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Triiodothyronine (T_3) action on aquatic locomotor behavior during metamorphosis of the bullfrog *Rana catesbeiana*

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ABSTRACT Thyroid hormones - particularly triiodothyronine, T_3 - play a critical role in the morphological transformations comprising metamorphosis in larval bullfrogs *(Rana catesbeiana)*. Traditional staging criteria for anuran larvae incompletely distinguish physiological and behavioral changes during growth. We therefore first developed a new parameter to describe larval growth, the developmental index (DI), which is simply the ratio between the tail length of the larva and its head diameter. Using the DI we were able to identify two distinct populations classifying the larvae during growth along a continuous linear scale with a cutoff value of DI at 2.8. Classification based on the DI, used in this study, proved an effective complement to existing classifications based on developmental staging into pre- or pro-metamorphic stages. Exposure to T_3 in the water induced a rapid (beginning within 5 min) and significant decrease (~20-40%) in locomotor activity, measured as total distance traversed and velocity. The largest decrease occurred in more developed larvae (DI< 2.8). To determine correlated changes in the neuromuscular junctions during metamorphosis and apoptotic tail loss, miniature endplate currents from tail muscle were recorded during acute exposure to a hypertonic solution, which simulates an apoptotic volume decrease. Our results support a role for T_3 in regulating larval locomotor activity during development, and suggest an enhanced response to volume depletion at the neuromuscular junction of older larvae (DI<2.8) compared to younger animals (DI≥2.8). We discuss the significance of the possible role of an apoptotic volume decrease at the level of the neuromuscular junction.

KEY WORDS: *apoptosis, tadpole-classification, AVD, MEPC, development*

Introduction

Thyroid hormones (THs) are lipophylic ligands that regulate cellular differentiation, development, cardiac function, and basal metabolism (Oppenheimer, 1987). THs are essential for normal development in most vertebrate species (Zhang and Lazar, 2000; Yen, 2001). In anuran amphibians THs secreted from the thyroid gland are especially important in regulation of metamorphosis. Moreover, THs steadily increase prior to metamorphic climax (see Burggren and Just, 1992; Becker, Stephens, Davey, Schneider and Galton, 1997; Callery and Elinson, 2000; Schreiber, Das, Huang, Marsh-Armstrong and Brown, 2001; Das, Schreiber, Huang and Brown, 2002; Buchholz, Hsia, Fu, and Shi, 2003). Additionally, there is a rapid increase in thyroid hormone receptors (TRα), reaching a maximum expression during the metamorphic climax itself (Yaoita and Brown, 1990). These events lead to precise regulation of the final morphological, physiological and behavioral changes generally associated with amphibian metamorphosis (Tata, 2006).

Amphibian metamorphosis has been described as having three general developmental periods (Dodd and Dodd, 1976). Pre-metamorphosis (pre-mp) is characterized by a period of development and growth of the larva prior to formation of a functional thyroid gland. Pre-mp extends approximately from Stage (St) 42 to 53 (Nieuwkoop and Faber, 1967). Pro-metamor-

Abbreviations used in this paper: AVD, apoptotic volume decrease; DI, developmental index; MEPC, miniature end-plate current; Pre-mp, pre-metamorphosis; Pro-mp, pro-metamorphosis; T3, triiodothyronine.

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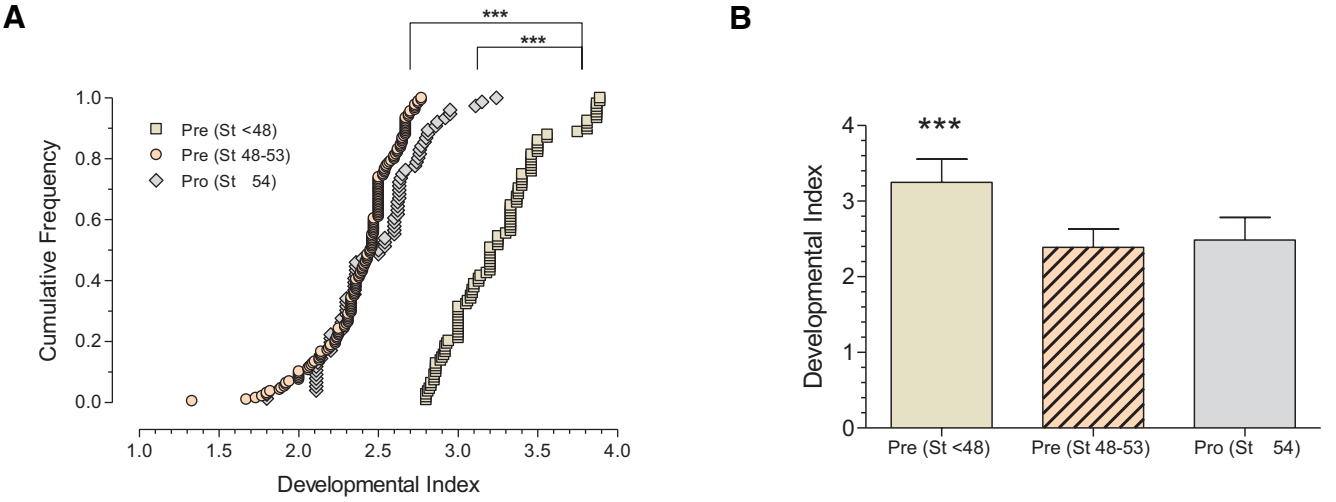

