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Effects of temperature on embryonic and early larval growth and development in the rough-skinned newt (*Taricha granulosa*)

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ABSTRACT

We investigated the effects of temperature on the growth and development of embryonic and early larval stages of a western North American amphibian, the rough-skinned newt (*Taricha granulosa*). We assigned newt eggs to different temperatures (7, 14, or 21 °C); after hatching, we re-assigned the newt larvae into the three different temperatures. Over the course of three to four weeks, we measured total length and developmental stage of the larvae. Our results indicated a strong positive relationship over time between temperature and both length and developmental stage. Importantly, individuals assigned to cooler embryonic temperatures did not achieve the larval sizes of individuals from the warmer embryonic treatments, regardless of larval temperature. Our investigation of growth and development at different temperatures demonstrates carry-over effects and provides a more comprehensive understanding of how organisms respond to temperature changes during early development.

1. Introduction

Temperature is among the most indelible and long-studied factors influencing growth and development in animals (Angilletta et al., 2004; Gillooly et al., 2002; Huey and Stevenson, 1979; Lillie and Knowlton, 1897; Zuo et al., 2012). Temperature can play especially important roles at early life-history stages, where it is known to profoundly influence embryonic and larval growth and development (Brown et al., 1992; Howe, 1967; Pepin, 1991), and have important fitness consequences for later life (Blanckenhorn, 2000; Chamaille-Jammes et al., 2006; Huey and Berrigan, 2001).

One of the ways that temperature can influence organisms throughout their lives is through carry-over effects, where the effects of temperature experienced at one life-history stage pass to the next discrete life stage. Carry-over effects of embryonic temperature have been observed in a wide variety of taxa (reviewed by Hopkins et al. (2014)), ranging from arthropods (Ernsting and Isaaks, 1997; Geister et al., 2009; Giménez, 2006) and tunicates (Thiyagarajan and Qian, 2003) to fish (Johnston et al., 1998; Martell et al., 2005, 2006) and reptiles (Brooks et al., 1991; Elphick and Shine, 1998; O'Steen, 1998).

Amphibians are excellent models for studying the effects of temperature at, and across, early development. Temperature has long been known to affect rates of embryonic and larval growth in these animals (Moore, 1939; Wilbur and Collins, 1973; Harkey and Semlitsch, 1988; Newman, 1989; Smith-Gill and Berven, 1979; Álvarez and Nicieza, 2002), and the discrete life stages of amphibians make them attractive models for the study of carry-over effects. Carry-over effects have been found in amphibians exposed to ultraviolet radiation (Belden and Blaustein, 2002; Pahkala et al., 2001), salinity (Wu et al., 2012; Hopkins et al., 2014), and acidic conditions (Räsänen et al., 2002), but the carry-over effects of temperature across early life-history stages are still largely unknown. Given that the world is undergoing unprecedented anthropogenic change (Steffen et al., 2007), including global climate change (IPCC, 2014), and amphibians are known to be particularly sensitive to changes in their environments (Hopkins, 2007), investigating the implications of temperature shifts on the development and growth of amphibians at and across early life-history stages is crucial for conservation efforts (Walther et al., 2002).

We investigated the effects of temperature on embryonic and early larval growth and development in the rough-skinned newt (*Taricha granulosa* Skilton; Caudata: Salamandridae), a common

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amphibian inhabiting ponds and streams along the west coast of North America. We predicted that increasing temperatures would result in more rapid growth and development, and that the temperature at which embryos developed would affect growth and development after hatching, providing evidence for carry-over effects.

2. Materials and methods

2.1. Animal collection, housing, and egg deposition

Gravid *T. granulosa* females (16) were collected from Hunter Creek, Curry County, Oregon (42°22′07.30′′N, 124°24′16.64′′W) by dip net, minnow trap, or by hand in May 2013. Animals were housed individually at Utah State University in plastic containers filled with 200 ml filtered water. The containers were kept at 14 °C and newts were fed blackworms (*Lumbriculus* spp.) ad libitum.

