

Role for Corticoids in Mediating the Response of *Rana pipiens* Tadpoles to Intraspecific Competition

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ABSTRACT Competition is known to decrease growth and development rate in tadpoles, but the physiological basis for this phenomenon is poorly understood. We hypothesized that competition results in increased production of stress hormones and that these hormones are responsible for the suppression of growth and development. To test this hypothesis, we measured whole-body corticosterone content in premetamorphic Leopard frog (*Rana pipiens*) tadpoles raised at two different population densities and three different food levels. Whole body corticosterone content was elevated in tadpoles subjected to either limited food (at low density) or high density. Within the low and intermediate food treatments, high density reduced tadpole growth and slowed development. Limited food slowed growth and development at all densities. Blocking corticoid synthesis by treating tadpoles with metyrapone (MTP) reversed the growth suppression caused by high density (tested in the intermediate food level treatment) but did not alter the effect of density on development rate. MTP treatment did not alter the depressive effect of limited resources on growth or development. Our results suggest that elevated corticoid biosynthesis mediates the negative effect of increased population density (i.e., increased intraspecific competition) on tadpole growth. *J. Exp. Zool.* 292:32–40, 2002. © 2002 Wiley-Liss, Inc.

Competition is well known to affect components of individual fitness in amphibian larvae. Increased competition intensity has been shown to decrease larval growth rate, development rate, survivorship, and size at metamorphosis (Brockelman, '69; Wilbur and Collins, '73; Wilbur, '77; Smith, '87; Berven and Chadra, '88; Scott, '90). Both a longer larval period and a smaller size at metamorphosis can delay adult reproductive maturity, decrease size at first reproduction, and in some cases decrease adult survival to first reproduction (Berven and Gill, '83; Smith, '87; Semlitsch et al., '88). Longer time to metamorphosis also may increase larval exposure time to aquatic predators (Wilbur, '80; Werner, '86) or decrease the chance of metamorphosing before a quickly-drying pond disappears (Newman, '92).

Despite abundant evidence of competition's influence on individual fitness, the physiological mechanisms that translate environmental density or resource cues into growth and developmental responses have not been identified. Much is known about the physiological regulation of amphibian development, but the link has not yet been made between this knowledge and that of the phenomenological effects of competition. Such a link would enhance our understanding of amphibian physiological ecology and our ability

to predict competitive outcomes in complex systems.

Amphibian metamorphosis is controlled by thyroid hormone, which induces tissue-specific metamorphic changes (Kikuyama et al., '93). Steroid hormones produced by the amphibian interrenal glands (homologous to avian and mammalian adrenal cortex) also may play a central role in controlling metamorphosis. The production of corticoids by the interrenal glands increases during metamorphosis, more or less in parallel with thyroid hormone, and corticoids are known to synergize with thyroid hormone during pro-metamorphosis to accelerate metamorphic changes (Frieden and Naile, '55; Gray and Janssens, '90; Kikuyama et al., '93; Hayes and Wu, '95). However, corticoids may inhibit or not affect hind limb development of early stage (pre-metamorphic) tadpoles (Kobayashi, '58; Hayes et al., '93), and treatment with ex-

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ogenous corticosterone slows tadpole growth throughout development (Wright et al., '94; Hayes et al., '93). This growth inhibitory effect of corticoids is common throughout vertebrate taxa, as these hormones serve to mobilize stored fuels and increase metabolism under emergency situations; thus the designation 'stress hormones' (Chester-Jones et al., '72; Selye, '73; Munck et al., '84; Schreck, '93; Wingfield, '94).