Fig. 1. Distribution of *R. catesbeiana* larvae based on the developmental index (DI) and relationship to the traditional developmental stages of pre-metamorphosis (pre-mp) and pro-metamorphosis (pro-mp). (A) *The ratio of tail length to head diameter, the DI, was measured in all larvae. The cumulative frequency of all DI values is shown. Two patterns of distribution are seen, around a DI value of 2.8. Larvae having a DI < 2.8 included both pre-mp (St 48-53, circles) and pro-mp specimens (St ≥54, diamonds), while the group of larvae with DI ≥ 2.8 was composed exclusively of pre-mp specimens (St<48, squares).* **(B)** *Differences in DI between the two populations (DI < 2.8 and DI ≥ 2.8) were statistically significant (p=0.001). For the group having DI < 2.8, we found no significant differences between specimens at stages pre-mp or pro-mp. The mean values ± SD (n) are, for pre-mp (St <48) 3.247±0.3070 (108); for pre-mp (St 48-53) 2.387±0.2450 (185); and for pro-mp (St ≥54) 2.486±0.2989 (76).*

phosis (pro-mp) occurs when the developing thyroid gland begins to secrete THs, extending approximately from St 54 to 57 when limb buds become apparent (Nieuwkoop and Faber 1967). Finally, during the climax of metamorphosis (metamorphosis proper, mp) TH blood concentrations peak and then fall after St 62, concurrent with front limb eruption, intestinal remodeling, and resorption of the tail and gills (Regard, Taurog, and Nakashima, 1978).

In anuran amphibians, two of the most prominent morphological changes are the loss of the tail by apoptosis and the growth of limbs (Wassersug, 1989). Indeed, most studies on the changing locomotion have focused on the gross morphology of the muscular structures rather than on the physiological characteristics of the involved muscles. Surging T_3 levels during metamorphosis have been shown to produce rapidly acting non-genomic effects (independent of gene transcription and protein synthesis) on the neuromuscular junctions of larval tail muscle (Rojas, Bonilla, Báez, and Lasalde, 2003). It is conceivable that T_3 might have a direct, rapidly acting effect on locomotor activity in larval *Rana catesbeiana*, and that this effect would be developmentally stage-specific. As shown here, the exposure of larvae to T_3 induced a reduction in the free-swimming motility of the animals that was significantly correlated to the developmental stage.

Metamorphosis is also a time of massive programmed cell death (apoptosis) of tail musculature. Apoptosis generally begins with impaired cell volume regulation and associated loss of cellular function (Okada and Maeno, 2001). THs are heavily implicated in this apoptosis during amphibian metamorphosis (Huang, Marsh-Armstrong and Brown, 1999; Yoshida, Okada, Kinoshita, Hara, Sasaki, *et al.,* 2002; Brown *et al.,* 2005). THs influence presynaptic activity at neuromuscular junctions during larval development (Rojas, Bonilla, Báez, and Lasalde, 2003), but the mechanism is not yet well understood, and any linkage back to TH-mediated apoptosis has not been studied. Since cell volume regulation is a primary target for apoptosis, we hypothesized that developing larvae would exhibit different sensitivities to disruptions in cell volume regulation according to the developmental stage. We tested this hypothesis by inducing volume changes in the neuromuscular junctions of tail muscles by exposure to hypertonic solutions, and indeed, the increase in MEPC frequency was significantly different between young and old larva.

Results

Developmental index (DI)

We have frequently observed large variations among individual tadpole larvae in size, weight, and physiological and behavioral responses, irrespective of the developmental stage. Indeed, animals classified pre-mp (Dodd and Dodd, 1976), seem more prone to exhibit these variations between individuals. This situation prompted a re-examination of the available criteria used to classify larvae. Among possible alternatives to traditional developmental staging (Nieuwkoop and Faber, 1967), we considered the use of simple body measurements as an index of growth. We selected the ratio between tail length and head diameter, which we call the developmental index, DI, since both structures are critically and simultaneously altered during development. In this report we use the DI for the first time to assess its usefulness in studies of anuran behavior and cellular phenomena.

The DI was measured in tadpoles from about 4-8 cm total length and each animal was also staged according to Nieuwkoop and Faber (1967). A frequency histogram of DI values showed two distinct maxima, with averages of 2.42 ± 0.247 (261) and 3.247 ± 0.307 (108) (mean ± SD, N), and minimum value at 2.8.

Figure 1A shows all DI values plotted against the cumulative frequency. The two distinct populations can be observed. A comparison was made between the DI values and the classifica-

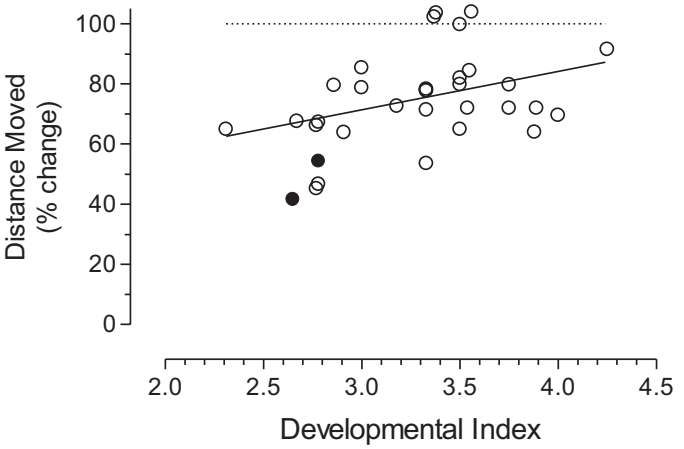

Fig. 2. Effect of 24 h T_3 exposure on locomotor activity in larvae of *R. catesbeiana*. *Locomotor activity is expressed as the percent change in total distance moved during exposure to T_3 as compared to control, plotted against the DI value of the larvae. For each larva, control locomotor activity was measured for 24 h, followed by a 24 h test period with 250 nM T_3 added to the water. The majority of the larvae show a reduction in locomotor activity in the presence of T_3. It can be seen that larvae having DI < 2.8 show the greatest reduction in locomotor activity with T_3. The majority of T_3 treated larvae showed significantly ($p < 0.01$) reduced displacement as compared to controls. Closed circles represent larvae with limb buds.*