Females were injected with 10 µl luteinizing hormone releasing hormone ([des-Gly10, D-His(Bzl)6]-LHRH ethylamide; Sigma #L2761, Sigma-Aldrich, St. Louis, MO, USA) to induce oviposition onto pieces of polyester fiber. Eggs were collected within 12 h of deposition and placed in different cups with 200 ml of filtered water that were designated to one of three environmental control chambers (7 °C, 14 °C, or 21 °C) using an equal-probability method to ensure that each female's offspring were represented equally in all treatments. The temperature treatments chosen for this study reflect the natural variation the animals experience at the site from which they were collected (Hopkins, unpublished data). Generally, water temperatures are cooler toward the beginning of the breeding season in spring and warmer throughout the summer as the offspring grow and develop. However, there is considerable variation even within a small reach of the sample stream driven by water depth, microhabitat complexity, and weather patterns. Eggs and larvae of *T. granulosa* can be subject to the temperatures chosen for this study in the wild, depending on local conditions.

2.2. Temperature treatments and measuring growth and development

For each individual egg, time (in days) from oviposition to hatching was recorded, and any egg that failed to hatch was removed from the experiment. Once hatched and free-swimming, the larvae were placed in individual cups with 200 ml of filtered water. At least 60 larvae from each embryonic temperature treatment were re-designated to larval treatments (7 °C, 14 °C, or 21 °C), again using an equal-probability method so that any effect of the female would be balanced across treatments. The combination of embryonic and larval temperatures created a total of nine treatments with at least 20 individuals (and a maximum of 26) in each treatment and a total of 200 larvae. Specifically, $N_{7,7\ (\text{embryonic temperature, larval temperature})}=24$, $N_{7,14}=20$, $N_{7,21}=22$, $N_{14,7}=23$, $N_{14,14}=22$, $N_{14,21}=22$, $N_{21,7}=26$, $N_{21,14}=20$, and $N_{21,21}=22$.

Each larva was measured and staged immediately after hatching and then weekly for four weeks, using a stereo-microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). Total length was recorded using an ocular micrometer. Larvae were staged following the standard salamander early life-history developmental staging protocol of Harrison (1969). Once the larvae grew beyond the Harrison staging protocol, the Watson and Russell (2000) larval staging scheme was utilized. However, the numbering system for Harrison (1969) was continued instead of using the Watson and Russell (2000) numbering system. The equivalent of stage 7 in Watson and Russell's (2000) system was stage 46 in Harrison's protocol (1969), so stage 8 according to Watson and Russell's (2000) system was considered to be stage 47 in this study.

Although larvae open their mouths at stage 44, they retain residual yolk until stage 46 (Harrison, 1969). Supplemental feeding (which could confound the effects of temperature alone on growth and development) was thus unnecessary for the time frame of this study, which continued for four weeks if the group mean did not reach stage 46. Animals were exempt from further analysis if the mean developmental stage for the treatment group reached stage 46. Larvae were euthanized in 5% MS-222 at the conclusion of the experiment.

2.3. Statistical analyses

The effect of temperature on time to hatching was assessed using Friedman's Test (blocking on individual female) due to a lack of normality in these data. Where an overall significant difference was found, we compared the effects of different treatments using Dunn's Multiple Comparisons. The effect of embryonic temperature on length and developmental stage at hatching was assessed using two-way ANOVAs with female incorporated as a random effect, and post-hoc Tukey-adjusted multiple comparisons. Because we examined length and development of all larvae for three weeks post-hatching, and larvae at 7 and 14 °C for four weeks post-hatching, we ran two separate analyses: the first analyzing the effects of embryonic temperature, larval temperature, time, and their interactions on larval growth (total length) and development (developmental stage) for all three larval temperatures for three weeks post-hatching, and the second analyzing these effects for 7 and 14 °C for four weeks post-hatching. Both models were mixed model two-way factorial incomplete random block designs with repeated measures modeled using a first-order autoregressive structure. Individual female was treated as a random block effect, and within each female values for multiple larvae in each treatment combination were averaged and the means were subsequently used as response data. Depending on fecundity, each female contributed 0–5 offspring to each treatment, with a mean of 1.62. Where an overall significant effect of embryonic temperature was found, we conducted post-hoc comparisons using the "simulate" adjustment to determine differences in embryonic treatment at each week at each larval treatment (Edwards and Berry, 1987). Statistical analyses were completed in SAS v9.3 (Cary, North Carolina, USA), and significance was set at $\alpha=0.05$.

