods) for all treatments from the Metamorphosis Experiment in Fig. 2-5A. CONTROL *D. excentricus* larvae reached metamorphic competence 18.3 ± 1.8 (one S.E. of mean) days after fertilization. Competence was reached in HIGH TH (10^{-9}M thyroxine) larvae in less than half that time (7.7 ± 0.67 days after fertilization); and was accelerated in LOW TH (10^{-11}M thyroxine; 14.3 ± 0.67 days after fertilization). An ANOVA with Post Hoc comparison using Bonferroni corrections revealed a significant difference between HIGH TH and CONTROL ($p = 0.003$), but not between LOW TH and CONTROL ($p = 0.40$). Larvae from INHIBITOR (1.67×10^{-3}M thiourea) did not reach the 30% settlement threshold over the course of the experiment. This thiourea-induced delay was rescued in the MIXED treatment (10^{-9}M thyroxine plus 1.67×10^{-3}M thiourea), where 30% settlement was reached after 12.3 ± 1.8 days. Note that settlement was most synchronous in HIGH TH (Fig. 2-5A).

In the plasticity experiment, we found that food also had significant effects on timing to metamorphic competence in *D. excentricus* larvae (Fig. 2-6A), as has been previously reported (Hart and Strathmann 1994). Under high food conditions (HF), larvae settled earlier than in low food (LF) (Fig. 2-6A). In the two treatments in which we combined food with high thyroid hormone concentrations (HFHT, LFHT), the larvae settled sooner than in any of the other treatments (Fig. 2-6A). The timing of settlement was similar in LFLT and HF, indicating that hormones can mimic the effect of food.

In addition, we report a significant decrease in juvenile size (test diameter) at settlement in HIGH TH (162.50 + 30.10μm; $n = 56$, $p < 0.001$) and MIXED (227.94 +

48.84μm; n = 10; $p < 0.001$) relative to the CONTROL (290 + 21.31μm; n = 9) in the Metamorphosis Experiment. We found no significant difference between CONTROL and LOW TH (281.56 + 45.91μm; n = 11; $p = 0.585$). Note that these differences in juvenile size (Fig. 2-5B) were largely due to differences in time to settlement (Fig. 2-5A).

In the Plasticity Experiment, the results with juvenile size (Fig. 2-6B) are comparable to the Morphometrics Experiment (data not shown) and the Metamorphosis Experiment (above). In general, juveniles from the high thyroid hormone (thyroxine) treatments (HFHT, LFHT) were significantly smaller then juveniles from most other treatments. In the low and no hormone treatments (HFLT, LFLT, HF, LF), LF and LFLT juveniles were significantly smaller than HF juveniles. However, addition of low levels of thyroid hormone to the low food treatment (LFLT) did not change juvenile size when compared to LF.

In summary, food and hormone both lead to accelerated growth to metamorphosis, and a combined low hormone and low food treatment mimics the acceleratory effects of high food. Taken together, these results indicate a connection between the metamorphic effects of food and thyroid hormone in echinoid larvae. Still, while increased food levels lead to settlement at an increased juvenile size (Hart and Strathmann 1994), high levels of thyroid hormone leads to settlement at a reduced size when compared to appropriate controls.

Endogenous Hormone Synthesis

To test whether thiourea effects on *D. excentricus* larvae were direct or indirect (via thiourea effects on algae) we transferred rudiment stage 3 *D. excentricus* larvae (see Table 2-1) to one of four treatments in the absence of food (i.e., no algae): 10^{-9}M

thyroxine (TH); CONTROL; 10^{-3}M thiourea (INHIBITOR); 10^{-3}M thiourea plus 10^{-9}M thyroxine (MIXED). We detected a subtle but significant delay in settlement in INHIBITOR [26.33 + 2.52% (one S.E. of the mean) settled on days 14-18] when compared with the CONTROL (37.33 + 4.59%) (two tailed t-test: t28, - 2.099 < 0.05; Fig. 2-7). TH larvae settled much earlier than in the CONTROL (80 + 5.77% by day 10). In the MIXED treatment, the thiourea-induced settlement delay was rescued with exogenous TH (66.67 + 12.02% by day 10). These results indicate that *D. excentricus* larvae can synthesize thyroid hormone (or a thyroid hormone-like compound; but see Materials and Methods) endogenously. However, this endogenous thyroid hormone production is not sufficient to allow *D. excentricus* larvae to reach metamorphic competence in the complete absence of larval food; such larvae do not develop beyond the appearance of pentaradial symmetry in the rudiment (rudiment stage 1, see Table 2-1; data not shown).

Furthermore, the finding that these inhibitor effects are rescued by the addition of excess thyroxine indicates that thiourea is an effective, specific blocker of thyroid hormone production and/or signaling in *D. excentricus* larvae.

Mortality and Abnormalities

We detected no significant difference in mortality between any of the treatments and the control using a two-sample Kolmogorov-Smirnov test in the Morphometrics Experiment (less than 11.2% average daily mortality in all treatments; note that "mortality" also includes any larvae lost during transfer) the Plasticity Experiment (less than 4.4% average daily mortalities) and the Metamorphosis Experiment (less than 6.5% in all treatments). We also detected no significant difference in abnormality in the

Morphometrics Experiment between any of the treatments and the control using a two-sample Kolmogorov-Smirnov test (less than a 5.8% average daily occurrence in all treatments) and comparable levels of abnormality were observed in the Plasticity Experiment.

Discussion

Although complex, metamorphic life histories have evolved independently in a wide variety of animal phyla, many of these life cycles appear to have converged upon a common mechanism: the life cycle transitions are controlled by hormones (reviewed in Matsuda 1987). The best-studied cases involve the derived, metamorphic life histories of holometabolous insects (bees, beetles, butterflies etc.) and amphibians (frogs, salamanders etc.), which are orchestrated, respectively, by steroid and thyroid hormones (reviewed in Nijhout 1994; Rose 1999). Furthermore, the evolution of novel life histories within these two disparate animal groups appears to involve alterations in the regulation of these hormones. For example, larval reproduction in flies (Hodin and Riddiford 2000), direct development in frogs (Callery and Elinson 2000; Hanken et al. 1997; Jennings and Hanken 1998) and neoteny in salamanders (Frieden 1981; Galton 1992; Kühn and Jacobs 1989; Yaoita and Brown 1990) all entail major alterations in the timing of hormonal release and/or the cellular hormonal response. Although little information is available about metamorphic roles of hormones in most other phyla, a striking case of parallel evolution has recently been reported in amphibians and echinoderms (sea urchins, starfish and their kin): both groups utilize thyroid hormone as a regulator for metamorphosis (defined here broadly to include the lengthy development of the juvenile rudiment in echinoderms). Whether thyroid hormones are involved in the evolution of novel life histories in echinoderms, as they are in amphibians, has not been previously explored.

As is true in amphibians, several planktotrophic sea urchins (Chino et al. 1994; Johnson 1998), two sand dollar species (Hodin et al. 2001; Saito et al. 1998; Suyemitsu et al. 1997) and a starfish (Johnson and Cartwright 1996) complete metamorphosis early in the presence of exogenous thyroxine (T4), one form of thyroid hormone. Intriguingly, the major source of the hormone for feeding echinoderm larvae appears to be the planktonic algae that they consume (Chino et al. 1994). We propose that thyroid hormones are an environmental signal that larvae use as an indicator of nutritional state (see Pfennig 1992) for a strikingly parallel case in amphibians). We will discuss this hypothesis below in the context of phenotypic plasticity, juvenile size, and life history evolution in echinoids.

Thyroid Hormone as a Cue for Adaptive Phenotypic Plasticity in Echinoid Larvae

Phenotypic plasticity is ontogenetic variability within a genotype due to naturally occurring or experimentally imposed environmental heterogeneity (reviewed in Schlichting and Pigliucci 1998). An adaptively plastic response depends upon the ability of an organism to sense an environmental cue (such as day length, temperature, etc.), allowing it to respond appropriately to changing conditions (reviewed in Schlichting and Pigliucci 1998). Larval food concentrations in the marine environment undergo large temporal and spatial fluctuations (Fenaux et al. 1994; Morgan 1995; Pechenik 1987). Several laboratory and field studies on echinoid larvae (Fenaux et al. 1994; Hart and Strathmann 1994; Strathmann et al. 1992) have demonstrated that when food is limiting, a shift occurs towards investment into growth and development of larval rather than juvenile structures, resulting in an increase in feeding efficiency. When larvae live in an enhanced food environment, by contrast, investment into larval structures is reduced and the juvenile rudiment develops at a faster rate, an apparently adaptive response to avoiding the dangers inherent in the plankton (reviewed in Morgan 1995).

Here we report differential investment into juvenile versus larval structures in *D. excentricus* larvae that were exposed to different levels of exogenous thyroid hormones (specifically thyroxine) in either low or high food conditions. Excess amounts of exogenous thyroid hormone (henceforth "TH") induce a morphometric and metamorphic response in *D. excentricus* larvae similar to the effect of excess food (Fig. 2-8). Indeed, *D. excentricus* larvae exposed simultaneously to low food and low TH levels resemble *D. excentricus* larvae exposed to high food levels in the absence of excess TH. Furthermore, we show that larvae reared with the TH synthesis inhibitor thiourea resemble larvae raised in reduced food conditions. These similar developmental responses lead us to conclude that echinoid larvae use TH levels as an indicator of food availability in the plankton (see "Thyroid Hormones As an Indicator of Larval Nutrition," below). This notion that exogenous TH can induce an adaptively-plastic morphometric response similar to the effects of diet is not without precedent. Pfennig (1992) showed that TH (thyroxine) treatment of spadefoot toad tadpoles (*Scaphiopus multiplicatus*) induces a carnivorous larval morph, a response also induced by a diet switch from a commercial omnivorous fish food to shrimp (known to contain T2 - a form of TH).

We propose that in echinoderm larvae, TH acts in a signaling system that activates juvenile development while repressing larval growth. To further test this hypothesis, we can examine the dose response of starved larvae to TH, to see if TH alone can produce ontogenetic effects similar to ingested food. We can also pre-treat algae with TH synthesis inhibitors to try to produce well-fed larvae that respond as if they are being starved.

Although the plastic response of echinoderm larvae to environmental food levels has been suggested to be adaptive, no study to date (including our own) has directly addressed the fitness consequences in juveniles of this induced plastic response. Hart and Strathmann (1994) noted that direct fitness comparisons between larvae with the long-arm (delayed metamorphosis) and short-armed (accelerated metamorphosis) phenotypes suffer from the confounding effect of food treatment. These authors proposed to uncouple the plastic response from the food effects by generating larvae with the high food (short arm) morphology at a low food level (using TH), and the reverse (using inhibitors). Due to the confounding effects of inhibitors on the algae themselves (not shown, but described below), the latter experiment is unfortunately not feasible. As for the use of TH, since it also results in precocious settlement at a small size (via accelerated rudiment growth), its usefulness in testing the specific adaptive hypothesis proposed by Hart and Strathmann (Hart and Strathmann 1994) is also questionable. Still, in our plasticity experiment, we identified what may be an ideal protocol for a fitness comparison akin to the one that Hart and Strathmann proposed. Our analysis of the low food (LF) and low food/low hormone (LFLT) treatments revealed that the latter developed to metamorphosis faster via the short arm (high food-like) phenotype, with no ultimate difference in juvenile size (but with presumed differences in stored nutrition; see below). The consequence of feeding efficiency on fitness can now be tested by directly comparing post-larval performance in juveniles derived from the LF and LFLT treatments. We predict that LF-derived juveniles would grow more quickly after settlement than LFLT-derived juveniles, indicating a selective advantage to the low food (long arm, delayed settlement) phenotype.

Differences Between Thyroid Hormone and Food Effects: Stomach Size

We found that stomach size is differently affected by food and hormone treatments. Several food limitation experiments including our own indicate that larvae from low food treatments have shrunken stomachs, while their well-fed counterparts have enlarged stomachs. By contrast, our TH-treated *D. excentricus* larvae had reduced stomachs, while inhibitor-treated larvae had enlarged stomachs relative to controls, and these effects were dose-responsive [note, however, that it is only our measurements of absolute stomach size (not shown) which show this different pattern between food and hormone; when calculated relative to rudiment size, the effects of food and hormone are quite similar; see Fig. 2-4].

Unlike the larval arms and the juvenile rudiment, which are larval- and juvenile-specific structures respectively, the larval stomach in echinoids also acts as a nutritional storage organ, and is inherited by the juvenile (Chia and Burke 1978). Since TH-treated larvae have reduced arm growth, they presumably have lower feeding rates than do control larvae (Hart and Strathmann 1994). Still, such larvae invest more energy into juvenile rudiment development than controls, which must come at the expense of nutritional storage in and growth of the stomach. Thiourea-treated larvae, by contrast, delay juvenile rudiment development, and therefore can be hypothesized to invest more of their nutritional intake into stomach storage and growth.

Thyroid Hormones As an Indicator of Larval Nutrition: Implications for Juvenile Size in Echinoids

Many marine invertebrates have a complex life history, often involving a feeding planktonic larval stage, which has an entirely distinct morphology, behavior and habitat from the benthic adult. While some progress has been made in recent years in identifying

possible settlement cues (reviewed in Hadfield 2000), very little is known about the mechanisms by which larvae attain the competence to respond to such settlement cues. In some insects, developmental transitions (molting and metamorphosis) have been shown to involve attainment of a critical size, and that structures such as stretch receptors in the cuticle are involved in sensing body size (reviewed in Nijhout 1994; Nijhout 1999).

TH levels might be a crucial component of the signal for metamorphic competence in echinoids and possibly other echinoderms as well. We infer from our results, as well as those of Chino et al. (1994) and Heyland and Hodin (2004) the following mechanistic scenario. The TH that is present in planktonic algae accumulates in the larval body during feeding. During this period, the accumulating TH is directing the growth and development of the juvenile rudiment. Then, competence is reached after internal TH levels reach a particular threshold and the larva becomes responsive to specific settlement cues. By exposing larvae of *D. excentricus* to excess exogenous TH at different food concentrations, competence was reached earlier than in the corresponding controls. However, the shorter larval period of experimental larvae resulted in the acquisition of less total nutrition than in control larvae, and earlier metamorphic competence was attained by a significantly accelerated development of the juvenile rudiment. As a consequence, juveniles from the experimental high TH treatment, for example, metamorphosed at about half the normal size (juvenile test diameter). This degree of juvenile size reduction is unprecedented for planktotrophic echinoids reared under any condition. Moreover these results suggest that larval TH levels, rather than juvenile size itself, is a critical indicator of metamorphic competence.

Our unpublished data (with A. Reitzel) for the sand dollar *Leodia sexiesperforata* further supports this suggestion that competence is connected to larval TH levels. *L. sexiesperforata* larvae develop from eggs with approximately 6 times the energy content of *D. excentricus* eggs, but still need to feed in order to reach competence (L.R. McEdward lab, unpublished data). However, when we treated starved *L. sexiesperforata* larvae with TH, they completed metamorphosis and formed living juveniles (see chapter 3). These data suggest not only that TH in the absence of exogenous food is sufficient to induce development through metamorphosis in *L. sexiesperforata*, but also that TH levels are somehow connected to the attainment of metamorphic competence.

The length of the larval period and juvenile size are two traits that can potentially shape the evolution of life history strategies in marine invertebrates. Due to high mortality in the plankton, reduction of the planktonic period should generally be advantageous (for review see Morgan 1995; Rumrill 1990). However, a reduced larval period could lead to a reduced juvenile size (or a reduction in stored energy, as discussed above), which might have negative fitness consequences. Although such fitness consequences have been previously discussed (Emlet et al. 1987; Kolding and Fenchel 1981; Pechenik et al. 1998; Perron 1986; Strathmann 1974; Strathmann and Vedder 1977) experimental tests are relatively rare (but see Emlet and Hoeghguldberg 1997; Gosselin and Qian 1997). Our results demonstrate for the first time that metamorphosis at a substantially reduced juvenile size is mechanistically possible in planktotrophic echinoid larvae (for data on lecithotrophic larvae see Emlet and Hoeghguldberg 1997; Okazaki and Dan 1954). This finding lends support to the notion that such juveniles would suffer reduced fitness, possibly by lowering their competitive ability and/or

increasing their vulnerability to predation. Such hypotheses can now be tested using TH as an experimental tool.

Our conclusion that echinoid larvae use TH levels as an indicator of larval nutrition depends upon the finding that the production of TH-like compounds is widespread among disparate algal taxa (Tsuchiya and Ito, reported in Chino et al. 1994; Eales 1997; Ragan 1981; Tsuchiya 1969). Production of such a compound has not been reported for *Dunaliella tertiolecta*, the unicellular green alga that we used as larval food in this study. However, our unpublished results with the TH synthesis inhibitor thiourea demonstrated a reversible, dose-responsive effect of this inhibitor on *D. tertiolecta* growth rates, providing strong indirect evidence that this alga produces TH-like compounds. A greater diversity of phytoplankton species should be investigated for their relative TH contents, since these would represent the naturally occurring sources of hormones available to planktotrophic larvae such as *D. excentricus*.

Thyroid Hormones and Life History Evolution in Echinoderms

One of the most striking life history patterns in marine invertebrates in general, and echinoderms specifically, is that similar looking adults can be produced from radically different ontogenetic routes: feeding (planktotrophic) development from relatively small eggs, or non-feeding (lecithotrophic) development from large eggs via entirely dissimilar larvae (reviewed in Strathmann 1985). While this life history diversity seems to indicate a substantial flexibility of development, further analysis suggests that there might be constraints on the evolution of lecithotrophy from planktotrophic ancestors (see also Hodin et al. 2001).

Lecithotrophic taxa are found in all five extant echinoderm classes, with the class Crinoidea (sea lilies and feather stars) being entirely lecithotrophic. In the four other

echinoderm classes, numerous evolutionary transitions from planktotrophy to lecithotrophy have been hypothesized (reviewed in Wray 1996). Still, the taxonomic distribution of lecithotrophs within these classes appears decidedly non-random. In the echinoids, for example, most of the 20 or so (Emlet 1995; Wray 1996), Wray pers. comm. 2001} independently evolved instances of lecithotrophy are concentrated in a few orders, notably the orders Clypeasteroida (sand dollars and sea biscuits), Spatangoida (heart urchins) and Cidaroida (pencil urchins). By contrast, two of the largest and most diverse orders of regular sea urchins, the orders Diadematoida and Echinoida, have no and relatively few independently-evolved lecithotrophs, respectively (McEdward and Miner 2001).

We suggest that one factor that might explain such a non-random distribution of lecithotrophs is differences among taxa in the ability of their larvae to synthesize TH endogenously. We hypothesize that endogenous synthesis of TH is a necessary pre-adaptation for the evolution of lecithotrophy. Those taxa that plesiomorphically have the ability to synthesize this hormone endogenously would be expected, therefore, to undergo parallel evolutionary transitions from planktotrophy to lecithotrophy more frequently.

Endogenous synthesis of TH has been reported for one lecithotrophic echinoderm, the sand dollar (order Clypeasteroida) *Peronella japonica* (Saito et al. 1998; Suyemitsu et al. 1997). This, along with our data on the planktotrophic sand dollar *D. excentricus* (this study) and the facultative feeding sea biscuit Clypeaster rosaceus (unpublished with A. Reitzel), suggests that clypeasteroid larvae have the capacity (albeit limited in the case of Dendraster) for endogenous TH synthesis. By contrast, two planktotrophic sea urchin species from the order Echinoida (which has relatively few lecithotrophic taxa) do not

show obvious inhibitor effects, suggesting that they produce little if any TH endogenously (Chino et al. 1994). These data support our hypothesis that endogenous TH production can be considered a pre-adaptation for the evolution of lecithotrophy.

A broad taxonomic survey of lecithotrophs and their planktotrophic relatives (using an approach that we took here for the inhibitor experiment) should reveal differing effects of TH synthesis inhibitors on juvenile morphogenesis and development to metamorphic competence. We predict that planktotrophic species from the orders Clypeasteroida, Cidaroida and Spatangoida will consistently show more pronounced inhibitor effects than their counterparts in the orders Diadematoida and Echinoida. Our recent unpublished data (in collaboration with T. Capo) with *Diadema antillarum* larvae provides some support for this prediction.

The above scenario begs the following question: if ability to synthesize TH endogenously first evolved before the origin of lecithotrophy in these groups, then what could be a selective advantage of internal hormone synthesis itself? One possible advantage could be to modify the phenotypically plastic response that larvae have to ingested THs. Such larvae might exist in a relatively predictable planktonic environment, such that there is no adaptive value to maintaining a great degree of plasticity. The internal production of TH could dampen the plastic effects of temporary food fluctuations in the environment, as well as those that we see in the laboratory. Furthermore, planktotrophic echinoderm larvae developing from relatively large eggs could undergo metamorphosis more quickly, thereby escaping the dangerous planktonic environment (Morgan 1995; Rumrill 1990), only if they do not depend upon feeding in the plankton to obtain all of the required TH. These hypotheses make a testable prediction: the ability to

synthesize TH endogenously is inversely proportional to the scope of plasticity, and directly proportional to egg size in a given taxon. Experiments with clypeasteroid species representing a 10 fold range in egg volume (*D. excentricus*, C. rosaceous and P. japonica) offer initial support for this prediction. Addressing this hypothesis further could provide the synthesis of ecological and ontogenetic mechanisms necessary to account for any such patterns in life history evolution.

Table 2-1. Temporal occurrence of characters in the juvenile rudiment, and definition of rudiment stages. + indicates presence of juvenile character; - indicates absence of juvenile character. Note that body skeletal plates include first multi-branched spicules (as indicate in drawing). See also Figure 2-1B for an image showing some of these juvenile characters.

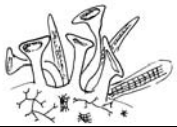
Rudiment stage	Stage 1	Stage 2	Stage 3	Stage 4
				
Pentaradial symmetry	+	+	+	+
Primary podia	-	+	+	+
Adult skeleton	-	+	+	+
Body skeletal plates	-	-	+	+
Tube feet	-	-	+	+
Spines and/or spine primordia	-	-	-	+

Table 2-2. Qualitative summary of ANOVA results for morphological character measurements as a function of rudiment stage for all experimental treatments (Morphometrics Experiment, approx. 13°C) compared to CONTROL (see Table 2-1 for our definitions of juvenile rudiment stages). The numerical data can be found in Table 2-4. ↓ indicates that the morphological character measurement was significantly ($p < 0.05$) smaller than in CONTROL. ↑ indicates that the morphological character measurement was significantly ($p < 0.05$) larger than in CONTROL.

Character	Rudiment Stage	Treatment			
		HIGH TH (10^{-9}M T4)	LOW TH (10^{-11}M T4)	LOW INHIBITOR (10^{-4}M thiourea)	HIGH INHIBITOR (10^{-2}M thiourea)
Postoral arm length	1			↓	↓
	2	↓		↓	↓
	3	↓			
	4	↓		↓	N/A
Postdorsal arm length	1			↓	↓
	2	↓	↑		↓
	3				
	4	↓		↓	N/A
Body midline length	1		↓	↓	
	2	↓			
	3	↓			
	4	↓			N/A
Stomach size	1				
	2	↓			↑
	3	↓			↑
	4	↓			N/A
Rudiment size	1	↑		↓	
	2				
	3				
	4				N/A

Table 2-3. Analysis and comparison of growth trajectories between the HIGH TH [10^{-9}M thyroxine], CONTROL and HIGH INHIBITOR [10^{-3}M thiourea] and for each morphological character measurement relative to the rudiment size (Morphometrics Experiment, approx. 13°C). For each independent replicate, the Pearson correlation coefficient between the morphological character measurement and the rudiment size was analyzed and compared between the treatments and the control using ANOVA with simple contrast. Mean ± one S.E. mean (p-value).

Character	*Treatment*		
	High TH	Control	High Inhibitor
Postoral arm length versus Rudiment size	-0.65 ± 1.02E-02* ($p < 0.01$)	0.52 ± 0.12	0.60 ± 0.11 ($p = 0.56$)
Postdorsal arm length versus Rudiment size	-0.68 ± 1.46E-02* ($p < 0.01$)	0.57 ± 0.11	0.59 ± 3.37E-02 ($p = 0.82$)
Body midline length versus Rudiment size	-0.63 ± 7.20E-02* ($p < 0.01$)	0.78 ± 3.06E-02	0.90 ± 6.51E-03 ($p = 0.10$)
Stomach size versus Rudiment size	-0.49 ± 0.18* ($p < 0.01$)	0.45 ± 0.20	0.82 ± 5.20E-02 ($p = 0.14$)

Table 2-4. ANOVA with simple contrast of morphological character measurements (see Fig. 2-1) as a function of rudiment stage for all experimental treatments compared to CONTROL (Morphometrics Experiment, approx. 13°C). The comparison is based on observed means. Values represent the mean difference between morphological character measurements (negative values: smaller than CONTROL; positive values: larger than CONTROL). ** $p < 0.025$; * $p < 0.05$

Values ± one S.E. mean		Rudiment Stage			
Treatment	Character	1	2	3	4
HIGH TH [10^{-9}M thyroxine]	Postoral arm	-26 ± 17	-110 ± 18**	-146 ± 39**	-214 ± 24**
	Postdorsal arm	-27 ± 24	-63 ± 24**	-76 ± 49	-211 ± 26**
	Bodymidline	2 ± 10	-49 ± 19**	-74 ± 21**	-162 ± 21**
	Stomach size	-13 ± 11	-44 ± 20*	-104 ± 23**	-107 ± 21**
	Rudiment size	24 ± 10**	-11 ± 23	-50 ± 28	-18 ± 23
LOW TH [10^{-11}M thyroxine]	Postoral arm	-25 ± 13	-8 ± 18	9 ± 37	2 ± 26
	Postdorsal arm	-20 ± 19	52 ± 24*	22 ± 47	54 ± 29
	Bodymidline	-16 ± 8*	-22 ± 19	-16 ± 20	-13 ± 23
	Stomach size	-16 ± 9	-23 ± 19	-29 ± 22	-30 ± 22
	Rudiment size	-2 ± 8	-14 ± 22	-22 ± 26	37 ± 25
LOW INHIBITOR [10^{-4}M thiourea]	Postoral arm	-53 ± 14**	-57 ± 18**	-75 ± 41	-53 ± 24*
	Postdorsal arm	-50 ± 20**	2E-02 ± 24	-51 ± 51	-72 ± 26**
	Bodymidline	-27 ± 9**	1 ± 19	13 ± 21	10 ± 21
	Stomach size	-17 ± 9	19 ± 20	8 ± 24	53 ± 20**
	Rudiment size	-21 ± 8**	-3 ± 23	-2 ± 29	16 ± 23
HIGH INHIBITOR [10^{-2}M thiourea]	Postoral arm	-54 ± 13**	-93 ± 15**	-73 ± 41	N/A
	Postdorsal arm	-144 ± 18**	-112 ± 20**	-71 ± 51	N/A
	Bodymidline	-12 ± 8	31 ± 16	38 ± 21	N/A
	Stomach size	6 ± 9	64 ± 16**	55 ± 24*	N/A
	Rudiment size	-11 ± 8	17 ± 19	-1 ± 29	N/A

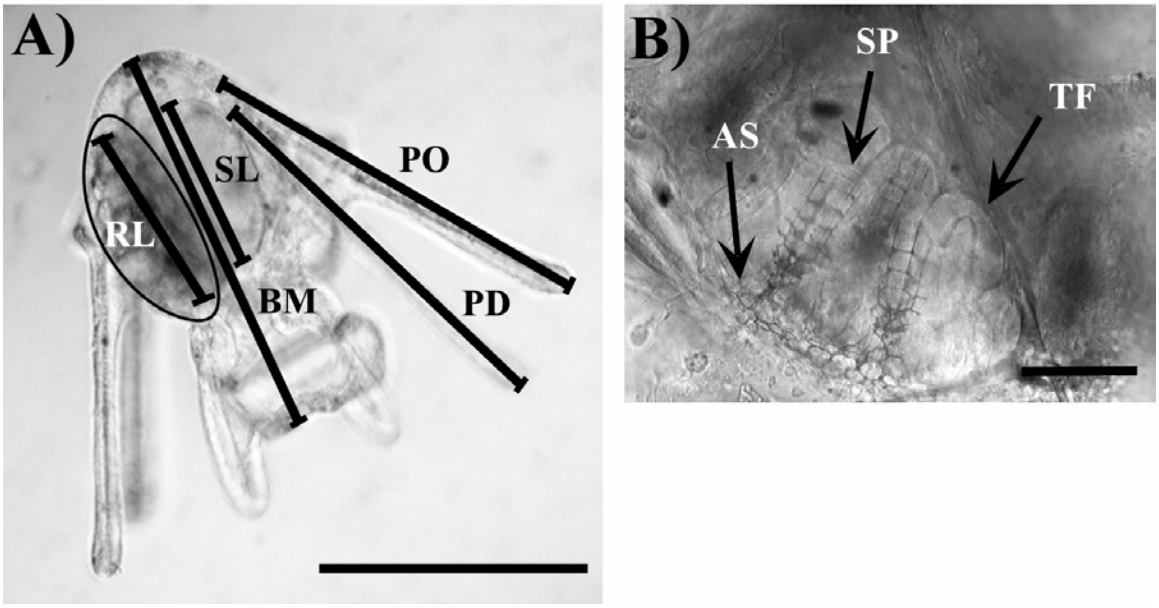


Figure 2-1: Larval and adult characters analyzed in this study. A) Pluteus larva and B) close-up image of the juvenile rudiment (="echinus rudiment"; circled in A) in the planktotrophic larva of *Dendraster excentricus*. In the Morphometrics and Phenotypic Plasticity Experiments (see Materials and Methods), 5 larvae were randomly chosen and the indicated structures were measured for each replicate. PO = postoral arm; PD = postdorsal arm; BM = body midline; SL = stomach length [stomach size (SS) is a combined measure of length and width - width measured but not indicated; for details see Material and Methods]; RL = juvenile rudiment length [juvenile rudiment size (RS) is a combined measure of length and width - width measured but not indicated; for details see Material and Methods]; AS = adult skeleton; SP = juvenile or adult spine; TF = tube foot. PO, PD, BM, SL and RL were measured, AS, SP, TF (and other adult structures not indicated here) were used to stage the development of the juvenile rudiment (see Table 1). Bar in A) equals 300µm, in B) 40µm.