tion of the larvae according to their developmental stage (Dodd and Dodd, 1976). Interestingly, all larvae in group DI ≥ 2.8 corresponded to animals staged at pre-mp, while the larvae in group DI < 2.8 included specimens that were either pre-mp or pro-mp (Fig. 1A). Differences between these two DI populations (DI ≥ 2.8 and DI < 2.8) were found to be statistically significant ($p < 0.001$, Figs. 1A and B). Further analysis revealed that the pre-mp specimens in group DI ≥ 2.8 were at stages 48 or less (Nieuwkoop and Faber, 1967), while pre-mp specimens in group DI < 2.8 were at St 48-53. Pro-mp larvae (in group DI < 2.8) corresponded to St 54 and above. No significant difference was found between the pre-mp and pro-mp larvae in the group having DI < 2.8.

Basal locomotor activity

Locomotor activity was measured under control conditions for all larvae tested, with a standard observation period of 2 h. No significant differences existed in the levels of control locomotor activity ($p > 0.1$, two-tailed Mann Whitney t-test), as expressed by mean displacement (distance moved). Larvae with DI < 2.8 swam an average length of 22.8 ± 1.2 m during 1 h of observation (n=120), while larvae with DI ≥ 2.8 swam an average length of 18.8 ± 0.9 m during 1 h of observation (n=67).

Effect of T_3 on buccal movements

Buccal movements of the larvae were counted under control conditions and after the addition of T_3 to the water. Larvae acutely exposed to 250 nM T_3 showed significantly accelerated buccal movements in less than 10 min of treatment. No significant differences in these rapid-onset buccal responses to T_3 were observed across the DI values tested, which were all DI < 2.8. The results indicated that in the first 10 minutes, the number of buccal movements per minute increased about 11% of the resting values, that is, from 78.8% ± 0.91 to 87.83% ± 1.5 (n=14).

Effects of T_3 on locomotor activity

Locomotor activity was evaluated under control conditions and after the addition of T_3 to the bath to a final concentration of 250 nM. The durations of exposure to T_3 were 24h or 2h (see Methods).

Larvae treated for 24 h with 250 nM T_3 decreased their locomotor activity, as measured by distance moved, as compared to controls. This decrease in locomotor activity occurred across all DI values (Fig. 2). Figure 3A shows the mean reduction in distance moved for pooled data across all DI (grouped as DI ≥ 2.8 and DI < 2.8) expressed as % change. Both larvae with DI ≥ 2.8 and those with DI < 2.8 showed significant reductions in distance moved in the presence of T_3 by 51.18% ± 9.20 (7) and 75.97% ± 4.20 (26), respectively.

Larvae treated for 2 h with 250 nM T_3 also exhibited a

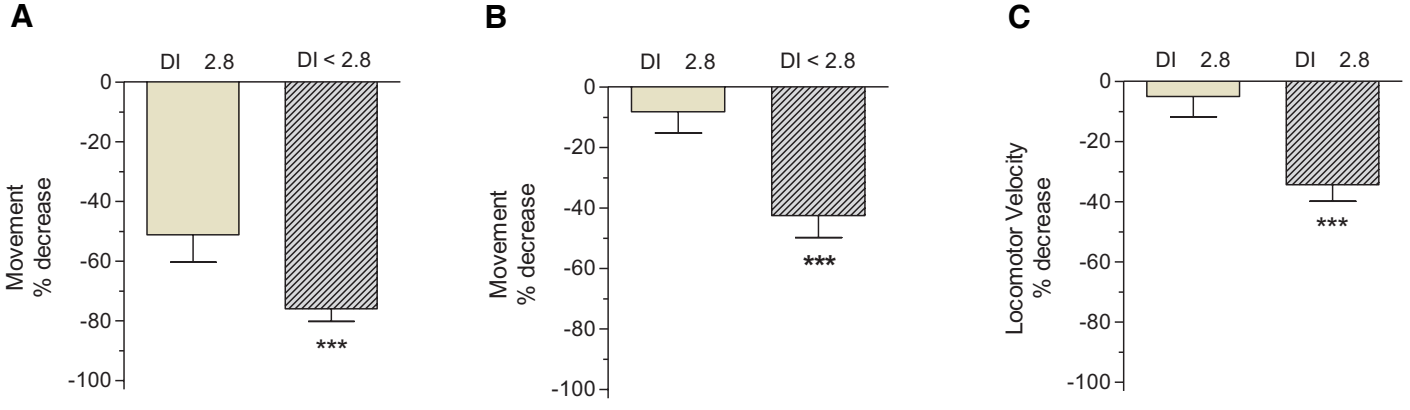

Fig. 3. Effect of T_3 exposure duration on locomotor activity in larvae of *R. catesbeiana*. **(A)** *Mean reduction in percent change of total distance moved produced by 24 h exposure to T_3. Larvae with DI < 2.8 exhibited the greatest reduction in movement.* **(B)** *Mean reduction in percent change of total distance moved produced by 2 h exposure to T_3. Larvae with DI < 2.8 show more reduction in movement.* **(C)** *Mean reduction in percent change in average velocity produced by 2 h exposure to T_3. Larvae with DI < 2.8 showed significantly greater reduction in velocity. Significant differences ($p < 0.01$, t-test) between populations are indicated by asterisks.*

