Thyroid Hormones Accelerate Larval Skeletogenesis in Sea Urchins (*Strongylocentrotus purpuratus*) Through a Membrane Receptor-Mediated MAPK Signalling Cascade

by

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ABSTRACT

THYROID HORMONES ACCELERATE LARVAL SKELETOGENESIS IN SEA URCHINS (*STRONGYLOCENTROTUS PURPURATUS*) THROUGH A MEMBRANE RECEPTOR-MEDIATED MAPK SIGNALLING CASCADE

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Thyroid hormones are critical regulators of development and metabolism in vertebrates. In indirect-developing sea urchins, there is strong evidence that thyroid hormones accelerate growth of the juvenile rudiment, a necessary structure for metamorphosis. The juvenile rudiment contains skeletal elements required for survival as an adult sea urchin. I investigated the effects of thyroid hormones on development and skeletogenesis (*de novo* generation of skeleton) in sea urchins. As well, I investigated a mechanism through which thyroid hormones act on skeletogenesis. Thyroid hormones accelerate initiation of skeletogenesis in several stages of *Strongylocentrotus purpuratus* larval development, including during gastrulation, formation of the posterodorsal arms, and development of the juvenile rudiment. Thyroid hormones bind to a membrane receptor in skeletogenic primary mesenchyme cells. Pharmacological inhibition suggests that this membrane receptor is an integrin and triggers a mitogen activated protein kinase cascade acting through ERK1/2 which phosphorylates transcription factors regulating skeletogenesis, including Ets1 and Alx1.
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INTRODUCTION

Skeletogenesis, the process by which skeleton is formed de novo, has been a particular focus of a great deal of research on *S. purpuratus*. Skeletogenesis in the embryo is well studied, with nearly the entire relevant gene regulatory network driving and regulating this process having been identified (Longabaugh, 2012). As well, thyroid hormones have long been known to accelerate development in larval sea urchins (Chino et al., 1994; Heyland and Hodin, 2004; Saito et al., 1998). If thyroid hormones act to accelerate development, it is reasonable that they might act on skeletogenesis. Given the well-studied nature of skeletogenesis, it is possible to determine the mechanisms through which thyroid hormones act, by inhibiting pathways regulating skeletogenesis or measuring gene expression in response to TH. Here, I examine the effects of thyroid hormones on larval skeletogenesis in an attempt to discover the mechanism or mechanisms by which thyroid hormones regulate sea urchin development.

Non-genomic and genomic mechanisms of TH action

Thyroid hormones (TH) accelerate development and metamorphosis in a variety of organisms, including amphibians and fish (Galton, 1992; Gomes et al., 2015). There are two main categories of thyroid hormone signaling mechanisms which have been found in vertebrates: genomic and non-genomic (Figure 1). While regulatory effects of thyroid hormones have been described in invertebrates, including sea urchins, molluscs, and cnidarians, no mechanism has been characterized, as of yet (reviewed in Taylor and Heyland, 2017).

Genomic mechanisms involve the nuclear thyroid hormone receptor (TR) binding to hormone response elements (TRE) within the promoter region of many genes (reviewed in Cheng et al., 2010). In the absence of TH, the TR inhibits transcription by binding to corepressors. When TH is present, the TR remains bound to the TRE but a conformational change causes it to bind coactivators instead and promote transcription. In vertebrates, this is the canonical system of TH signaling. The thyroid hormone, 3,5,3’,5’-tetraiodothyronine (T4, also known as thyroxine), is released from the thyroid and deiodinated to 3,3’,5-triiodothyronine (T3, also known as L-thyronine) both at and before reaching target tissues. T3 is then transported to the nucleus, where it binds TR, promoting transcription. In zebrafish, T2 has also been identified as a ligand of TR (Orozco et al., 2017), and in Amphioxus (Cephalochordata), Triac, a
deaminated form of T3, is the active hormone binding the nuclear thyroid hormone receptor (Paris et al., 2008).

Nongenomic mechanisms of thyroid hormone action are more diverse (reviewed in Davis et al., 2016). Generally, they are characterized by a more rapid response time with the mechanism being the phosphorylation and consequent direct activation of proteins. Nongenomic mechanisms can generate a response in seconds or minutes. In vertebrates, virtually all cells are capable of responding to TH in a non-genomic fashion (Davis et al., 2008), for example via TH binding to a membrane receptor, integrin αVβ3. In this case, T4 binds to a binding site within the RGD binding pocket of integrin αVβ3, triggering a mitogen-activated protein kinase signaling cascade. This process has been shown to induce angiogenesis (Bergh et al., 2005; Davis et al., 2016), regulate sodium channels during development of the nervous system (reviewed in Lin et al., 2012; Yonkers and Ribera, 2008), and induce proliferation of osteoblasts (Scarlett et al., 2008), among other functions (Davis et al. 2016). Other non-genomic mechanisms include binding to cytoplasmic or membrane-bound TR, as well as some unidentified mechanisms in mitochondria.

**Sea urchin life history**

Among invertebrates in which thyroid hormones have been found to regulate development, sea urchins are an excellent model for investigating the effects and mechanism. *Strongylocentrotus purpuratus* is often used as a model organism for studying development and skeletogenesis. It is easy to raise and care for pluteus larvae, producing thousands of larvae with a modicum of effort. It is also the subject of a vast body of literature on development and embryogenesis reaching back to the 19th century. The sea urchin was originally chosen as a model for embryogenesis because it has a transparent embryo, allowing many processes to be easily observed in vivo. The *S. purpuratus* genome has been sequenced (Consortium et al., 2006) and transcriptomes of larval and adult sea urchins are available and well annotated (Samanta et al., 2006; Tu et al., 2012). Another advantage is that there are closely related sea urchins with both direct and indirect modes of development, allowing comparison and examination of the evolutionary events that lead to the evolution of life histories and life history transitions (Raff, 1992).
Sea urchins use a variety of life history strategies. Some urchins invest considerable resources in their eggs, providing them with the nutrients required to develop directly to adulthood. Others, such as *S. purpuratus*, invest fewer resources in a greater number of eggs, which hatch into a feeding larval stage (Figure 2). Urchins with feeding larvae undergo a free-swimming “pluteus” stage where the pelagic larva eats plankton, disperses, and prepares for settlement. As the larva reaches metamorphic competence, it grows a juvenile rudiment. This rudiment contains the structures necessary for metamorphosis and will eventually absorb the resources of the larva and emerge as a juvenile sea urchin (Warner et al., 2012).

Biomineralization is an important and common process for providing hard biological materials (Kirschvink and Hagadorn, 2000). As an adaptation it can provide protection and structure or assist in feeding or locomotion. Organisms in almost every phylum of the metazoa produce biominerals. Especially notable for their extensive skeletal use of biomineralization are Echinoderms, Vertebrates, Arthropods, and Molluscs. Echinoderms are the closest relatives to the Vertebrates that produce a mineralized skeleton. Although their unique skeletal morphologies and fossil records indicate Echinoderms probably evolved biomineralization independently (Knoll, 2003), it has been proposed that they share many components of a common toolkit for producing biominerals including carbonic anhydrases and collagens with vertebrates (Livingston et al., 2006).