Circulating corticoid concentrations are elevated in many taxa in response to environmental factors including food deprivation (birds: Cherel et al., '88; Kitaysky et al., '99), extreme weather (birds: Wingfield et al., '97), exercise (mammals and birds: Schreck, '93), crowding (fish: Schreck, '81), social dominance by a conspecific (fish and mammals: Louch and Higgenbotham, '67; Sapolsky, '87; Overli et al., '99), and hibernation (reptiles: Dauphinville et al., '90). However, relatively little is known about environmental influences on corticoid production in amphibians. As in other taxa, corticoids regulate metabolic processes in amphibia and are elevated in response to acute stressors such as handling or confinement (Licht et al., '83; Zerani et al., '91; Moore et al., '94). Both circannual and circadian rhythms in circulating concentrations of corticoids have been measured in amphibians (Licht et al., '83; Jolivet-Jaudet et al., '84; Thurmond et al., '86; Hopkins et al., '97), suggesting some influence of the environment on hormone production. Hayes ('97) measured elevated corticoid content in toad tadpoles held at high densities, and previous work of ours demonstrated a functional role for the neuroendocrine stress axis in mediating the developmental response of Western spadefoot toad tadpoles to pond drying (see Denver, '97, '98). To our knowledge, no other studies have examined directly the influence of environmental factors on corticoid production in amphibians.

We tested the hypothesis that the growth- and developmental responses of *Rana pipiens* (northern leopard frog) tadpoles to intraspecific competition are mediated through the production of corticoids. We raised tadpoles at different population densities and food levels, measured whole-body corticosterone content, and observed the effect of blocking corticoid synthesis on the tadpoles' growth and developmental responses to competition.

MATERIALS AND METHODS

R. pipiens eggs were purchased from Carolina Biological Supply Co. (Burlington, NC). All experi-

ments were done in environmental chambers, maintained at 22°C and at 12L:12D.

Experiment 1: Effects of conspecific density and food level on whole-body corticosterone content

Premetamorphic tadpoles (Gosner stage 25; Gosner, '60) were placed into plastic tanks containing 4 L water, at a density of eight or 40 tadpoles per tank, and were fed a ground food mixture of 3:1 Purina Rabbit Chow:Tetramin Fish Flakes, at 18, 6, or 2% body mass per day (based on the mean mass of animals per tank, weighed weekly). Food levels of 10–15% body mass per day allow tadpoles to grow at rates similar to those observed in nature, and levels of 7.5% have been shown to induce resource competition among ranids (Werner, '92). Density and food treatments were fully crossed to give six treatments, with two replicate tanks per treatment. Treatment identifications are 8H, 8M, 8L, 40H, 40M, and 40L, corresponding to low (8) versus high (40) density and high (H), intermediate (M), or low (L) food levels, respectively. Tadpoles were fed on days one and three. On day four, ten tadpoles from each treatment (five from each replicate tank) were randomly selected and immediately anesthetized by submersion in 0.01% benzocaine. Tadpoles were weighed and frozen at -20°C for later extraction and analysis of whole-body corticosterone content by radioimmunoassay (RIA; see below).

Experiment 2: Effects of conspecific density, food level, and metyrapone on growth and development

Premetamorphic tadpoles (Gosner stage 25) were weighed and placed into 4 L plastic tanks at the densities and food levels described for Experiment 1. Additional treatments added metyrapone (MTP; Sigma-Aldrich, St. Louis, MO), a corticoid synthesis inhibitor, to some of these density/food combinations. Not all density/food combinations could be treated with MTP, due to limited availability of the compound, so we chose to add MTP to four of the six density/food combinations: 8H, 8M, 8L, and 40M. Comparison among the 8H, 8M, and 8L groups allowed us to observe the effect of decreased corticoid biosynthesis on growth or developmental responses to limited food levels. Comparison between the 8M and 40M groups allowed us to observe the effect of decreased corticoid biosynthesis on any responses to high density. Three replicate tanks were included for all ten treatments. Water in all tanks was changed twice

weekly, and tadpoles were fed every 1–3 days. MTP was dissolved in ethanol and added to the tanks following water change, to give a final concentration of 110 μM MTP in the water. Non-MTP tanks received ethanol only. Ethanol concentration in the water was 0.0043% of total water volume. MTP concentration was determined based on Hayes and Wu ('95), who found that 22 μM MTP reduced whole-body corticosterone content of toad tadpoles by 33%. Our goal was 100% reduction of *R. pipiens* whole-body corticosterone content.