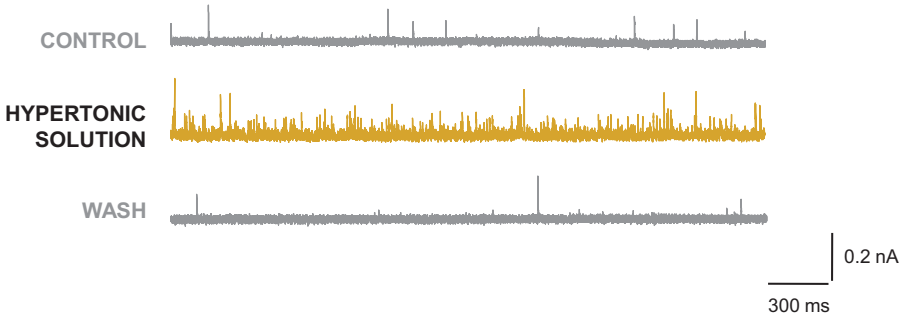

Fig. 4. Miniature endplate current (MEPC) recordings from a tail preparation of *R. catesbeiana* tadpole. *The application of a hypertonic saline solution (100 mM sucrose in normal saline, middle trace) produces a reversible increase in the frequency of the MEPCs. Sample traces shown were extracted from 900s acquisition recordings for control, hypertonic and wash protocols.*

reduction in distance moved as compared to controls. Larvae with DI ≥ 2.8 exhibited an 8.20% ± 7.10 (3) decrease in distance moved, whereas those with DI < 2.8 exhibited an 42.60% ± 7.30 (24) decrease in distance moved (Fig. 3B). We also observed that larvae treated acutely with T_3 for 2h generally exhibited a marked decrease in velocity as compared to controls (Fig. 3C). Larvae having DI < 2.8 showed a statistically greater decrease in velocity of 34.40% ± 5.50 (13) as compared to the 4.99% ± 6.80 (3) reduction in velocity for larvae with DI ≥2.8.

Miniature end-plate currents and tonicity

Spontaneous miniature end-plate currents (MEPCs) were recorded from neuromuscular junctions in isolated preparations of larval tail muscle. Basal MEPC frequency was measured in normal saline and changes in MEPC frequency were evaluated after application of a hypertonic saline solution to the bath.

Application of the hypertonic solution (saline containing 100 mM of sucrose) produced an increase in the frequency of the MEPCs in all the preparations tested (Fig. 4). However, preparations from animals with DI ≥ 2.8 showed only a small transient enhancement of MEPC frequency after exposure to the hypertonic solution (Fig. 5A), while animals with DI < 2.8 exhibited a large transient response to hypertonicity (Fig. 5B). The percent of change in the maximal MEPC frequency under hypertonic conditions as compared to controls was significantly larger in larvae with DI < 2.8 (1066% ± 547, n=5) than in larvae with DI ≥ 2.8 (553% ± 129, n=6).

Discussion

Developmental index

The complexity of physiological changes occurring in the brief period leading up to metamorphic climax in anuran amphibians has long been recognized. However, the distinction between rapidly occurring physiological and transitional events has been obscured by the use of staging tables that largely depend upon "absent/present" decision pathways (e.g., absence or appearance of limb buds) (Gosner, 1963; Nieuwkoop and Faber, 1967; see Burggren and Doyle, 1986). Indeed, precise alignments of