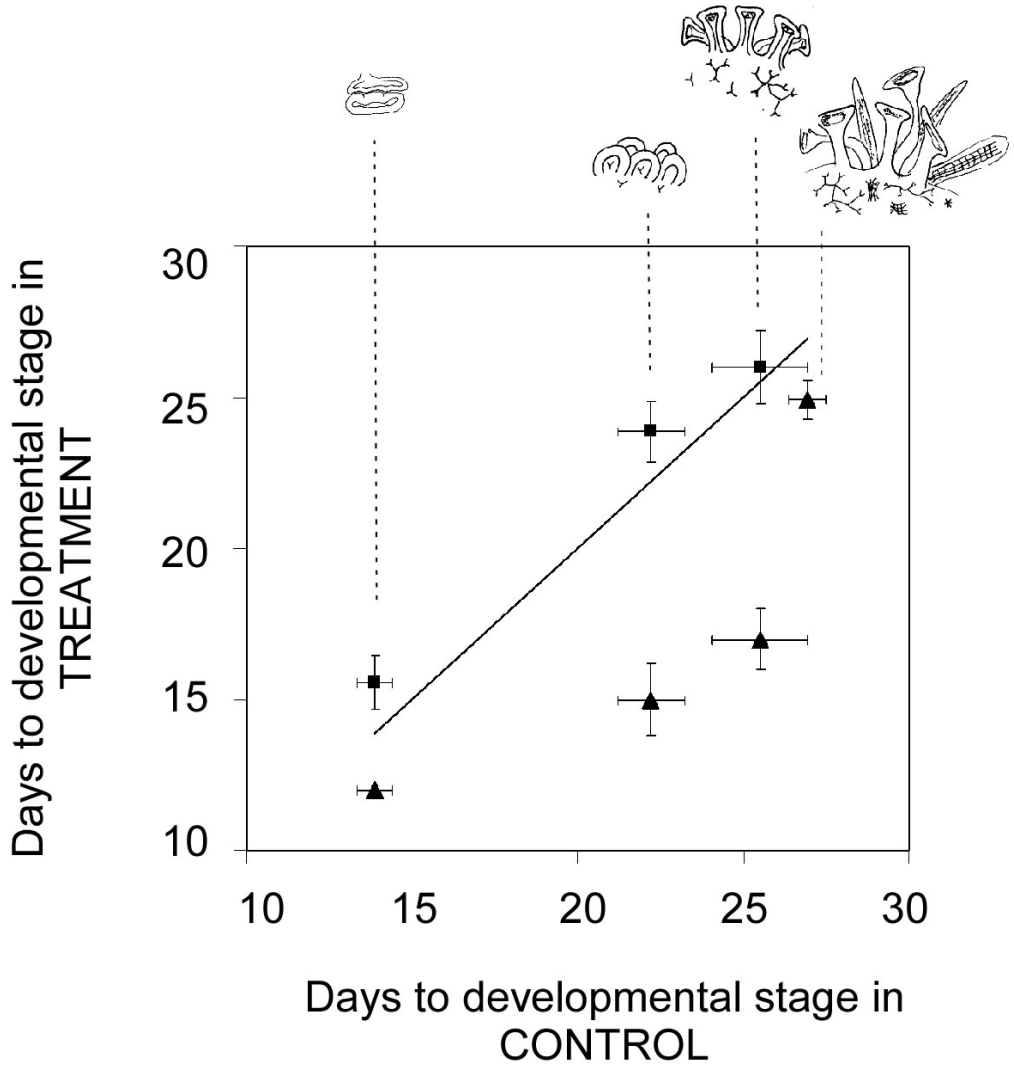


Figure 2-2: Thyroid hormone (thyroxine) accelerates and a thyroid hormone synthesis inhibitor (thiourea) delays the developmental sequence of *D. excentricus* larvae relative to the control (Morphometrics Experiment, approx. 13°C). Times to developmental stages 1-4 (drawn above the graph; see Table 1 and line drawings in this figure) for the control are plotted on the x-axis. Equivalent stages for thyroxine and thiourea are plotted on the y-axis. Points above the line indicate delayed development relative to the control; points below the line indicate accelerated development relative to the control. ▲ HIGH TH (10^{-9}M thyroxine) vs. CONTROL. ■ HIGH INHIBITOR (10^{-2}M thiourea) vs. CONTROL. Reference line indicates CONTROL vs. CONTROL. Error bars represent one S.E. mean.

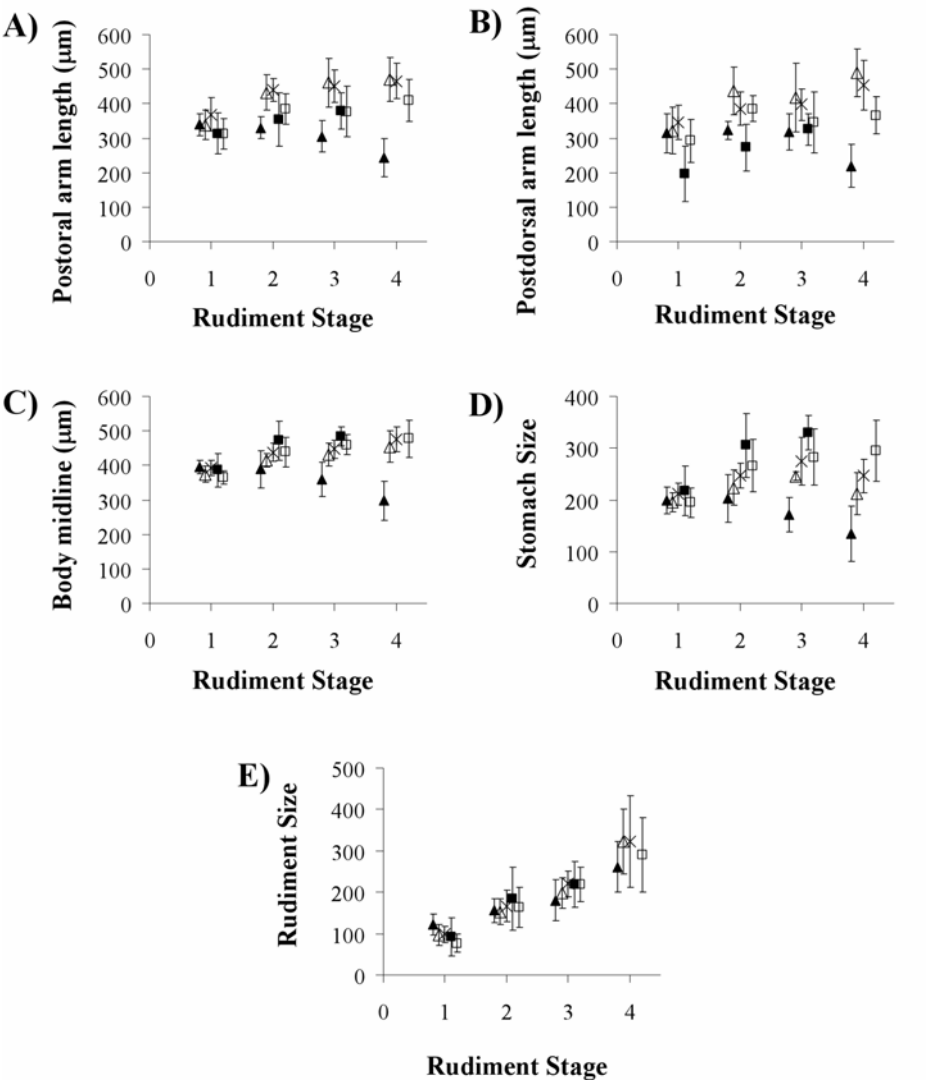


Figure 2-3: Heterochronic changes in relative allocation to larval and juvenile structures as a result of thyroid hormone (thyroxine) and thyroid hormone synthesis inhibitor (thiourea) treatments. A-E) Growth curves for larval characters in µm [postoral arm length (PO), postdorsal arm length (PD), body midline length (BM)] stomach size (SS) and juvenile rudiment size (RS) as a function of juvenile rudiment stage (stage) for all four experimental treatments and the control at 13°C (Morphometrics Experiment; see Figure 2-1A for a guide to structures; see Table 1 for rudiment stage criteria; see Materials and Methods for our method for calculating SS and RS). Measurement sets were taken at 12, 16, 23 and 28 days after fertilization. Error bars indicate ± one S.D. mean (S.D. is used here due to unequal sample size after staging of larval development). ▲ HIGH TH (10^{-9}M thyroxine); Δ LOW TH (10^{-11}M thyroxine); × CONTROL; □ LOW INHIBITOR (10^{-4}M thiourea); ■ HIGH INHIBITOR (10^{-2}M thiourea).

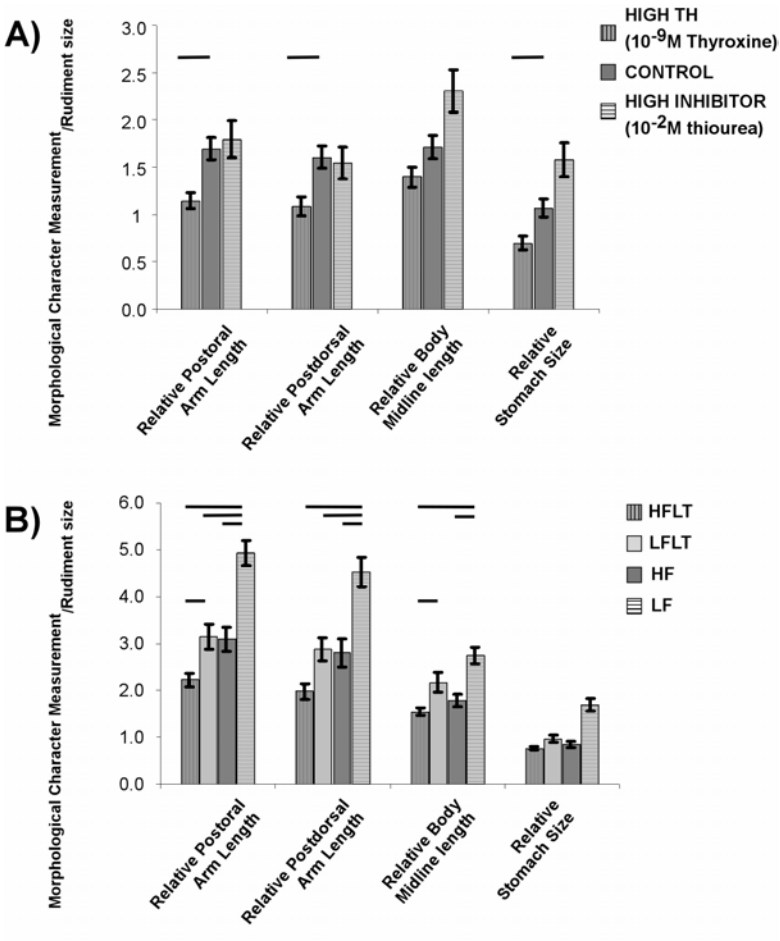


Figure 2-4: Thyroid hormone (thyroxine) and food have similar effects on the relative investment into larval versus juvenile growth in *D. excentricus* larvae. Morphometric analysis from Morphometrics Experiment (A) and Plasticity Experiment (B) at rudiment stage 3 + 4 combined (Table 1). Relative morphometric character measurements (relative postoral arm length, relative postdorsal arm length, relative body midline length and relative stomach size) are calculated by dividing the appropriate morphological character measurement (MCM) by rudiment size (see Materials and Methods and Figure 2-1). All values are means ± one S.E. of the mean. Horizontal bars indicate significant pair wise differences between mean values based on multivariate analysis of variance using Bonferroni corrections for multiple comparisons. Morphometrics Experiment: HIGH TH (10^{-9}M thyroxine); CONTROL; HIGH INHIBITOR (10^{-2}M thiourea). Plasticity Experiment: HFLT (6000 cells/ml *Dunaliella tertiolecta*; 5×10^{-11}M thyroxine); LFLT (2000 cells/ml *D. tertiolecta*; 5×10^{-11}M thyroxine); HF (6000 cells/ml *D. tertiolecta*); LF (6000cells/ml *D. tertiolecta*).

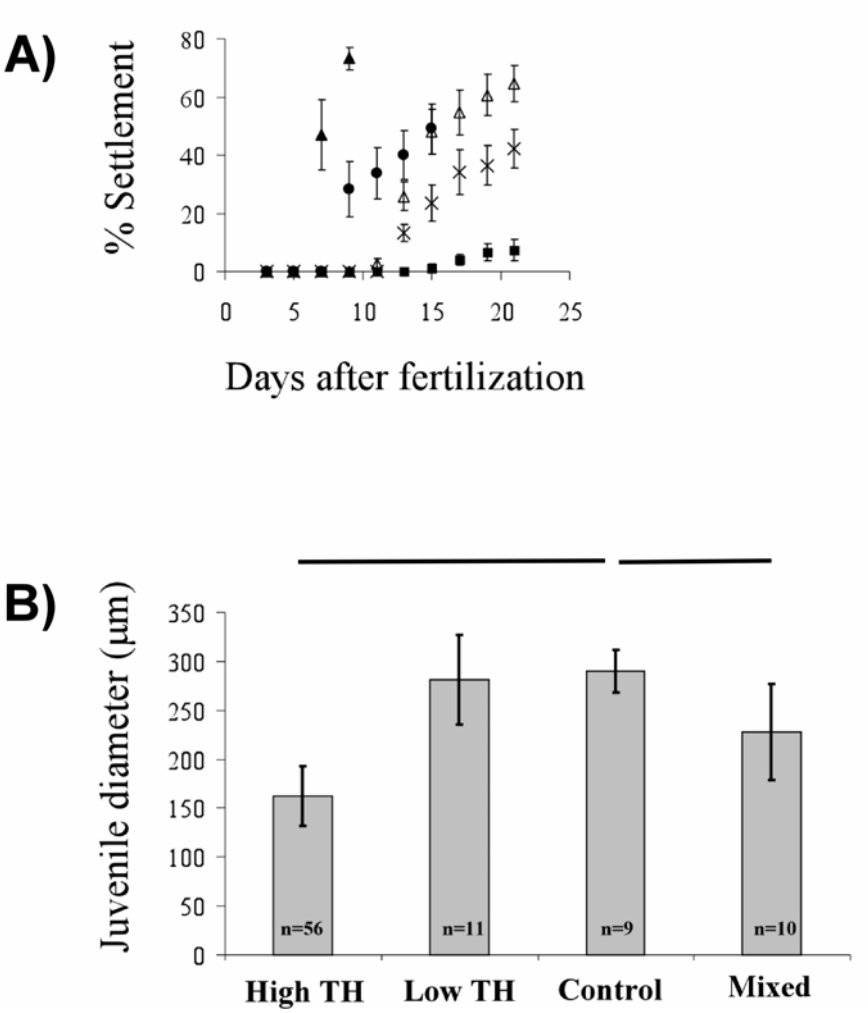


Figure 2-5: Exogenously applied thyroid hormone (thyroxine) results in earlier settlement of *Dendraster excentricus* larvae at a reduced juvenile size; the thyroid hormone synthesis inhibitor thiourea delays settlement (Metamorphosis Experiment, approx. 22°C). A) Percent settlement over time for all four experimental treatments and the control (error bars represent one S.E. mean). Note that in the MIXED treatment, the thiourea-induced delay of settlement is rescued by thyroxine. ▲ HIGH TH (10^{-9}M thyroxine); Δ LOW TH (10^{-11}M thyroxine); × CONTROL; ■ INHIBITOR (1.6×10^{-3}M thiourea); ● MIXED (10^{-9}M thyroxine plus 1.6×10^{-3}M thiourea). B) Juvenile (test) diameters (Juv. Diam.) at settlement for the four experimental treatments and the control. We only measured diameters for juveniles after settlement was greater than 30% in a given replicate (see Materials and Methods). Error bars represent one standard deviation; n = number of measurements per treatment. Similar results are seen at 13°C (not shown).

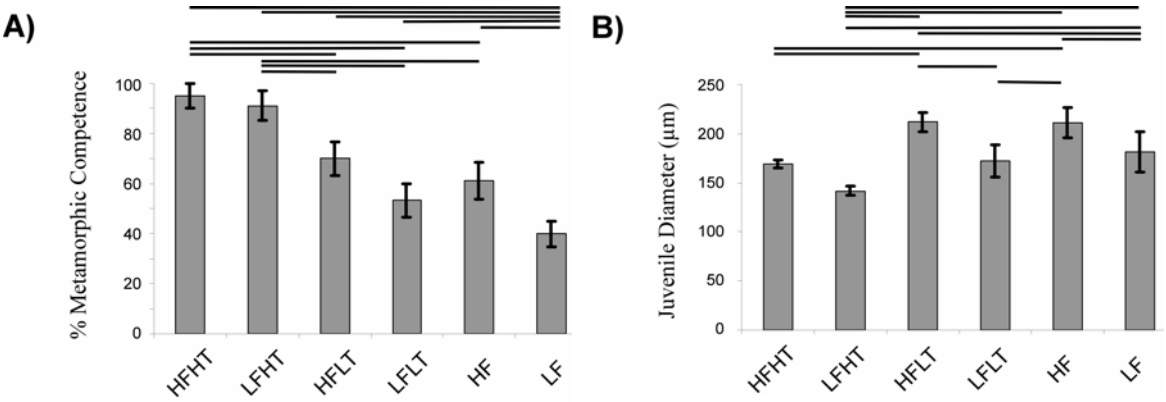


Figure 2-6: Excess food and thyroid hormone both accelerate development to metamorphosis; low food and thyroid hormone lead to settlement at a reduced juvenile size. Metamorphosis (A) and Juvenile Size (measured as juvenile diameter: Juv. Diam.) (B) in the Plasticity Experiment. Mean values ± one S.E. of the mean. Horizontal bars indicate significant difference between mean values ($p<0.05$) using univariate analysis of variance with Bonferroni corrections for multiple comparisons. HFHT (6000cells/µl ml *Dunaliella tertiolecta*; 5×10^{-10}M thyroxine) LFHT (2000cells/µl ml *D. tertiolecta*; 5×10^{-10}M thyroxine) HFLT (6000cells/µl ml *D. tertiolecta*; 5×10^{-11}M thyroxine) LFLT (2000cells/µl ml *D. tertiolecta*; 5×10^{-11}M thyroxine), HF (6000cells/µl ml *D. tertiolecta*), LF (6000cells/µl ml *D. tertiolecta*).HFHT (n=4), LFHT (n=6), HFLT (n=6), LFLT (n=6), HF (n=6), LF (n=6). n = numbers of replicate treatments, 5 individuals/replicate.

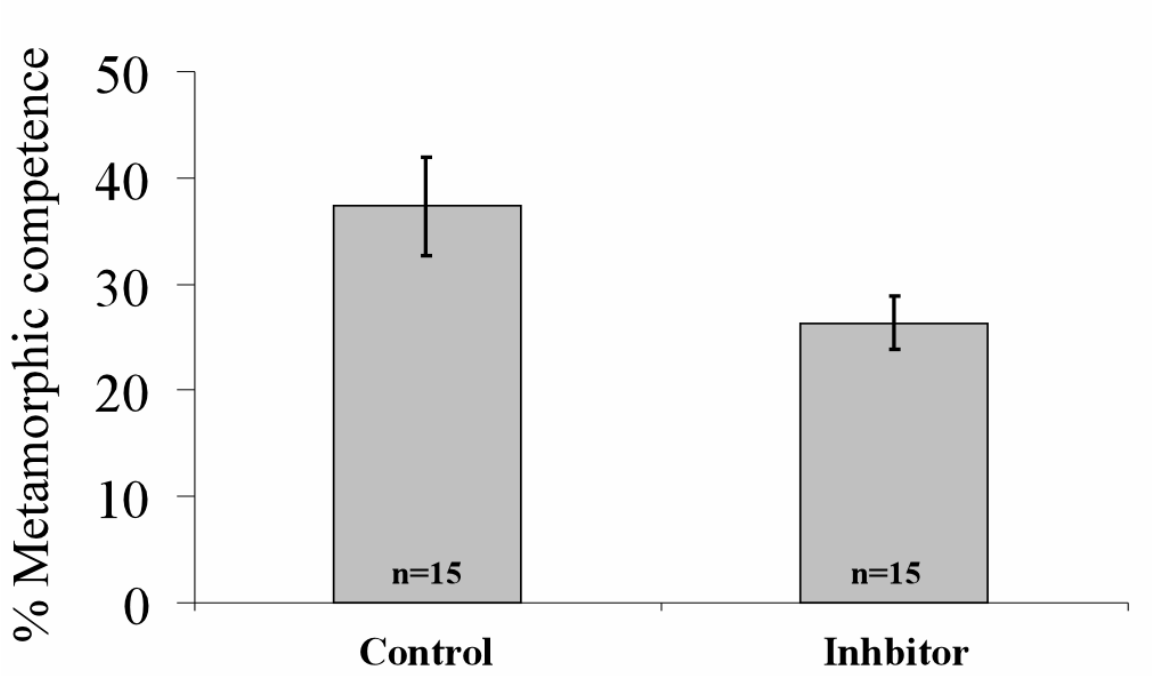


Figure 2-7: Thiourea delays settlement in the absence of food in *Dendraster excentricus* larvae. This suggests that *D. excentricus* larvae can synthesize thyroid hormones endogenously. Larvae were starved after rudiment stage 3 (see Table 1) and exposed to different treatments (Inhibitor Experiment, approx. 19°C). Relative to the starved control, settlement occurred earlier when 10^{-9}M thyroxine (TH; see the text) was present and settlement was delayed when larvae were treated with 10^{-3}M thiourea (INHIBITOR). This effect however was rescued with a mixed treatment: 10^{-9}M thyroxine plus 10^{-3}M thiourea (MIXED; see the text). Error bars represent one S.E. of the mean.

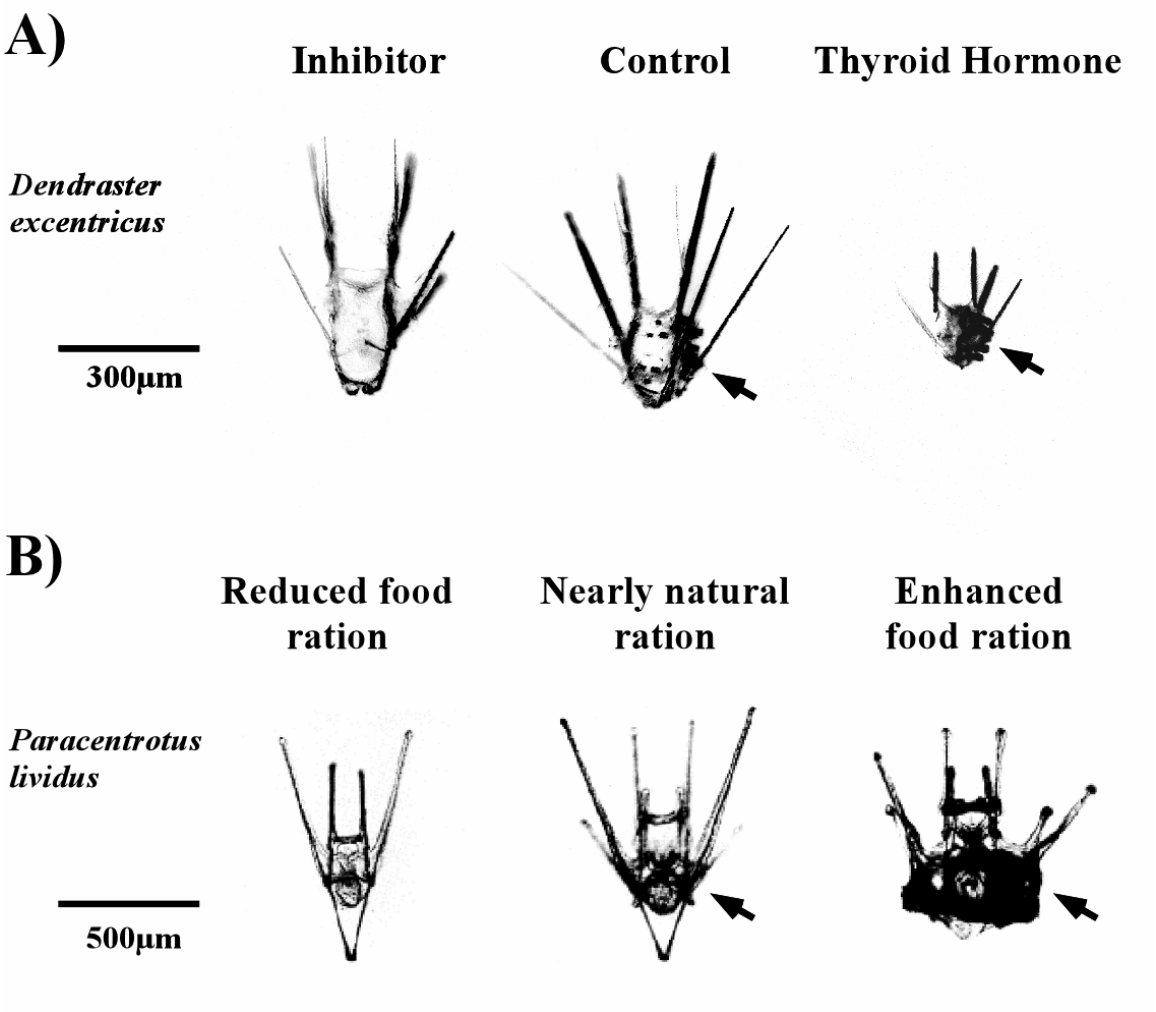


Figure 2-8: Similar morphological effects of thyroid hormone and food on echinoid pluteus larvae. A) Morphological effects of thyroid hormone (10^{-9}M thyroxine; HIGH TH) and a thyroid hormone synthesis inhibitor (10^{-2}M thiourea; HIGH INHIBITOR) on *Dendraster excentricus* larvae (23 days after fertilization at 13°C; Morphometrics Experiment). B) Morphological effects of reduced (left), nearly natural (middle) and enhanced (right) natural plankton on larvae of the sea urchin *Paracentrotus lividus* Lamarck [11 days after fertilization at approx. 20°C; pictures in B reproduced from Strathmann et al.(1992, Evolution 46(4) pp. 976-977)]. Arrows indicate the accelerated juvenile rudiments in the thyroid hormone-treated (A, right panel) and enhanced food (B, Right panel) larvae when compared to the control (A, middle panel) and nearly natural food (B, middle panel) larvae, respectively. Juvenile rudiments are not visible at this stage in inhibitor-treated (A, left panel) and reduced food (B, left panel) larvae.

CHAPTER 3
THYROID HORMONES DETERMINE DEVELOPMENTAL MODE IN ECHINODERMS

Introduction

Two extreme developmental modes are found among marine invertebrate taxa. At one end of the spectrum is larval planktotrophy, where females release many, small, energy poor eggs that require external nutrition to grow and develop to the benthic settlement stage. At the other extreme is lecithotrophy, where females produce few, large, energy rich eggs that require no additional nutrition from the environment to reach the settlement stage (McEdward and Janies 1997; McEdward and Miner 2001; Strathmann 1985; Thorson 1950).

Planktotrophy and lecithotrophy are often viewed as distinct optima in the adaptive landscape of maternal investment strategies (Christiansen and Fenchel 1979; Mortensen 1921; Roughgarden 1989; Sewell and Young 1997; Vance 1973a; Vance 1973b) recently reviewed in (Havenhand 1995; McEdward and Miner 2001). However, several recent studies have suggested that reproductive success can also be optimized at intermediate levels of maternal investment (Levitan 2000b; McEdward 1997). For example, McEdward (1997) presented a fecundity-time model that used the concept of facultative feeding to account for a continuum of reproductive strategies between planktotrophy and lecithotrophy. McEdward proposed to sub-divide the planktonic stage into a non-feeding, a facultative feeding and an obligate feeding period (Fig 3-1). Two key predictions of the McEdward model have found recent support from work on subtropical sand dollars and

sea biscuits [Echinodermata: Echinoidea: Clypeasteroida; (Eckert 1995; Herrera et al. 1996)]: 1) the length of the facultative feeding period is positively correlated with egg size, and 2) the length of the obligate feeding period is negatively correlated with egg size. For example, larvae from the sand dollar *Mellita tenuis* (family Mellitidae) develop from relatively small eggs (100-125µm), and have a short facultative feeding period and a long obligate feeding period. Larvae of the sand dollar *Leodia sexiesperforata* (family Mellitidae) develop from larger eggs (160-210µm), and reach relatively late developmental stages without feeding (i.e., they have a long facultative period). Still, both *L. sexiesperforata* and *M. tenuis* larvae are considered obligate planktotrophs. By contrast, larvae from the sea biscuit *Clypeaster rosaceous* (family Clypeasteridae; egg size 260-280µm) are functionally lecithotrophic in that larvae complete metamorphosis when starved. Since these larvae (unlike obligatorily lecithotrophic larvae) have the ability to feed, they are called facultative planktotrophic larvae (Emlet 1986; Miner et al. 2002, see Fig. 3-1). (Note that the egg size and juvenile size ranges mentioned here are data we collected over the past years from females we spawned in the laboratory. This variability in egg size however has also been previously mentioned (see Hadfield and Strathmann 1996; Miner et al. 2002). From an evolutionary point of view, facultative feeding has been hypothesized as an intermediate life history strategy between planktotrophy and lecithotrophy (Emlet 1986; Hart 1996). Still, the paucity of confirmed facultative-feeding echinoids (only *Clypeaster rosaceus* and the heart urchin *Brisaster latifrons* have been investigated to date*)* has made it difficult to rigorously test this hypothesis in a phylogenetic context (c.f. Hart et al. 1997).

Most authors agree that lecithotrophic development has evolved multiple times independently from planktotrophy within the echinoids and other marine invertebrate taxa (Hart et al. 1997; McEdward and Janies 1997; Strathmann 1985; Wray 1995);(but see Lacalli 1993; McHugh and Rouse 1998). Although many scenarios have been proposed to account for this evolutionary transition (e.g., George 1999; Jaeckle 1995; Levitan 1996; Strathmann et al. 1992; Turner and Lawrence 1979; Villinski et al. 2002; Wray et al. 1996) no laboratory or field manipulations (including egg size manipulations) have succeeded in transforming an obligate planktotroph into a functional lecithotroph. Here we test whether treatment of an obligatorily planktotrophic larva with the vertebrate thyroid hormone thyroxine (T4) is sufficient to induce this transformation.

Thyroid hormones such as thyroxine (T4) and 3,3',5-L-triiodothyronine (T3) are abundant in the planktonic algae that feeding larvae consume (Chino et al. 1994, see Materials and Methods) and empirical studies show that these hormones have profound effects on echinoderm life history traits, including developmental rate (Heyland and Hodin 2004; Hodin et al. 2001; Saito et al. 1998; Suyemitsu et al. 1997) and size at settlement (Heyland and Hodin 2004). Recent data also suggests that some echinoid larvae can synthesize thyroid hormones endogenously (Heyland and Hodin 2004; Saito et al. 1998; Suyemitsu et al. 1997). Such results indicate that obtaining TH from the environment is connected to the ability of feeding larvae to undergo the transition from larva to juvenile. But is the addition of TH sufficient for a large-egg planktotroph to complete metamorphosis and settle in the total absence of food?

Here we show that larvae of *Leodia sexiesperforata* can complete metamorphosis in the absence of any food if they are exposed to the thyroid hormone thyroxine (T4),

while the larvae of *Mellita tenuis* cannot. In other words, TH treatment is sufficient to turn an obligatorily planktotrophic species with sufficiently large eggs into a functionally lecithotrophic one. We will discuss these data in the context of life history theory, juvenile size and the evolution of alternative life cycles in echinoids specifically, and marine invertebrates in general.

Materials and Methods

We use the term thyroid hormones based on the evidence provided by Chino et al. (1994) and Saito et al. (1998) and our own measurements of both T4 and T3 in different echinoid species and four species of unicellular algae (see chapter 5; Fig. 5-5), using HPLC purification (high pressure liquid chromatography) and/or RIA (radio-immunoassay).

Animal Collection, Culturing of Larvae and Experimental Treatments

We collected adult *Mellita tenuis* (Clark, 1940) by SCUBA west of Cedar Key, Florida in May 2001 and adult *Leodia sexiesperforata* (Leske, 1778) by snorkeling off Long Key, Florida in June 2001. We maintained animals in aquaria with re-circulating seawater at the University of Florida (Gainesville) until we set up fertilizations (*M. tenuis* on May 17; *L. sexiespeforata* on June 5; one male and one female from each species), using previously-described procedures (see Strathmann 1987).

