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5 Increased larval density induces accelerated metamorphosis independently

6 of growth rate in the frog *Rana sphenocephala*

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19 Abstract.--We grew larval *Rana sphenoccephala* at different densities but maintained
20 equal mean growth rates among density treatments (via equal per capita food levels) in
21 order to test the hypothesis that larval density can influence metamorphic timing
22 independently of larval growth rate. Tadpoles at high density metamorphosed earlier
23 than tadpoles at low density despite growing at similar rates. Food reductions did not
24 accelerate metamorphosis. These results support the hypothesis that density can be a
25 sufficient cue to initiate metamorphosis independently of growth rate.

26

26 In amphibian larvae, metamorphic parameters affect fitness. The effects of density on
27 metamorphosis have been demonstrated to carry over into the juvenile period with the
28 result that larvae raised at low density generally metamorphose at larger sizes, survive
29 better, have greater lipid stores, higher resistance to parasites, have increased likelihood
30 to breed and produce larger clutch sizes (Scott, 1994; Beck and Congdon, 2000; Morey
31 and Reznick, 2001; Scott et al., 2007). Size at metamorphosis also influences age and
32 size at maturation (Smith, 1987; Semlitsch et al., 1988). Thus, it remains essential to
33 clarify how environmental cues can be transduced to the endocrinological initiation of
34 metamorphosis.

35 The influence of density on metamorphic size is so predictable that one can
36 reliably create differently-sized juvenile (newly transformed) amphibians for experiments
37 by growing larvae at varied densities, with the repeatable result that larvae at high density
38 become small juveniles and vice versa (Scott, 1994; Morey and Reznick, 2001). In
39 contrast, the effect of density on metamorphic timing is not so clearly understood.
40 Several models (e.g., Wilbur and Collins, 1973; Day and Rowe, 2002) suggest that a
41 decline in larval growth that would be associated with increased density can initiate
42 metamorphosis. In contrast, most observations from nature and experiments indicate that
43 slower growth that accompanies high larval density is associated with delayed
44 metamorphosis (Newman, 1987; Scott, 1990).

45 Because density and growth in amphibian larvae are typically confounded in
46 nature, we conducted an experiment wherein we varied density of larval *Rana*
47 *sphenocephala* while keeping per capita growth equal. In addition, we crossed this
48 density treatment with a growth treatment of (1) consistent growth or (2) a decline in

49 growth. Several studies have indicated that a decline in growth that occurs during late
50 larval development can result in a change in the timing of metamorphosis (e.g., Morey
51 and Reznick, 2000; Ryan and Semlitsch, 2003). Our null hypothesis was that variation in
52 density and growth regime would fail to result in variation in metamorphic size and
53 metamorphic timing.

54 MATERIALS AND METHODS

55 We obtained embryos of larval *Rana sphenocephala* from Sullivan Supply Company
56 (Middleboro, Tennessee) in October 2003. At this locality and at similar latitudes
57 (Johnson, 1992), *R. sphenocephala* breed and deposit eggs in the autumn, and tadpoles
58 overwinter and metamorphose the following June-July. Upon hatching (30 October),
59 tadpoles were placed into one of 72 plastic boxes (30 X 17 X 21 cm LWH) filled with
60 2600 ml of reverse-osmosis (RO) water.

61 The boxes were divided into groups of six blocks, with twelve boxes per block.
62 Each box was a replicate in a fully-factorial 2 X 6 design, where the factors were food
63 reduction (yes/no) and density (1, 2, 4, 8, 10, or 20 tadpoles per box). Thus, each box in
64 a block held 1, 2, 4, 8, 10, or 20 tadpoles, replicated twice in each block to provide a
65 constant food treatment and a food reduction treatment. Blocks contained 90 tadpoles,
66 for a total of 540 tadpoles. Boxes were maintained in a room with windows that provided
67 an ambient (Minot, North Dakota) light cycle. Temperature in the room was 20°C +/-
68 3°C.