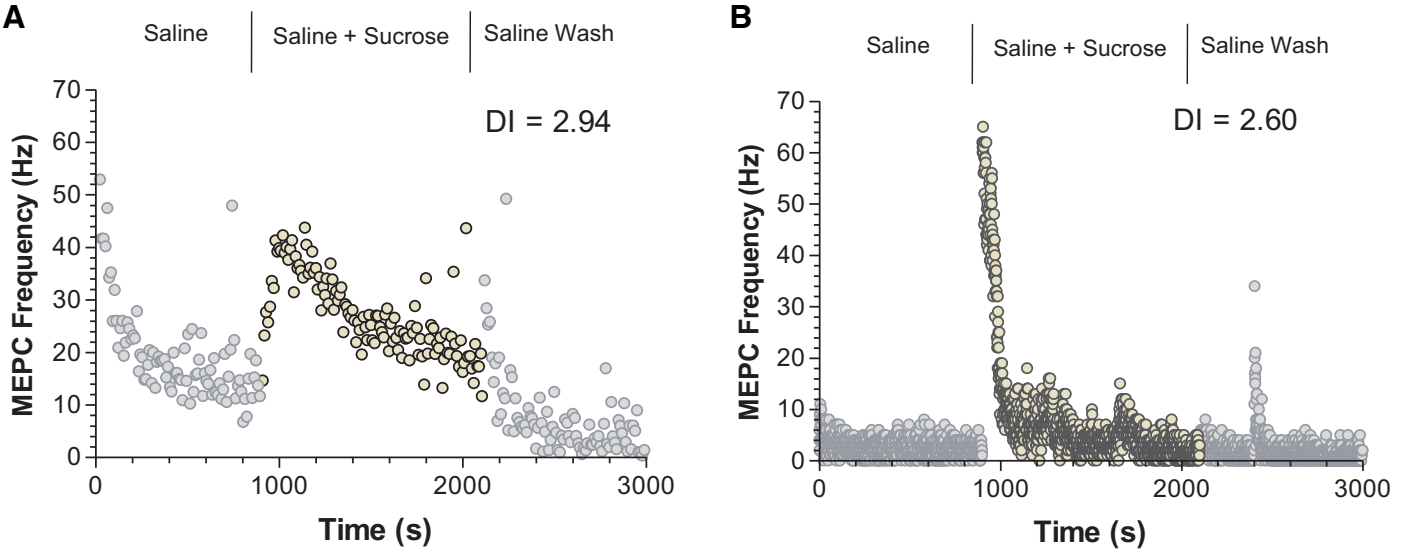

Fig. 5. Influence of developmental stage on the MEPC frequency response to hypertonic solutions in tail preparations of *R.catesbeiana* tadpoles. *MEPCs were recorded from neuromuscular junctions of the 3rd myotome during perfusion with normal saline, followed by hypertonic saline and subsequent wash with normal saline. MEPCs were averaged every 10 s.* **(A)** *Typical recording for a larva with DI≥ 2.8. Note the increase in MEPC frequency upon addition of hypertonic solution, which subsequently decays but does not reach basal levels. Upon washout, the MEPC frequency slowly returns to approximately basal levels. The DI value for this specimen was 2.94.* **(B)** *Typical recording for a larva with DI<2.8. The basal frequency of the MEPCs is substantially and very rapidly increased upon addition of the hypertonic solution, but this change is transient. However, a slight increment in frequency remains during the hypertonic solution, which is effectively reduced to basal levels upon washout. The DI value for this specimen was 2.60.*

physiologically important measures such as T_3 plasma concentrations with behavioral events, and the transition from tail-based to limb-based locomotion, have been difficult to achieve because of the inability to clearly distinguish between stages of development prior to metamorphosis. This limitation is especially apparent when relying on morphological landmarks that might occur at slightly different times in larvae of identical physiological stage.

To overcome this difficulty in correlating behavioral and electrophysiological phenomena associated with metamorphosis, we capitalized on the observation that transformation of the larvae during development involves a simultaneous reduction in tail length and an increase in head diameter. Measurement of the ratio of tail length to head diameter, the developmental index (DI), revealed two significantly different populations in a frequency histogram, those with DI≥2.8 and those with DI < 2.8. We evaluated whether these populations were correlated to the traditional staging levels of pre-mp and pro-mp. After classifying the animals into pre-mp or pro-mp, we found that all larvae having a DI ≥2.8 corresponded to the pre-mp classification, while those with DI <2.8 included individuals either at pre-mp or pro-mp. Thus, the classification of the larvae into two groups according to the DI value was not equivalent to the traditional classification into pro-mp or pre-mp. Indeed, the DI < 2.8 group associates pro-mp (St ≥ 54) animals with late pre-mp (St 48-53) animals, while early pre-mp (St <48) animals are distributed exclusively to the DI ≥ 2.8 group. Moreover, the use of tail length or head diameter alone as possible indices of developmental growth produced the same variable results as did traditional staging.

We have shown that changes in locomotor behavior and MEPC frequency in response to externally applied THs differ significantly between the DI ≥ 2.8 and DI < 2.8 groups of larvae. Within the context of this study, late pre-mp and pro-mp larvae respond similarly to applied THs, supporting a functional significance to the use of the DI and its possible application as a general growth parameter in anurans.

T_3 influence on locomotor activity

Buccal muscle activity patterns in larval bullfrogs have been evaluated during gill irrigation and feeding behavior (Larson and Reilly, 2003). We evaluated the buccal muscle activity patterns in the presence of T_3 in the rearing water, and changes in buccal movement frequency were observed 3-5 min after T_3 exposure, suggesting fast absorption of T_3. This observation is in agreement with changes in buccal activity patterns observed by others (Burggren and Doyle 1986).

Locomotor activity as measured by larval distance moved (displacement) in two dimensions, was reduced by both long duration (24 h) and acute (2 h) exposure to T_3 in the rearing water (Figs. 2 and 3). These T_3 effects were more pronounced in larvae with DI < 2.8. However, the evident T_3–induced reduction in locomotor activity in all larvae indicated that body shape was not a major factor in the T_3 response within our total range of DI values (see Liu, Wassersug, and Kawachi, 1996, 1997).

Acute exposure to T_3 for 2 h was sufficient to produce a pronounced inhibitory effect on locomotion (Fig. 3B). Although there is general agreement on the fact that most of T_3 effects are via thyroid receptor regulation of nuclear target genes, a number of reports suggest non-genomic effects of thyroid hormones (Rojas, Bonilla, Báez, and Lasalde, 2003; Saelim, John, Wu, Park, Bai, *et al.,* 2004). These non-genomic effects are of rapid onset (seconds to minutes), do not depend on intracellular binding of T_3 and nuclear thyroid receptors, and are independent of gene transcription and protein synthesis (Davis and Davis, 2002). The T_3 effect on buccal movement occurs in as little as 3-5 minutes, and locomotion is affected within 2 h (Fig. 3). These rapid actions of T_3 are likely related to non-genomic actions, possibly involving an effect on spontaneous neurotransmitter release at the neuromuscular junctions of larval bullfrogs. Exposure of the neuromuscular junction of the tail to T_3 for as little as 3-5 min sharply increases the spontaneous release of neurotransmitter (Rojas, Bonilla, Báez, and Lasalde, 2003). In addition, exposure of larvae to T_3, during 2 h or 24 h significantly reduced locomotor activity more in larvae with DI < 2.8, as compared to larvae with DI ≥2.8, supporting the idea that T_3 has a differential physiological effect during anuran stages of development.