After fertilization (fertilization success >98%), we distributed embryos into three glass jars filled with 2 liters 0.2µm Milipore^{TM} –filtered sea water (MPFSW). Hatching occurred by 12 hours post-fertilization for both species, after which we transferred larvae to 1 liter MPFSW at 10 larvae/ml. We then set up four replicate cultures per experimental treatment for each species as follows: FOOD treatment (6000 cells/ml of the unicellular green alga *Dunaliella tertiolecta*; no thyroxine), STARVED treatment (no food; no

thyroxine) and STARVED+T4 treatment [no food; 10^{-9}M thyroxine (Sigma)]. After settlement occurred in the FOOD treatment, we maintained cultures from the other treatments for a maximum of 2 weeks or until more than 50% of larvae had undergone settlement or died (June 7th for *M. tenuis* and June 19th for *L. sexiesperforata*). We changed water (using MPFSW) in the cultures every 48 hours, transferring larvae individually with glass pasteur pipettes. In the STARVED+T4 treatment we added fresh thyroxine (see below) at each water change, and in the FOOD treatment we added fresh algae. Culturing temperatures for larvae and adults were 26±1ºC for both experiments. We monitored abnormalities in larvae, but detected no differences among treatments.

We made up T4 stock solutions using the following protocol (modified from Chino et al. 1994): we diluted 0.0155 g L-Thyroxin (Sigma-Aldrich: T-1775) in 10ml 0.01N NaOH and slowly warmed up to 50ºC under continuous stirring. Once all traces of T4 were dissolved (approx. 10 minutes), we added 190ml of dH_2O and stirred again for 5 minutes. Aliquots of this 10^{-4}M solution were immediately frozen at -20ºC for future use. Before each water-change we thawed one aliquot of T4 stock solution, diluted it 1:100 in MPFSW, and added 1ml of that 10^{-6}M working stock to 1 liter of MPFSW (for a final concentration of 10^{-9}M), mixing well with mechanical agitation.

Biochemistry

We performed biochemical analyses (protein, lipid and carbohydrate) on newly spawned, fertilized eggs and newly metamorphosed juveniles. We prepared replicate samples in micro-grinders (100-1000 µl capacity, Fisher Scientific) removing any seawater with a micropipette. Note that we ran 4 replicates each of 25 eggs per sample for *L. sexiesperforata* and 40 eggs per sample for *M. tenuis*. For *L. sexiesperforata*

juveniles from the FOOD and STARVED+T4 treatments, we used 20 specimens per replicate for protein and lipid analysis, and 30 specimens per replicate for carbohydrate analysis. Each measurement was replicated 4 times for each biochemical species (see table 3-1).

We quantified protein with the Coomassie brilliant blue G-250 binding assay (Bradford 1976) with Bovine Serum Albumin (BSA) as the standard (0.0 – 7.5 µg carbon). We homogenized samples in 700 µl distilled water and transferred 600 µl aliquots from each replicate into 13 x 100 mm glass test tubes. We added 50µl of Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA) to each tube, mixed the contents thoroughly, and took spectrophotometric measurements (595 nm, 1cm path length). Bio-Rad's protein assay is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein. We calculated protein content as the weight of BSA (µg) yielding equivalent color change. To calculate total protein content for the original sample, we corrected for the difference in volume between the 600 µl aliquot and the full volume of distilled water used in the extraction.

We measured lipid content using the acid-dichromate oxidation technique (Parsons et al. 1984) with tripalmitin as the standard (0.0 – 50.0 µg carbon). We homogenized samples in 200 µl chloroform and methanol at a 2:1 (v/v) ratio. Next, we added 50 µl of distilled water to each grinder, and extracted the lipids by additional agitation and grinding with the pestle (Bligh and Dyer 1959). Phases were allowed to separate and we transferred 100µl aliquots of the organic phase from each sample into 13 x 100 mm acid-washed (0.3% acid dichromate) test tubes, and dried them using a dry bath incubator at 65ºC for 2 h. Lipids were oxidized with potassium dichromate (0.30%) in concentrated

sulfuric acid (400µl, 15 min, 105ºC). We diluted samples in 900µl distilled water and took spectrophotometric measurements (440 nm, 1 cm path length). We calculated lipid content as the weight of tripalmitin (µg) yielding equivalent reduction in dichromate oxidation. We calculated total lipid content for the original sample as described above for total protein content.

We assayed for carbohydrate content using the phenol-sulfuric acid method (Dubois et al. 1956) with dextran as the standard (0.0 – 20.0 µg carbon). We homogenized samples in 150 µl of distilled water and transferred 100µl aliquots of each into 1.5ml plastic centrifuge tubes. We then added 100µl of liquefied phenol (Fisher Scientific) to each tube, followed immediately by 500 µl of concentrated sulfuric acid (H_2SO_4). Tubes were capped, mixed, and left at room temperature for 10 min. All samples were heated in a dry bath incubator at 30ºC for 20 min and mixed thoroughly, and then measured spectrophotometrically (490 nm, 1 cm path length). The phenol-sulfuric acid method is a colorimetric test that quantifies production of a yellow-orange product from reducing sugars and polysaccharides. We calculated carbohydrate content as the weight of dextran (µg) yielding equivalent color change. We calculated total carbohydrate content for the original sample as described above for total protein content.

Morphological Analysis

For *L. sexiesperforata*, we sampled 5 larvae from each of 4 replicate cultures per treatment (see Table 3-1) at 25, 73, 126, 143 and 263 hours post-hatching (note that we sampled 10 larvae from one replicate from the STARVED treatment at 263 hours; all larvae from the other 3 replicates had died). We performed morphological analyses on larval characters (postoral arms: PO, postdorsal arms: PD, body midline: BM) the stomach size (SS; see below) and the juvenile character rudiment size (RS; see below)

(Fig. 3-2). We measured these characters in all cases in larvae that we had previously fixed in 4% paraformaldehyde (a maximum of 72 hours before measurement), dehydrated through an EtOH series (50%-100%), and cleared in Clove Oil (Sigma-Aldrich: C8392). We then measured the cleared larvae using a technique previously described and applied for similar purposes by McEdward (1985). We mounted larvae on a microscopic slide and viewed them using a compound Olympus microscope with an attached camera lucida. We identified specific larval landmarks (Fig. 3-2) on a digitizing tablet in order to retrieve the x- and y-information of the landmark. The z-information was retrieved with a rotary encoder attached to the fine focus knob of the microscope (see McEdward 1985). The data (digitized x,y,z information from each individual landmark; see Fig. 3-2) were exported into an Excel^{TM} spread sheet and we calculated the sizes of the morphological characters (see above) using general trigonometric analysis supported by Excel^{TM} macro commands. While PO, PD, and BM are all used as linear measurement, stomach size (SS) and rudiment size (RS) were calculated as the square root of the cross sectional area of an ellipsoid (using SL, SW and RL, RW respectively as the axes of the ellipsoid; see Fig. 3-2). We also staged development using one stage of the juvenile rudiment that is easily recognizable: the occurrence of first juvenile skeletal elements. We measured two morphological characters from freshly metamorphosed larvae: the spine length and the two largest test diameters that were orthogonal to one another using the system described above (microscope and digitizer). We then used these measured diameters to calculate juvenile size as an ellipsoid.

Settlement and Metamorphosis

As previously described (Heyland and Hodin 2004) we distinguish between settlement and metamorphosis. While metamorphosis is a longer-term process encompassing the morphogenetic transition from the bilateral larva to the pentameral juvenile, we define settlement operationally as the time when the larvae attach to the bottom of a glass jar and can resist a suction challenge with a Pasteur pipette. In all experiments we induced settlement by exposing larvae to 40mM excess KCl. If larvae settled within 6 hours we considered them to have settled. *L. sexiesperforata* larvae usually settled after 2 to 4 hours.

Statistics

We used SPSS^{TM} (version 11.0) for all statistical tests. We plotted each morphological character against rudiment size (RS) using scatter plots, and compared the distribution of points for the two treatments (STARVED+T4 and STARVED) using correlation analysis with subsequent Z- transformation for comparison of correlation coefficients. For comparison of juvenile size, spine length in juveniles, numbers of spines and biochemical composition of juveniles, we used 2-tailed independent samples t-test and MANOVA commands in SPSS^{TM} based on estimated marginal means. All p-values are from null hypotheses testing that the mean difference equals 0.

Results

We exposed larvae of *Leodia sexiesperforata* and *Mellita tenuis* to the following three treatments: FOOD, STARVED and STARVED+T4. For *L. sexiesperforata*, we present a complete analysis of egg size, energetic composition of eggs, larval development, settlement and energetic composition of post-metamorphic juveniles for the

STARVED+T4 and the FOOD treatments (Tables 3-1 and 3-2; Fig. 3-3). While we have a complete data-set on *Mellita tenuis* morphology for the FOOD treatment, we only present here the finding relevant in comparison to *L. sexiesperforata*: namely that larvae from *M. tenuis* did not develop to metamorphosis in the absence of food with or without excess thyroxine (STARVED treatment and STARVED+T4 treatment).

Upon induction of settlement with 40mM excess KCl at 170 hours post-hatching, 57.08 ± 6.25% (S.E.) of the *L. sexiesperforata* larvae from the STARVED+T4 treatment and more than 50% from the FOOD treatment settled (Table 3-2). No larvae from the STARVED treatment ever settled upon induction with KCl (Fig. 3-3B). These STARVED larvae died within 2 weeks (336 hours) in culture. In *M. tenuis*, >50% of the larvae from the FOOD treatment ettled upon induction with 40mM excess KCl 288 hours post-hatching. No larvae from the STARVED+T4 or STARVED treatments settled during the 21 days of this experiment (May 17 to June 7) in response to induction with KCl; by the end of the experiment, all larvae had died. In summary, we found that larvae of *L. sexiesperforata* have the capacity to complete metamorphosis in the absence of food only when excess thyroid hormone is present, while starved *M. tenuis* larvae cannot complete metamorphosis, either with or without excess hormone.

Post-metamorphic *L. sexiesperforata* juveniles from the STARVED+T4 treatment were significantly smaller than those from the FOOD treatment ($t_{1,18}$=-19.71; $p<0.001$). Juveniles from the STARVED+T4 treatment had significantly lower lipid (d=1.33µg±0.53; p=0.024), carbohydrate (d=0.28µg ±0.04; $p<0.001$) and protein (d=0.21µg±0.06; p=0.004) contents when compared with the FOOD treatment (d represents difference in energy content ± S.E. of the mean, derived from an analysis of

variance with marginal estimated means; see Materials and Methods) (Table 3-2, Fig. 3-3).

Although *L. sexiesperforata* juveniles derived from the STARVED+T4 treatment were smaller than those from the FOOD treatment, they otherwise appeared normal and functional (e.g., they were capable of moving their tube feet and spines, and could adhere to the walls of their culture bowls). To further assess juvenile development, we measured and counted their spines. We did not find any significant difference between average spine length from the STARVED+T4 treatment (61.45±17.12μm;n=9) compared to the FOOD treatment (76.05±25.80μm;n=9) ($t_{1,16}$=0.47; p=0.64), nor did we detect a significant difference ($t_{1,18}$=0.20; p=0.85) in spine number (14.3±0.73 STARVED+T4 *vs.* 14.5±0.72 FOOD treatment).

We investigated *L. sexiesperforata* larvae for their relative investment into larval structures (larval arm length and bodymidline length) or the stomach versus the juvenile rudiment (for a comparable analysis see Strathmann et al. 1992 and chapter 2). We consider the stomach to be both a larval and a juvenile structure since the larval stomach is partially retained in the juvenile (Chia and Burke 1978). Fig. 3-4 shows that developmental trajectories for PO, PD, BM and SS relative to RS are different between the STARVED and STARVED+T4 treatments. Pearson's correlation coefficients and two-tailed test value for the STARVED treatment were: RS-PO ($r_{1,13}$=0.42; p=0.16); RS-PD ($r_{1,13}$=0.85;p<0.01); RS-BM($r_{1,13}$=0.47;p=0.10); RS-SS($r_{1,13}$=-0.67;p=0.01). Pearson's correlation coefficients and two tailed test value for T4 treatment were RS-PO ($r_{1,12}$=-0.91;p<0.01); RS-PD ($r_{1,12}$=-0.65;p=0.02); RS-BM ($r_{1,12}$=-0.94;p<0.01); RS-SS ($r_{1,12}$=-0.98;p<0.01). We then compared these Pearson's correlation coefficients

from the STARVED+T4 STARVED treatments using Fisher's Z-transformation. Z values are as follows: RS-PO ($Z = 4.23$; $p<0.01$); RS-PD ($Z = 4.44$; $p<0.01$); RS-BM ($Z = 4.83$; $p<0.01$); RS-SS ($Z = 3.08$; $p<0.01$).

The aforementioned results show that development through metamorphosis was significantly accelerated with thyroid hormone treatment in *L. sexiesperforata*. This acceleration was accomplished both by a shift in investment from larval to juvenile structures (see above), as well as precocious development of juvenile structures. At 25 hours after hatching, 100% of the larvae from the STARVED+T4 treatment had begun to build juvenile skeletal structures, while none of the STARVED larvae had done so. Furthermore, only 25% of STARVED larvae had juvenile skeletal structures 126 hours post-hatching, 20% at 143 hours post-hatching and 33% at 263 hours post-hatching.

Discussion

We show that an obligatorily feeding larva has the ability to become metamorphically competent and settle in the absence of food when the thyroid hormone (TH) thyroxine is provided. Measurements of several morphological characters suggest a relative shift in energy allocation from larval to juvenile structures in hormone treated larvae compared to the control. This change in energetic investment is apparently what allows the hormone treated larvae to reach settlement in the absence of food. We will discuss the implications of these findings for: 1) the determination of developmental mode in echinoids; 2) the control of juvenile size in echinoids and 3) the evolution of lecithotrophy.

Egg Size and TH as Determinants of Developmental Mode in Echinoids

By definition, planktotrophic larvae require exogenous nutrition before they can undergo the metamorphic transition and settle to the benthos. Lecithotrophic larvae,

which either do not need to feed (facultative feeding mode) or cannot feed (non-feeding mode), develop from significantly larger eggs than their planktotrophic relatives, and lecithotrophic descendents have been hypothesized to have evolved many times independently from planktotrophic ancestors within the echinoids (sea urchins, sand dollars, and their kin) and other invertebrate groups (Hart et al. 1997; McEdward 1985; Strathmann 1985; Wray 1995); (but see Lacalli 1993; McHugh and Rouse 1998). These observations have led to the hypothesis that there is a taxon specific critical egg size that determines developmental mode in echinoids, and other marine invertebrates as well (Emlet et al. 1987; Mortensen 1921; Sewell and Young 1997); (reviewed recently in Havenhand 1995; McEdward and Miner 2001).

Still, if egg size is the only determinant of developmental mode in echinoids, then we would predict a critical egg size, above which we would find strictly lecithotrophic development [there has been widespread controversy concerning how well egg size represents maternal investment (for discussion see McEdward and Morgan 2001). We will use egg size here as a proxy for maternal energy investment since most readers are more familiar with this terminology]. We know from comparative and experimental data that no such critical threshold exists for echinoids. The larvae of the heart urchin *Brisaster latifrons* have the ability to feed, but can also complete metamorphosis in the absence of exogenous food (thus they are facultative feeders Hart 1996). But *B. latifrons* larvae develop from much larger eggs (345 μm diameter) than the non-feeding (obligatorily lecithotrophic) sand dollar species *Peronella japonica* (276 μm diameter, which is at the lower end of the egg size range for lecithotrophs in echinoids). Moreover, blastomere separation experiments done 50 years ago with *P. japonica* showed that their

non-feeding larvae can complete metamorphosis from half- (110 μm) or quarter sized (88 μm) embryos (Okazaki and Dan 1954); note that some differences in juvenile morphology were observed), far below the hypothesized critical egg size for lecithotrophic development in echinoids. Thus, egg size does not correlate strictly with developmental mode in echinoids.

Our data presented here provide further evidence that egg size is not the only factor determining developmental mode in echinoids, though it is clearly important. The addition of the thyroid hormone thyroxine (T4) can allow an obligatorily planktotrophic larva to complete metamorphosis in the absence of food. The ability to do so, however, is also influenced by egg size. Furthermore, the evidence to date suggests that different echinoid species differ in the ability of their larvae to synthesize thyroid hormones endogenously, with lecithotrophic larvae having a greater capacity for endogenous hormone synthesis than their planktotrophic relatives (Heyland and Hodin 2004; Hodin et al. 2001; Saito et al. 1998; Suyemitsu et al. 1997).

Together, these results provide a plausible explanation for why there is no clear threshold of egg size in echinoids that results in lecithotrophic development. Determination of development mode is most likely a combination of factors such as egg size and the ability to synthesize signaling molecules such as thyroid hormone endogenously. To further test this hypothesis we propose to apply a similar experimental design as we used in this study to a diversity of echinoderm species developing from different egg sizes. Moreover we propose to generate half or quarter-sized embryos from species such as *Leodia sexiesperforata* and the facultative feeding sea biscuit *Clypeaster rosaceus,* and test them for their ability to complete metamorphosis in the absence of

food. If any of these size-reduced embryos are unable to develop to settlement under these conditions (and we can be reasonably certain that reduced sized *L. sexiesperforata* embryos would be unable), we suggest exposing these larvae to thyroxine and then testing for their ability to complete metamorphosis.

Juvenile Size in Echinoids

Emlet et al. (1987), reviewed time to settlement and size at settlement from over 200 echinoid and asteroid species (for a comparable analysis see Levitan 2000). These meta-analyses revealed that: a) juvenile size among echinoid species with feeding larvae is relatively constant (approximately 380 ± 70 μm) over a 3-fold range in egg diameters (70-210 μm) b) time to settlement is highly variable in planktotrophic echinoid larvae; c) planktotrophic and lecithotrophic asteroids have variability in size at settlement, but less variability in time to settlement; and d) lecithotrophic asteroids show a strong positive correlation between egg size and juvenile size, while planktotrophic echinoids and asteroids do not.

Emlet et al. (1987) and Emlet and Hoegh Guldberg (1997) proposed that egg size might play a fundamentally different role in planktotrophic and lecithotrophic development. While planktotrophic larvae can potentially settle earlier when egg size is increased, lecithotrophic larvae, which already develop at the maximal developmental rate (McEdward 1997), could use increased energy from the egg to build a larger juvenile. Our data from *L. sexiesperforata* (as well as our previous experiments with the sand dollar Dendraster excentricus Heyland and Hodin 2004), show that thyroid hormone-treated planktotrophic larvae settle at a much smaller size than has been previously observed in the laboratory or in the field.

Empirical evidence has supported the hypothesis that better quality juveniles frequently have higher fitness by outperforming juveniles of poorer quality. These better quality juveniles, either assessed by higher energy content or by juvenile size, can result in increased juvenile growth rates (Miller and Emlet 1999; Phillips 2002; Roberts and Lapworth 2001), increased longevity (Emlet 1986; Emlet and Hoeghguldberg 1997), increased intra- and inter-specific competitive ability (Connell 1985), or a size refuge from predation (Gosselin 1997; Stoner 1990). Such costs associated with small juvenile size could explain why it would not be advantageous for larvae to metamorphose at the smallest size possible, despite the initial survival advantage associated with a shorter developmental time in the plankton (Lamare and Barker 1999; Rumrill 1990).

We propose the testable hypothesis that juvenile size in planktotrophic echinoids is constrained, and that this constraint can be experimentally relaxed by exposing larvae to thyroid hormones. Experimentally induced small juveniles could be exposed to different competition and predation regimes in order to measure their mortality in the natural environment when compared with control, full-sib juveniles. Such an experiment is presumably not possible by simply manipulating food level or food type, since such treatments provide only subtle variation in juvenile size (Hart 1996; Hodin et al. 2001; Sinervo and McEdward 1988).

Our data suggests that thyroid hormone from an exogenous source (phytoplankton in the field; addition of TH to the water in our experiments) signals to a feeding echinoid larva when it has reached the appropriate stage to undergo settlement (Heyland and Hodin 2004). This particular environmental signal could be more reliable than alternative signals such as the number of ingested particles because different food types (different

algae species) can vary significantly in caloric content (Hinegardner 1969; Jonasdottir 1994; Mcedward and Herrera 1999; Strathmann 1987; Strathmann 1971). We can also conclude that the caloric content per se is unlikely to be the signal for the attainment of competence to settle, since our TH-treated *L. sexiesperforata* larvae completed metamorphosis despite being deprived of an external energy source. Moreover, if caloric content alone were directly related to competence, then half and quarter embryos from *P. japonica* would not be able to complete metamorphosis. Nevertheless, it is unclear to us why larvae would not use caloric content as a cue. One possibility is that there is a strong correlation between caloric and thyroid hormone contents in planktonic algae, something that has not been investigated to date.

TH and the Evolution of Lecithotrophy

Hormones play critical roles in the development of insects (ecdysteroids and juvenile hormone) and amphibians (thyroid hormones) where they regulate complex morphological transitions such as larval and pupal molts and metamorphosis to the adult stage (reviewed in Nijhout 1994; Tata 1998). Furthermore, these same hormones have been hypothesized to underlie the evolution of alternative life history strategies within these two animal groups (insects: e.g. Hodin et al. 2001; Nijhout 1999; Truman and Riddiford 2002); (amphibians: e.g. Callery and Elinson 2000; Frieden 1981; Galton 1992; Hanken et al. 1997; Jennings and Hanken 1998; Kühn and Jacobs 1989; Rose 1999; Yaoita and Brown 1990). While there is considerable support for the hypothesis that, among echinoids, planktotrophy is the ancestral life history strategy and lecithotrophy is derived (Strathmann 1978; Strathmann 1985; Wray 1996); (but see Lacalli 1993), little information exists on the mechanisms that could have led to such a radical change in mode of life.

In echinoderms, several authors including ourselves have investigated the hormonal regulation of metamorphosis (Chino et al. 1994; Heyland and Hodin 2004; Hodin et al. 2001; Johnson 1998; Johnson and Cartwright 1996; Saito et al. 1998; Suyemitsu et al. 1997) and the possible involvement of these hormones in life history evolution as well (Heyland and Hodin 2004; Hodin et al. 2001). Thyroid hormones (T4 and T3) accelerate larval development through metamorphosis and lead to precocious metamorphosis and settlement in sand dollars (Heyland and Hodin 2004; Hodin et al. 2001; Saito et al. 1998; Suyemitsu et al. 1997) and sea urchins (Chino et al. 1994; Johnson 1998). While lecithotrophic sand dollar larvae seem to synthesize hormones endogenously (Saito et al. 1998; Suyemitsu et al. 1997), planktotrophic sea urchin larvae depend largely on hormones from phytoplankton, which contains significant amounts of T4 and T3 (Chino et al. 1994, see also material and methods). Such findings indicate that thyroid hormones may have played a critical role for the evolution of lecithotrophy in echinoids and potentially for other marine invertebrates as well (Heyland and Hodin 2004; Hodin et al. 2001).

As outlined above, the evolution of lecithotrophy from planktotrophy correlates consistently with an increase in egg size among echinoids (Emlet et al. 1987; Hart et al. 1997; McEdward and Janies 1997; Sewell and Young 1997; Strathmann 1985; Wray 1995). Moreover, echinoid species with facultatively planktotrophic larvae (such as *Clypeaster rosaceus* and *Brisaster latifrons*) have moderately large eggs, and can be viewed as evolutionary intermediates on the way to lecithotrophy. Our data show, however, that a lecithotrophic mode of development can be induced with thyroid hormone but without an increase in egg size.

Based on this information, and the facultative feeding model presented by McEdward (1997), we envision a scenario for how a derived lecithotrophic mode of development might have arisen. The relative length of the facultative feeding period of *C. rosaceus* is extended compared to closely related obligate planktotrophs such as *Mellita tenuis* and *L. sexiesperforata* (Fig. 3-1). Here we consider two proximate mechanisms by which the facultative feeding period could be extended: 1) by increasing egg size and 2) by accelerating development so that later developmental stages can be reached with the available maternal energy (note however that the latter option depends largely on the energetic costs associated with accelerated development and how these costs compare to the costs associated with staying in the plankton for an extended period of time). Based on our scenario, mechanism 1 requires increased maternal investment, while mechanism 2 could be achieved by up-regulation of endogenous thyroid hormone synthesis at a given egg size. Our morphological analyses in this and a previous study (Heyland and Hodin 2004) provide evidence that TH treatment induces an acceleration of development of juvenile structures relative to larval structures. The fact that this acceleration of development allows *L. sexiesperforata* larvae to eliminate the obligate feeding period suggests that up-regulation of endogenous TH synthesis (mechanism 2) may be the proximate mechanism underlying the evolution of lecithotrophy in planktotrophic species with sufficiently large eggs.

Still, in order to begin to tease apart the relative importance of egg size and endogenous TH synthesis on the relative lengths of the facultative and obligate feeding periods, as well as for the evolution of lecithotrophy, we propose the following. First, a broader range of echinoids (as well as other echinoderms) should be investigated for the

correlation between endogenous hormone synthesis, egg size, and the relative lengths of these feeding periods. Second, more large-egg planktrophs (from diverse taxa) should be exposed to TH treatments such as those reported here. Finally, we propose feeding larvae with algae pre-treated with inhibitors of TH synthesis, so that they will either contain very little or no hormone. We would predict, for example, that if larvae of *L. sexiesperforata* are fed with such algae, then they would be inhibited in their ability to reach the juvenile stage.

It has been known for a considerable time that feeding larvae with different algal species can result in different times to settlement in a variety of echinoids (Hinegardner 1969; McEdward and Carson 1987; Mcedward and Herrera 1999; Strathmann 1971) and, indeed, that some algae are unable to support growth through metamorphosis at all. Testing our hypothesis that these algae might differ in caloric content (as has been typically assumed) as well as in thyroid hormone content will have wide ranging implications, not only for the relationship between hormones and life history evolution, but also for our understanding of what exactly is required to make a juvenile out of a larva. Such studies would also offer what we believe is a more holistic picture of developmental hormones: namely, morphogenetic compounds in an appropriately ecological context.

Table 3-1. Egg size and egg energy are significantly greater in *Leodia sexiesperforata* than in *Mellita tenuis*. Values represent means ± S.E. of the mean. Egg diameters are from 20 individual eggs for each species. Egg energy contents for *M. tenuis* are from 40 eggs per independent replicate (5 replicates); egg energy contents for *L. sexiesperforata* are from 25 eggs per independent replicate (5 replicates). L=lipids; P=proteins; C=carbohydrates. All energy contents are in

Species	Egg Size (diameter in μm)	Egg Energy (μg)		
		L	P	C
Mellita tenuis	98.96 ± 1.9 μm	0.21 ± 0.0037	0.049 ± 0.0061	0.078 ± 0.025
Leodia sexiesperforata	205.03 ± 5.02 μm	0.63 ± 0.02	0.14 ± 0.0096	0.10 ± 0.0053

Table 3-2. Juveniles of *Leodia sexiesperforata* from the STARVED+T4 (10^{-9}M thyroxine; no food) treatment complete metamorphosis earlier at a smaller size and have significantly less energy (L=lipids; P=proteins; C=carbohydrates) than juveniles from the FOOD treatment (no excess thyroxine; 6000 cells/ml *Dunaliella tertiolecta*). Larvae from the STARVED treatment (no thyroxine; no food) did not reach the juvenile stage during the course of the experiment (see also Materials and Methods). Values indicate means ± one S. E. We used 20 juveniles per independent replicate for the protein and lipid tests, and 30 juveniles per independent replicate for the carbohydrate tests. For juvenile size, n= numbers of individual larvae measured; for energy, n=numbers of independent replicates (see Material and Methods).

Treatment	Age at settlement (hours after fertilization)	Juvenile size at settlement (mm^2)	Energy (µg)		
			L	P	C
STARVED+T4	142	3.15E-4 ± 6.84E-6 (n=10)	0.51 ± 0.14 (n=4)	0.21 ± 1.02E-2 (n=4)	6.90E-02 ± 7.20E-3 (n=4)
FOOD	170	4.83E-4 ± 5.10E-6 (n=10)	1.84 ± 0.59 (n=5)	0.41 ± 6.17E-2 (n=5)	0.35 ± 4.18E-2 (n=5)

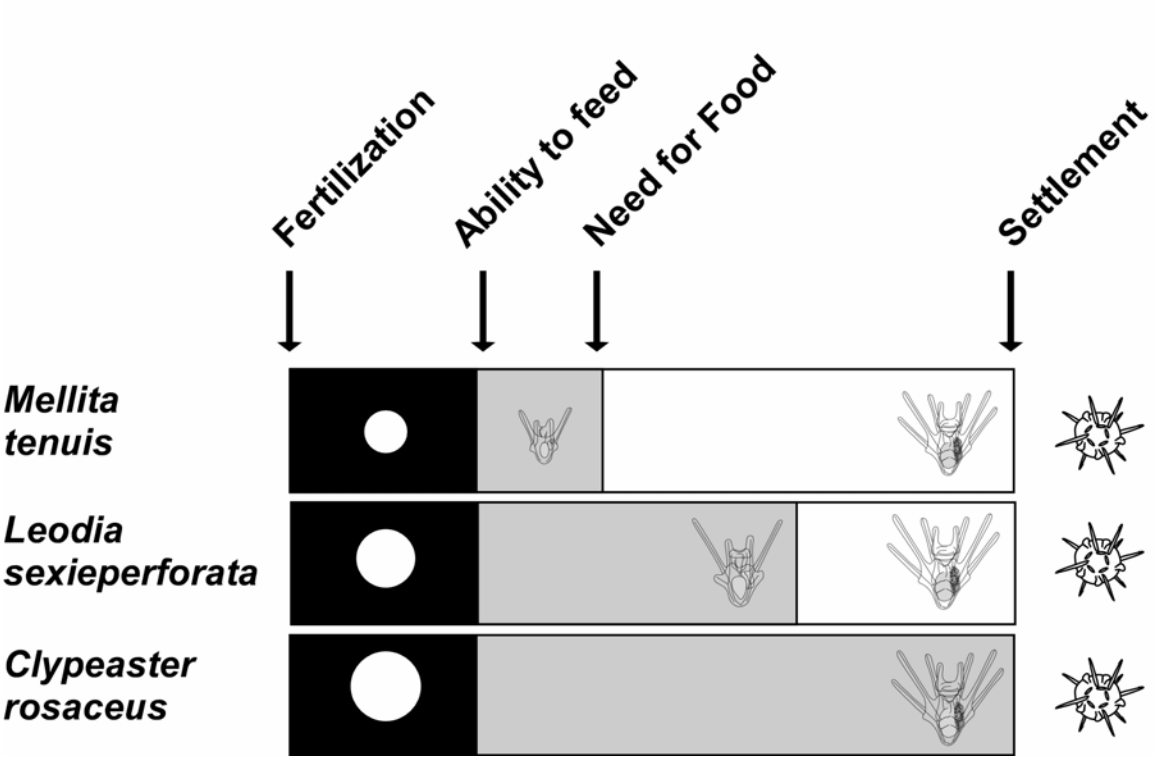


Figure 3-1. The concept of facultative feeding (modified from McEdward 1997) exemplified by three closely related echinoid species developing from different egg sizes. The planktonic period can be subdivided into three parts based on the ability and the need of larvae to feed: a non-feeding period (fertilization through ability to feed); a facultative feeding period (ability to feed through need to feed); and an obligate feeding period (need for food through settlement). *Mellita tenuis* develops from relatively small eggs (100-125µm) and its facultative period is very short. *L. sexiesperforata* develops from much larger eggs (160-210µm) and develops to early rudiment stages in the absence of exogenous food. *C. rosaceus* develops from 260-280µm eggs, yielding a true facultative feeding larva, which can develop to settlement in the absence of food (i.e., their facultative feeding period extends all the way to settlement). Unlike non-feeding larvae, however, *C. rosaceus* larvae have the ability to feed. Note that the lengths of the developmental periods in these three species as shown are relative values, and are not intended to indicate absolute time. Note also that juvenile diameters in the three species are similar (270-280µm; data not shown). Note that the egg size and juvenile size ranges mentioned here are from values that we measured in several different females spawned at different times (data not shown).