Most sea urchins produce biominerals in both phases of their ontogeny, the adult and the pluteus larva. Sea urchin biominerals are primarily calcium carbonate and magnesium, with the remainder composed of skeletal matrix proteins (Wilt, 1999). In the larva, the skeleton begins forming during embryogenesis. The long skeletal arms provide structural support for the ciliated band used in feeding, as well as protection from predators. The larval skeleton also shows notable adaptive phenotypic plasticity. When deprived of food, the arms grow longer and a higher, more efficient feeding rate can be observed (Boidron-Metairon, 1988; Hart and Strathmann, 1994).

In the adult, the major components of crucial protective and feeding structures are biomineralized. In addition to the obvious large skeletal test, the tube feet, spines, and teeth are also composed in part of biominerals. For the adult, skeletal structures aid in protection, supporting the rigid spines and test. As well, the teeth are essential for feeding in most species of
A skeletal ring exists in the tube feet which may be necessary for them to attach to surfaces (Smith, 1937).

In echinoids (sea urchins and sand dollars) the adult skeleton begins forming in what is known as the adult rudiment, growing within the larva before metamorphosis (Yajima and Kiyomoto, 2006). When the pluteus larva has all the required components for metamorphosis and is able to settle, it is considered metamorphically competent (discussed in Bishop et al., 2006). Before the pluteus larva settles and metamorphoses, many skeletal elements are ready to function, including the test, spines, and tube feet (Heyland and Hodin, 2014). The teeth, as well as muscle attachment sites, develop in the weeks following settlement. The tube feet are necessary for settlement and the test and spines are likely essential for protecting against predation. This makes it necessary to form skeletal structures while the echinoid is still in the larval stage, so they may be ready for use immediately upon settlement (Cameron and Hinegardner, 1974).

**Thyroid hormones regulate sea urchin development**

Thyroid hormones have been shown in some urchins to regulate development to metamorphosis (Figure 3). Irregular urchins (sand dollars) have been shown to exhibit accelerated development in response to T4 (Heyland and Hodin, 2004; Saito et al., 1998). As well, TH levels rise throughout development, peaking at metamorphic competence (Saito et al. 1998). It seems that the source for TH in some feeding urchin larvae may be exogenous, from the algae they consume (Heyland and Moroz, 2005). However, when exposed to thiourea (an inhibitor of TH synthesis), irregular urchins display delayed metamorphosis (Saito et al., 1998) and development to metamorphic competence (Heyland & Hodin, 2004). This suggests that thyroid hormone is both produced endogenously and/or derived exogenously in some echinoids. It has been hypothesized that sea urchin larvae obtain TH both from synthesis and from their algal food source, which can contain up to 1% iodinated compounds by weight (Heyland and Moroz, 2005).

In the presence of T4, the irregular echinoid *Dendraster excentricus* developed more quickly to metamorphic competence and showed an apparent shift in resources towards developing a juvenile rudiment (Heyland and Hodin 2004). This suggests T4 has an inhibitory effect on growth in the larval arms, but an acceleratory effect on skeletogenesis in the rudiment.
There are multiple ways TH can elicit pleiotropic effects on different cells and tissues. For example, in vertebrates, there are multiple thyroid hormone receptors which are expressed in different organs. Vertebrates have two main nuclear thyroid hormone receptors, TRα and TRβ. TRβ, for example, is expressed in the liver, retina, and brain, among other regions. As well, there is the membrane receptor, integrin αVβ3, which is expressed in nearly all vertebrate tissues, regulating angiogenesis, skeletogenesis, and neural development, for instance.

In the case of sea urchins, there are several possible explanations for the multiple effects of thyroid hormones. Sea urchins only have a single nuclear thyroid hormone receptor ortholog, so a difference between nuclear receptors can be eliminated as a possibility. In the simplest scenario, a membrane receptor could be responsible for the effect on skeletogenesis and a nuclear receptor responsible for the effect of T4 on larval arm retraction. The truth is potentially more complex. It is possible that a combination of signaling through both the membrane receptor and the nuclear receptor regulates developmental processes in sea urchins, as, for example, in vertebrate angiogenesis (Luidens et al., 2010).

**The skeletogenic gene regulatory network**

To investigate the mechanism by which thyroid hormones might act, it is necessary to understand the processes which they regulate. The developmental events and gene regulatory network leading to skeletogenesis in larval sea urchins are uncommonly well understood (Figure 4).

Primary mesenchyme cells (PMC) are mesenchymal cells which secrete skeleton in larval sea urchins. In *S. purpuratus*, at the 16 cell stage in the early embryo, the micromeres begin differentiating into PMCs. These PMCs then migrate to the blastocoel where they arrange into a ring (Decker and Lennarz, 1988). The location of this ring is coordinated by a signaling protein released by the ectoderm; vascular endothelial growth factor (VEGF) (Duloquin et al., 2007).

As the PMCs migrate to the ring, they merge in a syncytial network, losing their cell boundaries and becoming effectively one cell with many nuclei. Subsequently, the PMCs migrate to two clusters within the ring, guided by further ectodermal signals. Each PMC cluster initiates skeletogenesis, in the form of a tri-radiate spicule. The spicules undergo further elongation and branching to eventually form the larval skeleton.
The mechanisms of skeletogenesis in the juvenile rudiment are less well understood, but the morphology has been characterized, and a staging scheme developed in Heyland and Hodin (2014). Skeletogenesis in the rudiment is driven by secondary mesenchyme cells which undergo a transition to become skeletogenic late mesenchymal cells. These late mesenchymal cells are similar to primary mesenchyme cells (Yajima, 2007). As in early larval skeletogenesis, the late mesenchymal cells first form tri-radiate spicules. These spicules subsequently branch out to form either skeletal rings that will support the juvenile tube feet, or spine primordia from which the juvenile spines will extend. At the same time, the juvenile test begins developing beside the larval gut.

In recent years much information has been published on the genetic underpinnings of larval skeletogenesis in *S. purpuratus*, especially the gene regulatory network in the primary mesenchyme cells. At the root of PMC differentiation are two repressors, the transcription factors *pmar1* and *hesC* (Revilla-i-Domingo et al., 2007). Essentially, *hesC* is expressed in all cells and represses the skeletogenic fate. Activation of *pmar1* represses *hesC*, allowing the transcription factors *Tbr*, *Ets1*, and *Alx1* to be transcribed. *Ets1* and *Tbr* activate the promoters for *Tgif*, and possibly also for *Erg* and *Hex*. *Tgif*, *Erg*, and *Hex* are part of a self-amplifying toggle that stabilizes the cell fate as skeletogenic (McCauley et al., 2010). This loop acts in concert with the extracellular signal VEGF and a MAPK signaling cascade to promote a number of genes known to be necessary for skeletogenesis, such as the skeletal matrix protein SM50 (McCauley et al., 2010). A MAPK (ERK1/2) cascade phosphorylates the key regulatory transcription factors, *Ets1* and *Alx1* and is required for skeletogenesis to occur (Röttinger et al., 2004).

Investigation of thyroid hormone regulation of skeletogenesis then, should begin with an examination of the effects of THs on PMC spicule formation, MAPK signaling cascades, and transcription factors regulating skeletogenesis such as *Ets1*.