Neuromuscular activity during hypertonicity depends on larval stage

Tail resorption in anuran amphibians occurs immediately prior to metamorphosis and the appearance of the adult, tail-less morphotype. This structural remodeling results from regulated apoptosis mediated through T_3 (Yaoita and Brown, 1990), and has been proposed is preceded by a loss of cell volume regulation. In a previous study, we demonstrated that T_3 acutely affects neuromuscular junction physiology in anuran larvae (Rojas, Bonilla, Báez, and Lasalde, 2003). We now consider how changes in cell volume during development might affect MEPC generation at the neuromuscular junction. In the current experiments we tested the hypothesis that the neuromuscular junction should respond differentially to hypertonic solutions according to developmental stage. Our results showed that MEPC frequency increased in response to hypertonicity (Fig. 4), and that the magnitude of the increase was a function of advanced larval stage (Fig. 5). The reduction in cell volume produced by exposure to hypertonic solution mainly affects nerve terminals in the developmentally advanced larvae (DI < 2.8), which is consistent with the idea that T_3-induced apoptosis is possibly altering the neuromuscular junction through impairment of cell volume regulation. This proposal is also supported by the direct non-genomic action of T_3 on larval neuromuscular junction which is highly specific (Rojas, Bonilla, Báez, and Lasalde, 2003).

We have introduced a novel index for describing larval *Rana catesbeiana* growth, the DI, which is useful for evaluating complex behaviors, such as locomotion, as well as cellular phenomena, such as spontaneous transmitter release at the neuromuscular junction. Further evaluation of the DI and its possible applicability to other anuran species is warranted and may provide interesting information on behavioral and physiological phenomena during metamorphosis. In *Rana catesbeiana* larvae, buccal activity quickly increased in the presence of T_3 in the rearing water. This result argues for a rapid uptake of T_3 through the skin and a significant effect on the control of neural activity of the muscles producing the buccal movement.

In summary, locomotor behavior under control conditions, evaluated as total distance traversed, was found to be similar across all DI values tested; the addition of T_3 to the rearing water produced a decrease in locomotion that was dependent on the DI value, older animals (DI<2.8) being more susceptible to the effect

of T_3. The velocity of displacement was also reduced in similar proportion to that observed for distance traversed. In neuromuscular preparations from the tail, exposure to hypertonic saline solutions increased MEPC frequency as expected, but the magnitude of the increment depended on the DI of the larvae, with older larvae (DI<2.8) showing a greater response to hypertonicity. A possible mechanism for these effects may involve a direct, rapid action of T_3 on the neuromuscular junction, as we have reported previously (Rojas Bonilla, Báez, and Lasalde, 2003), as well as T_3 induced changes in volume regulation, as part of an apoptotic volume decrease.

Materials and Methods

Animal handling and maintenance

Larval bullfrogs, *Rana catesbeiana*, 4-8 cm total length, were obtained from Carolina Biological Supply (Miami, FL). Animals were maintained in water tanks (≤ 20 larvae per tank) at 22°C and under a 12:12 light:dark cycle. They were fed twice per week with commercial tadpole chow (Carolina Biological Supply, Miami, FL). Larvae were handled, maintained, used and disposed of according to the standards described in the NIH Guide for the Care and Use of Laboratory Animals and the Guidelines for the Use of Animals in Neuroscience Research. All animal handling procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Universidad Central del Caribe.

Developmental index

In larval *Rana catesbeiana* approaching metamorphosis, the head increases in diameter as the tail decreases in length. We measured tail length and head diameter, either manually or digitally with the program *Scion Image for Windows* (Scion Corp., Release Beta 4.0.2 MD). These measurements allowed us to calculate the ratio between tail length and head diameter, developmental index DI, which was used to classify the larvae on a linear scale. Tail and head measurements were obtained for 740 larval bullfrogs.

Behavioral measurements

Behavioral experiments were conducted at the Behavioral Testing Facility of the Universidad Central del Caribe (http://www.btfucc.org). Measurements were made at 22-24°C in an appropriately lighted room during the light cycle. Test animals were placed inside an acoustic chamber to avoid external noise or any other perturbation. The experimental chamber consisted of four open opaque acrylic cylinder containers (19 cm diameter, 1.5 L total volume). A single larva was placed in each container, which had been filled with 500 mL of distilled water - enough water to completely cover the larva's body but allowing almost no vertical movement (Z≈0). We assumed that only very minor alterations in the acquisition trajectory would result from displacement in the Z coordinate, therefore, trajectory analyses were carried out only in the X and Y coordinates (2D) to yield net body movement over time.

Effect of T_3 on buccal activity

T_3 was externally administered to larval *R. catesbeiana* by adding a fixed amount of a stock solution to the bath water for a final concentration of 250 nM T_3.

We measured buccal movement frequency in control conditions and after exposure to T_3 in order to estimate the time needed to see an effect. In brief, tadpoles were placed in containers as described above and allowing for a 30 min acclimatization period. Buccal movements were then carefully recorded using a video camera attached the stereoscope microscope or by direct visualization for 15 sec periods per minute for a total of 30 min. After this time T_3 was added to the water in the container and rapidly mixed to a final concentration of 250 nM, with little or no larval perturbation. Buccal movements were then evaluated for an additional 30 min. All larvae used in this experiment had DI < 2.8.

Protocols for T_3 exposure

Two experimental protocols were implemented to evaluate T_3 action on larval locomotion: exposure for 24 h to T_3, or exposure for 2 h to T_3. All tests were performed in groups of four larvae since that was the maximum number of animals that we could record simultaneously. In the first protocol, four larvae classified by their DI were placed individually in four containers for 2 h of acclimatization followed by 24 h of continuous control recording of their locomotion. Following acquisition of the control data, three of the four larvae were treated with 250 nM of T_3 (Sigma Aldrich, St Louis, MO, USA) and patterns of locomotion were again measured for 24 h. After the first 24 h control period the fourth larva was exposed to the vehicle only, and served as the sham control for the next 24 h recording. Thus, each test larva served as its own control. The second protocol was identical to the first, except for the duration of the control period (2 h) and exposure period (2 h).