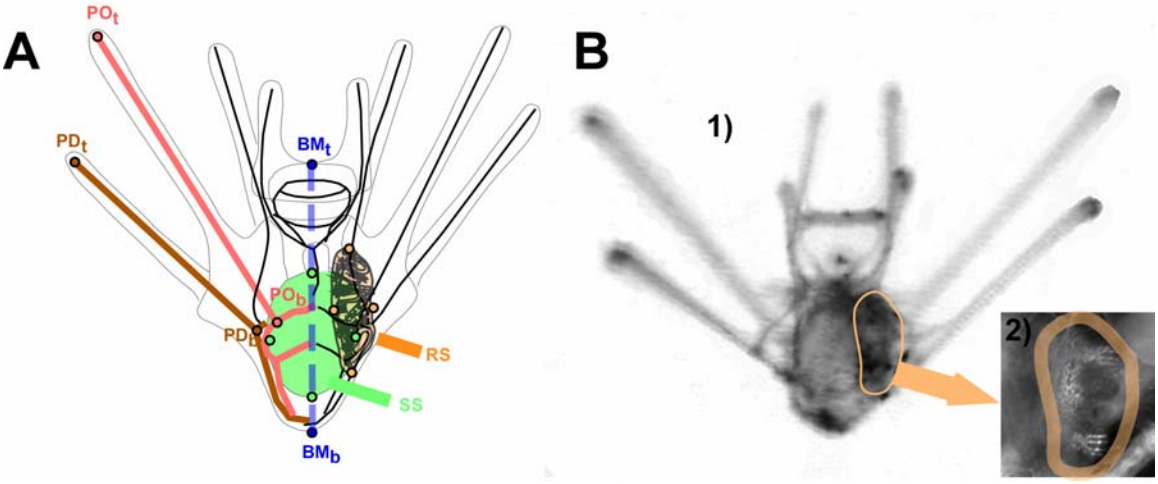


Figure 3-2. Morphological structures in *Leodia sexiesperforata* larvae. A) cartoon of the *L. sexiesperforata* larva shown in B1. 5 morphological characters are emphasized in color: postdorsal arms (PD; brown); postoral arms (PO; red); body midline (BM; blue); stomach size (SS; green); rudiment size (RS; orange). Lower case b stands for base and lower case t stands for tip. We calculated SS and RS as the surface of an ellipsoid in which SL, SW, RL, RW (not indicated) are the diameters of the ellipsoid. SL=longest diameter of stomach; SW= shortest diameter of stomach; RL=longest diameter of rudiment; RW=shortest diameter of rudiment. B2) dark field close-up image of the echinus rudiment (developing adult structures) of the larva shown in B1. The two bright spots indicate adult skeletal elements (skeletal plates and spines in this case).

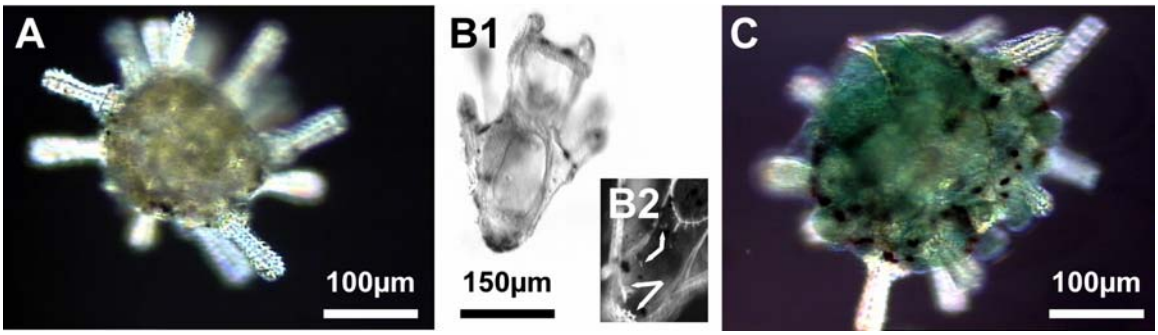


Figure 3-3. Starved larvae of *Leodia sexiesperforata* can complete metamorphosis in the absence of food when treated with thyroxine (A; STARVED+T4 treatment) while larvae reared in the absence of food and thyroxine cannot (B1; STARVED treatment; B2 is a dark-field close-up image from the larva in B1). Larvae fed with a normal food ration of 6000 cells/ml *Dunaliella tertiolecta* in the absence of excess hormone (FOOD treatment) settled at a much larger size (C) than larvae from the STARVED+T4 treatment (A). The STARVED larva in image B1 developed early juvenile skeletal structures (arrows in B2). Note that the larval structures in STARVED are partially resorbed (compare the larva in B1 to the healthy-looking larva depicted in Figure 3-2).

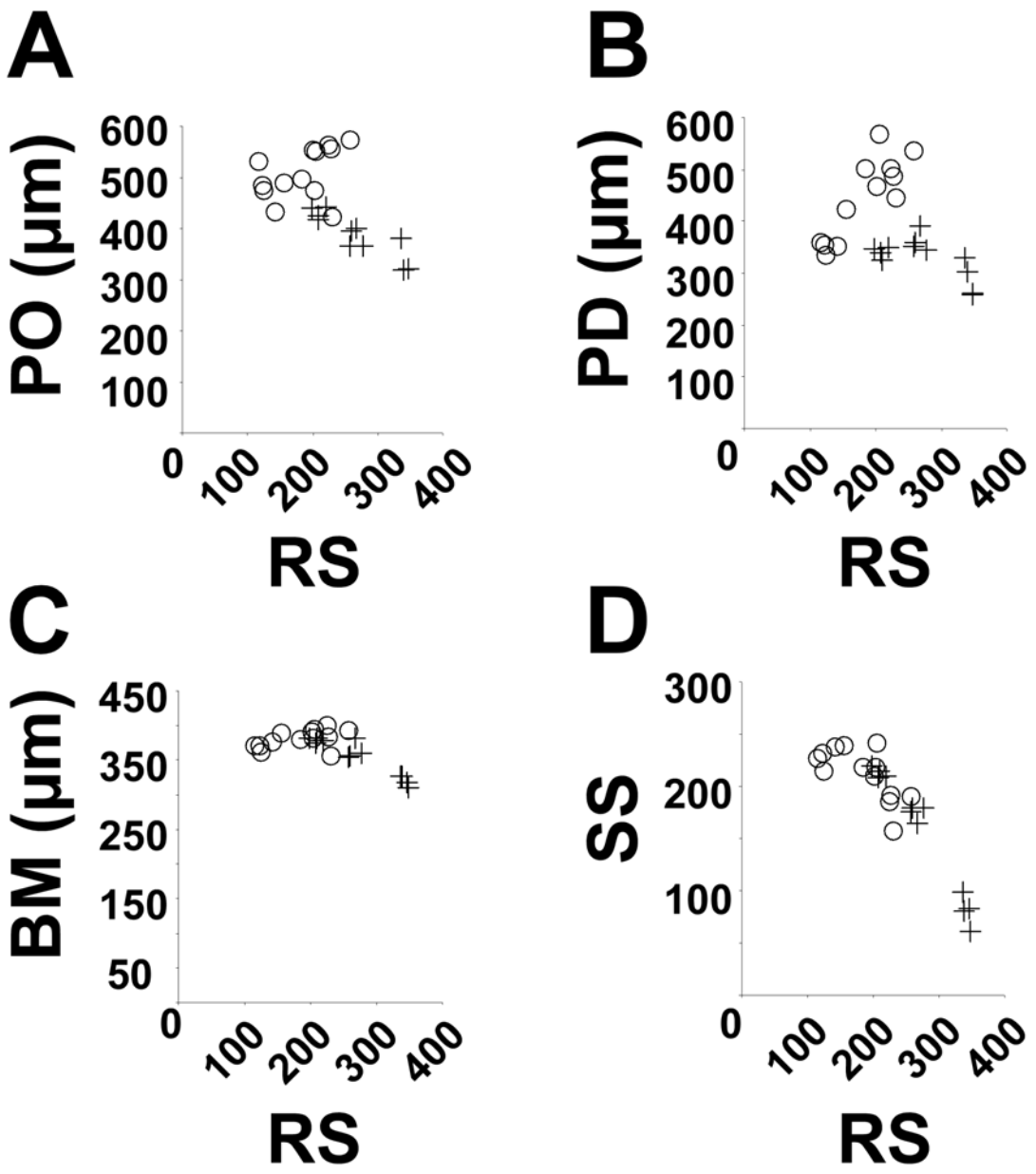


Figure 3-4. Relative allocation of energy is 'switched' from larval to juvenile structures when larvae are treated with exogenous hormone. (a-d) Correlation of morphological characters (y-axis) against rudiment size (x-axis). For results of correlation analysis see the text. O STARVED; + STARVED+T4.

CHAPTER 4
ENDOGENOUS THYROID HORMONE SYNTHESIS IN NON-FEEDING LARVAE OF THE SAND DOLLAR CLYPEASTER ROSACEUS

Introduction

Development in diverse organisms such as multicellular algae, fungi, cnidarians, echinoderms, insects, amphibians is often characterized by a dramatic transitional period between distinct life history stages involving changes in morphology, physiology and habitat. One such a transition in animals (Metazoa) is known as metamorphosis: a radical change in mode of life occurring between the larval and the juvenile or adult stage that has evolved many times independently (Hadfield 2000; Wray 1995). Our knowledge about the signaling mechanisms involved in the coordination of such complex life histories originate primarily from a few selected phyla, namely insects and amphibians, which use steroid and thyroid hormones respectively to coordinate postembryonic development and metamorphosis (Denver et al. 2002; Nijhout 1994).

Ten years ago, Chino et al. (1994) reported the presence of thyroid hormones in sea urchin larvae and their involvement in development and metamorphosis. Thus, some light was shed on an additional phylum that uses hormonal signaling to coordinate development and life history transitions. Several studies have since reported the presence of THs or their effects on growth and development in a variety of echinoderm species. The thyroid hormone thyroxine (T4) accelerates development to metamorphosis in sea urchins (Chino et al. 1994; Johnson 1998), sand dollars (Chino et al. 1994; Heyland and Hodin 2004; Hodin et al. 2001; Saito et al. 1998; Suyemitsu et al. 1997) and starfish

(Johnson and Cartwright 1996) and is, as was shown in sea urchins by Chino et al.(1994) and Hodin et al. (in prep.) many times more potent then T3 in these effects.

Non-feeding larvae have evolved many times independently from feeding larvae in several marine invertebrate groups, and specifically within the echinoids (sand dollars, sea urchins and sea biscuits) (Strathmann 1985; Wray 1995). While selection for egg size (Hart 1995; Levitan 1996; Levitan 2002; McEdward 1997; Sinervo and McEdward 1988; Strathmann 1977; Vance 1973a; Vance 1973b), length of the larval period (Hart 1995; McEdward 1997; Sinervo and McEdward 1988; Strathmann 1977; Vance 1973a; Vance 1973b) and juvenile size (Heyland and Hodin 2004; Strathmann 1977) clearly represent important ecological factors driving this evolutionary alteration in life history (Life history evolution: LHE), the mechanistic underpinnings of the life history transition itself has remained essentially unknown. Recent work has implicated thyroid hormone (TH) like signaling during post-embryonic echinoid development as such a mechanism (Heyland and Hodin 2004; Hodin et al. 2001). By inducing a phenotypically plastic response of larval and juvenile structures in feeding larvae, Thyroid hormone (TH) treatment simulates heterochronic developmental shifts that occurred when non-feeding development evolved from feeding development (Heyland and Hodin 2004). The plasticity signal (TH) seems to originate from algae for feeding larvae . Non-feeding larvae would thus have lost this hormone source when they lost the ability to feed. If TH signaling is still involved in non-feeding development, then larvae must either have evolved the ability to synthesize hormones endogenously or receive it maternally. Saito et al. (1998) presented evidence for endogenous TH synthesis (rather than maternal loading), in the non-feeding larvae of the sand dollar *Peronella japonica*. Still, the

hormone synthesis mechanisms themselves, for *P. japonica* or any other echinoderm, remain to be clarified.

Here we investigate mechanisms of TH synthesis and its involvement in metamorphosis in the sea biscuit *Clypeaster rosaceus*. This species has a unique life history in that it produces facultative feeding larvae: they have the ability to feed but do not need to feed in order to reach metamorphosis (Emlet et al. 1987). If TH is necessary for development to metamorphosis for these larvae, we predict that they must have evolved the ability to synthesize enough hormone endogenously in order to reach metamorphic competence (i.e., they have become independent from the exogenous hormone source). Alternatively larvae may also rely on maternally derived hormone or may not require the hormone at all. We present however evidence that *C. rosaceous* larvae do synthesize thyroxine (T4; a thyroid hormone) from incorporated iodine. Then we provide evidence for the presence of thyroid peroxidase (TPO), a critical enzyme involved in thyroid hormone synthesis in vertebrates. Our pharmacological studies show that metamorphosis is inhibited by thiourea, a thyroid hormone synthesis inhibitor, but can be rescued by adding exogenous thyroxine. This latter result strongly indicates that endogenous hormone synthesis is required for the attainment of metamorphic competence in this facultatively feeding species. Finally our developmental analysis reveals that thiourea also induces a phenotypically plastic response of arm and rudiment growth, further emphasizing the role of TH as a signal for phenotypic plasticity. We discuss these new data in an evolutionary context of the role of thyroid hormones in echinoderm and deuterostome development.

Materials and Methods

Larval Culturing

Adult *Clypeaster rosaceus* were collected at a depth of 3–7m during September 2000, October 2001 and September 2003 at Long Key Channel, Florida and used for the following experiments: morphometrics experiments (October 23rd – November 3rd 2000); metamorphosis experiment (December 4th – December 13th 2001); iodine experiment (October 2nd – 13th 2003). Adults were maintained in aquaria with recirculating seawater (18–20 °C). On October 23rd 2000, December 4th 2001 and October 2nd 2003 we spawned one female and one male by shaking the adult animal until gametes were released. Eggs were fertilized with a dilute suspension of sperm (1:10,000) in a 2000ml glass beaker. Hatching occurred within 12 hours. Then larvae were diluted to 1 larva / 5ml MFSW (millipore-filtered seawater; 0.2µm) and cultured in 1 liter glass jars (filled with 800ml MFSW) for the morphometrics experiment and metamorphosis experiment, and in gallon jars (filled with 3800ml MFSW) for the iodine experiment. No food was provided in any of the experiments. Water was changed on days 1, 3, 5, 7 for both the morphometrics and metamorphosis experiment and every 3 days for the iodine experiment.

Experimental Designs

Morphometrics and metamorphosis experiment: Experimental treatments were set up after hatching and each treatment was repeated four times. Experimental treatments were as follows: morphometrics experiment [THYROXINE (10^{-9}M thyroxine), HIGH INHIBITOR (10^{-3}M thiourea), LOW INHIBITOR (10^{-5}M thiourea), RESCUE (10^{-9}M thyroxine + 10^{-3}M thiourea), CONTROL (MFSW)]; metamorphosis experiment [THYROXINE (10^{-9}M thyroxine), RESCUE (10^{-9}M thyroxine + 10^{-3}M thiourea),

THIOUREA 10^{-3} (10^{-3}M thiourea), THIOUREA 10^{-4} (10^{-4}M thiourea), THIOUREA 10^{-5} (10^{-5}M thiourea), THIOUREA 10^{-6} (10^{-6}M thiourea), THIOUREA 10^{-7} (10^{-7}M thiourea), CONTROL (MFSW)]. Larvae were exposed to the chemical treatments immediately after hatching and until the end of the experiment. In the morphometrics experiment 10 larvae per replicate were removed from cultures for morphometrics analysis at 36, 72, 100 and 140 hours. For the metamorphosis and the morphometrics experiment larvae were counted and checked for developmental abnormalities such as asymmetries and dwarf phenotypes at each water change. The latter phenotype is characterized by an extremely small body size, usually three to four times smaller than other larvae. We prepared thyroxine (Sigma-Aldrich: T-1775) as described in (Chino et al. 1994), and thiourea [Sigma-Aldrich T7875; a thyroxine synthesis inhibitor which acts by blocking thyroid peroxidase (TPO) activity] in MFSW at appropriate concentrations.

Iodine experiment: In the iodine experiment we exposed larvae (October 9th, day 7) for 18 hours in 12 well plates to the following experimental treatment [THIOUREA 10^{-3} (10^{-3}M thiourea), THIOUREA 10^{-5} (10^{-5}M thiourea), THIOUREA 10^{-7} (10^{-7}M thiourea)]. We placed 30 randomly chosen larvae into each well, containing 4ml of solution. All solutions were made up in SW^{125} (MPFSW with I^{125} at 51937 dpm; Carrier free specific activity of I^{125} was 642.8GBq/mg).

Morphometric Analysis

We sampled 5 larvae from each of 4 replicate cultures per treatment and performed morphological analyses on larval characters (postoral arms: PO, postdorsal arms: PD, body midline: BM) the stomach size (SS; see below) and the juvenile character rudiment size (RS; see below). We measured these characters in all cases in larvae that we had

previously fixed in 4% paraformaldehyde (a maximum of 72 hours before measurement), dehydrated through an EtOH series (50%-100%), and cleared in Clove Oil (Sigma-Aldrich: C8392). We then measured the cleared larvae using a technique previously described and applied for similar purposes by McEdward (1984). We mounted larvae on a microscopic slide and viewed them using a compound Olympus microscope with an attached camera lucida. We identified specific larval landmarks on a digitizing tablet in order to retrieve the x- and y-information of the landmark. Landmarks used here are the same as used in chapter 3 and illustrated therein. The z-information was retrieved with a rotary encoder attached to the fine focus knob of the microscope (see McEdward 1984). The data (digitized x, y, z information from each individual landmark) were exported into an Excel ^{TM} spread sheet and we calculated the sizes of the morphological characters (see above) using general trigonometric analysis supported by Excel ^{TM} macro commands. While PO, PD, and BM are all used as linear measurement, stomach size (SS) and rudiment size (RS) were calculated as the square root of the cross sectional area of an ellipsoid (using SL, SW and RL, RW respectively as the axes of the ellipsoid). We staged development using a modification of the previously discussed scheme used for *Dendraster excentricus* (chapter 2). We considered larvae to be in stage 1 when spikes were present on the body rods, at least rudiments of post-dorsal arms were present and the hydrocoel started to flatten. Stage 2 is characterized by the formation of the dorsal arch. In stage 3 first adult skeletal elements are present such as spicules, skeletal plates and fused pentaradial skeletal elements. At stage 4, juvenile arm spines were visible in the juvenile rudiment. Juvenile size was measured as the average of two maximal test diameters (not including the spines).

Metamorphic Competence

We tested for metamorphic competence over the duration of 4 days, on December 11th, 13th and 14th 2001. We randomly chose 90 pre-metamorphic larvae from each treatment and distributed them randomly into 3 replicates in 15cm petri-dishes at 30 larvae per dish. After 6 hours we scored the number of post-metamorphs (pre-metamorphic larvae that had undergone the metamorphic transition defined as the moment when tube feet stick out of the larva and it attaches firmly to the bottom of the culture dish). Based on our definition metamorphic competence was only reached when more than 30% of larvae per replicate underwent the metamorphic transition.

Iodine Incorporation and TLC (Thin Layer Chromatograms)

After the exposure, larvae were transferred from each well into a separate tube and washed 5 times with fresh MFSW until the radioactivity in the supernatant was below 30dpm. Between each wash larvae were centrifuges at 1980g for 3 minutes and kept on ice. In order to test whether I^{125} that the larvae had incorporated was built into T4, we prepared samples for thin layer chromatography (TLC). We added 1ml of ice cold MeOH to each sample after it was counted (see below) and let it stand at 4ºC over night. After vortexing all samples at full strength for 2 minutes we centrifuged them at 1980g for 10 minutes and collected the supernatant. Then we dried them down in a speed-vac to complete dryness and spiked samples with 100ul non-radioactive 10^{-4}M T4 (Sigma-Aldrich T-1774). Note that we prepared an additional negative control at this point that contained 100ul of the original SW^{125} plus 1ml of ice cold MeOH that was processed in the same way as all the samples. The dry pellet was resolved in 30ul 0.01N NaOH. Note that usually not all the salt crystals resolved. All 30ul (excluding the crystals) were

loaded on a TLC plate (Whatman LK5D silica gel 150A with fluorescence marker; Whatman #4851-840) and run for 1.5 hours in a 2-methylbutanol/*t*-butyl alcoholy/25%NH 3/acetone, 7:14:14:56, vol/vol solvent. We visualized the cold T4 marker under UV light on a BioRad ^{TM} Flour-S MultiImager system and radioactive bands on a Molecular Dynamics ^{TM} Phosphorimager SI. Overlaying the UV image with the on from the phosphor imager allowed us to compare the radioactive bands to our T4 standard. Samples containing I^{125} were counted on a ssMPD instrument (BioTraces, Inc., Herndon, VA) in standard mode. In standard mode, digital signal processing is used to distinguish the I^{125} decay specific characteristics from those of background events to give a background equivalent to 5 DPM of I^{125} with about 45% efficiency.

Immunohistochemistry

Clypeaster rosaceus larvae and juveniles at different developmental stages were fixed for 20 minutes at room temperature in 4% paraformaldehyde in PBS and then post-fixed in 100% ice cold MeOH for 10 minutes. Specimens were then washed three times in PBS to remove all the paraformaldehyde and MeOH and either stored at 4°C in PBS or immediately processed. We transferred specimen into PBT (0.3%), washed them three times (10 minutes each wash) and incubated them then for 30 minutes in blocking solution (5% normal goat serum). After this incubation specimens were washed several times in PBT and then incubated with the primary antibody (anti-T4 serum raised in mouse 1:200 (SIGMA) in PBT (0.3%) and 5% normal goat serum) over night at 4°C or 2 hours at room temperature (20°C). Then specimens were removed from primary antibody, washed three times in PBT and incubated with the secondary antibody (anti mouse Alexaflour ^{TM} 488 from Molecular Probes ^{TM} at 1:200) over night at 4°C or at

room temperature for 2 hours. Finally specimens were washed in PBS and mounted in Vectorshield ^{TM} to view with a confocal microscope.

Biochemical Analysis

We assayed total energy (joules per individual) in eggs and juveniles using the acid dichromate oxidation with the micro-modification by McEdward and Carson (1987) of the procedure described by Parsons(1984). Briefly, we placed individuals in 13 mm test tubes (Fisher Scientific^{TM}) and removed excess seawater using a micropipette. All samples were rinsed in distilled water then pipetted dry. We next added 100µl of concentrated phosphoric acid, vortexed, and dried samples for fifteen minutes at 105°C. After samples cooled to room temperature, we added 200µl 0.3% acid dichromate, vortexed thoroughly, and heated again for fifteen minutes at 105°C. We then added 350µl of distilled water, vortexed the individual tubes, and allowed them to cool to room temperature. Finally, we measured sample absorbance at 440 nm and calculated energy content based on a glucose standard (0 to 2 joules). For our analysis, we had 4 replicates for each experimental treatment using 30 specimen (eggs or juveniles) per replicate.

Statistical Analysis

All comparisons between treatments were done using MANOVA or students t-test commands in SPSS ^{TM}. Results are presented in the text and the tables in the following format: mean difference (treatment value minus control value) ± one S.E.; p-value. If the mean difference is positive this means that the value in the experimental treatment was larger than the value in the control. All p-values are from null hypotheses testing that the mean difference mentioned above equals 0.

Results

Thiourea Inhibits and Thyroxine Accelerates Attainment of Metamorphic Competence

We tested for metamorphic competence of *Clypeaster rosaceus* pre-metamorphic larvae on three days. On December 11th (7 days after fertilization) more than 30% from the THYROXINE and the RESCUE treatment had reached metamorphic competence. Larvae from the thiourea treatments and the CONTROL had not reached the 30% metamorphic competence limit at that time (Fig. 4-1A). The ANOVA results using Bonferroni correction for the pairwise comparisons are: THYROXINE-CONTROL (63.17±8.08%; $p<0.001$); RESCUE-CONTROL (76.51±8.08%; $p<0.001$). On December 13th and 14th (9-10 days after fertilization) only larvae from the CONTROL and THIOUREA treatments 10^{-7}M, 10^{-6}M and 10^{-5}M had reached the 30% threshold when we combined the data from those two days, and metamorphic competence was significantly lower in THIOUREA 10^{-3}M and THIOUREA 10^{-4}M compared to the CONTROL (Fig. 4-1B). The ANOVA results using Bonferroni correction for the pairwise comparisons are: THIOUREA 10^{-3}M-CONTROL (-50.96±7.85%; $p<0.001$) THIOUREA 10^{-4}M-CONTROL (-38.47±7.85%; $p<0.001$). We measured juvenile diameters and biochemical composition of juveniles. We did not find any difference in juvenile size and biochemical composition between any of the treatments using MANOVA with Bonferroni correction. Table 4-1 presents the mean values and S.E. of juvenile sizes as a function of treatment and egg size.

In summary these data demonstrate an acceleration of development in the thyroxine treatment compared to the control. Moreover a significantly higher percentage of larvae reached metamorphic competence in the THYROXINE and RESCUE treatments.

Thiourea strongly inhibited metamorphic competence in a dose responsive way. However no effect of this acceleration was seen in terms of juvenile size or juvenile biochemical composition.

Thiourea and Thyroxine Induce Phenotypically Plastic Response of Larval and Juvenile Characters But Neither One Leads to a Shift in Developmental Timing in Clypeaster Rosaceus

In a separate experiment we investigated how thiourea and thyroxine affects the larval morphology during development. In order to estimate relative investment into larval versus juvenile structures, we plotted each morphological character as a function of rudiment size (Fig. 4-2A-D). These graphs show that larvae from the inhibitor treatments (THIOUREA 10^{-3}M and THIOUREA 10^{-5}M) invested relatively more into larval structures and the stomach than into juvenile structures compared to the control. Larvae from the THYROXINE and the RESCUE treatment however invested relatively more energy into juvenile than larval structures. We tested these comparisons statistically by calculating the Pearson's correlation coefficient for each morphological character against the rudiment size per treatment and replicate. These correlation coefficients were then compared using ANOVA with Bonferroni correction. For postoral arm length the THIOUREA 10^{-3}M treatment led to a significant higher investment into larval structures relative to juveniles structures [-0.235±0.06 ($p=0.024$)], while the THYROXINE [1.19±0.09 ($p<0.001$)] and RESCUE [1.041±0.09 ($p<0.001$)] treatments led to a significantly higher investment into juvenile structures relative to larval structures. For body midline length the THYROXINE [1.177±0.08 ($p<0.001$)] and RESCUE [1.216±0.08 ($p<0.001$)] treatments led to a significantly higher investment into juvenile structures relative to larval structures. For stomach size the THYROXINE [1.298

±0.09μm^2 ($p<0.001$)] and RESCUE [1.152±0.10μm^2 ($p<0.001$)] treatments led to a significantly higher investment into juvenile structures relative to larval structures. No differences in investment were found concerning postdorsal arm length.

Additionally to the morphometrics analysis we tested whether experimental treatment induced shifts in developmental timing using heterochrony plots (Fig. 4-3; see also chapter 2 for similar analysis in *Dendraster excentricus*). THYROXINE and RESCUE treatments accelerated developmental timing slightly whereas the THIOUREA 10^{-3}M treatment decelerated development. However none of the direct comparisons turned out to be significant using ANOVA with Bonferroni correction and estimated marginal means due to unequal sample size.

Mortality and Abnormalities in Cultures

No significant differences in survival and developmental abnormalities between the treatments were found until the age of 196 hours post fertilization when the survival rate was significantly lower in the 10^{-3}M THIOUREA (3.12±0.9%; $p=0.04$) treatment and the RESCUE treatment (3.41±0.9%; $p=0.02$) compared to the control. 244 hours after fertilization the survival rate was significantly lower in the 10^{-3}M THIOUREA treatment compared to the CONTROL.

Clypeaster Rosaceus Larvae Incorporate Radioactive Iodine and Build Thyroxine

Here we tested if *Clypeaster rosaceus* larvae (5 days post-fertilization) incorporate radioactive iodine (I^{125}) and use it to synthesize thyroxine. Additionally, we tested if the incorporation and/or synthesis of thyroxine can be inhibited by thiourea. We found that iodine is incorporated in *Clypeaster rosaceus* larvae and that this incorporation can be inhibited by thiourea in a dose responsive way (Fig. 4-3A). We then identified

radioactive thyroxine on the TLC plate, which means that the incorporated radioactive iodine was used to synthesize T4 (Fig. 4-4). Although our band from the cold T4 (identified under UV light) corresponds exactly to the radioactive band seen under the phosphor imager, the running distance Rf of 0.3 for thyroxine is smaller than what is usually expected under these conditions. We assume however that this discrepancy is due to the different running conditions in our setup. In summary we were able to show that thiourea does inhibit thyroid hormone synthesis in *Clypeaster rosaceus* larvae. Furthermore our data seem to suggest that the inhibition of thyroxine synthesis occurs on the level of the iodine uptake.

Presence of Thyroxine and TPO (Thyroid Peroxidase) in Perimetamorphic Stages of Clypeaster Rosaceus

The enzyme critical for thyroid hormone synthesis in vertebrates is thyroid peroxidase (TPO), and this gene is also found in the sea urchin species *Lytechinus variegatus* (see chapter 5; accession number). For *Clypeaster rosaceus* we used a monoclonal antibody directed against human TPO (green in A-I) and a polyclonal antibody directed against thyroxine (red) to analyze the distribution of these molecules in developmental stages of the sea biscuit (Fig. 4-5). We also used a nuclear stain (blue) to visualize cells in larvae. Finally, we used the monoclonal antibody 1E11 (kindly provided by R. Burke; green in J-L). This antibody recognizes a 65kD protein called Synaptotagmin p65 and has been extremely useful to visualize invertebrate nervous systems (Burke pers. com.). Images in Fig. 4-5 are larval (A-F) and juvenile (G-L) stages. 2 days old larvae (A-C) do not show any TPO staining. T4 however appears to be presented in cells of the ciliated band (B). C) shows a close-up of ciliated band cells stained with thyroxine antibody. Pre-metamorphosis (D-F) larvae 8 days) show distinct

staining patterns for TPO at the base of the spines (E) and in the stomach region (F). Thyroxine still appears in the ciliated band although staining is clearly less intense in stages closer to metamorphosis (E compared to F). Post-metamorphosis 10 days; G-I), TPO staining at the base of the juvenile spines is still present but thyroxine staining has almost disappeared. I) represents a close-up of TPO cells at the base of the spines. Images J through L represent staining patterns using the antibody 1E11 on post-metamorphic juveniles (10 days) indicating that the region at the base of the spines consists of nervous tissue. Please note that in chapter 5 we present the full length sequence of the TPO orthologs LvTPO (accession number; cloned from *Lytechinus variegatus* larvae). A related gene was also found in the yet to be finished genome from another sea urchin *Strongylocentrotus purpuratus* (chapter 5). Finally we cloned this gene from the mollusk *Aplysia californica* AcaTPO (accession number). Note that LvTPO shares the majority of all functional TPO domains with human TPO and at least for *Lytechinus variegatus* we have shown co-localization between TPO RNA probe using in situ hybridizations and monoclonal anti human TPO antibody as used in this study (see chapter 5).