**Questions and hypotheses**

There are two questions I attempt to address in this thesis: what are the effects of thyroid hormones on skeletogenesis in sea urchins, and what is the mechanism by which they occur? To characterize the effects of TH on sea urchins, I exposed the larvae of *S. purpuratus* to a variety of thyroid hormones and quantified the rate of skeletogenesis after TH exposure using larval
staging techniques developed in Heyland and Hodin (2014), as well as by tracking the presence or absence of skeletal spicules. I hypothesized that TH would accelerate skeletogenesis in larval sea urchins.

As well, I hypothesized that TH in sea urchins might act non-genomically, previous efforts having failed to show TH binding to the sea urchin TR ortholog (A. Heyland, unpublished data). Seeing some homology between sea urchin skeletogenesis and vertebrate angiogenesis, I further hypothesized that TH acts by binding to an integrin membrane receptor, triggering a MAPK cascade, phosphorylating and activating transcription factors regulating skeletogenesis. I tested my hypotheses by exposing gastrulae to both THs and pharmacological inhibitors of MAPK and integrin αVβ3.

MATERIALS AND METHODS

Adult and larval urchins

Adult urchins (S. purpuratus) were shipped from Monterey, CA, where they were collected by diving and subsequently kept in tanks of artificial seawater at the Hagen Aqualab (University of Guelph, ON). The adults were fed a diet of kelp (Macrocystis pyrifera, and Kombu spp.) every 2-3 days. Temperature was maintained at 12-14°C and salinity at 31 g/L.

Urchins were spawned by injecting 0.5-2 mL of 0.5 M KCl, depending on the size of the urchin. Sperm was collected dry by pipette. Females were inverted over a beaker of filtered artificial seawater (FASW) to collect eggs. Eggs were passed through a 120 μM filter to remove debris, before being washed twice with FASW. Sperm was diluted in 1 mL FASW, before being added slowly to the beaker of eggs until fertilization success as determined by presence of a fertilization envelope reached >90%. Fertilized eggs were subsequently washed once more with FASW to remove excess sperm and allowed to develop at 12°C in a 1 L beaker until hatching.

Hatched embryos were transferred to 2 L beakers at a density of 1 larvae/mL. Sea urchin larval cultures were maintained at 12-14°C with salinity at 31-33 ppt. Cultures were stirred constantly and kept on a 12:12 light cycle. The cultures were cleaned and had the water replaced three times weekly. At the same time, cultures were fed Rhodomonas sp. at a density of 6000 cells/mL.
**Hormone preparation**

Thyroid hormones, including T4, T3, rT3, T2, Tetrac, and Triac were dissolved in DMSO at 100 mM before being diluted to 1 mM in FASW (Thyroid hormones ordered from Sigma-Aldrich). Aliquoted hormones were stored at -20°C for up to 12 months. To expose larvae, THs were diluted in FASW to the required concentration and larvae were placed in that FASW. FASW with DMSO and/or FASW with rT3 and DMSO was used as a vehicle control in every experiment.

**Morphological experiments**

Larvae were exposed to THs and the morphological effects were observed and quantified. In all cases, larvae were imaged using polarized light microscopy on a Nikon TI Eclipse compound microscope to enhance visibility of skeletal elements.

Both gastrulae and 12 day-old larvae were used to quantify the effects of TH on skeletogenesis. Initiation of skeletogenesis was scored by the presence or absence of spicules in either the border-ectoderm/dorsal-ventral margin intersection in gastrulae, or at the base of the newly forming postero-dorsal arms in 12 day-old larvae. In the acute experiments, larvae were kept in 6-24 well plates at a density of 20 embryos/mL for gastrulae, or 1 larvae/mL for 12 day-old larvae. In the 12 day-old groups, water was changed and larvae were fed 6000 c/mL every 2 days. The gastrulae were kept a maximum of 26 hours post hatching and did not require feeding or water changes.

Late stage larvae were used to determine the effects of THs on rudiment development and larval arm retraction. Late stage larvae were kept in the same fashion as 12 day-old larvae. The development of the rudiment was subsequently scored by the staging scheme developed in Heyland and Hodin (2014). Both soft tissue and skeletal elements were assessed as described in Heyland and Hodin (2014).

**Fluorescently labeled THs**

Thyroid hormones, including T4, T3, and rT3 were conjugated with rhodamine to produce fluorescently labeled thyroid hormones. Synthesis was carried out as in Cheng et al. (1979), with several exceptions. Thyroid hormones were mixed with equimolar TRITC (mixed
isomers, Sigma-Aldrich 95197-95-8) to conjugate rhodamine to the amino group of the thyroid hormones. Reaction was stirred and allowed to proceed for 1 hour at room temperature. Products were purified by column chromatography and checked by thin layer chromatography (solvent used for both chromatographies was ethyl acetate:methanol:water, 5:2:3 volume, as in original protocol). Solvent was then evaporated, leaving behind acceptably pure rhodamine conjugated hormones. Rhodamine-reverse thyronine (RH-rT3) was synthesized by substituting rT3 for T3 in the reagents.

Live larvae were exposed to rhodamine-thyroxine (RH-T4), rhodamine-thyronine (RH-T3) or rhodamine-reverse thyronine (RH-rT3) at the biologically relevant concentration of $10^{-8}$ M. They were incubated for 30 minutes. Larvae could then be fixed for immunohistochemistry, or imaged live. Larvae were imaged under 557 nm light, the excitation wavelength of TRITC. Filters were used to examine only the emission wavelength of TRITC (Nikon Bandpass filter cube, excitation 540 nm, emission 605 nm).

qRT-PCR

Gastrula were lysed in TRIzol reagent at -20°C overnight. RNA extraction was carried out using a Direct-zol™ RNA MiniPrep kit, following the manufacturer’s instructions (Zymo Research). Concentration and RNA purity were checked using Nanodrop 8000 Spectrophotometer (Thermo Scientific). RNA was copied to cDNA with an Applied Biosystems cDNA synthesis kit as per the provided protocol and stored at -20°C until use. qRT-PCR was performed on a StepOne Plus with SYBR Green dye. Ets1 (SPU_002874) primer concentrations were optimized using a test mix of cDNA from 2 day, 12 day, and 25 day-old larvae. 400 nM was found to be the most effective concentration for both forward and reverse primers (Primers ordered from Invitrogen, Ets1 Forward 5’ CGCAAAAAACAAACCCCAAGAT 3’, Reverse 5’ TCTGCAGGTCACAGACGAAG 3’, Rizzo et al. 2006). Transcript levels were compared to Ubiquitin (Ubq, NM_214533) as a housekeeping gene, using the ΔΔCt method (ΔΔCt reviewed in Schmittgen and Livak 2008) with a primer concentration of 300 nM (Ubq Forward 5’ CACAGGCAAGACCATCACA 3’, Reverse 5’ GAGAGAGTGCGACCATCCTC 3’).
**Immunohistochemistry**

Gastrulae were stained with a custom 6a9 antibody (mouse monoclonal, obtained from the Ettensohn lab at Carnegie Mellon University) to examine primary mesenchyme cell membranes. M8159 (mouse monoclonal, Sigma-Aldrich) was used to quantify MAPK (ERK1/2) phosphorylation. Gastrulae were fixed for whole mount immunohistochemistry in methanol at -20°C for 5 minutes before being transferred to PBS. Fixation in paraformaldehyde was found to be unnecessary and damaging to larval tissues. After immunohistochemistry, larvae were imaged under fluorescent light with a Cy5 filter (Nikon Bandpass filter cube, excitation 620 nm, emission 700 nm).