69 Every three days, tadpoles were removed from boxes, the boxes were cleaned,
70 refilled with 2600 ml of fresh RO water, and tadpoles were replaced into the boxes. The
71 total mass of tadpoles in each box was determined once per week, beginning on day 18

72 and ending on day 193. Mass was determined by placing all tadpoles from a box in an
73 aquarium net, counting and recording tadpole number, blotting dry to remove excess
74 water, and weighing the total tadpole mass to the nearest mg using a top-loading balance.

75 Feedings occurred immediately after water changes (i.e., every three days).
76 Feedings consisted of a per capita 25 mg aliquot of a finely ground 1:1 mixture of rabbit
77 chow and fish food flakes, e.g., the one-tadpole treatment received a 25 mg aliquot
78 whereas the 10-tadpole treatment received a 250 mg aliquot at each feeding.

79 As tadpoles grew, aliquots were increased. On day 25 (November 24), aliquots
80 were increased to 50 mg per capita, and on day 95 aliquots were increased to 75 mg per
81 capita. Because a minimal size is required for competence to metamorphose (Wilbur and
82 Collins, 1973), we waited until day 144 (March 22) to administer a food reduction
83 treatment. At this date, all tadpoles were prometamorphic and larger than the minimal
84 metamorphic size of *R. sphenoccephala* from wild populations (Butterfield et al., 2005).
85 Thus, at day 144, one-half of the treatments were subjected to a food reduction that
86 returned these treatments to a per capita 25 mg aliquot. Metamorphs (defined as forelimb
87 emergence) were weighed and larval period (days) was recorded. Metamorphs first
88 appeared on day 195 (May 12) and all tadpoles had metamorphosed by day 240 (26
89 June). These metamorphic dates are approximately coincident with estimates from wild
90 populations at similar latitudes (Johnson, 1992).

91 In order to confirm expected growth effects, we used a one-way MANOVA to
92 examine the effect of density on the vector of average mass recorded each week, and then
93 compared these masses at each date using a one-way ANOVA. Then, we analyzed mean
94 metamorphic timing (in days) and mean metamorphic mass (in mg) of all transforming

95 tadpoles with a two-way MANOVA with density and food reduction as treatment effects.
96 Data for metamorphic timing were inversed-transformed (Alford and Harris, 1988) and
97 data from metamorphic mass were log-transformed (Sokal and Rohlf, 1995). We used
98 $\alpha = 0.05$ as significance criterion, and Wilks' λ was used as our multivariate test statistic.

99 RESULTS

100 Because the one-tadpole and two-tadpole density treatment groups grew at slower rates
101 they were removed from further analyses. The other density treatment groups (i.e., 4, 8,
102 10, and 20 tadpoles) grew at similar mean rates (Fig. 1). Mortality was low (< 5%) and
103 similar across all groups.

104 Food reduction did not result in a reduction in larval period (Table 1). There was,
105 however, a significant effect on metamorphic mass of food reduction: food-reduced
106 groups had significantly lower metamorphic size (Fig. 2).

107 There was a significant difference in larval period due to variation in density:
108 tadpoles at higher density had shorter larval periods, i.e., exhibited accelerated
109 metamorphosis (Table 1). Density did not affect metamorphic mass (Fig. 3).

110 DISCUSSION

111 Because the causes (e.g., pond desiccation) and consequences (decreases in
112 growth rate) of high larval density are usually confounded, our goal was to grow tadpoles
113 living at different densities at equal mean rates in order to test the hypothesis that
114 variation in density causes variation in metamorphic timing. Our results indicate that
115 density can influence metamorphic timing in *R. sphenoccephala* tadpoles. While there was
116 not a significant difference in body size (our intended result), metamorphic timing

117 differed significantly between the 4-tadpole treatment and the 20-tadpole treatment, with
118 the 20-tadpole treatment density metamorphosing earlier.