Discussion

Among the echinoderms, evidence for TH related function originates from at least three of the five classes, sea urchins and sand dollars (Echinoidea), brittle stars (Ophiuroidea) and sea stars (Asteroidea), while conclusive evidence has so far only been provided from echinoids (Chino et al. 1994; Heyland and Hodin 2004; Hodin et al. 2001; Johnson 1998; Saito et al. 1998; Suyemitsu 2000). Several authors including ourselves have shown that thyroid hormones accelerate development to metamorphosis (Chino et al. 1994; Heyland and Hodin 2004; Hodin et al. 2001; Johnson 1998) ultimately leading to an earlier attainment of metamorphic competence (Heyland and Hodin 2004, see also

chapter 2, 3 and 5) in sea urchins and sand dollars. In fact in chapter 3 we have shown that thyroxine is necessary and sufficient for metamorphosis in the sand dollar *Leodia sexiesperforata*. Intriguingly, the source of hormone can be either endogenous (Heyland and Hodin 2004; Saito et al. 1998; Suyemitsu 2000) or exogenous from the phytoplankton upon which larvae feed (Chino et al. 1994). While we discuss TH from exogenous sources in detail in chapter 2 and will discuss it in the following chapter 5 further this study provides evidence for endogenous thyroid hormone synthesis in facultatively feeding larvae of the sea biscuit *Clypeaster rosaceus*. Moreover our data suggest that THs act as an endogenous plasticity signal, further indicating that THs may have been involved in the evolution of non-feeding development. These results allow us to draw conclusions in two critical evolutionary contexts: the evolution of thyroid hormone function in deuterostomes and the evolution of non-feeding development within the echinoderms.

Do Echinoids Have an Endostyle Homologue?

While several components of the TH signaling system are present in all chordates, increasing evidence suggests that urochordates (ascidians) and cephalochordates (lancelets) share critical elements of this signaling pathway. The endostyle, present in urochordates, cephalochordates and larval lampreys, is primarily involved in filter feeding (Ogasawara 2000; Ruppert et al. 1999). Based on morphological, developmental and molecular evidence it is also the best candidate for the ancestor of the vertebrate thyroid gland (Ogasawara 2000; Ogasawara et al. 1999) although some alternative scenarios were recently discussed (Mazet 2002). Still, the fact that the endostyle expresses the thyroid peroxidase (TPO) gene and synthesizes THs in urochordates and cephalochordates is consistent with the thyroid gland homology hypothesis.

Thyroxine has been localized and measured in larvae of the ascidian *Ciona intestinalis* (Patricolo et al. 2001) and TH synthesis inhibitors can block post-settlement juvenile morphogenesis in the ascidian *Boltenia villosa* (Davidson et al. 2002a). Fredriksson and colleagues (1993) identified a putative nuclear receptor for T3 in ascidians which has a similar affinity (K_d) to those found in other chordates. However, the maximal binding capacity was much lower (Fredriksson et al. 1993). Although evidence for thyroid related function in cephalochordates is sparse there is evidence for TPO gene (expressed in the endostyle) and several other TH signaling related compounds (reviewed in Mazet 2002). However, what is the evidence for endostyle or thyroid homologues structures in the other deuterostome groups, the echinoderms and the hemichordates?

While adult hemichordates do have several characters in common with chordates such as gill slits and dorsal nerve chord (reviewed in Ruppert et al. 1999) adult echinoderms lack all of these characters. Neither of the two phyla possesses an endostyle and the search for endostyle homologues structures appears to be still open. Ruppert et al. (1999) rejected the previously hypothesized hypobranchial ridge as a possible homologous structure to the chordate endostyle, and suggested the epibranchial ridge instead based on morphological and developmental evidence.

Both, echinoderms and hemichordates develop via planktonic larvae characterized by elaborate ciliated bands and a radical metamorphosis that transforms them into the benthic adult form at the end of their larval life. The ventral ciliated band of hemichordate tornaria and echinoderm auricularia larvae has been previously discussed as a homologue to the chordate endostyle (Morgan 1891). Garstang (1894) and later

Ruppert et al. (1999) further considered the possibility that the endostyle precursor in larvae of ancestral echinoderms and hemichordates might have been simply a ciliated band associated with the pharynx. Based on our immunohistochemical results using an anti-thyroxine antibody, both sea biscuit (*Clypeaster rosaceus*) and sea urchin (*Lytechinus variegates*; see chapter 5) larvae (including late stage larvae) appear to have thyroxine in the cells of their ciliated band. TPO activity however is clearly not localized in the ciliated cells (see Figure 4-5). So if the ciliated band of echinoid larvae is homologous to the chordate endostyle, it appears that, at most, its function as a feeding organ was retained, and that TH synthesis and/or metabolism in echinoids has followed significantly different evolutionary routes.

TH Synthesis Appears in Nervous System Related Structures in Clypeaster Rosaceus

TH function has been confirmed for all vertebrates investigated. These hormones control and coordinate crucial processes such as growth, metabolism, development and homeostasis. Vertebrate THs signal both via nuclear hormone receptors (transcription factors regulating gene function in response to the hormonal signal, reviewed in Yen 2001), and via non-genomic pathways, not involving any de novo protein synthesis (reviewed in Hulbert 2000). The thyroid gland is the main organ responsible for TH synthesis: a reaction involving the fusion of two tyrosines with several iodine atoms (McLachlan and Rapoport 1992). The sodium iodine symporter (NIS) located in the epithelial cells of the thyroid incorporates iodine (I) which is transported into the lumen of the follicular cells along with thyroglobulin (TG), the protein delivering tyrosine for TH synthesis (Dunn and Dunn 2001). Thyroid peroxidase (TPO) then catalyzes two sequential reactions, the iodination of tyrosine residues provided by TG and the synthesis

of T4 (thyroxine) and T3 (3,3',5'triiodo-L-thyronine) from two iodotyrosines (Dunn and Dunn 2001). The primary THs synthesized in the thyroid however is T4 and not T3. THs are then transferred via the blood stream to various tissues where they elicit their physiological functions.

Both iodine uptake and thyroxine synthesis are completely blocked in *C. rosaceous* by the goitrogen thiourea, suggesting a function of TPO in iodine uptake and/or iodination (binding of iodine to proteins and specifically to tyrosine residues). Note that in chapter 5 we report on identical effects of thiourea on iodine uptake and TH synthesis in the sea urchin *Lytechinus variegatus*. In this species (as well as in the feeding sand dollar *Dendraster excentricus*; not shown) we also showed that $KCLO_4$, a competitive inhibitor of vertebrate NIS function, does not have any effects on iodine uptake. These findings not only provide additional support for a role of TPO in iodine transport, but also suggests that the effects that we report for thiourea are not simply generalized toxic effects on larvae , but are specific to certain classes of inhibitors.

While TPO is present in specific cell clusters at the base of the juvenile spines pre- and post settlement, it seems to be largely absent during early development in *Clypeaster rosaceus* (Figure 4-5). In concert with our findings of metamorphic inhibition by thiourea (further discussed below), these TPO expression profiles point to a role of endogenous thyroid hormone synthesis in juvenile development and metamorphosis, rather than in phenotypic plasticity in younger stage larvae (which may be more attributable to exogenous THs; further discussed below).

The TPO cell clusters mentioned above appear to co-localize with a region containing nervous tissue, as we were able to confirm with the pan neuronal antibody

1E11 (kindly provided by R. Burke). TH function in the nervous system or nervous system related structures is well known in all vertebrates. THs are critical for nervous system development in mouse, human and several other vertebrate species (Bernal 2002). However, although TH synthesis and/or TPO activity in the central nervous system had never been reported, iodine uptake and iodination does occur in the choroid plexus (Spitzweg et al. 1999), the production site of the cerebrospinal fluid (CSF). TH seems to be provided to the brain via passage of the blood-brain (CSF) barrier using transthyretin as a possible carrier (Palha et al. 2002; Schreiber et al. 1990). By contrast, our results presented here, and our recent evidence from the mollusk *Aplysia californica* (chapter 2), suggests that TPO activity does occur in invertebrate nervous system and/or nervous system related tissues.

TH Function in Echinoids, a Mechanism Involved in Development to Metamorphosis

The synthesis and function of hormone in juvenile structures elucidates the role of TH in larval development and metamorphosis. The larval body is transformed into a juvenile over the course of the larval period, which can last for several weeks in many echinoderm species. For the transformation from a larva to juvenile, a number of physiological and ontogenetic processes need to be coordinated with the internal and external environment. It has been previously emphasized that many marine invertebrate species rely on external cues and internal neuronal conductance during their fast metamorphic transition and settlement (reviewed in Hadfield 2000), which, in echinoderms, occurs after the juvenile has been essentially fully-formed inside the larva. In this context we consider TH signaling in echinoid larvae as a potent system coopted to read environmental signals, such as for example food abundance (see discussion of

phenotypic plasticity below and in chapter 2), and subsequently respond to them appropriately (e.g., via alterations in developmental trajectories; discussed below and in chapter 2).

The scenario outlined above is not without precedent. Bishop and Brandhorst (2001) previously described nitric oxide (NO) signaling in metamorphosis in the sea urchin *Lytechinus pictus*. Nitric oxide synthase (NOS), catalyzes the conversion L-arginine to L-citrulline, and NO is a product of this reaction. Several NOS inhibitors induced settlement in competent larvae, indicating that NO acts as a repressor of settlement. In this context it is critical to emphasize the difference between competence, metamorphosis and settlement. As we pointed out in chapter 2 (Heyland and Hodin 2004) metamorphic development consists of several distinct phases. The juvenile is essentially present at the time of competence and all that is required for the metamorphic transition is a specific settlement cue. Therefore all three terms refer to distinct life history phases. Although the identity of natural settlement inducers remain largely unknown, it is likely that such a cue would signal via this inhibitory NO pathway. Using a universal antibody directed against NOS, (Bishop and Brandhorst 2001) detected NOS in neurons of larval and juvenile tissues, such as the tips of the larval arms, the pre-oral hood and the lower lip of the larval mouth as well as the juvenile tube feet. These findings suggest that both larval and juvenile structures may be responsible for suppression of metamorphosis. Together with our findings on TH signaling (Fig. 4-4, Heyland and Hodin 2004,) as well as TPO and TH localization (Fig. 4-5) in peri-metamorphic stages, larvae appear to be a system with two distinct physiological entities: the ephemeral larval structures equipped with a multitude of sensory structures that are able to communicate with the external

environment, and the juvenile structures capable of interpreting the information provided. The result is a highly complex signaling system coordinating larval development and metamorphosis with a multitude of environmental signals. While this system appears to dominate the development to metamorphic competence once this state is reached, systems like NO come into action and orchestrate the metamorphic transition using external settlement cues as the initiator.

TH Function: A Mechanism for the Evolution of Non-feeding Development

Non-feeding development has evolved many times independently within the echinoids from feeding development (Strathmann 1985; Wray 1995). While feeding larvae use their elaborate convoluted ciliated bands that run around skeletal arms for feeding, non-feeding larvae have lost many of these structures as well as the ability to feed. The sea biscuit *Clypeaster rosaceus* fills an intermediate position in between these two extreme modes of development, in that larvae are facultatively feeding: their ciliated bands can capture food particles and a stomach is present, however metamorphosis can be reached in the complete absence of food (Emlet et al. 1987).

Many scenarios have been discussed concerning how non-feeding development evolved from feeding development (review in Hart 2002). Egg size can be a strong predictor of developmental mode in echinoderms and other taxa, in some cases (such as the echinoids), however, this correlation is less strong (Emlet et al. 1987; Levitan 2000a), suggesting that there are other factors involved as well (further discussed in chapter 2 and 3). Ecological considerations such as trade offs between egg size and egg number, differences in survival in the plankton, limitations to dispersal, as well as differences in juvenile growth and mortality in feeding and non-feeding taxa have thus far dominated the discussion about the evolution of alternative life history modes in marine

invertebrates (reviewed in previous chapters and (Hart 2002), while mechanistic (ontogenetic) changes have received relatively little attention. Our data presented here once again highlight thyroid hormone signaling as a possible mechanism involved in this evolutionary transition.

Our data together with previously published results (Saito et al. 1998) suggest that within the clypeasteroids, non-feeding larvae evolved the ability to synthesize THs endogenously. Still, feeding sand dollar larvae (Heyland and Hodin 2004; Hodin et al. 2001) have the ability to synthesize hormone endogenously as well, although not sufficiently to develop to metamorphosis in the absence of food (Heyland and Hodin 2004). Therefore, as previously mentioned, endogenous hormone synthesis might be a pre-adaptation for the evolution of lecithotrophy. By acquiring endogenous hormone production, feeding larvae could have gained a fitness advantage based on decreased time in the plankton (chapter 2, Chapter 3 and data presented here) and independence from phytoplankton availability. This fitness advantage, however, may be decreased by costs resulting from a reduced juvenile size. The diversity of life histories among echinoids specifically, and echinoderms in general, provide fertile ground for testing this hypothesis further. Different classes of echinoids seem to have evolved non-feeding development with different frequencies (Heyland and Hodin 2004; Hodin et al. 2001). In fact, some groups, such as Diadematoids, have no known non-feeding larvae. We propose to test for endogenous hormone synthesis in these groups and predict that taxa with lower frequency of non-feeding development have a limited ability to synthesize hormone endogenously.

Phenotypic Plastic Response is Maintained in Non-Feeding Development

THs mediate the expression of alternative phenotyps in larvae of the sand dollar *Dendraster excentricus* (chapter 2; phenotypic plasticity). When applied exogenously, the

hormone simulates high food conditions in that it leads to a shift in investment from larval to juvenile structures. Larvae reared in low food environments show a shift in investment in the opposite direction (from juvenile to larval structures), resulting in long larval arms that can capture more food and compensate almost entirely for the reduction of food particles in the environment (Hart and Strathmann 1994). For *Clypeaster rosaceus*, we show an increase in arm length when we treated larvae with the thyroid hormone synthesis inhibitor thiourea.

These similar responses of feeding larvae to low food conditions and non-feeding larvae to inhibitor treatment suggest that non-feeding larvae have maintained vestiges of the phenotypically plastic response to thyroxine. In feeding larvae the cue is exogenous, while it appears to be endogenous for non-feeding larvae. These findings further support our scenario outlined above, that ancestral feeding larvae gained the ability to synthesis all TH necessary for metamorphosis endogenously as a precondition to the evolutionary loss of larval feeding.

A question that needs further investigations is whether the phenotypically plastic response to endogenous hormone levels is a general characteristic of non-feeding larvae, or if it is only present in *Clypeaster rosaceus* with its specialized feeding mode (facultative feeding). Since these sea biscuit larvae have the ability to feed, a certain amount of plasticity could be retained for functional reasons. We observed previously that egg size in *Clypeaster rosaceus* can vary significantly (McEdward Lab unpublished observations). This could lead to a situation where, in some cases, insufficient maternal reserves are provided making feeding obligatory in order to reach metamorphosis. In such cases it would be advantageous for larvae to have a phenotypically plastic response

to food abundance. Unfortunately not enough information exists about facultative feeding and its functional significance. Experiments comparing particle ingestion rates in inhibitor-induced long arm larvae and control could answer some of these questions. Such experiments will allow us to further understand what role hormones play in the evolution of derived life histories in echinoderms.

Table 4-1. Total energy content of *Clypeaster rosaceus* eggs and newly metamorphosed juveniles (post-metamorphs) in µg and size (µm). Note that no significant difference in total energy content were found between juveniles from different treatments. For further details of analysis see text.

	Treatment	Total Energy (µg)	Size (µm)
Egg	-	1.67±0.24 (n=5)	293.81±2.17 (n=20)
Juveniles	CONTROL	0.60±0.02 (n=3)	279.47±4.88 (n=3)
	RESCUE	0.79±0.07 (n=3)	277.70±7.74 (n=6)
	THYROXINE (10^{-9}M)	0.58±0.08 (n=3)	282.21±5.37 (n=6)
	THIOUREA (10^{-6}M)	0.63±0.07 (n=3)	282.67±6.10 (n=3)
	THIOUREA (10^{-7}M)	0.52±0.06 (n=3)	250.24±4.20 (n=3)

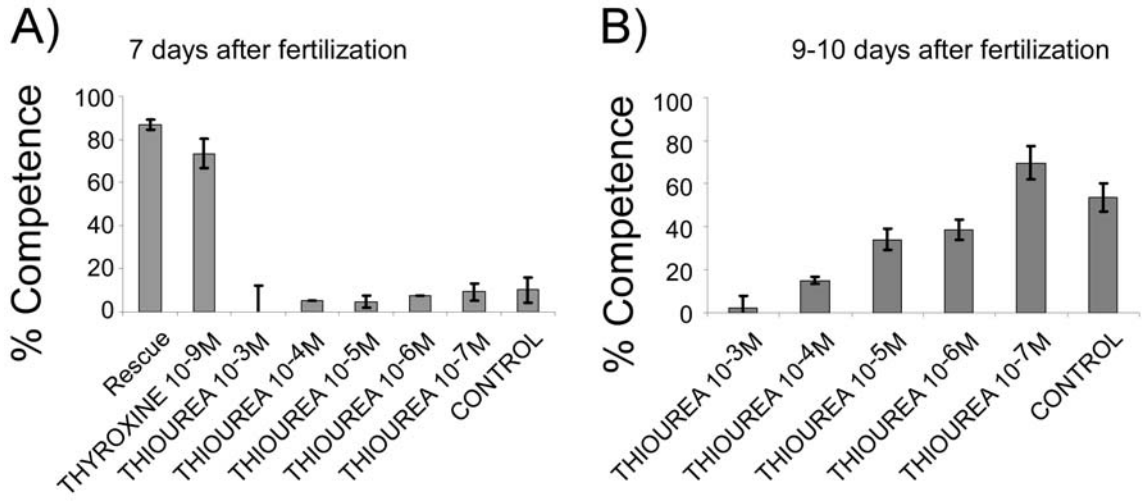


Figure 4-1. Thyroxine accelerates development to metamorphosis (A), while thiourea leads to a inhibition of metamorphosis in a dose responsive manner (B). Larvae of *Clypeaster rosaceus* were induced to metamorphose using 0.55M excess KCl on December 11th and December 13th/14th. (A) While the majority of larvae from the THYROXINE and the RESCUE treatment underwent the metamorphic transition upon induction (i.e., were competent; see text for definition) on December 11th, larvae from the INHIBITOR treatments and the CONTROL did not reach the threshold of 30% competence (see our definition in text). B) Two to three days later, however, larvae from the CONTROL and the 10^{-5}M , 10^{-6}M and 10^{-7}M thiourea treatments reached metamorphic competence while larvae from 10^{-3}M and 10^{-4}M thiourea did not.

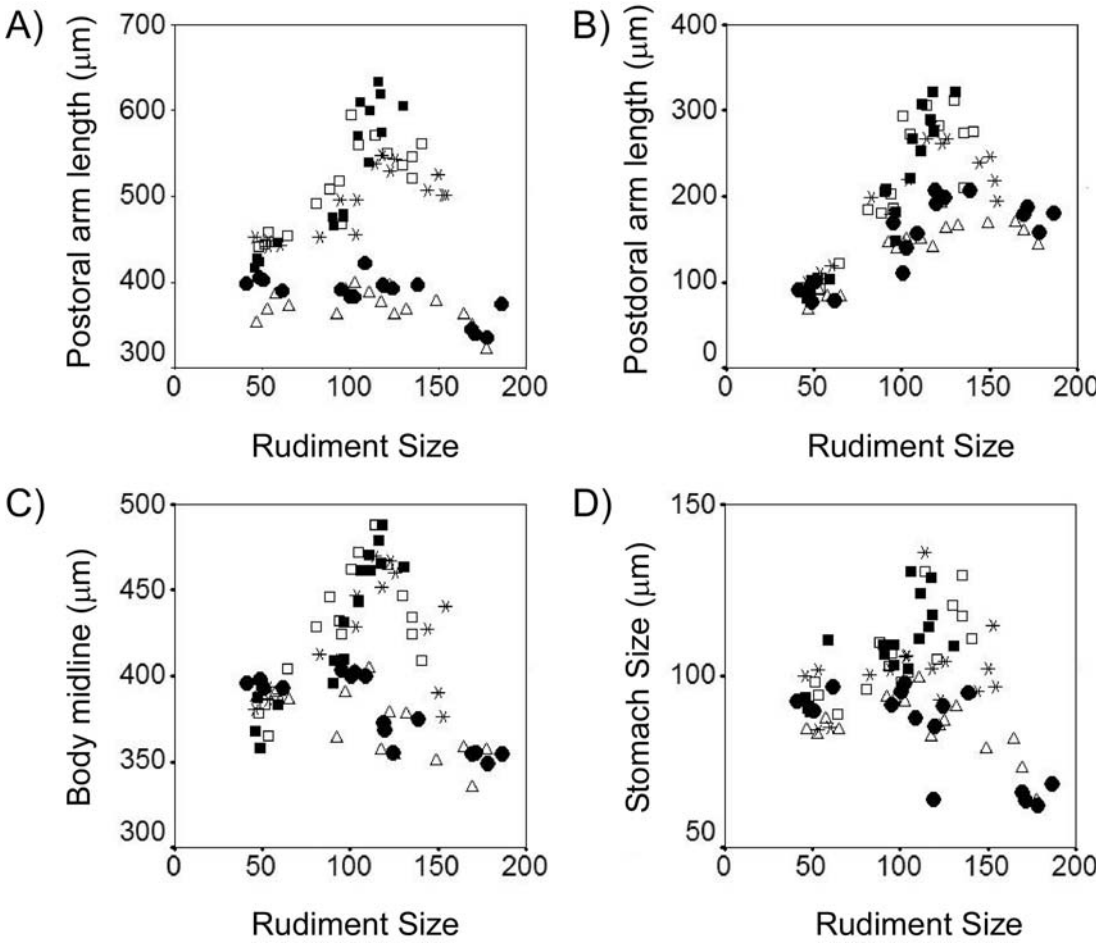


Figure 4-2. Thyroxine induces a shift of investment from larval to juvenile structures and thiourea induces a shift of investment from juvenile to larval structures. Larval structures (postoral arm length, postdorsal arm length and body midline) and the stomach size are plotted against the juvenile structure rudiment size. Statistical analysis was performed using correlation analysis in combination with MANOVA. The THIOUREA 10-3M treatment led to a significantly higher investment into larval postoral arm length relative to rudiment size in the case of postoral arm length, while the THYROXINE and RESCUE treatment led to a significantly higher investment into juvenile structures relative to larval structures concerning postoral arm length, body midline and stomach size (for analysis see text). Note that only data are shown for which both morphological measurements were present. ∗ CONTROL ● TYROXINE (10^{-9}M thyroxine) □ THIOUREA 10^{-3}M (10^{-3}M thiourea) ■ THIOUREA 10^{-5}M (10^{-5}M thiourea) ∇ RESCUE (10^{-9}M thyroxine + 10^{-3}M thiourea)

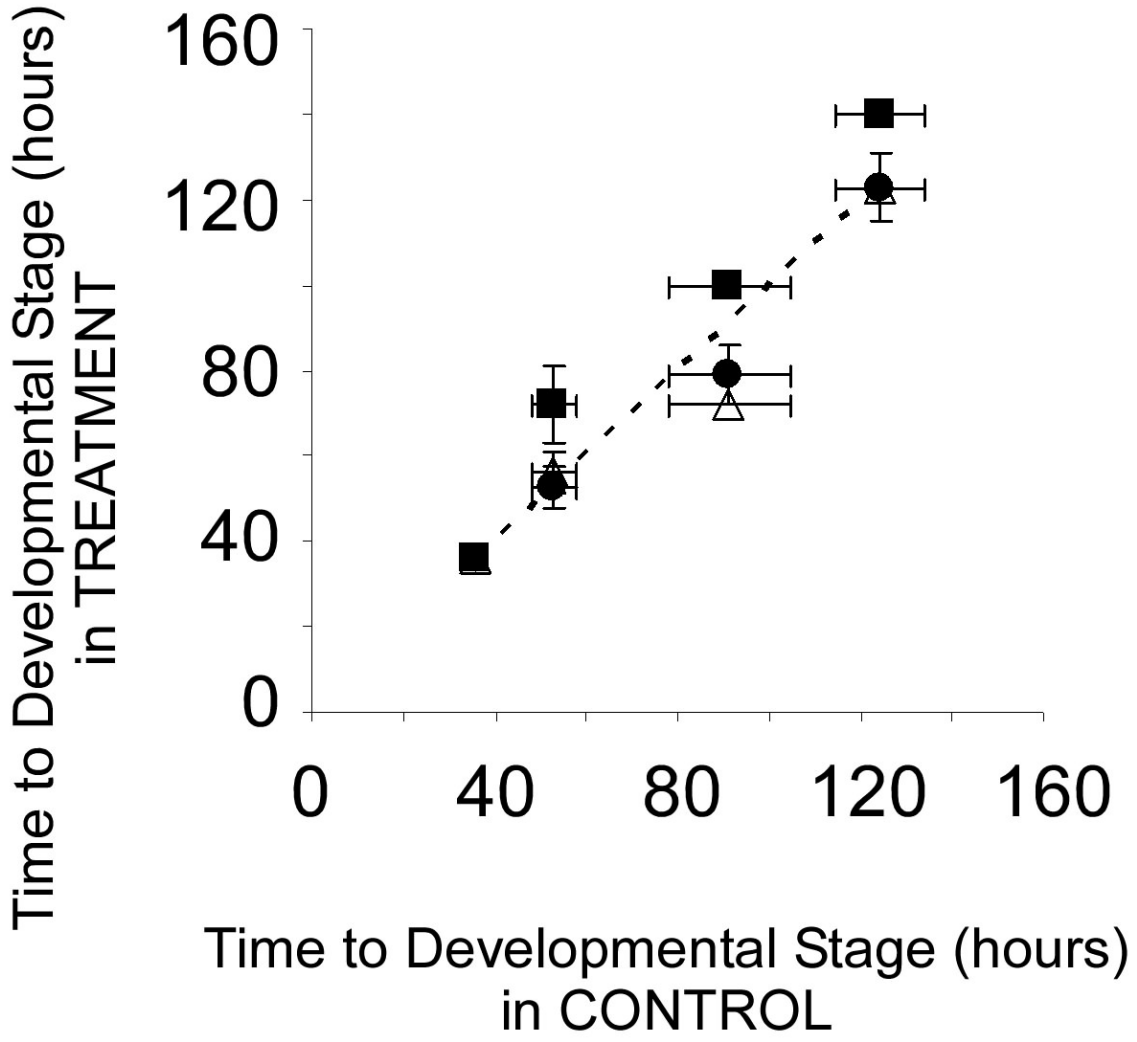


Figure 4-3. Thyroxine treatment does not lead to heterochronic shift in development in *Clypeaster rosaceus* facultative feeding larvae. Graph represents heterochrony plot for the following treatments: ● TYROXINE (10^{-9}M thyroxine); ■ THIOUREA 10^{-3}M (10^{-3}M thiourea) ∇ RESCUE (10^{-9}M thyroxine + 10^{-3}M thiourea). Each point in a heterochrony plot represents the mean value of time to a particular developmental stage. The y-axis information is the timing of larvae to a developmental stage from a given treatment. The corresponding timing of larvae to a developmental stage from the control is the X-axes information. Note that the error bars are S.E. of the mean based on four independent replicates. The dotted black line is the standard and represents developmental timing of control versus control. All points that lie below this black line represent accelerated development. All points above the black line represent decelerated development.

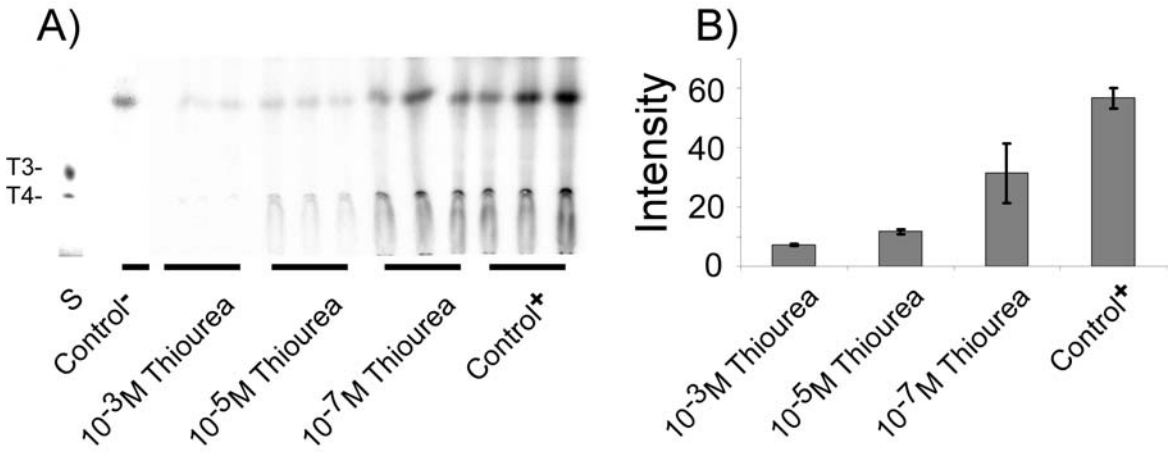


Figure 4-4. Larvae of *Clypeaster rosaceus* incorporate I^{125} and build thyroxine with it. A) TLC plate showing T4 (thyroxine) band but not T3 (3,3',5-L-triiodothyronine). B) Measurement of intensity of bands for all treatments and the control. Both iodine incorporation and thyroxine synthesis is inhibited by thiourea (A and B). Larvae were exposed to I^{125} for 18 hours in the presence (10^{-3}M Thiourea, 10^{-5}M Thiourea, 10^{-7}M Thiourea) and absence (Control^{+}) of the thyroid hormone synthesis inhibitor thiourea. The negative control (Control^{-}) consisted only of I^{125} and no larvae. Larvae were then extracted in MeOH over night and sample were dried down and loaded on TLC plates (A). S represents non-radioactive T4 (thyroxine) and T3 (3,3′, 5-triiodothyronine) standards that were run in the same and in different lanes and detected via UV light (see materials and methods). Intensity of bands was measured using imageJ^{TM} software. Note that the upper band represents I^{125} as seen in the control^{-}.