**Statistical analysis**

There are two sorts of dependent variables which were examined, binary and ordinal. Binary data described the presence or absence of a morphological feature such as a skeletal spicule. Ordinal data described the developmental stage of the larvae, according to the staging scheme put forward in Heyland and Hodin 2014. Independent variables included continuous variables such as time post fertilization, or concentration of a pharmacological compound, as well as categorial variables such as treatment group.

In cases where the dependent variable was binary and there was a single independent variable, a Pearson’s Chi Square test with a single degree of freedom was used to determine significant differences between groups. Each treatment group, including those at different concentrations or lengths of hormone exposure, was treated as an independent group. Treatment groups were compared to the control, and to each other treatment group using two-tailed two-sample Z tests, with a $p < 0.05$ considered to be significant, adjusted by Bonferroni correction using the default method in SPSS 25.

In cases where the dependent variable was binary and there were multiple independent variables, a generalized linear model (binary logistic regression) was used, with Bonferroni corrected pairwise post-hoc comparisons. Significance of the individual treatment groups relative to the control was tested, as well as whether treatment groups had a significant effect on the model, and whether the interaction between treatments was significant. Test statistics are reported as $W_n$, (Wald Chi-Square).
In cases where the dependent variable was ordinal (such as when a developmental staging scheme was used), the Mann-Whitney U test was used to determine significant differences between groups. The Mann-Whitney U test was chosen because it does not assume a normal distribution. This was necessary, because the developmental stages measured cannot be assumed to have an equal duration. The U statistic is reported.

Statistics were conducted in SPSS 25. Graphs were created in Excel 2010. Unless otherwise noted, proportions and means are reported as +/- the standard error.

RESULTS

Thyroid hormones accelerate skeletogenesis

In all ages of larvae tested (gastrulae, early stage, and late stage larvae), T4 was found to accelerate skeletogenesis in a dose dependent manner (Figure 5: gastrulae and early stage larvae; Figure 8: late stage larvae). T4 increased the rate of spicule formation in gastrulae and early pluteus larvae relative to both the control, and to the biologically inactive rT3 (Figure 5; 3.1-fold in gastrulae, $W_n = 48.7$, $p < 0.001$; 2.3-fold in early stage larvae, $W_n = 35.7$, $p < 0.001$). In the late stage larvae, the rudiment reached an average skeletal stage of 8.18, with growing juvenile spines and tube feet, as compared to the control with an average stage of 2.00: initial spicule formation (Figure 8, Mann-Whitney U = 99.0, $p < 0.001$). T4 was not found to significantly affect soft tissue development in the rudiment (Figure 8, Mann-Whitney U = 81, $p = 0.63$). High levels of T3 and T2 (100 nM, Figure 5, $W_n = 32.5$, 26.3 $p < 0.001$) were also found to accelerate spicule formation at the onset of skeletogenesis. No effect of Tetrac was found ($W_n = 0.4$, $p = 0.5$) while Triac inhibited skeletogenesis in gastrulae and early pluteus stages (Figure 5, $W_n = 4.1$ $p < 0.05$).

In the 12 day-old larvae, thyroid hormones were noted to cause aberrations in skeletal growth (Figures 6, 7). Ectopic skeleton (spicules forming outside the posterodorsal arms) was observed, as well as duplicate formation of the posterodorsal arms, and skeletal protrusions along the postoral arms. No specific location could be identified for ectopic skeleton formation, which occurred in a variety of positions. Only T4 and T3 caused skeletal abnormalities, with protrusions and duplicate posterodorsal arms being observed with higher exposure to T4 (Chi Square, $\chi^2 = 132.9$, 124.1, Z-test: $p < 0.001$), where nearly every larvae examined had a skeletal
aberration. Ectopic skeleton was seen at all levels of T4 exposure and the higher level of T3 exposure (Chi Square, $\chi^2 = 55.6$, Z-test: $p < 0.001$). No skeletal abnormalities were observed in the control or rT3 groups.

**Fluorescently labeled T4 shows T4 binding to PMC membranes**

Rhodamine conjugated T4 bound to primary mesenchyme cells, while RH-rT3 had no specific binding. Specifically, RH-T4 bound to the membrane and/or extracellular matrix of PMCs. Immunohistochemistry with 6a9, a sea urchin (*S. purpuratus*) specific antibody for the membrane of primary mesenchyme cells was used to confirm binding locations (Figure 9). RH-T4 colocalized with 6a9 staining, suggesting that RH-T4 does bind to the membrane of primary mesenchyme cells.

RH-rT3, the biologically inactive control, did not bind to any specific cells, or to specific regions within cells. It also did not colocalize with 6a9 antibody staining to any degree.

**Acute and chronic exposures to T4 show similar effects**

Larvae exposed to T4 for 1 hour showed a similar rate of acceleration of skeletogenesis when compared to larvae chronically exposed for 5 days. The higher concentration of T4 tested showed a significant acceleration of skeletogenesis relative to the control, with no discernable differences between acute and chronic exposures (Figure 10, acute 2.6-fold, chronic 2.2-fold relative to control, $\chi^2 = 15.9$, $p$ values < 0.01). The lower concentration tested was more variable with some larvae showing early skeletogenesis and others not producing spicules for the duration of the experiment. The lower concentration did not register as statistically significant, although trending towards an acceleration of skeletogenesis ($\chi^2 = 2.7$, $p = 0.10$).

**Inhibitors of ERK 1/2 show a MAPK-mediated mechanism**

When gastrulae were exposed to two inhibitors of MAPK signaling, SB203580, and PD98059, both with and without added T4, I found that T4 induced skeletogenesis was inhibited by PD98059, but not by SB203580.

SB203580, an inhibitor of p38, did not alter the effect of T4 on skeletogenesis (Figure 11, binary logistic regression, $W_n = 0.07$, $p = 1.00$) at any of the three concentrations tested (100
nM, 1 μM, 10 μM). The interaction between SB203580 and T4 was also not significant at any concentration tested (p = 0.07 to 1.00).

All concentrations of PD98059, an inhibitor of ERK1/2, significantly reduced the effect of T4 on skeletogenesis (Figure 12). The 0.5 μM and 5 μM concentrations caused a diminished effect of T4, such that it was not statistically different from the control (binary logistic regression, \( W_n = 0.00 \ p = 1.00 \)), with the phenotype being partially rescued by the highest level of T4 tested (\( W_n = 86.8, p < 0.001 \)). The highest level of PD98059 tested completely inhibited skeletogenesis (\( W_n = 153.9, p < 0.001 \)), with the effect being partially rescued by even low levels of T4 (\( W_n = 13.8, p < 0.001 \)). The interaction between T4 and PD98059 was significant (\( W_n = 121.5, p < 0.001 \)).