119 Studies on larval density have been of two kinds: density experiments (i.e.,
120 manipulations of actual number of larvae in a given volume) and desiccation experiments
121 (i.e., manipulations of same number of larvae in declining volumes). Density
122 experiments produced slower growth and delayed metamorphosis at smaller sizes (Morey
123 and Reznick, 2001; Loman, 2004; Resetarits et al., 2004), while desiccation experiments
124 produced accelerated metamorphosis at smaller sizes (Newman, 1988; Denver et al.,
125 1998; Kiesecker and Skelly, 2001).

126 Our study was a density experiment that deviated from the studies cited above by
127 keeping mean growth equal across density treatments. Our result of accelerated
128 metamorphosis is more similar to desiccation experiments. What this suggests is that the
129 retarded development in density experiments may have a nutritional basis, i.e.,
130 intraspecific competition for food prevents rapid development. In contrast, in our density
131 experiment larvae were freed from intraspecific competition and thus could accelerate
132 development. Perhaps the reduced per capita swimming volume experienced by tadpoles
133 at high density created similar stress conditions to those in desiccation experiments
134 wherein food access was not restricted at the same time as water volume was declining.
135 Thus, it is tenable that in actual ponds, the effect of density on metamorphic development
136 will be contingent on the nature of the larval habitat. If the pond's food resources remain
137 sufficient to support rapid growth, then development can be accelerated to escape a
138 drying pond. In contrast, a pond with poor growth opportunities for larvae is not likely to
139 be able to produce any metamorphs in the case of desiccation.

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195 **Figure legends**

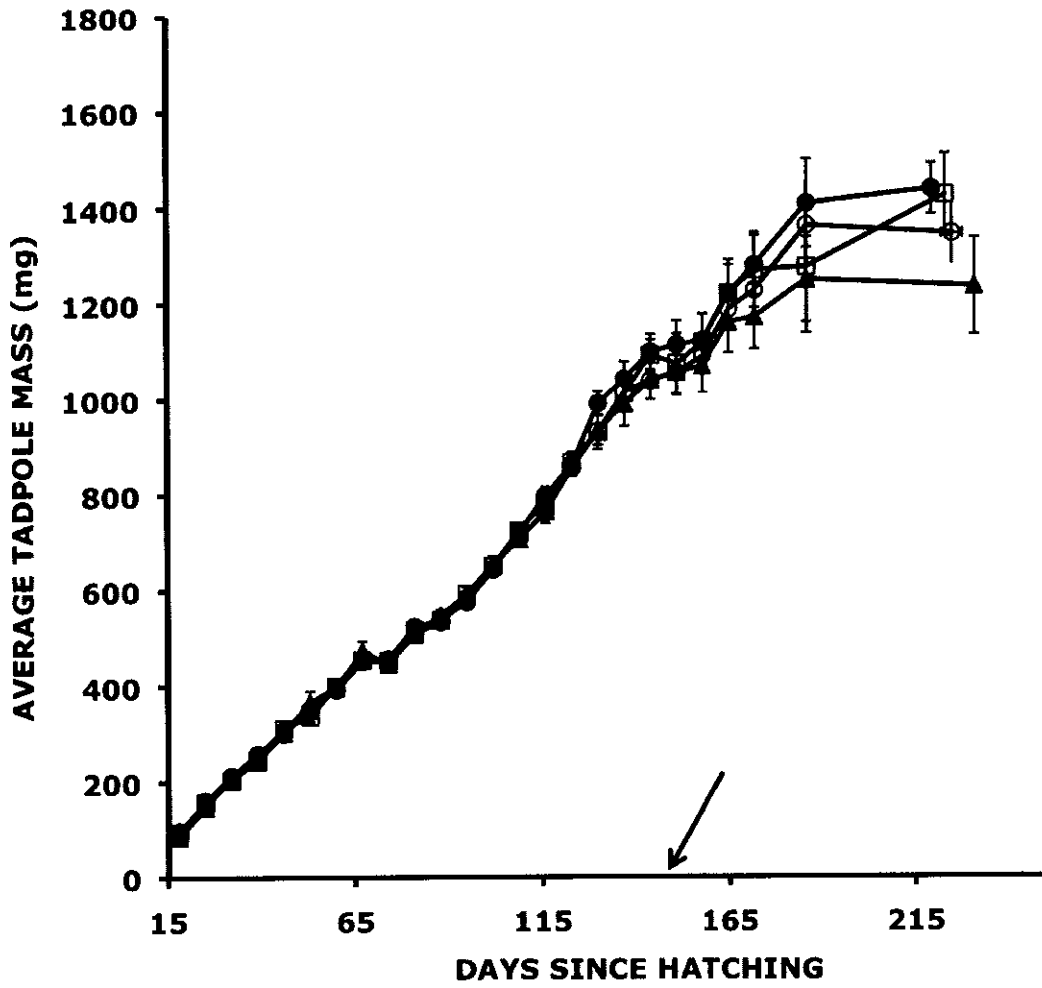
196 Fig. 1. Growth trajectories showing mean body size (\pm 1 SE) of tadpoles at density
197 treatments. Trajectories terminate at mean metamorphic mass and date of
198 metamorphosis. Closed triangles denote four tadpoles per box, open squares denote eight
199 tadpoles per box, open circles denote 10 tadpoles per box, and closed circles denote 20
200 tadpoles per box. Arrow indicates initiation of food reductions in half the food
201 treatments. Note the consequent increase in variance in tadpole masses in each density
202 treatment following food reduction.