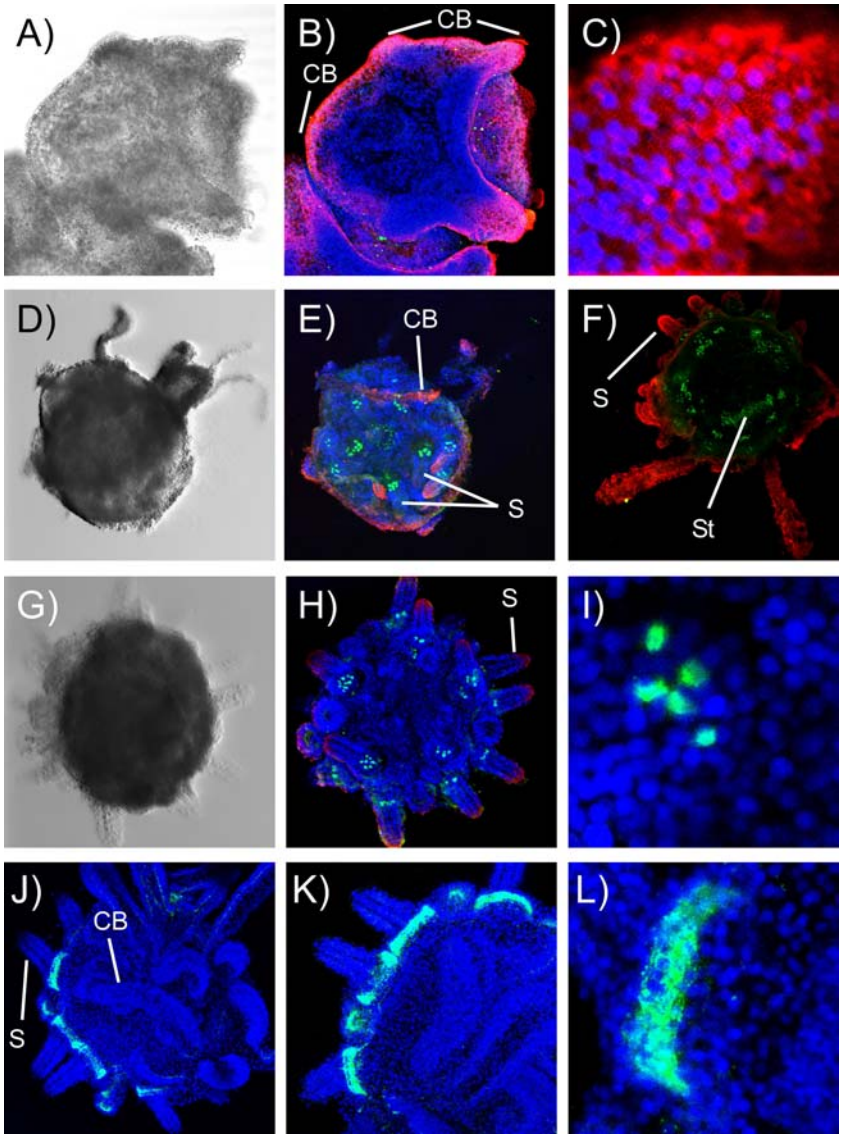


Figure 4-5. Immunohistochemical analysis of *Clypeaster rosaceus* developmental stages reveals presence of TPO (green in A-I) in potentially nervous system (green in J-L) related cell clusters at the base of the spines and presence of thyroxine (red) in the larval ciliated band. (A-C) 2 day old *Clypeaster rosaceus* larva stained with TPO (thyroid peroxidase) and T4 (thyroxine) antibody and the nuclear stain Draq5^{TM} (blue). (D-F) Pre-metamorphic (9 days) larvae stained with TPO and T4 antibody and nuclear stain. (G-I) Post-metamorphic juvenile (12 days) stained with TPO and T4 antibody and nuclear stain. (J-L) Post-metamorphic larvae stained with the nervous system marker 1E11 (kindly provided by R. Burke).

CHAPTER 5
PRESENCE OF THYROID PEROXIDASE ORTHOLOG IN THE SEA URCHIN *LYTECHINUS VARIEGATUS*. NEW INSIGHTS IN THE EVOLUTION OF THYROID HORMONE FUNCTION AMONG METAZOA

Introduction

Thyroid hormones (TH) are synthesized in the thyroid gland in vertebrates and have a multitude of functions in metabolism, growth, development and homeostasis. The primary form of TH synthesized in the thyroid gland is thyroxine (T4), which is then deiodinated to triiodothyronine (T3) (McNabb 1992) in the target tissue by deiodinases (DI). T3 can bind to nuclear hormone receptors transcription factors involved in a variety of cellular and developmental processes (reviewed in Yen 2001). One of the main players involved in thyroxine synthesis is thyroid peroxidase (TPO), an enzyme from the animal peroxidase family (Taurog 1999) that catalyzes the iodination of tyrosine residues from thyroglobulin (TG) and the fusion of two iodotyrosines to thyroxine. Iodine, the essential element for this reaction is incorporated into the thyroid by the sodium iodine symporter (NIS), a 65 kDa protein with 13 transmembrane domains (Dai et al. 1996). While TH synthesis by TPO and NIS is relatively well understood in vertebrates, little information exists on comparable TH synthetic mechanisms in other taxa.

Both ascidians (sea squirts) and cephalochordates (lancelets) possess an endostyle, a structure homologous to the vertebrate thyroid (Mazet 2002; Ogasawara et al. 1999; Ruppert et al. 1999) that has been shown to incorporate iodine. Moreover, TPO orthologues from both ascidians (CiTPO, Ogasawara et al. 1999) and lancelets

(Ogasawara 2000) are expressed in specific zones of the endostyle and may be involved in TH synthesis.

Iodine metabolism and TH related function is not restricted to the chordates. It has been previously described for disparate animal taxa such as hemichordates (Roche et al. 1961), bryozoans (Roche et al. 1964), cnidarians (Kingsley et al. 2001; Spangenberg 1971), insects (Davey 2000) and mollusks (Gerencser et al. 2002; Gerencser et al. 2003). In echinoderms (sea urchins, starfish and the like) increasing evidence shows that THs play critical role in development and metamorphosis (Chino et al. 1994; Heyland and Hodin 2004; Hodin et al. 2001; Johnson 1998; Johnson and Cartwright 1996; Saito et al. 1998) and might also be involved in life history evolution in this group (Heyland and Hodin 2004; Hodin et al. 2001). Thyroxine accelerates larval development (Chino et al. 1994; Heyland and Hodin 2004; Hodin et al. 2001; Johnson and Cartwright 1996) and leads subsequently to an earlier metamorphosis (Heyland and Hodin 2004). In chapter 3 we have shown that THs are not only necessary for feeding larvae (those larvae in echinoderms that needs to feed on phytoplankton in order to complete metamorphosis) but can also be sufficient for development through metamorphosis in the the subtropical sand dollar *Leodia sexiesperforata*. Still, the source or sources of hormone for echinoderm larvae remain controversial. Chino et al. (1994) first hypothesized that THs or TH-like compounds may primarily originate from unicellular algae in feeding sea urchin larvae. In a recent study (Heyland and Hodin 2004) we showed that feeding larvae from the sand dollar *Dendraster excentricus* may synthesize some hormone endogenously in addition to its exogenous source and Saito et al. (1998) showed that the non-feeding Japanese sand dollar species *Peronella japonica* contains detectable amounts

of T4 and T3. In both studies, the vertebrate goitrogen thiourea inhibited metamorphosis, suggesting the involvement of TPO related enzymes in thyroid hormone synthesis in non-chordate deuterostomes as well.

In order to further elucidate the mechanistic basis for TH signaling in echinoids we cloned a TPO ortholog from the sea urchin *Lytechinus variegatus* and analyzed its expression patterns using in situ hybridizations and immunohistochemistry. Our pharmacological analysis of TH and TH synthesis inhibitor effects on larval development further emphasizes a role of this enzyme in TH function and metabolism in the sea urchin.

Materials and Methods

Laval Culturing

Adult *Lytechinus variegatus* for the metamorphosis experiment were collected at Jupiter inlet Florida (26° 56’ 36’’N; 80° 4’ 24’’W) in October and November 2002 at low tide. Animals for the well plate experiment, molecular cloning of thyroid peroxidase gene and in situ hybridizations were collected off Long Key, Florida in February and October 2003 by snorkeling. Upon collection, animals were maintained in the Laboratory at 21-24°C in flow-through seawater.

We induced spawning by injection of 1ml 0.55M KCl solution in the gonad of adult urchins. Eggs were collected in MSFW and sperm were collected dry. After eggs had settled, excess water was replaced once with fresh MFSW and a 1:10,000 solution of concentrated sperm was added. One minute later eggs were viewed under the compound microscope to check for fertilization envelopes. Fertilization success was estimated by counting the number of successfully fertilized eggs out of 50 randomly sampled eggs. We only considered the fertilization as successful if fertilization success was more than 95%

(successfully fertilized/50). Larvae were maintained in gallon jars filled with 3.8 liter MFSW (millipore-filtered seawater; 0.2µm) at a concentration of 1 larva/5ml MFSW. Hatching occurred within 12 hours after fertilization. Larvae were then fed 4 cells/µl of the unicellular alga *Rhodomonas lens*. Water in cultures was changed every 2 days by reverse filtration (see Strathmann 1987). At each water-change fresh food [*T-ISO* (10'000cells/ml) or *R. lens* (3000cells/ml)] was added.

Experimental Designs

Metamorphosis Experiment

Adults for this experiment were spawned on November 18th 2002. Larvae in cultures were exposed to 4 concentrations of thyroxine (T4) and the control (no T4) two days after fertilization until metamorphosis. Primary T4 stocks [L-Thyroxin (Sigma); T-1776] were prepared at a concentration of 10^{-6}M, 10^{-7}M, and 10^{-9}M and then diluted 1: 10^{4} for the final concentrations of 10^{-10}M, 10^{-11}M, and 10^{-13}M (THYROXINE 10^{-10}M; THYROXINE 10^{-11}M; THYROXINE 10^{-13}M) in the culture jars (note that for this experiment we used 1 liter glass jars filled with 800ml MFSW). When larvae were considered competent for metamorphosis (definitions for metamorphosis in competence see chapter 2) water in 1l jars was reduced to 100ml by reverse filtration (see Strathmann 1987) and 4ml of 1M KCl was added resulting in 40mM excess final KCl concentration. Jars were screened for metamorphosed *L. variegatus* after 4 hours. This procedure was repeated three times: on November 30th, December 1st, December 3rd and December 5th. We estimated timing to metamorphic competence in larvae by calculating the cumulative percentage of metamorphosis for the dates listed above. Once the threshold of

20% cumulative percent metamorphosis was overcome we consider larvae in a replicate metamorphically competent.

Well plate Experiment

Eggs for the well plate experiment were fertilized April 2rd 2003 and the experiment was setup on April 11th 2003 (developmental stage E; Fig. 5-1). Note that before this exposure we cultured larvae for 24 hours in the complete absence of food to drain the stomachs of any algal food. We then distributed larvae into individual wells of 12-well plates filled with 4ml of MFSW exposed them to one of the following treatments. CONTROL (no chemicals added), THYROXINE (10^{-10}M thyroxine [Sigma: T-1776]), RESCUE (10^{-10}M thyroxine+10^{-3}M thiourea), THIOUREA (10^{-3}M thiourea), L-TYROSINE ($2*10^{-10}$M L-tyrosine [Sigma: T9040-9]), NaI ($4*10^{-10}$M NaI [Sigma: S2179]), L-TYROSINE+NaI ($2*10^{-10}$M L-tyrosine+$4*10^{-10}$M NaI), NaCl ($4*10^{-10}$M NaCl). At this point we took two images from each individual larva that was mounted alive on a microscope slide with sufficient MFSW. Each image was taken at a different magnification (10x, 20x) in order to be later able to measure stomach size and rudiment size respectively. Note that each treatment was replicated 12 times (i.e., one entire well-plate per treatment). On April 15th we removed each individual larva carefully from the well plate and photographed it in the same way as on April 11th. On April 16th we removed larvae from the well plate and flattened larvae underneath cover slides and imaged larval and juvenile skeletal structures. All images were analyzed using imageJ software (http://rsb.info.nih.gov/ij/). We measured postoral arm length (PO), post-dorsal arm length (PD), the stomach size (SS) and rudiment size (RS). SS and RS were calculated as the square root of the cross sectional area of an ellipsoid (using stomach

length, stomach width and rudiment length, rudiment width respectively as the axes of the ellipsoid).

I^{125} Experiment

In the iodine experiment we exposed larvae at developmental stage E (Fig. 5-1) for 8 hours in 12 well plates to experimental treatments. Note that before this exposure we cultured larvae for 24 hours in the complete absence of food to drain the stomachs from any algal food. We placed 50 randomly chosen larvae into each well, containing 4ml of solution (10^{-3}M thiourea and the control respectively). All solutions were made up in SW^{125} (MPFSW with I^{125} at 51937 dpm; Carrier free specific activity of I^{125} was 642.8GBq/mg). For each treatment we used 6 wells. After the exposure, larvae from three wells were pooled together in one test tube resulting in two independent replicates per treatment (150 larvae per replicate). Specimens were washed 5 times with fresh MFSW until the radioactivity in the supernatant was below 30dpm (counted on ssMPD instrument se below). Between each wash larvae were centrifuged at 1980g for 3 minutes and kept on ice.

To test whether I^{125} that the larvae had incorporated was built into T4, we prepared samples for thin layer chromatography (TLC). We added 1ml of ice cold MeOH to each sample after the sample was counted [samples containing I^{125} were counted on a ssMPD instrument (BioTraces, Inc., Herndon, VA) in standard mode. In standard mode, digital signal processing is used to distinguish the I^{125} decay specific characteristics from those of background events to give a background equivalent to 5 DPM of I^{125} with about 45% efficiency] and let it stand at 4ºC over night. After vortexing all samples at full strength for 2 minutes we centrifuged them at 1980g for 10 minutes and collected the

supernatant. Then we spiked the samples with 100µl non-radioactive 10^{-4}M T4 (thyroxine; Sigma-Aldrich T-1774) and T3 (3,3",5-Triiodo- L –thyronine; Sigma: T2877) and the concentrated then in a speed-vac to complete dryness. The dry pellet was resolved in 30µl 0.01N NaOH. Note that usually not all the salt crystals resolved. All 30ul (excluding the crystals) were loaded on a TLC plate (Whatman LK5D silica gel 150A with fluorescence marker; Whatman #4851-840) and run for 1.5 hours in a 2-methylbutanol/*t*-butyl alcoholy/25%NH 3/acetone, 7:14:14:56, vol/vol solvent. We visualized the cold T4 and T3 markers under UV light on a BioRad™ Flour-S MultiImager system and radioactive bands on a Molecular Dynamics™ Phosphorimager SI. Overlaying the UV image with the on from the phosphor imager allowed us to compare the radioactive bands to our THs standards.

Immunohistochemistry

L. variegatus larvae at different developmental stages (Fig. 5-1) were fixed for 20 minutes at room temperature in 4% paraformaldehyde in PBS and then post-fixed in 100% ice cold MeOH for 10 minutes. Note that before fixation we cultured larvae for 24 hours in the complete absence of food to drain the stomachs from any algal food. Specimens were then washed three times in PBS to remove all the paraformaldehyde and MeOH and either stored at 4°C in PBS or immediately processed. We transferred specimen into PBT (PBS with 0.3% triton-X), washed them three times (10 minutes each wash) and incubated them then for 30 minutes in blocking solution (5% normal goat serum) followed by several washes. We then incubated with primary antibodies [1:200 polyclonal rabbit anti-T4 serum (SIGMA and 1:400 monoclonal mouse anti-TPO serum (Research Diagnostics Inc. NJ, USA) in PBT over night at 4°C or 2 hours at room

temperature (20°C). Then specimens were removed from primary antibody, washed three times in PBT and incubated with the secondary antibody (anti mouse Alexaflour™ 488 from Molecular Probes™ at 1:200 and anti rabbit Alexaflour™ 547) over night at 4°C or at room temperature for 2 hours. After three more washes in PBT we transferred larvae into a 1:1000 dilution of the nuclear stain DRAQ5™ for 20 minutes. Finally specimens were washed in PBS and mounted in Vectorshield™ to view with confocal microscope (Zeiss™).

Gene cloning and In Situ Hybridizations

A pool of double stranded cDNA from larval stages was isolated and amplified following the protocols of the Clontech Smart-PCR cDNA synthesis kit (Clontech Laboratories) as described in (Matz 2003). We ligated cDNA to double stranded adaptors (Marathon cDNA Amplification Kit, Clontech). We selected conserved amino acid sequences among peroxidases to choose the sites for PCR primers We choose sense [TPO-F: ACIGCIGCITT(TC)(CA)GITT(TC)GGICA, corresponding to the amino acid sequence TAAFRFGH] and the anti sense [TPO-R: GGIA(AG)ICC(AG)TG(AG)TCIC(GT)I-CCIC(GT)(TC)TG, corresponding to the amino acid sequence QRGRDHGLP] degenerate primers based on Ogasawara et al. 1999. We then used rapid amplification of cDNA ends (RACE) to generate 5' and 3' PCR products following procedures previously described in (Matz et al. 2003). We cloned Gel purified PCR products into the pT-Adv Vector (Clontech TA cloning) and sequenced it at the Whitney Laboratory, St. Augustine (FL).

We fixed samples in 100 mM HEPES pH 6.9, 2 mM MgSO4, 1 mM EGTA for 24-48 hours and dehydrated them in 50% ethanol, then 80% ethanol (30 minutes each) and

stored at –20°C in 80% ethanol until use. We synthesized digoxigeninlabeled (DIG) antisense probes from linearized plasmids according to the protocols supplied with the DIG RNA Labeling kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). Whole mount in situ hybridizations were performed by a similar protocol to those of Swalla et al. (1994). We washed samples with phosphate buffered saline with 0.1% Tween 20 (PBT) then treated with 10 mg/ml Proteinase K in PBT at 37°C for 10 minutes. We stopped the reaction in 2 mg/ml glycine in PBT, and washed with PBT. Samples were post-fixed in 4% paraformaldehyde in PBS, washed with PBT and treated with 0.25% anhydrous acetic acid in 0.1 M triethanolamine (pH 8.0) prepared just before use. We hybridized samples overnight at 45°C with LvTPO anit sense full length probe and used the the sense probe as a negative control. We then washed with 2XSSC at 45°C and treated with 20 mg/ml RNase at 37°C. Samples were blocked in 0.1% blocking reagent in PBT, then incubated in 1/2000 anti-DIG-AP in PBT, both from the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals). AP detection buffer contained levamisole and NBT/BCIP. After the desired staining was reached, samples were rinsed in PBS. Samples were then mounted in benzyl alcohol:benzyl benzoate after being dehydrated through a series of ethanol washes: 30%, 50%, 80%, 90%, 100% along with two washes in benzyl alcohol:benzyl benzoate 1:1. Note that for all these procedures we cultured larvae for 24 hours in the complete absence of food to drain the stomachs from any algal food (cDNA synthesis and sample fixation).

Phylogenetic Analysis

We conducted a phylogenetic analysis of the nucleotide sequences of the catalytic domain using parsimony criterion as implemented in PAUP* (Swofford, D. L. 2002). Of 2308 characters 1883 were parsimony informative. All characters were treated as equally

weighted and unordered. Gaps were treated as missing data. The analysis was performed with a TBR heuristic search employing 10,000 random addition sequence replicates, holding 10 trees at each step of stepwise addition and otherwise default PAUP* settings. Taxa used in phylogeny and accession numbers: *Gaeumannomyces graminis*: fungal lineolate diol synthase; used as outgroup in the analysis (AF124979); Crayfish_PO: peroxinectine from the signal crayfish *Pacifastacus leniusculus* (JC4397); Urchin_OPO: sea urchin (*Lytechinus variegatus)* ovoperoxidases (AF03581); Sepia_PO: ink gland peroxidase from the cuttlefish *Sepia officinalis* (2320157A). Squid_PO: light organ peroxidase from the squid *Euprymna scolopes* (PN0667). Rat_TPO: thyroid peroxidase from rat (Pert_Rat); Human_TPO: thyroid peroxidase from human (Pert_Human); Human_SPO: salivery peroxidase from human (JC4935); Human_EPO: eosinophil peroxidase from human (PERE_Human); Human_MPO: myeloperoxidase from human (PERM_human); Human_Pdsn: Peroxidasin from human (D86983); Aplysia_PO: putative thyroid peroxidase from the mollusk *Aplysia californica*; Drosophila_Pdsn: peroxidasin from *Drosophila melanogaster* (S46224); Lancelet_TPO: putative thyroid peroxidase from the lancelet *Brachiostoma belcheri* (AB028841); *C. elegans*_Psdn: peroxidasin from *C. elegans* (CEF59F3). Urchin_TPO (LvTPO) putative thyroid peroxidase from the sea urchin *Lytechinus variegatus*; Ciona_TPO: putative thyroid peroxidase from *Ciona intestinalis* (AB022196); Halocynthia_TPO: putative thyroid peroxidase from *Halocynthia roretzi* (AB022197).

Thyroxine Measurements in Algae Samples

We reared replicate samples of algae of three species [*Dunaliella tertiolecta* (*D. tert.*), *T-ISO* and *Rhodomonas lens* (*R. lens*)] at the coastal research center (WHOI) in Woods Hole (MA) in summer 2002 in 25 liter containers using protocol previously

described by McEdward and Herrera (1999), although adapted for large scale use. Sterile starters for all three algae species were obtained from Dr A.D. Anderson's laboratory (Woods Hole Oceangraphic Institute, MA). We cultured starters in 1 liter containers until maximal growth phase was reached and then transferred one liter of this stock into 25liters of MFSW. For each species three replicate cultures were set up like that and grown under constant light conditions for 1 week at 20ºC. Before harvesting samples we took 25 random 1ml samples from the main stock and counted them using a hemocytometer. We then passed all 25 liters of algae suspension through an autoclaved filter unit (Millipore^{TM} 142mm Stainless-Steel filter holder) using an autoclaved Whatman^{TM} GF/C filter (1.2µm) and immediately froze samples in liquid nitrogen. For each algae species we collected a total of 4 replicates (4 times 25 liters) over the course of one month.

Algae samples were defrosted on ice, homogenized with a blender for 5 minutes in 2:1 Methanol:Chloroform and centrifuged at 3000rpm for 10 minutes at 4ºC. We then added 1/5th volume of 0.05% CaCl solution (in water), vortexed at full strength for 5 minutes and let phases separate over night at 4ºC. We collected the upper phase and re-extracted the lower phase 2 more times over night. Each upper phase was passed through a Amicon® Ultra-15 Centrifugal Filter Device (5Kd) and then dried down in a Speed-Vac^{TM}. Then the pellet was re-dissolved in 50µl 0.01N NaOH. All upper phases were pooled together and samples were stored at -80ºC until measured. Thyroxine measurements of algae samples were done in the fall 2003 using ELISA (Total Thyroxine (Total T4) ELISA Kit Alpha Diagnostic International, Inc.; TX, USA) following the manufacturers instructions. We used the following thyroxine concentrations for our

standards additionally to the standards provided: 31.04µg/dl, 15.52µg/dl, 7.76µg/dl, 3.88µg/dl, 1.94µg/dl, 0µg/dl. Note that all values are reported as hormone per g protein. The values on algal protein content were averaged from literature data (Jonasdottir 1994; Lavens and Sorgeloos 1996).

Data Analysis

Data were organized and analyzed in Excel^{TM} and SPSS^{TM}. Statistical comparisons between the experimental treatments and the controls were done using student's t-test, ANOVA with simple contrast or MANOVA. For all analyses we used SPSS^{TM}. Results are presented as: mean difference (treatment value minus control value) ± one S.E.; p-value. If the mean difference is positive this means that the value in the experimental treatment was larger than the value in the control. All p-values are from null hypotheses testing that the mean difference mentioned above equals 0.

Results

Characterization of LvTPO (*Lytechinus variegatus* Thyroid Peroxidase)

LvTPO has a total length of 703 amino acid residues. Figure 5-6 show the alignment with the catalytic domain of other peroxidase genes. In Figure 5-10 the entire alignment can be found. The catalytic domain of LvTPO extends from position 74 to 574 and includes all residues necessary for peroxidase function. These are proximal and distal histidine (110, 356), arginine (259) and asparagine (440). The Ca^{2+}-binding domain appears to be only partially conserved in *Lytechinus variegatus* [residues T(189), Y(191), D(193), S(195)]. While three residues are completely conserved [T(189), D(193) and S(195)] position 191 is Y in the sea urchin, instead of F as in other TPO genes described.

The phylogenetic analysis using 16 representatives from the peroxidase gene subfamilies [defined by (Daiyasu and Toh 2000)]. Two maximum parsimonious trees (MPT) were obtained (TL = 11390, CI = 0.4463, CI excluding uninformative characters = 0.4282, RI = 0.3776, RC = 0.1685). Strict consensus of the two trees is presented in Fig. 5-7. To assess branch support we computed 100 replicates of bootstrap based on heuristic search with 100 random addition sequences and otherwise default PAUP* settings. LvTPO clusteres together with the ascidian TPO (Halocynthia and Ciona) suggesting that this gene is a thyroid peroxidase. The sister clade however is *C. elegans* peroxidasin. This gene however does not cluster at all with any other peroxidasin gene.

Sea Urchin Thyroid Peroxidase (TPO) Homologue is Expressed in Larval and Juvenile Structures

LvTPO expression patterns using in situ hybridizations are presented in Fig. 5-8. RNA from the full length clone of this gene was detected to different degrees in the periphery of the stomach (St; Fig. 5-8A-C), cells in the larval arms (LA; Fig. 5-8B,C) and cells in the mouth region (M; Fig. 5-8B,C). We also report staining in juvenile structures such as the juvenile rudiment (R; Fig. 5-8B) and the pedicellariae (Pe; Fig. 5-8C). Immunohistochemical analysis using monoclonal anti human-TPO antibody (Fig. 5-9) confirmed presence of TPO protein in the stomach region and the base of the pedicellariae. Moreover we were able to detect thyroxine in cells of the ciliated band using polyclonal anti-thyroxine antibody.

Thyroxine Accelerates Development to Metamorphosis

Our results from the metamorphosis experiment (Fig. 5-2A) show that thyroxine accelerated development to metamorphosis (appearance of first adult skeletons, stage E; Fig. 5-1) and the timing of metamorphic competence (Fig. 5-2B). For a definition of

metamorphosis and competence see chapter 2. Number of adult skeletons was significantly higher in the THYROXINE 10^{-10}M (6.46±1.91; p=0.001) AND THYROXINE 10^{-11}M (4.05±1.91; p=0.038) treatment 9 days after fertilization compared to the control using ANOVA with simple contrast. Metamorphic competence was reached significantly earlier in the THYROXINE 10^{-10}M treatment compared to the control (-3.50±1.45 days; p=0.03). Note that we used the threshold value of 20% competence for this analysis (data not shown).

Thiourea Acts Antagonistically to Thyroxine

While we did not find direct inhibitor effects on morphology in the wellplate experiment our analysis shows antagonistic effects of the thyroid hormone synthesis inhibitor to thyroxine effects (Fig. 5-3). Thyroxine lead to a significantly stronger relative reduction of stomach size (-0.21±0.09; p=0.02), and absolute reduction of PO arm length (-168.98±43.17; p<0.001) and PD arm length (-169.90±74.20; p=0.02) and a significant relative increase in juvenile size (0.21±0.07; p=0.005) all relative to the control. We did not find however any significant difference between the THIOUREA treatment (10^{-3}M thiourea) and the CONTROL and the RESCUE treatment (RESCUE (10^{-10}M thyroxine+10^{-3}M thiourea) and the CONTROL indicating an antagonistic action between thiourea and thyroxine in terms of larval morphology. We also did find a significant relative decrease of stomach size (-0.19±0.09; p=0.04) in the NaI treatment.

Iodine Incorporation is Blocked With Thiourea But not KCLO4

From the I^{125} experiment (Fig. 5-4) we report that iodine uptake was blocked increasingly with increasing thiourea concentrations (Fig. 5-4A), another TH synthesis inhibitor, KClO4 however did not have any effect on iodine uptake in sea urchin larvae

(Fig. 5-4B). In another experiment using only one concentration of thiourea (10^{-3}M) larvae were then processed for TLC (thin layer chromatograph) after iodine incorporation and results from that experiment are reported in Fig. 5-4C. While CONTROL larvae synthesized T4, THIOUREA treated larvae did not synthesize any. Note that we were not able to detect any T3.

Three Algae Species Commonly Used as Larval Food Contain Different Amounts of Thyroxine

Figure 5-5 presents our findings on thyroxine in unicellular algae species. We measured different thyroxine concentration in three algae species: *Dunaliella tertiolecta* (*D. tert.*), *Rhodomonas lens* (*R. lens*) and *T-ISO* (isochrisis species). *D. tert.* Contained significantly more thyroxine then *R. lens* [237.96± 53.11µg*dl^{-1}(g Protein); p=0.013]. Note that all values were standardized for total average protein content of these algae species [*D. tert*. and *T-ISO* (Lavens and Sorgeloos 1996) *R. lens* (Jonasdottir 1994)].

Discussion

Our analysis of LvTPO (*Lytechinus variegatus* thyroid peroxidase) is based on the molecular cloning of this gene and observations on expression patterns throughout development using LvPTO full length probe and a human TPO antibody. We will first discuss the position of this gene among other members of the animal peroxidase superfamily, then discuss the distribution of this gene during development and finally link this information with our results on the pharmacological experiments using thyroxin and thyroid hormone synthesis inhibitor.

LvTPO, Another Invertebrate Member of the Animal Haem Peroxidase Super Family

Peroxidases are enzymes catalyzing oxygenation with hydrogen peroxide (Taurog 1999). While peroxidases from plants, fungi and animals show clear differences in their primary, secondary and tertiary structure their function appears to be very similar among organisms of these three kingdoms (Daiyasu and Toh 2000; Taurog 1999). The central reaction of all peroxidases investigated to date is the heterolytic cleavage of the iron-linked O-O bond leading to the formation of compound I (Poulos and Fenna 1994). Two histidines, a proximal and a distal one are critical for this reaction. Together with a few other well conserved residues such as arginine (259), aspartic acid (440) they are located within the about 500 amino acid residues long catalytic domain that is present in plant, fungal and animal peroxidases (Fig. 5-6; Fig. 5-10). In that respect LvTPO (*Lytechinus variegatus* thyroid peroxidase) is a typical peroxidase with all functional residues present.

The animal peroxidase superfamily (peroxidases found among animals) consists of a variety of different peroxidases such as Myeloperoxidase (MPO), thyroid peroxidase (TPO), salivery peroxidase (SPO), peroxidasin and many others. Compared to plant peroxidases the tertiary structure among animal peroxidases is much better conserved although the overall sequence similarity may be very low, representing a good example of how the tertiary structure can be far better conserved in evolution than the primary structure (Taurog 1999). The tertiary structure appears to be very similar primarily due to specific well conserved residues in the protein (Daiyasu and Toh 2000; Taurog 1999). For example the proximal and distal histidine and the arginine, aspartic acid and the Ca^{2+} binding domains mentioned above are completely conserved in deuterostome thyroid peroxidases (Fig. 5-6). In this respect LvTPO is an exception since it carried a tyrosine in

position 191 instead of a phenylalanine. The same change however has also been observed in other peroxidases such as Peroxidasin of human, drosophila and *C. elegans*, the ink gland peroxidase of sepia and light organ peroxidase in squid. The signal crayfish peroxinectine has a tryptophane in this position. One possible explanation for this change might be that the Ca^{2+} binding domain is not functional in LvTPO. Another possibility is that the Ca^{2+} binding domain is functional and the residue in position 191 is not critical for its function. The squid light organ is a bacteriogenic (symbiosis with bacteria) bioluminescence that exists due to the light emission from bacteria hosted in the mantle cavity (Young 1977, Nishiguchi et al. 2004). Tomarev et al. (1993) hypothesized that the putative squid peroxidase may be involved in modulating the population of bacteria inside the light organ. Bacteriocidal function is well known from other animal peroxidases, a general function associated with other peroxidases as well (Tomarev et al. 1993). Considering that this squid peroxidase show peroxidase activity without the phenylalanine residue in the Ca^{2+} binding domain, provides evidence that LvTPO might be functional as well. However a test for peroxidase activity will be required.