Larvae in the stages prior to metamorphosis were classified according to DI. Of the 187 larvae examined, 67 had a DI ≥2.8 and 120 had a DI < 2.8. This latter group included at least 8 animals with apparent complete limb bud development. These groups were evaluated in terms of locomotor activity after exposure to T_3.

Assessing locomotor activity

Locomotor activity of the larvae was assessed by a video tracking system designed specifically for the automation of behavioral experiments (*Ethovision©*, v. 3.0 Noldus, Netherlands). Larval locomotion was recorded using a digital camera connected to a computer, which detected each larval movement as a displacement with a sampling rate of 9 positions/sec (inter-point interval=111 ms).

Measured locomotor behavior parameters were determined as follows:

1. Displacement (movement): Distance traveled by the larva's center of gravity of the specimen between two sampling periods:

$$DM=\sqrt{(X_n-X_{n-1})^2+(Y_n-Y_{n-1})^2} \quad \textit{Eq. 1}$$

Where:
DM = Distance moved from sample n-1 to sample n.
X_{n-1}, Y_{n-1} = X, Y coordinates of objects at sample n-1.
X_n, Y_n = X, Y coordinates of objects at sample n.

2. Velocity: Distance traveled by the larva per unit time; i.e. the linear velocity in the X,Y plane (Eq. 2). Velocity was determined only during the time intervals in which the larva was actively moving. Thus, we generated the average velocity during displacement. The formula used was:

$$V=\frac{DM}{t_n-t_{n-1}} \quad \textit{Eq. 2}$$

Where:
V = velocity
DM = distance moved

Tail preparations for electrophysiological recording

Larvae were placed in cold (3-5 ºC) saline until no movement was apparent and then decapitated. The skin of the tail was carefully removed to expose the underlying muscle fibers and longitudinal slices were obtained for use in the electrophysiological experiments.

Focal recording of miniature endplate currents

MEPCs, resulting from spontaneous neurotransmitter release, were recorded from tail muscles of larval *Rana catesbeiana* having DI < 2.8 and DI ≥ 2.8. The caudal muscle fibers used were between 20-50 μm in

diameter and 200-500 μm in length, and the fiber surface is relatively clean allowing MEPCs to be focally recorded from discrete spots (Quiñonez, Romero and Rojas, 1996; Rojas, Bonilla, Báez, and Lasalde, 2003).

Electrophysiological recording

Miniature endplate current (MEPC) recordings were performed at the ends of the muscle fibers where the nerve terminals and acetylcholine receptors (AChRs) are located (Rojas, Bonilla, Báez, Lasalde-Dominicci, 2003). Typically, then, myotomes 3 to 4 of the rostral segment were used to record MEPCs. Frog saline composition (in mM) was NaCl 125, KCl 6, $CaCl_2$ 1.8, Hepes 10 with 100 nM TTX, adjusted to pH of 7.2 and with osmolarity of 255 mOsm/kg water. Hypertonic sucrose solution was prepared by adding sucrose to the normal frog saline up to a final sucrose concentration of 100 mM. All solutions were prepared daily.

Focal recording

Pipettes for focal recording were pulled using a P-87 puller (Sutter Instruments, Novato, California USA) and fire-polished using a microforge (Narashige Scientific Instrument Lab, Tokyo, Japan), to obtain a tip diameter between 9 and 14 μm. The pipettes were constructed using soft glass to avoid the channel rundown encountered when hard glass is used in embryonic muscle cells (Rojas and Zuazaga, 1988; Rojas, Bonilla, Báez, and Lasalde, 2003). Pipettes were filled with saline solution.

Focal recordings of MEPCs were obtained using pipette electrodes attached to an amplifier (Quiñones, Romero, and Rojas, 1996; Rojas, Bonilla, Báez, and Lasalde, 2003). A patch amplifier (GeneClamp 500, Axon Instruments Inc., Foster City, CA, USA) was used with a feedback resistor of 1 GΩ to record MEPCs in the macropatch configuration. The MEPCs were acquired using an A/D converter (Series E, National Instrument, Austin, Texas, USA) in a PC computer. The signals were continuously recorded using the program Electrophysiological Digital Recording WinEDR v2.3.9. (Dempster J., http://spider.science.strath.ac.uk/PhysPharm).

Solution exchange during MEPC recording

Experiments were conducted with a continuous superfusion of the solutions, allowing for rapid exchange with a set of valves. Stabilization of MEPC frequency was allowed for 10-15 min, after that a control recording was performed. The first 900 sec of acquisition of control MEPCs in normal saline were followed by 900 sec of acquisition in hypertonic saline (containing 100 mM sucrose). Finally, a 900 sec recovery period was obtained during washout with normal saline solution.

Statistical analyses

Statistical analyses were performed with SYSTAT (SPSS Inc. v10, Chicago, IL, USA) and GraphPad Prism (v.4 GraphPad Software Inc. San Diego, CA, USA). Mean values ± Standard Deviation (n) are shown. A Kruskal-Wallis test was used to compare populations. A fiduciary level of 0.1% was used for all statistical tests.

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References

BECKER, K.B., STEPHENS, K.C., DAVEY, J.C., SCHNEIDER, M.J. and GALTON, V.A. (1997). The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in *Rana catesbeiana* tadpoles. *Endocrinology* 138: 2989-2997.

BROWN, D.D., CAI, L., DAS, B., MARSH-ARMSTRONG, N., SCHREIBER, A.M. and JUSTE, R. (2005) Thyroid hormone controls multiple independent programs required for limb development in *Xenopus laevis* metamorphosis. *Proc. Natl. Acad. Sci. USA* 102(35):12455-12458.

BURGGREN, W.W. and DOYLE, M. (1986). Ontogeny of regulation of gill and lung ventilation in the bullfrog, *Rana catesbeiana*. *Resp. Physiol.* 66: 279-291.