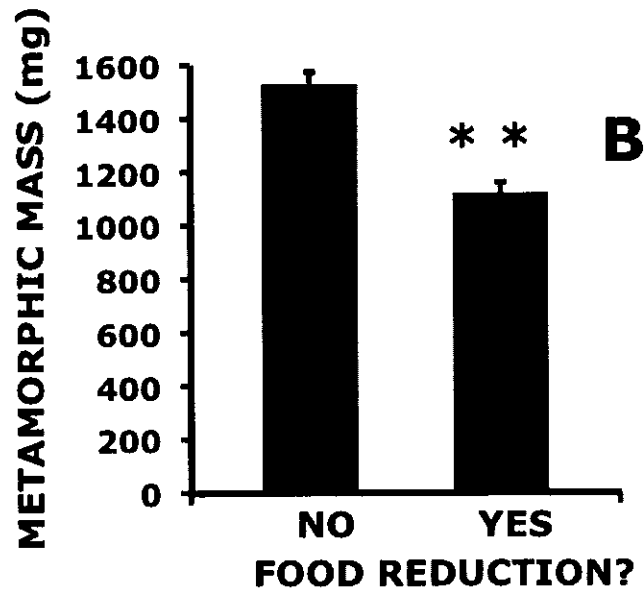
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204 Fig. 2. Summary of responses of larval *Rana sphenoccephala* to food reduction treatments.
205 (A) Larval period in days. (B) Mass at metamorphosis. Bars represent means + 1 SE. **
206 $P < 0.001$.

207

208 Fig. 3. Summary of responses of larval *Rana sphenoccephala* to density treatments. (A)
209 Larval period in days. (B) Mass at metamorphosis. Bars represent means + 1 SE. Letters
210 above bars indicate treatment group means not significantly different using LSD a
211 posteriori contrasts.