**PCR of Ets1 shows upregulation in response to T4**

A pooled sample of 1000-2000 gastrulae which were incubated in T4 for 90 minutes showed a large upregulation of Ets1, a transcription factor regulating skeletogenesis which is known to be phosphorylated and activated by MAPK (Figure 13; Röttinger et al., 2004). Gastrulae which were pre-incubated with PD98059 before being incubated with T4 did not show an upregulation of Ets1. No statistics were performed as a large number of individuals were pooled in a single RNA sample. Both levels of T4 in the absence of PD98059 increased expression levels of Ets1 by 46-fold or 6.9-fold respectively. This effect disappeared in the presence of PD98059.

**Effects of RGD on T4 activation of MAPK and promotion of skeletogenesis**

Gastrulae were either exposed to T4, RGD, or T4 and RGD and showed increased levels of phosphorylated MAPK and skeletogenesis in the presence of T4, an effect which was inhibited with the addition of RGD (Figures 14, 15). RGD is an inhibitor of T4 binding to integrin αVβ3 in vertebrates which competitively inhibits the binding pocket. Gastrulae exposed to T4 alone showed a dose-dependent acceleration of skeletogenesis, having more skeletal spicules after a 20 hour exposure (binary logistic regression, \( W_n = 41.6, p < 0.001 \)). The addition of low levels of RGD inhibited the effect of T4 on skeletogenesis, an effect which was rescued by the highest levels of T4 tested (Interaction between T4 and RGD, \( W_n = 2307.42, p < 0.001 \); comparison between control and RGD inhibited groups, \( p = 1.00 \); Comparison between control and T4 rescued groups, \( p < 0.01 \)). The addition of higher levels of RGD was sufficient to
suppress skeletogenesis in all gastrulae that I checked, an effect which was again rescued by higher levels of T4 (RGD inhibition of skeletogenesis: $W_n = 31.8, p < 0.001$, T4 rescue of skeletogenesis: $W_n = 41.6, p < 0.001$).

When the phosphorylation of MAPK (ERK1/2) in the presence of T4 or T4 and RGD was assessed using immunohistochemistry, T4 caused clear phosphorylation of ERK1/2 while RGD inhibited this effect. In the control, MAPK was phosphorylated at the vegetal pole of the gastrula, the region in which primary mesenchyme cells form. T4 caused a clear increase in MAPK (ERK1/2) phosphorylation levels across the entire gastrulae, but especially in the same vegetal region and in the basal membranes of epidermal cells. RGD prevented this effect of T4 on cells in the vegetal pole and primary mesenchyme cells, but did not fully mitigate the effect of T4 on the basal membrane of epidermal cells at lower concentrations. The highest concentration of RGD tested did fully prevent the effect of low levels of T4 on MAPK phosphorylation, though it was not able to inhibit the highest levels of T4 tested.

**DISCUSSION**

Thyroid hormones, specifically T4, T3, and T2 had an acceleratory effect on skeletogenesis as measured by skeletal spicule production. When the binding locations were investigated, I found that T4 bound to the membrane of primary mesenchyme cells in the developing gastrulae. Further, inhibitors of both integrin αVβ3 and MAPK(ERK1/2) prevented the acceleratory effect of T4 on skeletogenesis. Together, this evidence suggests T4 binds to an integrin, triggering a MAPK (ERK1/2) signal cascade, accelerating skeletogenesis.

**Differential effects of thyroid hormones**

In all experiments where multiple thyroid hormones were tested, T4 shows the strongest effect on skeletogenesis, while T3 and T2 show similar magnitudes of acceleration of skeletogenesis, but at higher concentrations, suggesting a lower binding affinity. Triac had an inhibitory effect on skeletogenesis, while Tetrac and rT3 show no effect on the rate of initiation of skeletogenesis. These results are consistent with how integrin-mediated TH signaling works in vertebrates, in that T4 has the highest binding affinity in comparison to T3 and Triac acts antagonistically to T4. This is also consistent with how TH signaling has been shown (so far) to work in other invertebrates. In nearly all cases tested, T4 is the most active TH. Cnidaria, for
example, do not even produce T3, but biomineralization is accelerated by T4 (reviewed in Taylor and Heyland 2017).

T4 accelerates skeletal stages in the rudiment dramatically, but has no noticeable effect on timing of soft tissue development during the initial stages of rudiment growth. The effect of T4 on soft tissue was checked to clearly isolate the effect that T4 has on rudiment development. Previous studies have shown that thyroid hormones accelerate rudiment development (Chino et al. 1994, Heyland and Hodin 2004). If both soft tissue and skeletogenesis was accelerated by T4, it would be possible that T4 was having an indirect effect on skeletogenesis by accelerating the development of the required soft tissue. The fact that soft tissue was not accelerated by thyroid hormones provides evidence that the acceleration of rudiment development found in previous work may be attributed to a direct effect of T4.

While early soft tissue development is not accelerated by T4, there are soft tissue elements in later structures which are accelerated by T4. The role of T4 in accelerating later rudiment structures may be either indirect, through the acceleration of skeletogenesis of elements which are necessary support structures for soft tissue development, or it may be more direct.

High levels of T4 or T3 can cause skeletal abnormalities such as protrusions from the larval arms, duplicate larval arms, or ectopic skeleton. Activation of skeletogenesis in migrating PMC cells, or cells not at usual sites of skeletogenesis, suggests that high levels of TH may be sufficient to initiate skeletogenesis without VEGF or other external signals. This only occurs with very high levels of TH, however, and it is likely that TH is not the sole initiator of skeletogenesis in sea urchin larvae. The second possibility explaining ectopic skeleton is that sufficiently high levels of TH may stimulate release of VEGF in ectoderm outside the developing arm tips. The third possibility is that VEGF or other external signals may be present outside the arm tips and with the addition of enough T4, a combination of signals activates skeletogenesis. Since I have already shown that TH stimulates MAPK phosphorylation of transcription factors controlling skeletogenesis it seems most likely that T4 is stimulating skeletogenesis either directly or in conjunction with other signals such as VEGF, rather than stimulating skeletogenesis indirectly, such as by triggering the release of VEGF.
Thyroid hormones bind to primary mesenchyme cells

Fluorescently conjugated thyroid hormones provided evidence that T4 binds to sea urchin gastrulae. In gastrulae, T4 was found to bind to the membrane, and/or the extracellular matrix of primary mesenchyme cells. Primary mesenchyme cells are the cells which build skeleton in larval sea urchins. The colocalization with 6a9, an antibody for the membrane of PMCs, was complete, varying only in intensity but not localization. The binding of RH-rT3, a rhodamine conjugated form of the non-biologically active form of T3, was entirely non-specific. The lack of specific binding of RH-rT3 suggests that the specific binding of RH-T4 reflects actual binding locations of T4. The T4 receptor might therefore be found in the PMC membrane or extracellular matrix. T4, then, is the cause of PMC staining and not residual rhodamine, or other interactions. I cannot exclude a nuclear receptor with these results, as it is possible that RH-T4 would not make it through the membrane and into the nucleus or cytoplasm. It is also possible that RH-T4 bound to a thyroid hormone transporter, and not to a functional receptor. The most likely possibility is that T4 binds to an integrin membrane receptor, as a result of the inhibitor experiments discussed later.