After 17 days at the different densities, food levels, and MTP treatments, all tadpoles were weighed, staged, and then returned to their respective tanks. At 52 days, as the first individuals began to reach metamorphic climax, all tadpoles were anesthetized by submersion in 0.01% benzocaine. Seven tadpoles from each treatment were randomly selected from the three replicate tanks, weighed, staged, euthanized, and frozen at -20°C for later extraction and analysis of corticosterone by RIA. All other tadpoles were weighed and staged only.

Tissue extraction and corticosterone RIA

The extraction procedure is described by Hayes and Wu ('95) and Denver ('98). Briefly, tissues were homogenized in ethyl acetate and the extracts fractionated by thin layer chromatography (TLC) to separate corticosterone from other lipids. The region of the TLC lane containing the corticosterone (as determined by calibration with both radiolabeled and radioinert corticosterone; see Denver, '98) was scraped and the silica collected into a borosilicate glass tube. The silica was extracted with ethyl ether, and the extract was dried under nitrogen and then resuspended in PBS-gelatin (PBS-G; 0.02 M, pH 7.3) for corticosterone RIA. The RIA was conducted as described by Licht et al. ('83). Anti-corticosterone serum was purchased from Endocrine Sciences (Calabasas, CA) and [^3H]-corticosterone from NEN Life Science Products, Inc. (Boston, MA). Samples from a single experiment were analyzed in a single RIA or in multiple RIAs on a single day. Inter- and intra-assay coefficients of variation were 12% and 10%, respectively, and were monitored by including a quality control standard (pooled rat plasma) in each RIA. Tadpole extracts exhibited parallelism in the RIA.

Data analysis and statistics

Data for corticosterone content in experiment 1 were analyzed by two-way ANOVA, with whole-

body corticosterone content as the response variable and food level and population density as treatments. For experiment 2, the effect of treatment on growth within non-MTP treatments was analyzed using two-way ANOVA, with relative growth as the response variable and food level and density as treatments. Multiple comparisons were not conducted on the main effects since the interaction terms were significant in all cases (see Results).

Calculation of relative growth

Relative growth was calculated by subtracting the mean initial mass of tadpoles within a tank from the mean final mass of tadpoles within that tank and dividing this difference by the mean initial mass. Measures of relative growth for animals in replicate tanks were averaged to provide an overall mean relative growth for the treatment ($n = 3$; sample unit was the tank mean). For effects of MTP on the growth response to competition, two one-way ANOVAs were conducted. The first compared relative growth among the six treatments: 8H no MTP, 8H with MTP, 8M no MTP, 8M with MTP, 8L no MTP, and 8L with MTP, to evaluate the degree to which MTP altered the growth response to limited resources. The second compared relative growth among the four treatments: 8M no MTP, 8M with MTP, 40M no MTP, and 40M with MTP, to evaluate the degree to which MTP altered the growth response to density. Pairwise comparisons following these one-way ANOVAs utilized a Bonferroni correction to account for multiple comparisons. For one-way ANOVAs and pairwise comparisons, we set $\alpha = 0.025$, to account for the fact that the two ANOVAs analyzed some common data.

Effect of treatment on development rate within non-MTP treatments was analyzed using two-way ANOVA, with Gosner stage as the response variable and food level and density as treatments. For effects of MTP on the developmental response to competition, two one-way ANOVAs were conducted as described for growth data, with developmental stage as the response variable.

To evaluate the degree to which MTP treatment reduced whole-body corticosterone content in chronically treated tadpoles, Student's *t*-test was used to compare corticosterone content of tadpoles from tanks with and without MTP treatment within each density/food level treatment.

Day 52 percent survivorship among treatments was analyzed with two-way ANOVA, with fraction survivorship (arcsine square root transformed)

as the response variable and density and food level as treatments. High mortality within the high density treatments precluded any quantitative analysis of day 52 growth and development data.

RESULTS

Experiment 1: Whole-body corticosterone content

Whole-body corticosterone content of *R. pipiens* tadpoles was elevated following short term exposure to both limited resources and increased density (Fig. 1). Two-way ANOVA showed significant main effects of food level ($F_{2,45} = 7.6$; $P = 0.001$) and density ($F_{1,45} = 9.6$; $P = 0.003$) and a significant interaction between the two ($F_{2,45} = 7.0$; $P = 0.002$).