BURGGREN, W.W. and JUST, J.J. (1992). Developmental changes in Amphibian physiological systems. *In: Environmental Physiology of the Amphibia. Editors, M.E. Feder and W.W. Burggren. University of Chicago Press, Chicago.*

BUCHHOLZ, D.R., HSIA, S.C., FU, L. and SHI, Y.B. (2003). A Dominant-Negative Thyroid Hormone Receptor Blocks Amphibian Metamorphosis by Retaining Corepressors at Target Genes. *Mol. Cell Biol.* 23: 6750–6758.

CALLERY, E.M. and ELINSON, R.P. (2000). Thyroid hormone-dependent metamorphosis in a direct developing frog. *Proc. Natl. Acad. Sci. USA* 97: 2615-2620.

DAS, B., SCHREIBER, A.M., HUANG, H. and BROWN, D.D. (2002). Multiple thyroid hormone-induced muscle growth and death programs during metamorphosis in *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA.* 99: 12230-12235.

DAVIS, P.J. and DAVIS, F.B. (2002). Nongenomic actions of thyroid hormone on the heart. *Thyroid* 12: 459-466.

DODD, M.H.I. and DODD, J.M. (1976). The biology of metamorphosis: In Physiology of the amphibian. *New York, Academic Press vol III,* p. 467-599.

GOSNER, K. (1963). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183-190.

HUANG, H., MARSH-ARMSTRONG, N. and BROWN D.D. (1999). Metamorphosis is inhibited in transgenic *Xenopus laevis* tadpoles that overexpress type III deiodinase *Proc. Nat. Acad. Sci.*, 96: 962-967.

LARSON, P.M. and REILLY, S.M. (2003). Functional Morphology of Feeding and Gill Irrigation in the Anuran Tadpole: Electromyography and Muscle Function in Larval *Rana catesbeiana*. *J. Morphol.* 255: 202-214.

LIU, H., WASSERSUG, R. and KAWACHI, K. (1996) A computational fluid dynamics study of tadpole swimming. *J Exp Biol.*199(Pt 6): 1245-1260.

LIU, H., WASSERSUG, R. and KAWACHI, K. (1997) The three-dimensional hydrodynamics of tadpole locomotion. *J Exp Biol.* 200(Pt 22): 2807-2819.

NIEUWKOOP, P.D. and FABER, J. (1967). Normal Table of *Xenopus Laevis* (Daudin). Hubrecht Lab., Utrecht; North-Holland Publishing Co., Amsterdam (2nd. edn).

OKADA, Y. and MAENO, E. (2001). Apoptosis, cell volume regulation and volume-regulatory chloride channels. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 130: 377-383.

OPPENHEIMER, J.H. (1987). Advances in our understanding on thyroid hormone action at the cellular level. *Endocrin. Rev.* 8: 288-308.

QUIÑONEZ, M., ROMERO, P.J. and ROJAS, L.V. (1996). Action of Protein Kinase A activators on the caudal neuromuscular Junction of toad tadpoles, recorded on synaptic spots. *Brain Res.* 737: 327-330.

REGARD, E., TAUROG, A., and NAKASHIMA, T. (1978). Plasma thyroxine and triiodothyronine levels in spontaneously metamorphosing *Rana catesbeiana* tadpoles and in adult anuran amphibia. *Endocrinol.* 102: 674-84.

ROJAS, L.V., BONILLA, L., BAEZ, S. and LASALDE-DOMINICCI, J.A. (2003). Thyroid Hormones Regulate the Frequency of Miniature End-Plate Currents in Pre- and Pro-metamorphic Stages of the Tadpole Tail. *J. Neurosci. Res.* 71: 670-678.

ROJAS, L. and ZUAZAGA C. (1988). Influence of the patch pipette glass on single acetylcholine channels recorded from *Xenopus* myocyte. *Neurosci. Lett.* 88: 39-44.

SAELIM, N., JOHN, L.M., WU, J., PARK, J.S., BAI, Y., CAMACHO, P. AND LECHLEITER, J.D. (2004). Nontranscriptional modulation of intracellular Ca^{2+} signaling by ligand stimulated thyroid hormone receptor. *J. Cell Biol.* 167: 915-924.

SCHREIBER, A.M., DAS, B., HUANG, H., MARSH-ARMSTRONG, N. and BROWN D.D. (2001). Diverse developmental programs of *Xenopus laev*is metamorphosis are inhibited by a dominant negative thyroid hormone receptor. *Proc. Natl. Acad. Sci. USA* 98: 10739-10744.

TATA, J.R. (2006) Amphibian metamorphosis as a model for the developmental actions of thyroid hormone. *Mol. Cell Endocrinol.* 246: 10-20.

WASSERSUG, R.J. (1989). Locomotion in amphibian larvae (or why aren't tadpoles built like fish) *Am. Zool.* 29: 65-84.

YAOITA, Y. and BROWN D.D. (1990). A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. *Genes & Dev.* 4: 1917-1924.

YEN, P.M. (2001). Physiological and Molecular Basis of Thyroid hormone Action. *Physiol. Rev.* 81: 1097-1142.

YOSHIDA, H., OKADA, Y., KINOSHITA, N., HARA, H., SASAKI, M., SAWA, H., NAGASHIMA, K., MAK, T.W., IKEDA, K. and MOTOYAMA, N. (2002). Differential requirement for Apaf1 and Bcl-X(L) in the regulation of programmed cell death during development. *Cell Death Differ.* 9: 1273-1276.

ZHANG, J. and LAZAR, M.A. (2000). The mechanism of action of thyroid hormones. *Annu. Rev. Physiol.* 62: 439-66.