Intriguingly, animal peroxidase genes appear as evolutionary modules in that peroxidase activity through the catalytic domain may have been added to another gene with a completely different function over evolutionary time. MPO and peroxinectine (isolated from crayfish blood cells) for example have cell adhesion function additionally to the peroxidase activity (Taurog 1999). Another interesting example is peroxidasin, a protein cloned from human cells, *Drosophila melanogaster* and *C. elegans*, that combines peroxidase activity with some developmental functions still to be elucidated (Nelson et al. 1994). Additionally to peroxinectine and peroxidasin several other invertebrate

members of animal peroxidases were cloned from the sepia ink gland (Palumbo and Jackson 1995), the squid light organ (Tomarev et al. 1993) and sea urchin eggs (LaFleur et al. 1998). LvTPO and AcaTPO (TPO homologue from *Aplysia californica*; Heyland et al. in prep.) are therefore a valuable contribution to this emerging dataset that might give new insights into the evolution of the animal peroxidase family.

Other members of the peroxidase superfamily are the so called ovoperoxidases, cloned from the sea urchins *Lytechinus variegatus* (LvOvo; accession number AF035381; (LaFleur et al. 1998), *Hemicentrotus pulcherrimus* (HpOvo; accession number BAA19738; (Nomura et al. 1999) and *Strongylocentrotus purpuratus* (SpOvo; accession number AF035380; (LaFleur et al. 1998). These enzymes are involved in blocking polyspermy during fertilization (LaFleur et al. 1998). Our sequence alignments (Fig. 5-6; 5-10) clearly distinguish LvTPO from LvOvo. Moreover the latter has only been shown to be expressed during fertilization.

Putative thyroid peroxidase from *Lytechinus variegatus* is significantly shorter than other TPOs (606 amino acid residues versus 900-1000 residues for most other identified thyroid peroxidases). The catalytic domain however is completely present. Moreover we cloned recently another putative TPO homologue from the sea hare *Aplysia californica* (AcaTPO). This TPO is even shorter with a total length of 579 residues. Taurog (1999) mentioned that all plant peroxidases are strongly reduced in size compared to animal peroxidases. Evidence for a reduction in size between vertebrate and invertebrate TPOs comes from the recently cloned lancelet TPO [Brachiostoma belcheri (BbTPO, accession number AB028841.1)] that has a total length of only 764 residues. Moreover, all vertebrate thyroid peroxidases have additional functional domains not necessarily related

to peroxidase function while BbTPO, AcaTPO and LvTPO lack these domains completely. This emphasizes further the modular structure of peroxidase genes and suggests that while these genes were probably linked to other function in vertebrates and lower chordates, this never happened among echinoderms and mollusks.

The phylogenetic analysis places LvTPO in close proximity to the ascidian TPOs showing that this gene is very similar to thyroid peroxidase genes. *C. elegans* peroxidasin, the sister clade to LvTPO could however also be a TPO, since no functional studies have been done to date and since it does not cluster at all with any other peroxidasin gene. Moreover Nelson et al. (1994) showed that this gene can link tyrosine residues and can, at least in vitro, iodinate protein.

TPO Function in Sea Urchin Larvae

Iodine appears to be limited in the terrestrial environment (Eales 1997). In marine environments however it occurs in much higher concentrations than on land (Davey 2000; Eales 1997). Our data presented provide evidence that sea urchin larvae can exploit this source of iodine. Larvae incorporate iodine efficiently and the same is known from many marine algae and several marine animals (reviewed in Eales 1997). Lampreys are the most basal vertebrates that posses a thyroid gland (Youson 2003). In larval lamprey, THs are synthesized in the endostyle, the homologue to the vertebrate thyroid (Ogasawara 2000; Ogasawara et al. 1999; Ruppert et al. 1999). This structure collects iodide and binds it to protein, forming T4 and T3 (Salvatore et al. 1959; Suzuki and Kondo 1971). The endostyle of ascidians and cephalochordates concentrates radio-labeled iodide from seawater, and in several species of these groups the endostyle is the site of biosynthesisfor THs (Dunn 1974; Fredriksson et al. 1993; Monaco et al. 1978; Suzuki and Kondo 1971; Tong et al. 1962). The endostyle of ascidians and amphioxus

expresses iodide peroxidase ortholog genes such as CiTPO (*Ciano intestinalis* TPO; (Ogasawara 2000; Ogasawara et al. 1999); HrTPO (*Halocynthia roretzi* TPO; (Ogasawara 2000; Ogasawara et al. 1999) and BbTPO (*Branchiostoma belcheri* TPO; (Ogasawara et al. 1999). In Bryozoans iodine uptake was blocked by a competitor of iodine transport, KSCN (potassium thiocyanate), but neither thiourea nor $KClO_4$ (potassium perchlorate) had any effect on iodine uptake (Roche et al. 1964).

Among vertebrates, TH synthesis can be inhibited through several pathways. Goitrogenic compounds (e.g., thionamides) may act as competitive substrates for the iodide peroxidase and subsequently inhibit covalent binding of iodide to thyroglobulin (TG: A thyroid protein that is the precursor to iodine-containing hormones and is typically present in the colloid of thyroid gland follicles) (DeGroot LJ 1962; Rosenberg 1952). In addition, some monovalent anions such as SCN-, CLO4-, and NO3- can inhibit iodide transport into the thyroid (Wyngaarden et al. 1953). KClO4, for example, is a potent competitive inhibitor of the sodium-iodine symporter localized in the thyroid follicular cells (Dai et al. 1996). $KClO_4$ however did not inhibit iodine uptake in sea urchin larvae and our unpublished observations indicate that $KClO_4$ also does not have any inhibitory effect on metamorphosis, while thiourea did. These findings suggest that TPO might be involved in iodine incorporation in sea urchin larvae.

The thyroid gland of vertebrates evolved as the central structure for TH synthesis and acts as a storage organ for iodine and thyroxine (Eales 1997) (T3 is also synthesized but in much smaller amounts and the main source for T3 is deiodination of T4 in the target tissue). If we assume that TPO is involved in TH synthesis in sea urchin larvae there does not seem to be a central structure for TH synthesis. We found TPO activity in

larval structures (mouth region and larval arms), the stomach and juvenile structures such as the base of the pedicellariae. Thyroxine on the other hand is clearly distributed in the larval ciliated band and no indications were found for presence of thyroxine in juveniles. Intriguingly all of these structures except the stomach region are part of the larval and juvenile nervous system as had been previously documented (Burke 1983a; Burke 1983b). Echinoderm larvae possess a very diffused nervous system with a only a few centralized 'organs' such as the apical ganglion. Our findings of TPO in these nervous system related structures emphasizes a role of hormone in metamorphosis and moreover seem to suggest that both the larval and the juvenile nervous system are involved in TH signaling during larval development. Physiological processes need to be coordinated between the larval and juvenile compartment together with the environment during development. TH synthesis in both larval and juvenile structures seems to provide the right platform for such a function: the larval arms (ciliated cells) and the stomach can process external stimuli from the environment, and the rudiment can process internal stimuli from other parts of the larva such as the stomach and the larval arms. Our findings of thyroxine distribution in the ciliated bands together with our findings on the role of TH as a plasticity signal (chapter 2) support such a function.

Still, in *Lytechinus variegatus* feeding larvae, the primary site of TPO expression appears to be the stomach region. While its presence in nervous system clusters needs further investigation and more comparative data, the presence of TPO in cells of the stomach is a good indication for TPO function in iodine and/or TH uptake from food. If larvae do incorporate hormone or hormone precursors from ingested algae cells TPO could play a critical role in this uptake. This hypothesis is further supported by our

findings of thyroxine in three unicellular algae species. To further test this idea it would be critical to test whether TPO activity in the stomach is actually inhibited by thiourea using both in situ hybridization and immunohistochemistry.

So far our evidence for LvTPO being a TPO however only originates from comparative data and in situ hybridization. It will be critical in the future to purify this protein and do a test for peroxidase activity. Moreover we will have to look at actual RNA levels during development.

Thyroid Hormone From Endogenous and Exogenous Sources: Implications for the Evolution of Non-Feeding Development

The response to thiourea is remarkably different between sand dollar and sea urchin feeding larvae (see also chapter 2 and chapter 4) and suggests a much higher capacity for endogenous hormone synthesis in sand dollars than in sea urchins. Development in *Lytechinus variegatus* larvae discontinued after food was removed (CONTROL treatment in well-plate experiments). Moreover, another experiment on sea urchins done by Chino et al. (1994) did not show any effects of thiourea on metamorphosis. Development in *Dendraster excentricus* larvae by contrast progressed significantly after food was removed (Chapter 2) and thiourea inhibited development to metamorphosis significantly.

In contrast to the relatively weak or absent inhibitor effects on *Lytechinus variegatus*, we found clear antagonistic effects of thiourea to exogenously applied thyroxine. While larvae responded to thyroxine by increased arm and stomach reduction, increased rudiment growth and early metamorphosis, these effects were absent when larvae were exposed to both thiourea and thyroxine (RESCUE treatment). The antagonistic action of thiourea to thyroxine might indicate that thiourea is blocking the uptake of exogenous hormone into the larva. Such an inhibition can explain the arrest of

growth and development in the RESCUE treatment and is in favor of our hypothesis that feeding sea urchin larvae receive the majority of hormone or hormone precursor from exogenous sources and have a very limited ability for endogenous hormone synthesis. Testing whether radioactively labeled tyrosine or thyroxine is incorporated and whether this incorporation can be inhibited by thiourea could further elucidate this hypothesis.

Still larvae did incorporate iodine and our results provide evidence that radioactive iodine was used to synthesize thyroxine. While we already discussed endogenous hormone synthesis as a mechanism involved in the evolution of lecithotrophy among sand dollars in the previous chapter our new findings now also provide evidence for such a mechanism in sea urchins and therefore possibly in all echinoids. Non-feeding development appears to have evolved several times independently among regular urchins as well and endogenous hormone synthesis may be an important pre-condition for this evolutionary transition.

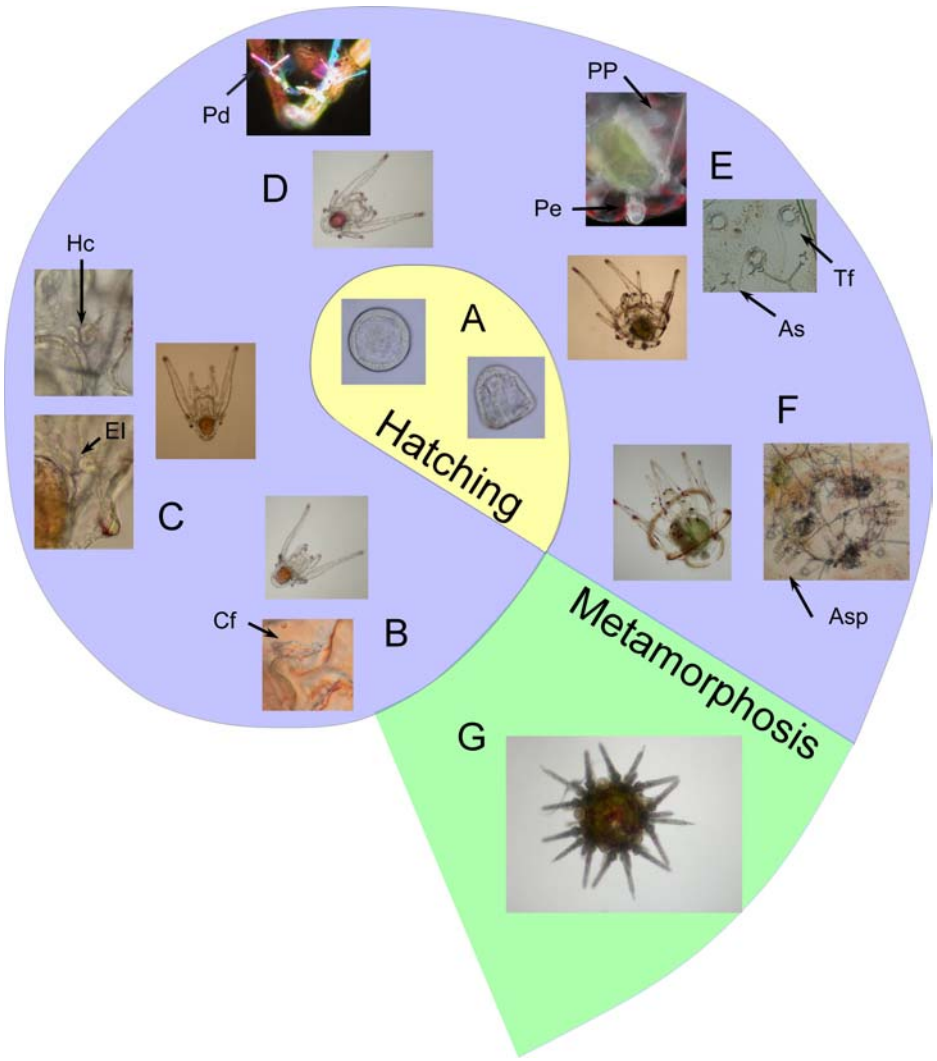


Figure 5-1. Embryonic and larval development of the sea urchin *Lytechinus variegatus*. Larvae (B) hatch after embryonic development (A) and develop continuously (B-F) to metamorphosis that transforms them into juveniles (G). (B-F) represent stages with distinct larval and juvenile characters. (B) Cf: coelom formation; (C) EI: Ectodermal invagination; Hc: Hydrocoel and ectodermal invagination contact; (D) Pd: Postdorsal arms start forming; (E) PP: Primary Podia, Pe: Pedicelariae; Tf: Tube feet with skeletal rings; As: Adult spicules; (F) Asp: Adult spines

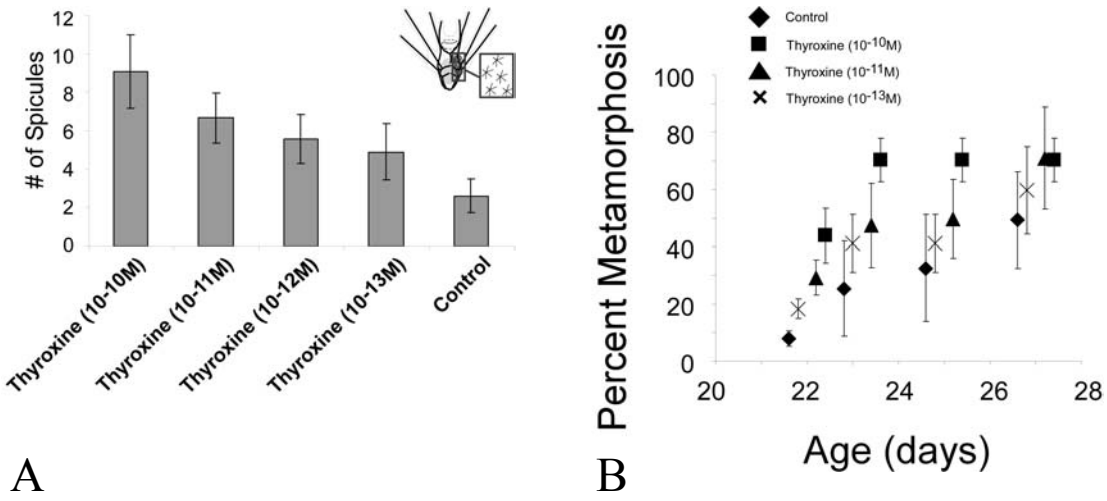


Figure 5-2. Thyroxine accelerates larval development (A) and metamorphosis (B). A) Larvae of *Lytechinus variegatus* were treated with different concentrations of thyroxine during larval development. We observed a significant increase in number of spicules (first adult skeletons; Stage E see Figure 5-1). B) Thyroxine also significantly accelerated development to metamorphosis (analysis see text).

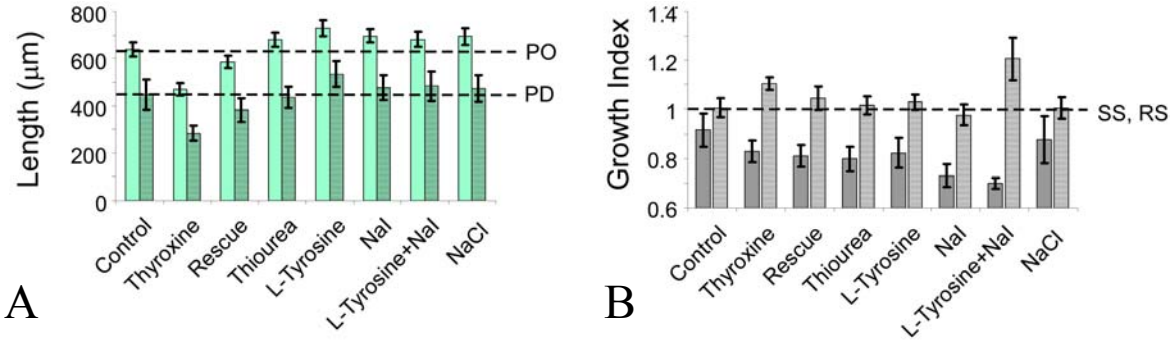


Figure 5-3. Thiourea acts antagonistically to thyroxine and iodine simulates the effects of thyroxine on stomach size (SS) We exposed larvae to 7 different treatments and the control in order to test whether iodine and/or tyrosine simulate the effect of thyroxine on larval characters (A), the stomach (B) and the juvenile rudiment (B) and whether thiourea has an inhibitory effect on these structures as it was found in other echinoid larvae. Thiourea did not have any effects on larval and juvenile structures while thyroxine did. The rescue treatment (thyroxine+thiourea) however was not significantly different from the control either. We conclude from these findings that thiourea acts antagonistically to thyroxine. We also found that NaI significantly decreases stomach size and so does thyroxine (for details on statistical analysis see text. Bright green: postoral arm length, dark green: postdorsal arm length, dark gray: stomach size, bright grey: rudiment size. Growth index indicates SS or RS after experimental exposure divided by SS or RS before exposure respectively. Dashed lines indicate the o-change isoclines. For PO and PD arm length this corresponds to the length of the arms in the CONTROL while it corresponds to 1 for stomach size and rudiment size (indicating no change).

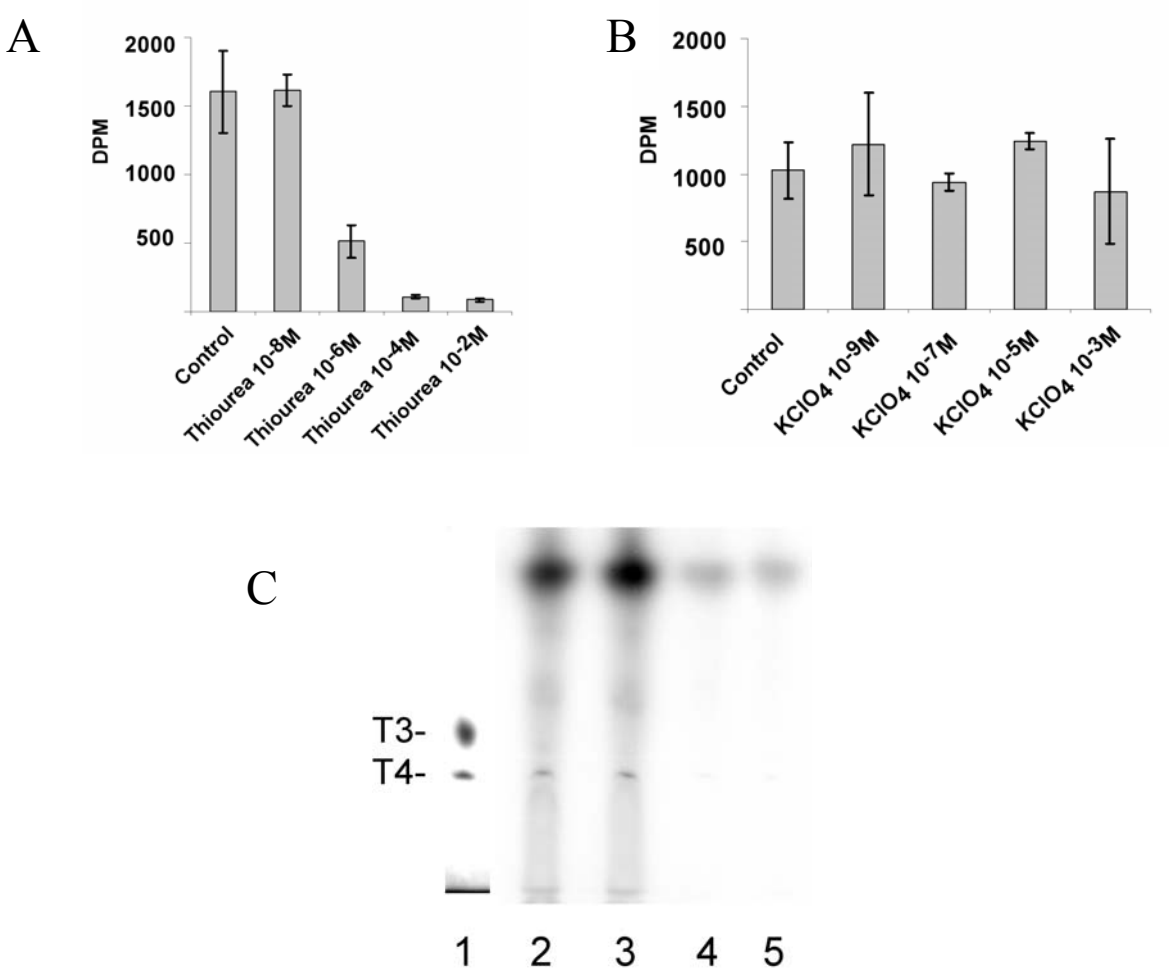


Figure 5-4. Thiourea inhibits iodine (I^{125}) uptake in Lytechinus variegates (A) while KCLO4 (another thyroid hormone synthesis inhibitor) has no effect on iodine uptake (B). Incorporated iodine is used to synthesize thyroxine (C; lane 2 and 3). In the thiourea treatment no thyroxine was synthesized (C; lanes 4 and 5). We exposed larvae of *Lytechinus variegatus* to different concentrations of the thyroid hormone synthesis inhibitors thiourea and $KClO_4$ to test whether iodine uptake can be inhibited. We found that thiourea can completely block iodine uptake while $KCLO_4$ did not have any effects. DPM are radioactive decays per minute counted. We then extracted thyroxine from larval samples and tested for presence of radioactive thyroxine using TLC (thin-layer chromatography). C) lane 1: non-radioactive standards of thyroxine (T4) and T3 (3,3",5-Triiodo- L –thyronine); lane 2,3: CONTROL (no thiourea added); lane 4,5: THIOUREA (10^{-3}M thiourea added). Note that the large black spot at the running front is I^{125}.

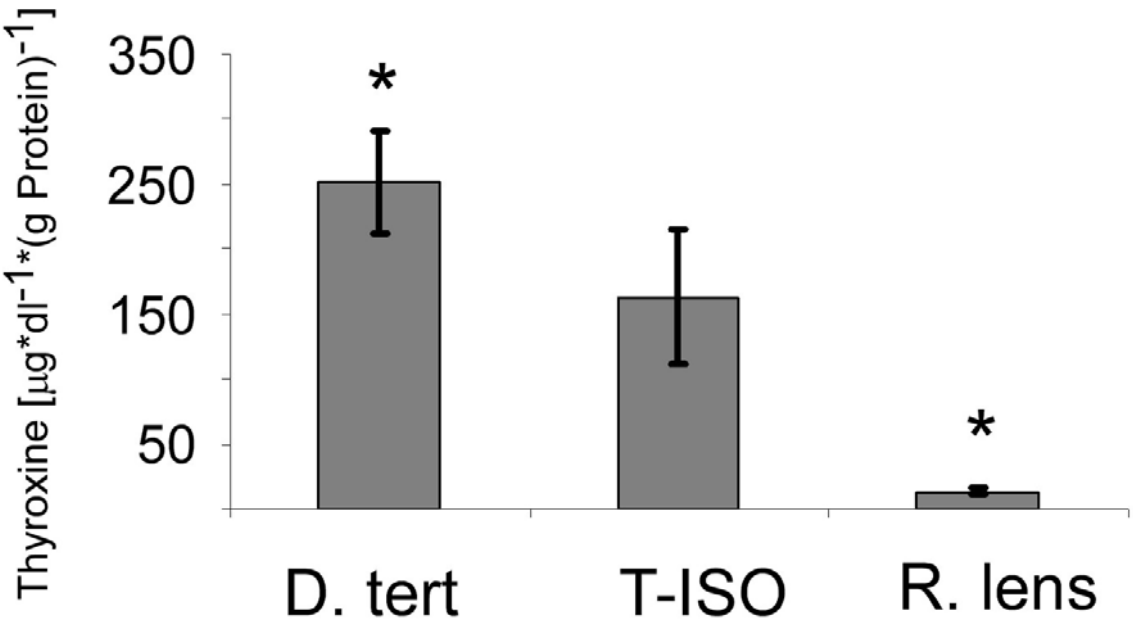
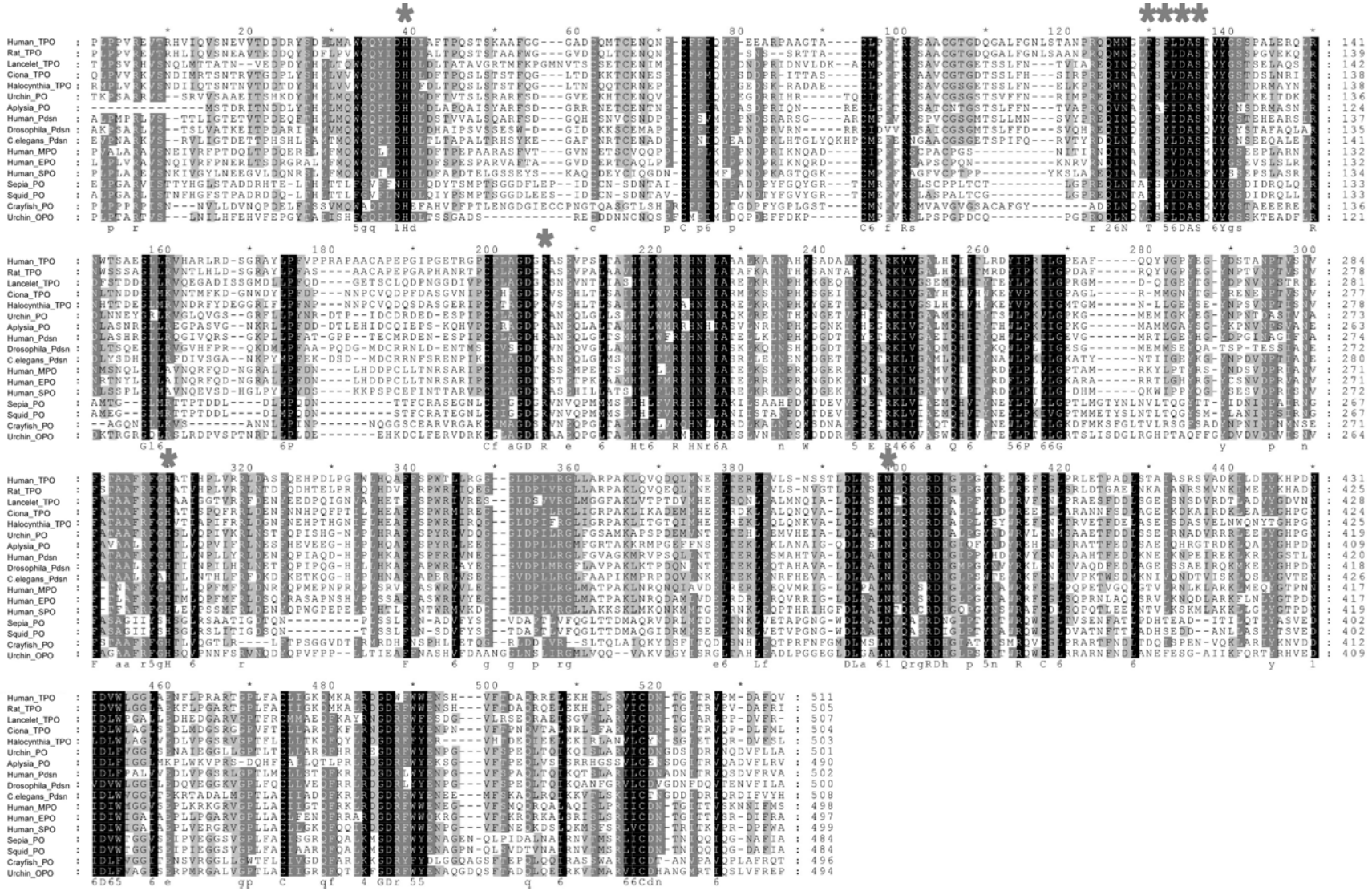


Figure 5-5. Three algae species commonly used to feed echinoderm larvae contain significant amounts of thyroxine. Values are ug*dl^{-1}*(g protein)$^{-1}$. Some algae however contain significantly more thyroxine then others. The difference between *Dunaliella. tertiolecta* and *Rhodomonas lens* is significant for $p<0.05$ (marked with asterix).