Experiment 2: Growth

Lower food levels caused lower growth rates within both densities (Fig. 2, top panel). Within the intermediate and low food treatments, high conspecific density decreased tadpole growth after 17 days, while within the high food group, higher density was associated with greater growth (Fig. 2, top panel). Two-way ANOVA showed a significant main effect of food level ($F_{2,12} = 153$; $P < 0.00005$) but no main effect of density ($F_{1,12} = 2.1$; $P = 0.17$), with a significant interaction between food level and density ($F_{2,12} = 35.3$; $P < 0.00005$).

Inhibition of corticoid biosynthesis by MTP did not affect growth within the low density treatment at the three food levels (Fig. 2, middle). However, while MTP did not ameliorate the depressive effect of limited resources on growth, it did ameliorate that of density. In the absence of MTP, high conspecific density depressed growth compared to

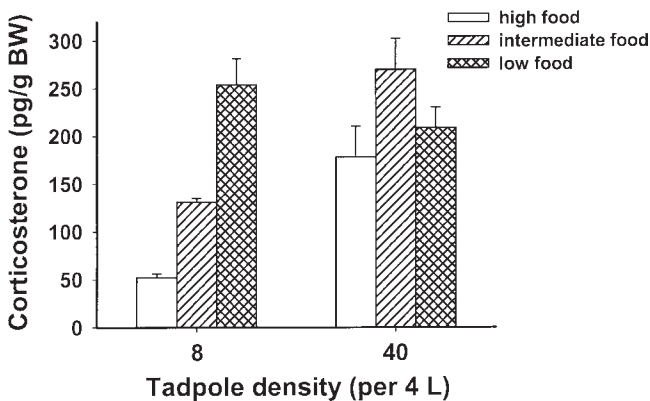


Fig. 1. Whole-body corticosterone content of *R. pipiens* tadpoles after four days at varying densities and food levels. Error bars represent standard errors of the mean of individual tadpoles ($n = 8-10$; 8M group, $n = 4$, due to radioimmunoassay values outside of optimal binding range).