In vertebrates, THs affect mesenchymal cells via both genomic and non-genomic pathways. Non-genomically, THs can bind to the membrane of fibroblasts, a mesenchymal cell responsible in part for angiogenesis (Bergh et al., 2005). Some intriguing parallels exist between vertebrate vascular mesenchyme derived cells and sea urchin primary mesenchyme cells (Figure 16). In vertebrates, a combination of VEGF and TH-mediated MAPK (ERK1/2) signaling regulate angiogenesis through phosphorylation of Ets proteins (Ferrara et al. 2003, Tomita et al. 2003). Sea urchin skeletogenesis also requires VEGF and phosphorylation of Ets1, a regulatory system which was originally only activated in the adult urchin, but later became co-opted to regulate larval skeletogenesis (Morino et al., 2012). There are differences, notably the lack of certain transcription factors in the vertebrate mechanism, as well as the negative feedback regulating the VEGF receptor, VEGFR. While the purpose of some transcription factors differ between the two systems, my evidence suggests these systems are conserved modules of mesenchymal cell regulation which have evolved divergent functions in sea urchins and vertebrates.
Evidence for a non-genomic mechanism

MAPK cascades achieve peak phosphorylation in 3-15 minutes (Kholodenko and Birtwistle, 2009) as compared to TH binding to the nuclear thyroid hormone receptor which often requires hours to days to have a regulatory effect (amphibian metamorphosis is a classic example, see Tata, 2006 for review). Some effects observed, namely initiation of skeletogenesis in gastrulae, occur and are affected by TH exposure on smaller time-scales than would be expected for a nuclear receptor. In gastrulae, a 20 hour exposure drastically accelerates skeletogenesis. As well, acute exposure of only 1h to high levels of TH is sufficient to cause acceleration of skeletogenesis in 12 day-old larvae. Finally, Ets1 and other expression level changes are observed in gastrulae after only 1-1.5h exposures (Figure 13). Together, this suggests a non-genomic mechanism of TH action, for example via phosphorylation.

My experiments with fluorescently labeled T4 provided evidence that T4 binds to the membrane of primary mesenchyme cells. A non-genomic mechanism of thyroid hormone action would proceed via either a membrane receptor or cytoplasmic receptor. The binding of T4 to PMCs along with the discussed homologies to vertebrate mesenchymal cell regulation suggest a role for a membrane bound integrin in TH regulation of skeletogenesis.

Finally, there have been previous efforts to characterize TH binding to a putative nuclear receptor in sea urchins (V. Laudet, A. Heyland, pers. com.). A variety of THs have been tested, but none have been found to bind to the nuclear receptor. Similarly, attempts to characterize the thyroid hormone nuclear receptor in other invertebrates have failed (reviewed in Taylor and Heyland 2017).

Thyroid hormones regulate skeletogenesis through MAPK

Two MAPKs have been found to be relevant to skeletogenesis in sea urchins, ERK1/2, and p38. Of those, ERK1/2 is necessary for skeletogenesis to occur and is known to act by phosphorylating Ets1, a transcription factor regulating skeletogenesis. While SB203580, an inhibitor of p38, did not prevent the effect of T4 on skeletogenesis (Figure 11), PD98059, an inhibitor of ERK1/2, did prevent the acceleration of skeletogenesis by T4. Moreover, groups treated with low to medium levels of PD98059 in the presence of all but the highest levels of T4 did not accelerate skeletogenesis significantly. This data suggests that ERK1/2 is a necessary
intermediary for the skeletogenesis caused by T4 at physiologically relevant concentrations of T4. Interestingly, the highest levels of PD98059 tested prevented skeletogenesis entirely, an effect which was partially rescued by T4. This confirms that MAPK (ERK1/2) signaling is essential for skeletogenesis in sea urchins, as was previously found by Rottinger et al. (2004).

ERK 1/2 phosphorylates Ets1, thereby regulating a variety of genes which are differentially expressed in primary mesenchyme cells (Rafiq et al., 2014). I performed qRT-PCR of Ets1, which, after a short 90 minute exposure to T4, showed Ets1 to be greatly upregulated. This effect vanished in the presence of PD98059, meaning the effect of T4 on the upregulation of Ets1 is also MAPK (ERK1/2) mediated. This suggests that the phosphorylation of Ets1 and its subsequent activation leads to the autoinduction of Ets1, in a positive feedback loop, either directly caused by Ets1 or indirectly by downstream transcription factors. Further, it implies involvement of TH in the upper regulatory controls of skeletogenesis, namely the transcription factor controlling the timing of initiation. This is reinforced by morphological results of T4, T3, and T2 in gastrulae, and 12 day-old larvae, where I found no acceleration of skeletal elongation, only an acceleration of initial spicule formation. Thyroid hormones, mainly T4, but also T3 and T2, therefore trigger initiation of skeletogenesis by triggering a MAPK cascade phosphorylating Ets1.

**Thyroid hormones act through an integrin-mediated mechanism**

What then, is the membrane receptor to which thyroid hormones bind in the sea urchin? In vertebrate mesenchyme cells involved in angiogenesis, T4 binds to the αV subunit of integrin αVβ3. A potential homolog of the integrin αV subunit has been identified (Susan et al., 2000), that is expressed throughout larval development, including during gastrulation and later larval stages. In vertebrates, T4 binds to the same binding pocket as RGD peptide on the integrin αV subunit. When tested in *S. purpuratus*, RGD had an antagonistic relationship with T4 activity. These findings provide evidence for thyroid hormone binding to an integrin in sea urchins.

I found that RGD peptide prevented or inhibited skeletogenesis, depending on the concentration used. This effect was rescued by higher levels of T4, but not by lower levels. The data are consistent with a competitive inhibition similar to that shown in vertebrates (Bergh et al. 2005). It is still possible that RGD, being a signaling peptide in its own right, has other effects on
skeletogenesis. However, subsequent tests with immunohistochemistry of phosphorylated MAPK suggest otherwise.

In the control group, primary mesenchyme cells express phosphorylated MAPK (ERK1/2). This is consistent with previous work on ERK signaling during gastrulation (Fernandez-Serra et al., 2004), and indicates that ERK is activated in PMCs during normal gastrulation. However, addition of T4 greatly increases the quantity of phosphorylated MAPK in PMCs, as well as causing some MAPK phosphorylation in the basal membranes of all ectodermal cells. This suggests that T4 may have effects in the developing embryo which are not limited to primary mesenchyme cells and skeletogenesis. Regardless, addition of RGD diminishes the intensity of the phosphorylated MAPK signal, suggesting that it is preventing the activity of T4, specifically its ability to trigger a MAPK cascade. Even the highest level of RGD applied did not completely eliminate ERK signaling. This suggests that thyroid hormones may not be the only signal causing the phosphorylation of ERK during gastrulation. It did, however, prevent the activity of T4 and, together with the arguments from homology, this is strong evidence that T4 works through an integrin membrane receptor in sea urchins.