Figure 5-6: Alignment of LvTPO catalytic domain with 16 other members of the animal peroxidase family. Conserved residues are marked with an asterix (see text for details). We aligned the catalytic domains from the following organisms: *Gaeumannomyces graminis*: fungal lineolate diol synthase; used as outgroup in the analysis (AF124979); Crayfish_PO: peroxinectine from the signal crayfish *Pacifastacus leniusculus* (JC4397); Urchin_OPO: sea urchin (*Lytechinus variegatus)* ovoperoxidases (AF03581); Sepia_PO: ink gland peroxidase from the cuttlefish *Sepia officinalis* (2320157A). Squid_PO: light organ peroxidase from the squid *Euprymna scolopes* (PN0667). Rat_TPO: thyroid peroxidase from rat (Pert_Rat); Human_TPO: thyroid peroxidase from human (Pert_Human); Human_SPO: salivery peroxidase from human (JC4935); Human_EPO: eosinophil peroxidase from human (PERE_Human); Human_MPO: myeloperoxidase from human (PERM_human); Human_Pdsn: Peroxidasin from human (D86983); Aplysia_PO: putative thyroid peroxidase from the mollusk *Aplysia californica*; Drosophila_Pdsn: peroxidasin from *Drosophila melanogaster* (S46224); Lancelet_TPO: putative thyroid peroxidase from the lancelet *Brachiostoma belcheri* (AB028841); *C. elegans*_Psdn: peroxidasin from *C. elegans* (CEF59F3). Urchin_TPO (LvTPO) putative thyroid peroxidase from the sea urchin *Lytechinus variegatus*; Ciona_TPO: putative thyroid peroxidase from *Ciona intestinalis* (AB022196); Halocynthia_TPO: putative thyroid peroxidase from *Halocynthia roretzi* (AB022197).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Squid_PO
Crayfish_PO
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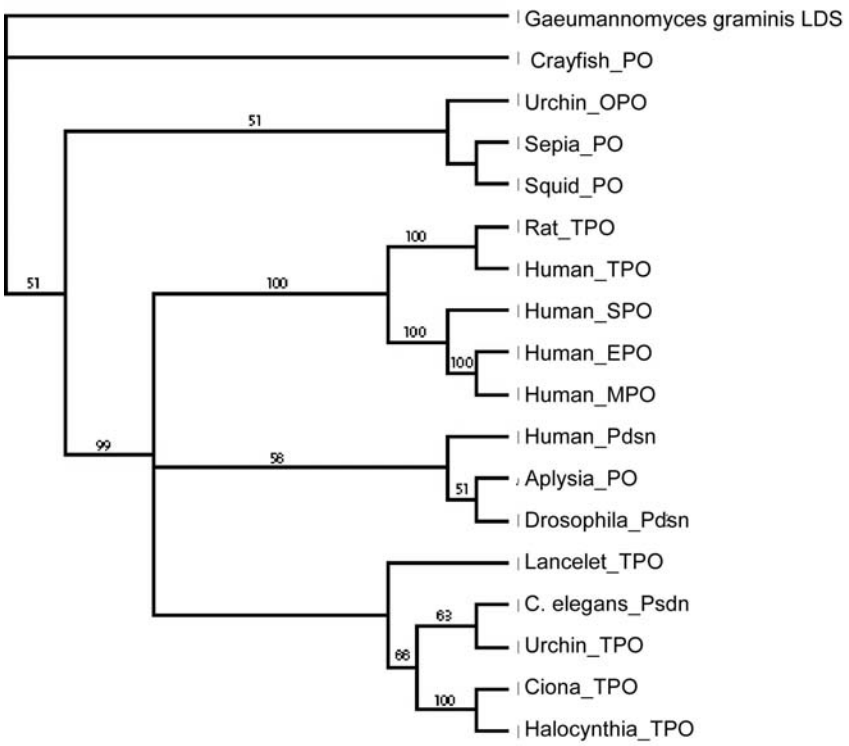


Figure 5-7. Phylogenetic analysis for LvTPO with 16 other peroxidase genes suggests that sea urchin TPO is a thyroid peroxidase. We used parsimony with subsequent bootstrap analysis. Strict consensus of the two MPTs resulted from parsimony analysis of the nucleotide sequences of the catalytic domain. Numbers above the branches are bootstrap values (100 replicates). Bootstrap values below 50% are not shown. Taxa used in phylogeny and accession numbers: *Gaeumannomyces graminis*: fungal lineolate diol synthase; used as outgroup in the analysis (AF124979); Crayfish_PO: peroxinectine from the signal crayfish *Pacifastacus leniusculus* (JC4397); Urchin_OPO: sea urchin (*Lytechinus variegatus)* ovoperoxidases (AF03581); Sepia_PO: ink gland peroxidase from the cuttlefish *Sepia officinalis* (2320157A). Squid_PO: light organ peroxidase from the squid *Euprymna scolopes* (PN0667). Rat_TPO: thyroid peroxidase from rat (Pert_Rat); Human_TPO: thyroid peroxidase from human (Pert_Human); Human_SPO: salivery peroxidase from human (JC4935); Human_EPO: eosinophil peroxidase from human (PERE_Human); Human_MPO: myeloperoxidase from human (PERM_human); Human_Pdsn: Peroxidasin from human (D86983); Aplysia_PO: putative thyroid peroxidase from the mollusk *Aplysia californica*; Drosophila_Pdsn: peroxidasin from *Drosophila melanogaster* (S46224); Lancelet_TPO: putative thyroid peroxidase from the lancelet *Brachiostoma belcheri* (AB028841); *C. elegans*_Psdn: peroxidasin from *C. elegans* (CEF59F3). Urchin_TPO (LvTPO) putative thyroid peroxidase from the sea urchin *Lytechinus variegatus*; Ciona_TPO: putative thyroid peroxidase from *Ciona intestinalis* (AB022196); Halocynthia_TPO: putative thyroid peroxidase from *Halocynthia roretzi* (AB022197).

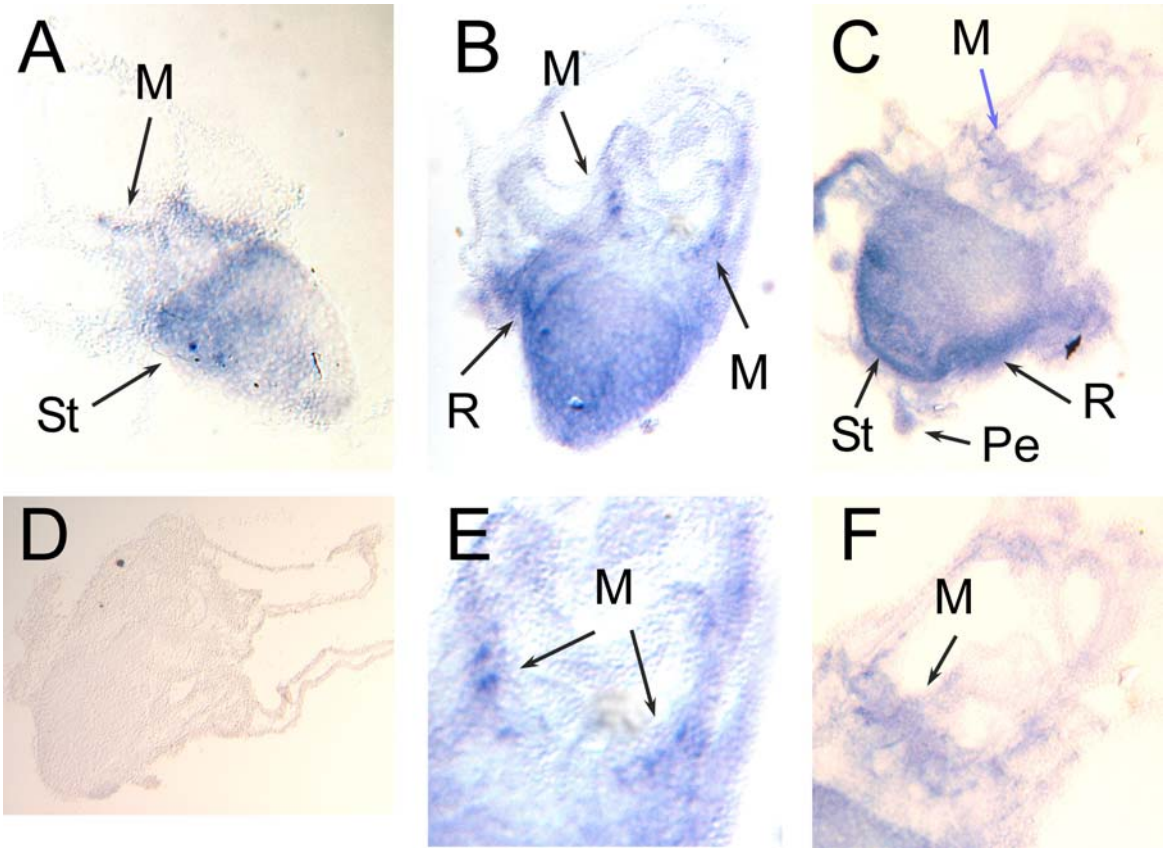


Figure 5-8. LvTPO (*Lytechinus variegatus* thyroid peroxidase) mRNA is present in different larval and juvenile structures. We used RNA anti-sense probes from the full length clone of LvTPO developmental stages to look at the distribution of this gene during development. Detection of TPO gene using full length LvTPO probe at different developmental stages. A) Pluteus larvae at stage B (see figure 5-1) show TPO expression in the stomach region and the mouth region; B) Pluteus larvae at stage D,E (beginning rudiment formation see figure 5-1)show staining in the ectoderm of the juvenile rudiment. C) Pluteus larvae at developmental stage E,F (figure 5-1) show TPO expression in the stomach region the mouth region and the rudiment. D) Control pluteus larva at developmental stage D, E using sense LvTPO probe. E) Close-up of the mouth region from the larva shown in (B). Cell cluster are stained in the region of the larval nervous system (see text for further explanations). F) Close up of larva shown in (C) staining of the mouth region in more diffuse than in earlier stage (see E).

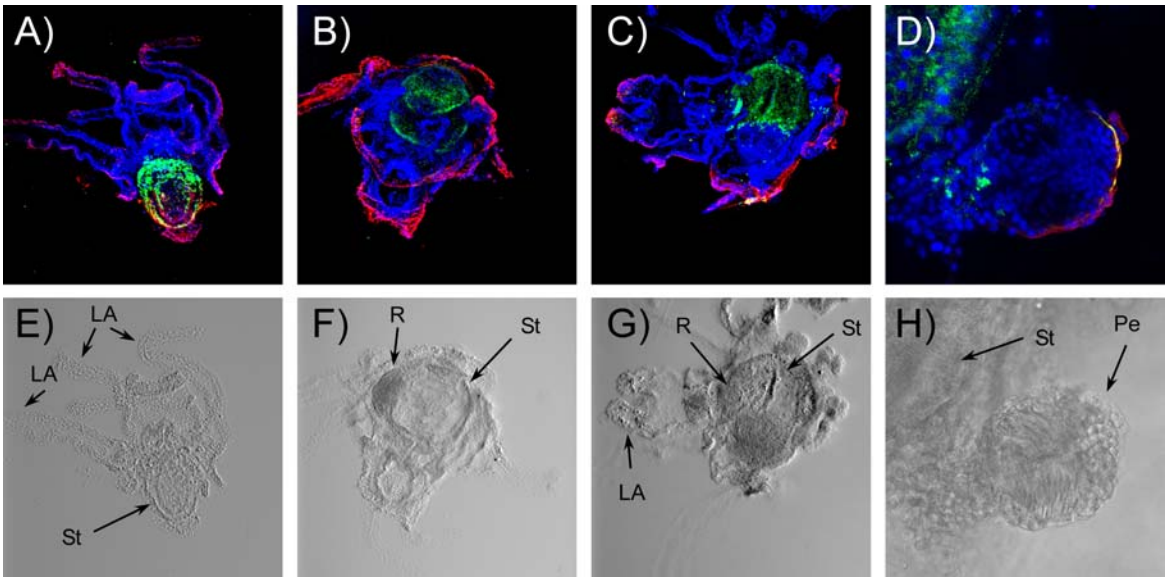
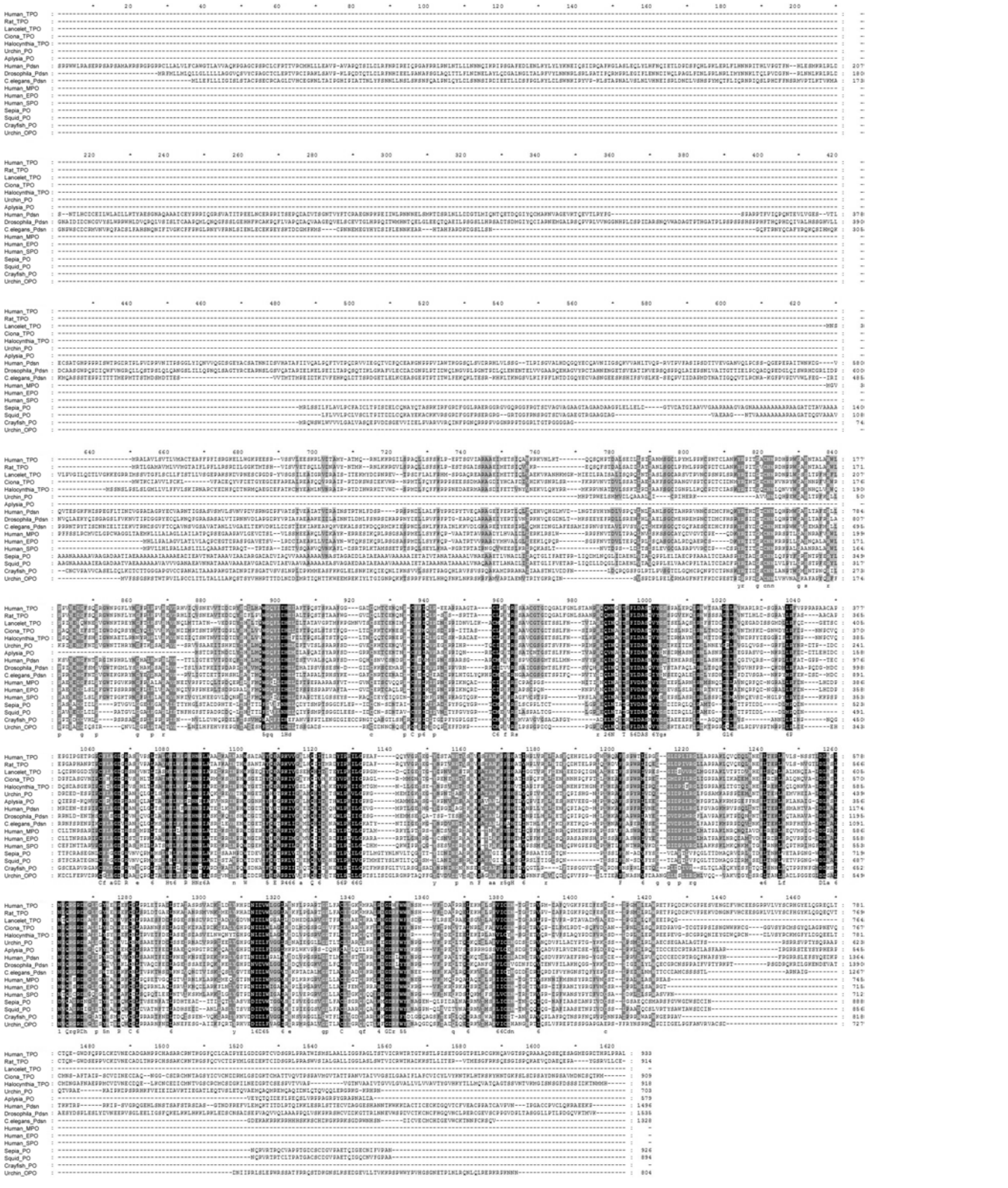


Figure 5-9: TPO protein is found in the stomach region and later in development at the base of the pedicellariae. We used monoclonal anti human TPO antibody to detect TPO in different developmental stages. TPO protein (green) appears to be present primarily in the stomach region (St) during development. We were able to detect small cell clusters however at the base of the pedicellariae (Pe), a juvenile and adult structure that develops before metamorphosis. Thyroxine (red) was distributed in the ciliated band the runs around the larval arms (LA). Nuclei are stained blue. A) Pluteus larva before beginning of rudiment formation (stage B see figure 5-1). B,C) Pluteus larva with juvenile rudiment (R) present (stage E and F). D) Close-up of pedicellariae (Pe) of pre-metamorphic larva (stage F). Images E through H are the corresponding DIC images to A through D.

Figure 5-10. Complete Alignment of LvTPO (*Lytechinus variegatus* thyroid peroxidase) with other 16 member of the animal peroxidase family. *Gaeumannomyces graminis*: fungal lineolate diol synthase; used as outgroup in the analysis (AF124979); Crayfish_PO: peroxinectine from the signal crayfish *Pacifastacus leniusculus* (JC4397); Urchin_OPO: sea urchin (*Lytechinus variegatus)* ovoperoxidases (AF03581); Sepia_PO: ink gland peroxidase from the cuttlefish *Sepia officinalis* (2320157A). Squid_PO: light organ peroxidase from the squid *Euprymna scolopes* (PN0667). Rat_TPO: thyroid peroxidase from rat (Pert_Rat); Human_TPO: thyroid peroxidase from human (Pert_Human); Human_SPO: salivery peroxidase from human (JC4935); Human_EPO: eosinophil peroxidase from human (PERE_Human); Human_MPO: myeloperoxidase from human (PERM_human); Human_Pdsn: Peroxidasin from human (D86983); Aplysia_PO: putative thyroid peroxidase from the mollusk *Aplysia californica*; Drosophila_Pdsn: peroxidasin from *Drosophila melanogaster* (S46224); Lancelet_TPO: putative thyroid peroxidase from the lancelet *Brachiostoma belcheri* (AB028841); *C. elegans*_Psdn: peroxidasin from *C. elegans* (CEF59F3). Urchin_TPO (LvTPO) putative thyroid peroxidase from the sea urchin *Lytechinus variegatus*; Ciona_TPO: putative thyroid peroxidase from *Ciona intestinalis* (AB022196); Halocynthia_TPO: putative thyroid peroxidase from *Halocynthia roretzi* (AB022197).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Drosophila_Pdsn
C.elegans_Pdsn
Human_MPO
Human_EPO
Human_SPO
Sepia_PO
Squid_PO
Crayfish_PO
Urchin_OPO

CHAPTER 6
SYNTHESIS

Thyroid Hormones (TH) and the Evolution of Non-Feeding Development

Life history transitions in a wide variety of animal and non-animal taxa are coordinated by hormones, and similar hormones have been repeatedly coopted in independently-evolved life history transitions in disparate taxa (an example of homoplasy). Derived life histories, such as vivipary in plants and the evolutionary loss of larval feeding in animals, involve alterations in these same hormones.

Hormonal signaling systems are by nature modular. Several of their components can be used independently in various contexts and especially their receptors (nuclear hormone receptors) consist of highly conserved domains that were assembled for hormonal signaling function in many different metazoan lineages many times independently. The modular nature of hormonal signaling systems predisposes them to be used in diverse developmental and evolutionary contexts. Studies of model systems will continue to provide an ever-expanding collection of molecular and cellular techniques to study organismal ontogenies. But only a comparative approach will allow for the synthesis of ecology, evolution and development that will illuminate patterns in organismal life histories. Here I pursued such a comparative approach to test the hypothesis whether thyroid hormones play a role in the evolution of alternative life histories in echinoids.

There are five extant classes within the phylum Echinodermata: Echinoidea (sea urchins and sand dollars); Asteroidea (starfish); Ophiuroidea (brittle stars and basket

stars); Holothuroidea (sea cucumbers); and Crinoidea (feather stars and sea lilies). Most studied echinoderms have a free swimming larval stage that disperses from the parental location, undergoes metamorphosis and settles to the benthos as a pre-reproductive juvenile. A minority of echinoderms brood their offspring. Those echinoderms with dispersing larvae can be further subdivided into those that feed as larvae and those that do not.

Similar morphology and feeding biology among larvae of echinoderms (and even their sister group the hemichordates) suggests that non-feeding development has evolved independently from feeding development on many occasions. The mechanisms underlying this life history transition however remain largely unexplored. My analysis on TH signaling in echinoid larvae suggests that thyroxine plays a critical role in metamorphosis and the evolution of non-feeding development in this group.

The source of hormone appears to be either endogenous (endogenous synthesis) or exogenous (phytoplankton) for echinoid larvae. Hormones from exogenous sources simulate the phenotypically plastic response of *D. excentricus* larvae to varying food conditions. Feeding larvae tend to grow longer arms when food is limiting. Since this response results in a higher feeding efficiency, larvae can at least partially compensate for the lack of food in the environment. If food is abundant, however, this response is reversed, larval arms are short and larvae invest relatively more into juvenile development. This shift in investment leads to a heterochronic shift in development, ultimately resulting in earlier metamorphosis. My data provide strong support for the hypothesis that algae-derived thyroxine (one form of thyroid hormone) is the cue for this phenotypically plastic response in feeding larvae, and that the larvae use ingested TH

levels as an indicator for larval nutrition, ultimately signaling the attainment of metamorphic competence. Such heterochronic shifts have been hypothesized to provide an important basis for evolutionary life history transitions in a variety of organisms such as amphibians, fish and insects. In this way, TH signaling in echinoid larval development appears as a key player in echinoid life history evolution. I then pursued this hypothesis that further, taking advantage of the immense life history diversity in this echinoderm class.

Feeding larvae have the ability to synthesize some THs endogenously, in addition to their exogenous source. I provided direct evidence for endogenous hormone synthesis in the NW Atlantic toxopneustid (regular) sea urchin *Lytechinus variegatus* (using thin layer chromotography) and indirect (reversible, pharmacological) evidence for it in the NE Pacific sand dollar *D. excentricus*. Interestingly, the effects of the TH synthesis inhibitor thiourea were much more profound in *D. excentricus* larvae than in *L. variegatus* larvae.

Moreover, TH appears to be not only necessary but also sufficient for development to metamorphosis in feeding larvae of the subtropical sand dollar *Leodia sexiesperforata*. These larvae undergo the metamorphic transition in the complete absence of food when exposed to exogenous hormone, while unexposed larvae did not. By contrast, *Dendraster excentricus* larvae, which develop from much smaller eggs than those of *Leodia sexiesperforata*, cannot reach metamorphosis in the absence of food, with or without thyroxine exposure.

In order to determine if non-feeding larvae have enhanced capacity for endogenous hormone synthesis relative to feeding larvae, I investigated TH synthesis capacity and

thiourea effects in the subtropical sea biscuit (a sand dollar relative) *Clypeaster rosaceous*. Larvae of this species develop from slightly larger eggs than those of *L. sexiesperforata*, and are actually facultative feeders, as they retain the ability to feed but can reach metamorphosis in the absence of food. Thin layer chromatographs in starved larvae indicated strong endogenous synthesis capacity, while the thiourea effects were the most profound of all the species examined.

Taken together, along with previously published studies by Chino et al. (on regular urchins) and Saito et al. (on the non-feeding sand dollar *Peronella japonica*), these findings suggest that the degree of endogenous hormone synthesis correlates with: 1) feeding mode (feeding versus non-feeding) and/or egg size (among the sand dollars and sea biscuits) as well as 2) phylogeny (regular sea urchins versus sand dollars/sea biscuits). Furthermore, once sufficient maternal resources are provided (egg size) the evolutionary transition from feeding to non-feeding development appears to be driven primarily by the availability of TH as I was able to confirm experimentally on *L. sexiesperforata* larvae. The dependence of feeding larvae on exogenous hormone sources, the hormonal induction of alternative larval phenotypes (phenotypic plasticity) and the capacity of non-feeding larvae to synthesize all the necessary hormone for attainment of metamorphic competence endogenously, support my hypothesis that upregulation of TH or TH-like compounds preadapted certain echinoid taxa (such as the sand dollars/sea biscuits) for the evolution of non-feeding development. This hypothesis can be further tested using a broader comparative survey of endogenous TH synthesis among echinoid and other echinoderm taxa. If this pre-adaptation scenario proves correct, it indicates that differences in the ability to synthesize the hormone internally may account for trends in

the evolutionary loss of larval feeding in echinoids specifically and possibly echinoderms in general.

Hormone From Exogenous Sources

Plant steroids and terpenes are widespread, ancient, and function as insect feeding deterrents in many cases (Li and Chory 1999; Pare and Tomlinson 1999). Indeed, insects cannot synthesize ecdysteroids without first obtaining an external source of sterols through feeding (Nijhout 1994). These findings raise the intriguing possibility that ancient insects first used plant derived chemicals as modulators of LHTs, and only later evolved the ability to synthesize particular hormonal compounds (such as juvenile hormones, which are sesquiterpenes) internally (Hodin in prep.). The evolution of thyroid hormone (TH) function in animals could have followed a similar route. The ancestral function of thyroid hormone could have been as feeding deterrents in algae and/or plants (Eales 1997), and the signaling functions in animals might, therefore, have been acquired secondarily. This situation could create positive selection to supplement exogenous supplies by endogenous synthesis, thus lessening the animal's dependence on feeding for these newly-acquired signaling functions.

A comparative analysis of thyroid hormone synthesis capacity in basal chordates provides some tantalizing evidence supporting this theory. The endostyle as it occurs in urochordates, cephalochordates and lampreys is a good candidate for a homolog of the vertebrate thyroid (Ogasawara 2000; Ogasawara et al. 1999; Ruppert et al. 1999) (but see Mazet 2002). One obvious morphological feature of the endostyle is its proximity to the digestive system. In fact, in urochordates and cephalochordates, the endostyle retains the function of a feeding organ with at least one band of ciliated cells(Ruppert et al. 1999). In lampreys, the endostyle acts in many respects more like an exocrine than an endocrine

gland: it secretes endogenously synthesized T4 into the alimentary canal, where it can be reabsorbed by the gut wall and then converted into T3 (Youson 1997). Results from amphibians further suggest that tadpoles have the ability to absorb TH-like compounds from their nutrition, subsequently leading to an acceleration of development to metamorphosis (Pfennig 1992). As described in the text, echinoderm larvae also obtain THs from their diet of planktonic algae (Chino et al. 1994), ultimately resulting in their attainment of metamorphic competence (Heyland and Hodin 2004).

Furthermore, ingested THs seem to be the signal for phenotypic plasticity in both sand dollar (Heyland and Hodin 2004) and spadefoot toad (Pfennig 1992) larvae. These findings suggest that the effect of ingested TH on larval morphogenesis could have been an ancestral mechanism through which TH was coopted, in both the amphibian and echinoderm lineages, as a stimulator of metamorphosis. Similar instances of cooption are found in many vertebrate parasites, including some flatworms and arthropods, which synchronize their life cycles with those of their hosts by responding to their host's hormones (reviewed in Matsuda 1987; Nijhout 1994). Finally, rotifers (*Asplanchna spp.*) utilize an ingested vitamin E metabolite (α-tocopherol) to regulate their parthenogenetic-to-sexual LHT and associated morphogenetic alterations (Gilbert and Thompson 1968). These diverse examples of dietary and other exogenous hormone sources should cause us to re-consider the commonly-understood definition of hormones as strictly endogenously synthesized signaling molecules.

Insights Into Mechanisms of TH Synthesis

The response of a wide variety of animals to iodine and thyroid hormone like compounds emphasizes the potential role of thyroid hormones as ancient signaling modules coopted for a variety of processes. The use of hormone from exogenous and

endogenous sources by echinoid larvae provides a good platform to further investigate components involved in this signaling system.

TH signaling in all vertebrates investigated to date involves a battery of components critical for ligand synthesis and translation of the signal into a physiological response. The thyroid peroxidase (TPO) catalyzes three essential reactions involved in TH synthesis. Thyroxine (T4) is de-iodinated in the target tissue to tri-iodotyrosine that binds to TRs with much higher affinity then thyroxine. In this study I focused on the identification of a TPO ortholog from sea urchins.

TPO belongs to the gene family of animal peroxidases. Additionally to their function in TH synthesis, other peroxidases are involved in disparate functions such as cell adhesion, fertilization and development. The central module of all peroxidases from plants and animals is the catalytic domain. This domain is also present in LvTPO (*Lytechinus variegatus* thyroid peroxidase), a peroxidase I cloned in this study, and for which I analyzed the distribution in feeding (*L. variegatus*) and non-feeding (actually starved *C. rosaceous*) larvae during larval development. While LvTPO appears to be relatively closely related to two other thyroid peroxidases present in ascidians, functional evidence suggests an involvement of LvTPO in iodine uptake and potentially TH synthesis. Its role in sea urchin larvae is particularly interesting since the gene appears to be expressed in a variety of larval structures and the stomach, in addition to juvenile structures in *C. rosaceous*. These findings suggest that the enzyme could serve a dual function in iodine and hormone uptake from the water, algae and TH synthesis, respectively.

The presence of other critical signaling components such TRs an de-iodinases in sea urchins still remains unknown. Future research in the field of TH related function in marine invertebrates and specifically echinoderms however will help to elucidate some of these questions.

Every organism has to process a multitude of internal and external signals at any given life history stage, whether it is an egg, an embryo, a larva or an adult. Signaling systems particularly suitable for these functions can be independently coopted in disparate taxa and modified for their particular function over evolutionary time. Highly modular signaling systems are more likely to be useful under various conditions, and would be expected to be favored over time. I propose that TH signaling represents such a system and that the analysis of homoplasious TH related functions such as TH synthesis via thyroid peroxidase and involvement of TH signaling in metamorphosis will help us to better understand the mechanistic basis of life history evolution in a variety of taxa. Our understanding on the physiological mechanism of TH-like signaling in echinoid larvae however will largely depend on future research elucidating more components of the TH signaling pathway such as TH-receptors or other receptors that TH can bind to and deiodinases. I expect thyroid hormone receptor like protein to be present in echinoid larvae although alternative signaling pathways can not be ruled out at this point such as non-genomic signaling mechanisms as they have been recently identified in vertebrate nervous system and circulatory system signaling. Finally I would like to emphasize the importance of identifying specific transporter proteins in echinoid larvae such as thyroglobulin and transthyretin.

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BIOGRAPHICAL SKETCH

Andreas Heyland graduated from the gymnasium in Frauenfeld (Switzerland) in 1993 with a specialization in mathematics and natural sciences. After finishing his undergraduate studies in biology at the University of Zurich he started his master's project in 1997 at the marine biological laboratory in Banyuls sur mer (University of Paris VI, Paris, France) under the supervision of Dr. Paul I. Ward from the University of Zurich and Dr. Jean-Claude Duchene from the marine biological laboratory in Banyuls sur mer. He defended the thesis entitled "Behaviour and mutual influencing of two polychaete species with different life-cycles during settlement" in 1998 at the University of Zurich. After this first working experience at a marine biological station with marine invertebrates, Andreas participated in two summer courses at the Friday Harbor Laboratories (University of Washington in Friday Harbor , WA, USA) in 1999: "Comparative Embryology of Invertebrates" and "Evolution of Development". The overwhelming diversity of larval forms and life history strategies that the students could study during these two courses contributed to Andreas's decision to do his Ph.D. with Dr. Larry McEdward from the University of Florida, FL, USA, one of the teachers of EvoDevo class and a leading specialist in larval ecology, development and life history evolution. Inspired by the Friday Harbor courses and his advisor Larry McEdward, Andreas started his Ph.D. at the University of Florida in the department of Zoology in 2000. Larry McEdward passed away unexpectedly in the summer 2001 and David Julian from the University of Florida became Andreas's new advisor with whom he defended

his dissertation in the spring 2004. Andreas received numerous small grants that helped him to accomplish his dissertation work. In addition he had the opportunity to present and discuss his dissertation work at several national and international meetings (SICB, Echinoderm Conference, Systematics and Evolution meeting, Neuroscience meeting) and was invited as a seminar speaker to the University of Florida (Gainesville, FL, USA), the Woods Hole Oceanographic Institute (Woods Hole, MA, USA), the Natural History Museum in Cambridge (Cambridge, UK) and the Friday Harbor Laboratories (Friday Harbor, WA, USA). Part of the experimental work was carried out at the Smithsonian Marine Station in Fort Pierce (FL, USA), the Friday Harbor Laboratories (University of Washington, WA, USA), the Rosenstiel School (University of Miami, Miami, FL, USA), Woods Hole Oceanographic Institute (Woods Hole, MA, USA), Marine Biological Laboratory (Woods Hole, MA, USA), and the Whitney Laboratory (University of Florida, FL, USA). Finally he was invited to the metamorphosis symposium at the Systematics and Evolution meetings in Patras (Greece) in 2002. After graduation Andreas will work as a postdoctoral fellow with Dr. Leonid Moroz at the Whitney Laboratory in Saint Augustine (University of Florida, FL, USA). His project focuses on the evolution of thyroid hormone signaling among the metazoa with specific emphasis on its function in the mollusk nervous system development.