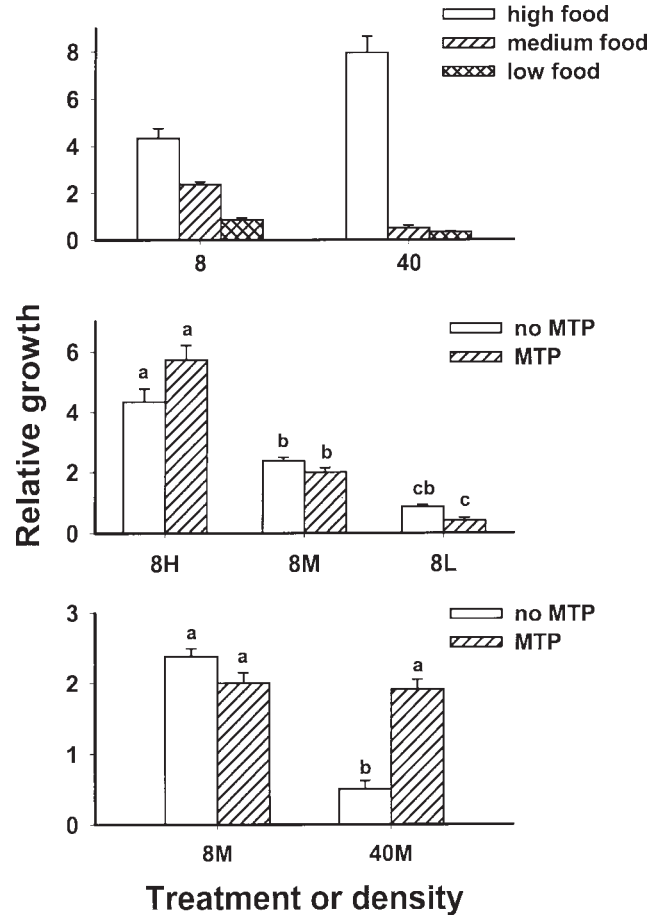


Fig. 2. Relative growth of *R. pipiens* tadpoles after 17 days at varying densities and food levels. Top panel: treatments without metyrapone (MTP). Middle panel: effect of MTP on growth among varying food levels, at the same density ('no MTP' bars are identical to those in top panel). Bottom panel: effect of MTP on growth between different densities, at the same food level ('no MTP' bars are identical to those in top panel, and 8M MTP group is identical to that shown in middle panel). See Materials and Methods for treatment abbreviations and for the calculation of relative growth. Different letters above the bars represent means that are significantly different from one another ($P < 0.025$). Means of bars with the same letter do not differ. Error bars represent standard errors of the mean of three replicate tanks.

the low density group, but in the presence of MTP, growth in the high density and low density groups did not differ (one-way ANOVA $F_{3,8} = 40$; $P < 0.00005$; Fig. 2, lower panel).

Experiment 2: Development

High density slowed tadpole development within the intermediate food treatment but was associated with accelerated development within the high food group (Fig. 3, top panel). Lower food levels slowed development within both densities (Fig. 3,

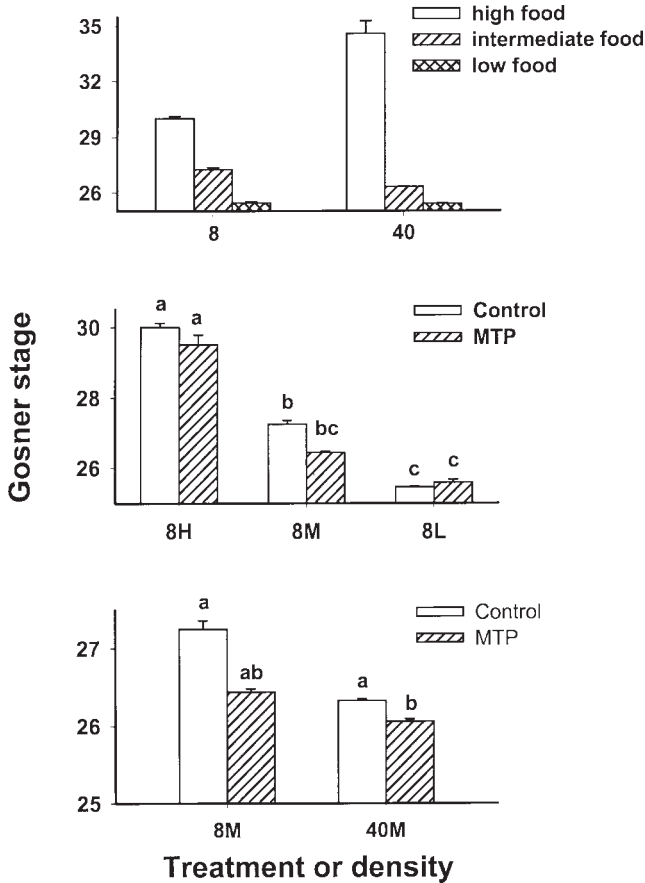


Fig. 3. Developmental stage (Gosner, '60) of *R. pipiens* tadpoles after 17 days at varying densities and food levels. Top panel: treatments without metyrapone (MTP). Middle panel: effect of MTP on development among varying food levels, at the same density ('no MTP' bars are identical to those in top panel). Bottom panel: effect of MTP on density between different densities, at the same food level ('no MTP' bars are identical to those in top panel, and 8/M MTP group is identical to that shown in middle panel). See Materials and Methods for treatment abbreviations. Different letters above the bars represent means that are significantly different from one another ($P < 0.025$). Means of bars with the same letter do not differ. Error bars represent standard errors of the mean of individual tadpoles ($n = 22$ – 116 ; sample sizes vary due to differences in treatment density and mortality).

top panel). Two-way ANOVA showed a significant main effect of food level ($F_{2,330} = 348$; $P < 0.00005$) but no main effect of density ($F_{1,330} = 0.55$; $P = 0.46$), with a significant interaction between food level and density ($F_{2,330} = 7.8$; $P = 0.0005$). Blocking corticoid synthesis with MTP did not alter the negative effect of limited resources on development (Fig. 3, middle panel). MTP treatment did not reverse the negative (though nonsignificant) effect of density on development but instead appeared to slow development compared to the non-

MTP groups (one-way ANOVA $F_{3,217} = 12$; $P < 0.00005$; Fig. 3, lower panel).