**Evolution of thyroid hormone signaling**

Thyroid hormones, including T3 and T4, have been synthesized since before metazoans evolved (Heyland and Moroz, 2005; Phatarphekar et al., 2013). Given the ubiquitous presence of iodinated compounds and proteins, some marine organisms consisting of up to 25% iodine by weight, it is likely that most animals have evolved mechanisms to respond to thyroid hormones. Sponges, for example, can consist of up to 10% iodinated compounds, mainly in the form of thyroid hormones (Roche, 1952). A large number of invertebrate species from diverse phyla respond to TH (Figure 3, for review see Taylor and Heyland 2017, Eales, 1997). Moreover, recent experimental data has uncovered new evidence for TH signaling mechanisms and regulatory functions in molluscs, sea urchins, ascidians, and amphioxus (for example, Huang et al., 2015; Paris et al., 2008; Patricolo et al., 2001; Saito et al., 2012).

Evidence has been scant for genomic mechanisms of thyroid hormone action. In molluscs and sea urchins, repeated efforts to characterize a nuclear receptor have failed. Sea urchin TR has been cloned and it was not activated by T4, T3, reverse T3, Tetrac, or Triac (A. Heyland, V. Laudet, pers. com.). In Molluscs, T4, T3, and TRIAC did not cause the putative TR to promote
transcription as detected by a luciferase assay. One possibility is that the thyroid hormone which activates these receptors has not yet been found, and is a metabolite such as T2. Another possibility is that mollusc and potentially other invertebrates including echinoderms, TR is an orphan receptor, or binds another ligand, and that TH signaling proceeds mainly via non-genomic mechanisms.

Given the ambiguity surrounding genomic mechanisms of TH action, it seems increasingly likely that non-genomic mechanisms may be responsible for TH regulation of developmental processes in some invertebrates. For example, skeletogenesis or metamorphosis have been reported to be regulated by T4 in various Cnidaria, including *Aurelia, Cassiopea* and some corals (Cabrales-Arellano et al., 2016; Nowak et al., 2009; Spangenberg, 1984). T4 is the only thyroid hormone present in Cnidaria (Tarrant, 2005), and is the most active hormone in sea urchins and molluscs. Notably, Cnidaria do not possess a nuclear thyroid hormone receptor ortholog. T4 is also the most active TH binding to integrin αVβ3 in vertebrates. With my results showing a T4 stimulated non-genomic mechanism regulating skeletogenesis in urchins, this model of non-genomic signaling in invertebrates is well supported.

Additionally, I propose that non-genomic mechanisms of thyroid hormone signaling may have evolved first. Cnidaria, a basal group, has no nuclear thyroid hormone receptor ortholog and responds to TH. Bacteria, including *E. coli*, have been shown to produce and be responsive to thyroid hormones and do not possess a nucleus, much less a nuclear thyroid hormone receptor (Phatarphekar, Buss, and Rokita 2013). I have shown that sea urchin skeletogenesis is regulated by thyroid hormones in a non-genomic fashion. It seems increasingly likely that this is the ancestral mode of TH signaling.

**CONCLUSION AND FUTURE DIRECTIONS**

The work presented here provides evidence that T4 acts in sea urchins through a membrane receptor-mediated MAPK (ERK 1/2) signaling cascade. I have also provided evidence that T4 may bind to an integrin ortholog. In combination with prior research, this suggests that the MAPK cascade triggered by T4 phosphorylates Ets1 and is necessary for skeletogenesis. This mechanism is likely a conserved module of mesenchyme cell regulation which acts in concert with VEGF.
Future work should characterize T4 binding to the integrin membrane receptor, possibly through radiobinding assays. The integrin should also be knocked out, and the effect of T4 on MAPK phosphorylation tested. This would determine the specific integrin subunits involved in sea urchin TH response, which would allow for an examination of the evolutionary history of TH signaling.