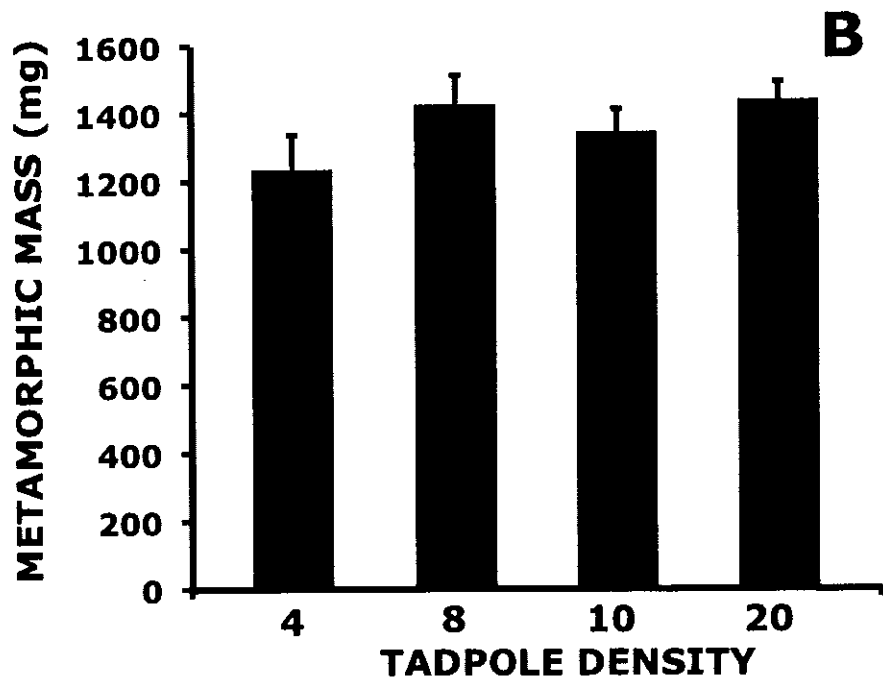
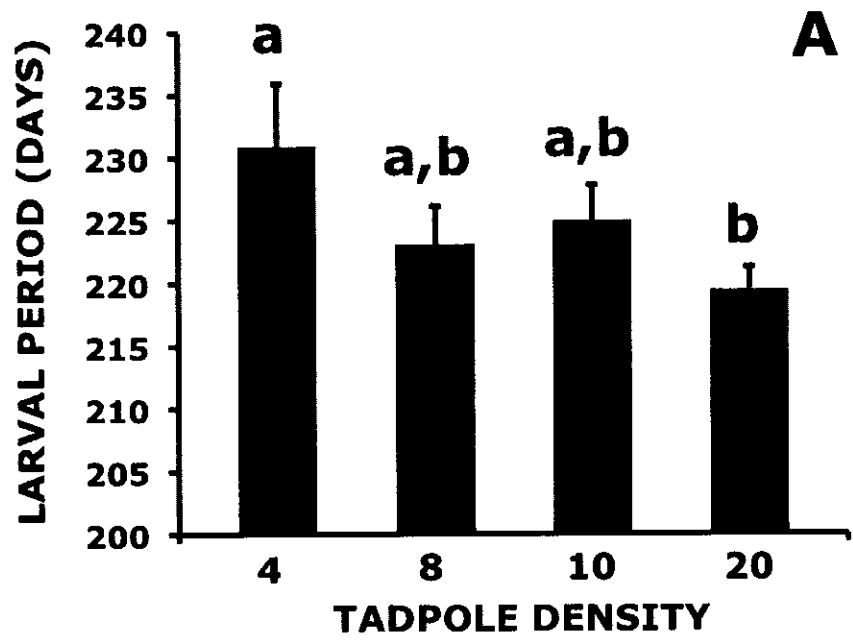


Table 1. Summary of multivariate and univariate analyses of the effect of treatments on log-transformed metamorphic mass (mg) and inverse-transformed larval period (days). Mean squares for metamorphic mass are $X \times 10^{-2}$, mean squares for larval period are $X \times 10^{-7}$. For the multivariate statistics, $df = 6,400$ for density and density X food; $df = 2,400$ for food. For univariate statistics, $df = 3,201$ for density and density X food, and $df = 1,209$ for food. For metamorphic mass, error $MS = 2.01 \times 10^{-2}$; for larval period, error $MS = 1.27 \times 10^{-7}$.

Univariate statistics

Source	<u>Multivariate statistics</u>			<u>mass at metamorphosis</u>			<u>larval period</u>		
	Wilks' λ	F	P	MS	F	P	MS	F	P
Density	0.955	1.56	0.158	3.22	1.605	0.189	4.01	3.15	0.026
Food	0.824	21.28	<0.001	65.20	32.48	<0.001	4.13	3.24	0.073
Density X Food	0.970	1.03	0.404	1.60	0.796	0.497	1.03	0.812	0.488