Experiment 2: Corticosterone content with MTP treatment; day 52 survivorship

Corticosterone content of MTP-treated tadpoles was lower than that of untreated tadpoles after 52 days of treatment but was still measurable (Fig. 4). Survivorship at day 52 was significantly lower in high density groups compared to low density (Fig. 5, two-way ANOVA: density $F_{1,12} = 26$, $P < 0.0005$; food $F_{2,12} = 0.39$, $P = 0.69$; interaction $F_{2,12} = 1.6$, $P = 0.24$). While high mortality precluded a quantitative analysis of growth and developmental responses, the patterns we observed at day 52 within low density groups were qualitatively similar to those presented for day 17.

DISCUSSION

We have shown that short term exposure (four days) to either increased conspecific density or limited resource levels elevated whole-body corticosterone content in *R. pipiens* tadpoles. This corticosterone elevation appears to be responsible for the depressive effect of density on growth but not on development, and does not mediate the effect of food limitation on growth or development. This study is the first to identify a physiological factor that varies with increased intensity of com-

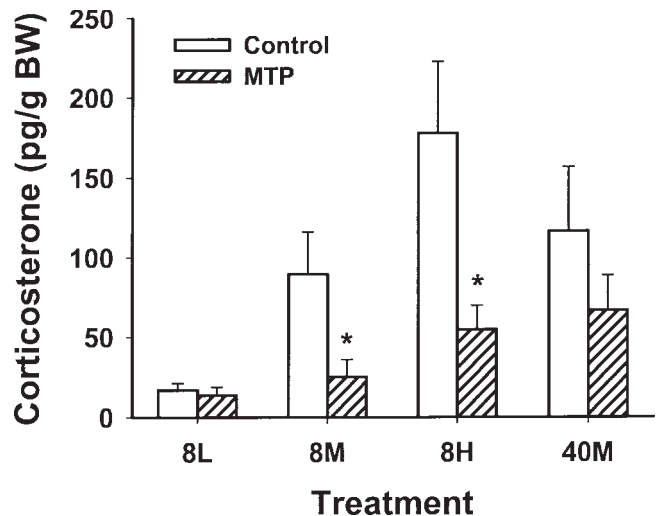


Fig. 4. Whole-body corticosterone content of *R. pipiens* tadpoles after 52 days at varying densities and food levels, with or without metyrapone (MTP). Asterisks (*) indicate significant difference of MTP-treated group from non-MTP group within each density/food level treatment ($P < 0.05$). Error bars represent standard errors of the mean of individual tadpoles ($n = 6$ – 7 ; 8L/MTP group, $n = 4$, due to radioimmunoassay values outside of optimal binding range).

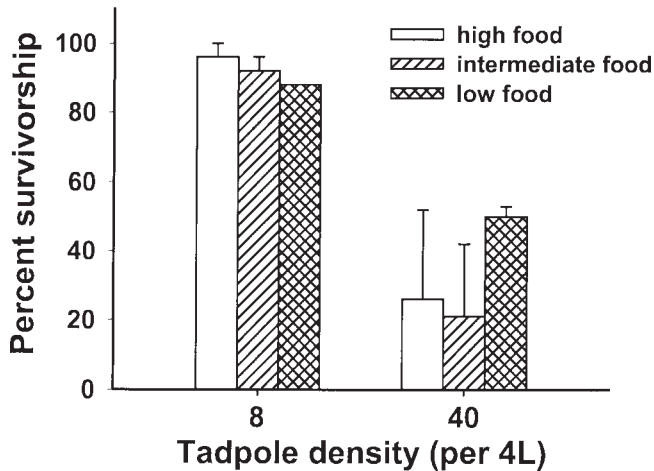


Fig. 5. Percent survivorship of *R. pipiens* tadpoles after 52 days at varying densities and food levels. Error bars represent standard errors of the mean of three replicate tanks.

petition in larval amphibians. Hayes ('97) reported an elevation in whole-body corticosterone content of *Bufo boreas* tadpoles held at five-fold higher densities than controls, with ad libitum food levels. In the current study, we separated competition into the two components of increased density and resource limitation, and found that each was associated with elevated corticosterone content.