TH signaling appears to be prevalent in metazoans. With a non-genomic mechanism for T4 activity now shown in sea urchins, other invertebrates also present promising targets for investigation of non-genomic thyroid hormone signaling, especially molluscs and cnidarians. In both those cases, T4 is the most active hormone and regulates development and biomineralization. In the case of Cnidaria, no nuclear receptor is present (Tarrant, 2005). A successful characterization of TH signaling in Cnidaria might confirm the hypothesis that non-genomic TH signaling, potentially through integrin membrane receptors, is the ancestral mode.
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Figure 1. Genomic and non-genomic mechanisms of thyroid hormone action in vertebrates. T4 is released by the thyroid gland, before being deiodinated into T3. In non-genomic signaling, T4 binds directly to an integrin membrane receptor before triggering a MAPK cascade, or T3 binds to cytoplasmic TR, phosphorylating PI3K. In genomic signaling, T3 binds to TR, activating transcription by recruiting co-activators to TRE regions in the genome. Alternative ligands, including Triac, Tetrac, T2, T3, and T4 may also sometimes activate or inhibit these pathways, depending on cell type and species. Pictured here is the most commonly biologically relevant ligand to each pathway.
Figure 2. Sea urchin indirect life history. Pictured here are the major stages of sea urchin life history during which skeletogenesis occurs. Post-hatching, the embryo grows larval arms, reaching the free-swimming pluteus stage. The pluteus grows posterodorsal arms, becoming the 6-arm larva. Prior to attaining metamorphic competence, the larva grows a juvenile rudiment with skeletal structures necessary for survival as a juvenile, including adult spines and tube feet. Also pictured is the metamorphosed juvenile, and the adult sea urchin.
Figure 3. Thyroid hormone evolution. NR: Nuclear receptor, TR: Thyroid hormone receptor, Ecd.: Ecdysozoa. In all cases, the presence of a feature in a phylum does not indicate the presence of that feature in all species of that phylum. NR are ancestral to all animals. TR, however, is found only in bilaterians. Hemichordates do not possess an endostyle that participates in TH synthesis, so the endostyle is depicted as common to Chordata. A thyroid gland with TH producing thyroid follicles is unique to vertebrates. While thyroid hormone synthesis is present in many phyla, the mechanism differs and the use of thyroglobulin is unique to vertebrates. Sea urchins (Echinodermata) do not have an endostyle or thyroid gland, but do possess an ortholog of TR. The phylogenetic tree was constructed from the Open Tree of Life project (Hinchliff et al. 2015) and reproduced from Taylor and Heyland 2017.
Figure 4. Gene regulatory controls of skeletogenesis. Two main inputs are known to be necessary for skeletogenesis in sea urchins, VEGF secreted by ectodermal cells, and a MAPK (ERK1/2) cascade with an unknown trigger. Ets1 and Alx1 are transcription factors, and are the most important upper regulatory controls of skeletogenesis in sea urchins, controlling almost half of the genes differentially expressed in primary mesenchyme cells (Rafiq et al. 2014). Their activity is essential for skeletogenesis to occur. Both Ets1 and Alx1 are activated or upregulated by MAPK (ERK1/2), as well as to some degree by VEGF.
**Figure 5. Rate of initiation of skeletogenesis after exposure to thyroid hormones.** Gastrulae were exposed for 20 hours, from 24-44 hours post fertilization (n=40-81). 6 armed larvae were exposed for 4 days, from 10 days post fertilization to 14 days post fertilization (n=63). Larvae were scored at regular intervals on the presence or absence of skeletal spicules; either hourly (gastrulae) or daily (6 armed larvae). Spicule deposition rate was normalized to the control. In both stages examined, T4, T3, and T2 accelerated skeletogenesis in a dose-dependent fashion, while Triac inhibited skeletogenesis. (Binary logistic regression with Bonferroni corrected p values. *: p < 0.05, **: p < 0.01, ***: p < 0.001, indicates significant difference from control group)
Figure 6. Representative images of ectopic skeleton and skeletal protrusions after 4 day exposure to thyroxine (100 nM). Images were taken with differential interference contrast microscopy (DIC). Frequently, after exposure to high levels of THs, larvae develop skeletal abnormalities. In panel A, skeletal rings, as well as a duplicate posterodorsal arm can be seen. Panel B shows an ectopic spicule, as well as abnormal branching from the posterodorsal arm. Panel C shows several ectopic spicules. Panel D shows a number of unusual skeletal protrusions on the post-oral arm, as well as presumptive primary mesenchyme cells in the process of laying down skeleton.
Figure 7. Skeletal abnormalities caused by T4 and T3 over a 4 day exposure. Only T4 and T3 caused skeletal abnormalities, with protrusions and duplicate posterodorsal arms being observed with higher exposure to T4 (Z-test, p < 0.0001), where nearly every larvae examined had a skeletal aberration. Ectopic skeleton was significantly more present in all levels of T4 exposure and the higher level of T3 exposure (Z-test, p values = <0.0001 to 0.004). No skeletal abnormalities were observed in the control or rT3 groups.
Figure 8. The effect of thyroxine on rudiment development. Pictured above are late stage larvae kept either without T4 (A) or with 100 nM T4 for 5 days (B). T4 exposed larvae have significantly more developed skeletal elements in the rudiment, as well as shortened larval arms. Larvae at soft tissue stage 1 (C) or skeletal stage 0 (D) were exposed to T4 for 5 days (n = 12, for staging scheme, see Heyland and Hodin 2014). T4 drastically accelerated skeletogenesis in the rudiment (Mann-Whitney, p < 0.0001) as well as accelerating other markers of metamorphic competence, including arm retraction and tube feet protrusion, but did not accelerate early soft tissue development (Mann-Whitney, p = 0.63). This suggests that the rudiment of late stage larvae may become responsive to T4 only as skeletal development begins.
Figure 9. Colocalization of fluorescently labeled T4 (RH-T4), labeled rT3 (RH-rT3), and primary mesenchyme cells (PMCs). Gastrulae were incubated with RH-T4 and RH-rT3 for 30 minutes prior to fixation in methanol. Following fixation, immunohistochemistry with 6a9 antibody was used to stain the membrane of PMCs. RH-T4 binds specifically to the membrane of PMCs, while RH-rT3 does not, suggesting a specific binding site for T4 in the PMCs.
Figure 10. 12 day-old larvae after acute or chronic exposure to T4. In the acute exposure groups, larvae were exposed to rT3 or T4 for 1 hour before being washed thoroughly and placed in clean seawater (n = 21). The chronic exposure groups were exposed for 3 days (n = 82). Larvae from both groups were imaged daily, and the presence or absence of spicules in the developing posterodorsal arms was noted. Acute exposure (1h) to thyroxine has similar effects to chronic exposure over 4 days, with high levels of T4 causing a significant increase in spicule initiation in both groups (Z-test, p values < 0.01).
Figure 11. Effect of T4 on skeletogenesis is not inhibited by SB203580, a MAPK (p38) inhibitor. Gastrulae were pre-exposed to SB203580 before being exposed to T4 for 20 hours (n=20). Following exposure, spicule initiation was monitored hourly for 5 hours. The hypothesis that SB inhibits skeletogenesis was tested using a binary logistic regression (D = 11, df = 12). T4 increased the rate of skeletogenesis (Bonferroni corrected p < 0.0001), while SB had neither a significant effect on skeletogenesis nor any interaction with the effect of T4 (p = 0.996).
Figure 12. The effect of T4 on skeletogenesis is inhibited by PD98059, a MAPK (ERK1/2) inhibitor. Gastrulae were pre-exposed to PD98059 before being exposed to T4 for 20 hours (n=100). Following exposure, spicule initiation was monitored hourly for 5 hours. The hypothesis that PD98059 inhibits the effect of T4 on skeletogenesis was tested using a binary logistic regression (D=232, df=110). T4 increased the rate of skeletogenesis, while PD98059 inhibited the effect of T4 on skeletogenesis (Bonferroni corrected p values < 0.0001). The highest levels of PD prevented skeletogenesis completely, an effect which was rescued by T4 (Bonferroni corrected p = 0.0012). This suggests that T4 acts through a MAPK (ERK1/2) cascade.
Figure 13. qPCR of Ets1 after T4 exposure in the presence of a MAPK (ERK1/2) inhibitor. T4 exposure causes upregulation of Ets1, a regulator of skeletogenesis. This effect is blocked by PD98059, an inhibitor of MAPK. Each group is a single pooled sample of an estimated 1000-2000 gastrulae. Mean ΔΔCt is displayed with standard error of technical replicates.
Figure 14. The effect of T4 on skeletogenesis is inhibited by RGD, a small signaling peptide and inhibitor of T4 binding to integrins. Gastrulae were pre-exposed to RGD before being exposed to T4 for 20 hours (n=20). Following exposure, spicule initiation was monitored hourly for 5 hours. The hypothesis that RGD inhibits the effect of T4 on skeletogenesis was tested using a binary logistic regression (D=49, df=15). T4 increased the rate of skeletogenesis, while RGD inhibited the effect of T4 on skeletogenesis (Bonferroni corrected p < 0.05). The highest levels of RGD prevented skeletogenesis completely, an effect which was rescued by T4 (Bonferroni corrected p < 0.05). A significant interaction was detected between RGD and T4, suggesting T4 may bind to an integrin membrane receptor.
Figure 15. Immunohistochemistry of phosphorylated MAPK after T4 exposure and T4 exposure in the presence of RGD peptide, a competitive inhibitor for integrin binding. Gastrulae were exposed to T4 or T4 and RGD for 90 minutes prior to fixation and staining. T4 increases levels of phosphorylated MAPK in the primary mesenchyme cells, as well as the basal membrane. RGD blocks the activation of MAPK in response to T4, but can be rescued with high levels of T4.
Figure 16. Proposed mechanism of thyroid hormone action in sea urchins and the homologous system in vertebrates. In sea urchins thyroid hormones bind to an integrin on the surface of primary mesenchyme cells, triggering a MAPK cascade. MAPK phosphorylates Ets1 and Alx1, activating them. Ets1 and Alx1 upregulate regulatory controls of skeletogenesis, leading to initiation of skeletogenesis. In vertebrates, thyroid hormones bind to integrin αVβ3 on the membrane of fibroblasts, triggering a MAPK cascade. MAPK phosphorylates Ets, promoting angiogenesis. Some details differ, notably the lack of Alx in the vertebrate mechanism, as well as the inhibition of VegfR by lower regulatory controls.