Competition is well known to inhibit growth and reproduction, and our results are consistent with these findings. That tadpoles in the 40H treatment exhibited higher growth rates than those in the 8H treatment could represent an Allee effect (Allee, '31), whereby increased density facilitates growth up to some threshold density and inhibits growth thereafter. Other studies have also found positive effects of density over some density ranges in tadpoles (Brockelman, '69; Travis and Trexler, '86). Wilbur ('77) observed such an effect in toad tadpoles, with a negative correlation between growth and density becoming apparent only at densities above 40 tadpoles per 2 L. Beiswenger ('75) concluded that facilitation among toad tadpoles occurred through physical interactions that stirred and suspended the food, making it more available for consumption. While this stirring mechanism has not been studied in *R. pipiens* tadpoles, several studies suggest that physical interactions are important mediators of competitive growth effects in this species (Rugh, '34; Gromko et al., '73). If the stirring mechanism were acting in the current experiment, food levels in the 40M and 40L treatments might not have been sufficient for tadpoles to benefit from such physical interactions, while

food levels in the 40H treatments may have been sufficient for this mechanism to act. Travis and Trexler ('86) found negative density effects in toad tadpoles within environments of poor physical quality but positive density effects in high-quality environments, and others have shown negative density effects to be more pronounced in physically stressful environments (Watkinson, '82). In our experiment, density negatively affected growth at intermediate and low food levels, and our results with MTP at the intermediate food level suggest that the mechanism may involve the endocrine stress axis.

For tadpoles fed at the intermediate food level, the negative effect of density on growth was reversed by MTP treatment (Fig. 2, bottom panel), suggesting that the elevated corticosterone content associated with increased density (see Fig. 1) produced the observed growth depression. However, the deceleration of development observed in the 40M compared to the 8M group was not reversed by MTP (Fig. 3, bottom panel), suggesting that the developmental response was not mediated by corticoids. Instead, MTP slowed development at both densities. Treatment of pre-metamorphic tadpoles with corticoids inhibited growth but not development (Gray and Janssens, '90; Hayes et al., '93; Wright et al., '94; Hayes, '95), and our findings support a physiological role for corticoids in growth suppression caused by crowding when resources are limited. The inhibition of development by MTP in our study suggests that some amounts of endogenous corticoids are necessary for development. These results support the findings of Kikuyama et al. ('82), who concluded that endogenous corticoids are necessary to sustain tadpole development. They showed that treatment with amphenone B (a corticoid synthesis inhibitor) retarded T4-induced tail regression in toad tadpoles (under thiourea treatment to avoid possible effects of amphenone B on endogenous thyroid hormone concentrations), and that this effect was reversed by treatment with corticoids.

While the growth response to high density was reversed by MTP treatment, neither the growth nor the developmental response to limited resources was affected by MTP treatment, suggesting that corticoids do not mediate these responses to limited resources. If corticoids were mediating these responses, the values in the 8M-MTP and 8L-MTP treated groups should have approached those of the high food group at the same density (8H; see Figs. 2–3, middle panels). The response to limited resources is likely due to metabolic con-

straints involving insufficient nutrients for structural growth or development. Resource limitation is known to elevate plasma corticoid concentrations in other taxa, which serves to mobilize spare fuel in fasting animals (Selye, '73; Munck et al., '84; Wingfield, '94; Kuhn et al., '98). While MTP treatment should reduce such catabolic activity, overall growth and development could still be limited by insufficient resources.

While corticosterone did not mediate the developmental response to competition or the growth response to limited resources, the significant increase in corticosterone content with both limited resources and high density raise the possibility that other physiological or behavioral responses besides growth and development could be influenced by this corticosterone increase. For example, corticosterone is known to affect behavior in other taxa (Moore and Zoeller, '85; Silverin, '86; DeNardo and Sinervo, '94; Wingfield et al., '97), and the presence of competitors has been shown to influence tadpole behavior (Skelly, '92; Anholt and Werner, '95). Direct studies of corticosterone's effect on tadpole behavior, and the interaction of any effects with competitor presence, would clarify the degree to which elevated corticosterone mediates tadpole behavioral responses to competitors.

In addition to the information the current results provide about the mechanisms of competitive response, they may also yield insight concerning the factors that ultimately determine the timing of metamorphosis. Wilbur and Collins ('73) proposed that development rate, and thus the length of the larval period, responded to growth rate throughout the larval period, and that any effect of environmental factors (such as competition, predation, pond drying, etc.) on development rate would be mediated through effects on individual growth rate. Smith-Gill and Berven ('79) and Travis ('84) noted that rates of growth and development are imperfectly correlated and suggested that each rate varies independently in response to the environment. Our results are consistent with the latter hypothesis, as we were able to dissociate the developmental response to density from the growth response. While growth was accelerated by MTP treatment in the 40M group, development was unchanged compared with the non-MTP group. This pattern suggests that growth rate per se does not dictate development rate, but that perhaps some physiological factor (other than corticosterone; e.g., thyroid activity) alters development in response to environmental conditions. Further insight into theoretical questions about control of

metamorphic timing is likely to be gained through additional studies of endocrine responses to environmental conditions. Hormones interact in complex ways to orchestrate tadpole growth and development (Kikuyama et al., '93), with specific actions changing over development and in the presence of other hormones. Given the responsiveness of the hypothalamo-pituitary-interrenal (HPI) axis to competitive intensity and to pond drying (Denver, '97; '98), this axis may prove to be a common mediator of tadpoles' developmental responses to their environment.

Figure 4 demonstrates that MTP lowered whole-body corticosterone content after 52 days but did not produce 100% reduction. While higher MTP doses might further reduce corticosterone content, feedback mechanisms may prevent complete reduction of whole-body corticoids by treatment with a static dose of MTP. A reduction in corticoid production would reduce negative feedback on the hypothalamus/pituitary and thus increase stimulation of the interrenal glands (Axelrod and Reisine, '84; Keller-Wood and Dallman, '84; Munck et al., '84). Consequently, an increasing dose of MTP might be required to fully suppress corticoid production by this strengthening signal. The non-MTP-treated groups in Fig. 4 show a corticosterone pattern reversed from that shown in Fig. 1, due to the fact that, after 52 days, tadpoles at lower densities and higher food levels had reached later developmental stages than those at high density or low food levels. Whole-body corticosterone content increases as development progresses in *R. pipiens* and other ranid tadpoles (Krug et al., '83; Glennemeier and Denver, unpublished data). The short-term corticosterone response to competition shown in Fig. 1 was not confounded by developmental stage, as all tadpoles were at Gosner stage 25. Mortality at day 52 was higher in high density groups compared to low density groups, consistent with other studies of density-dependent larval survivorship (Wilbur, '80; Smith, '87).

CONCLUSIONS

We have identified a proximate mediator of the growth response to competition in *R. pipiens* tadpoles. Other mediators may include hormones associated with the endocrine stress axis (e.g., hypothalamic and pituitary hormones), as this axis has been shown to mediate responses to environmental change in tadpoles and other taxa (Wingfield et al., '97; Denver, '98; Overli et al., '99). Further knowledge of tadpole physiological ecology promises to provide a valuable tool for pre-

dicting the outcomes of species interactions. Processes such as predation and competition are known to interact in complex ways to influence fitness in larval amphibians (Werner, '91; Anholt and Werner, '95; Werner and Anholt, '96). The identification of one or more common physiological mediators through which tadpoles respond to predators, competitors, or other environmental factors may help to simplify hypotheses about how these processes interact and may improve predictions of community-level outcomes.

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