3. Results

3.1. Effect of embryonic temperature on hatching timing, body length and developmental stage at hatching

There was a significant effect of embryonic temperature on time to hatching (Friedman's $\chi^2=161.67$, $p<0.001$) (Fig. 1A). Eggs reared at 21 °C took the shortest time to hatch (mean ± SE = 15.41 ± 0.18 days), followed by eggs reared at 14 °C (29.82 ± 0.29 days) and 7 °C (109.3 ± 1.18 days) (Fig. 1A). There was a significant effect of embryonic temperature on body length ($F_{2,182}=4.74$, $p<0.01$), and developmental stage ($F_{2,182}=24.37$, $p<0.001$) at hatching, with larvae hatching significantly larger at 7 °C than at 14 °C (Tukey-adjusted multiple comparison, $p<0.01$) (Fig. 1B), and more developed at 21 °C than at 7 °C or 14 °C (Tukey-adjusted multiple comparisons, $p<0.001$) (Fig. 1C).

3.2. Effects of temperature on larval growth and development

There was a significant effect of embryonic temperature, larval temperature, time, and their interactions on larval newt growth

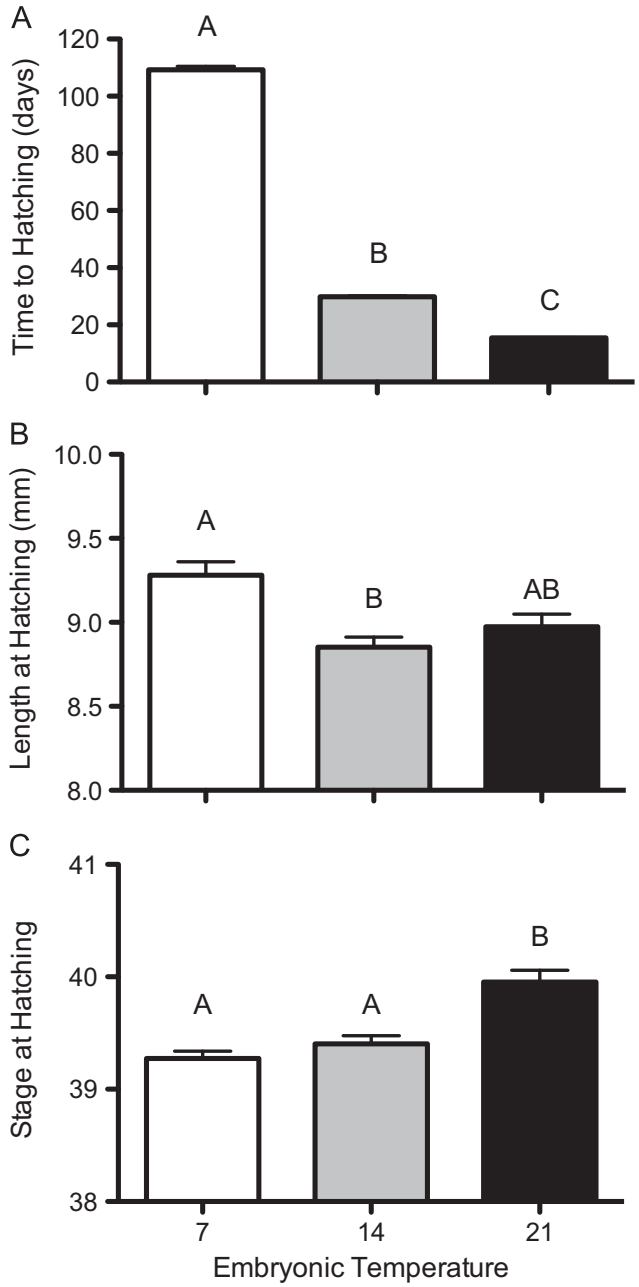

Fig. 1. (A) Time to hatching, (B) total body length at hatching, and (C) developmental stage at hatching of newt larvae reared as eggs in either 7 °C, 14 °C, or 21 °C. Asterisks or different letters indicate statistical differences among treatments ($p < 0.05$).

and development for both three and four week analyses (Table 1). Larval growth and development was slowest at 7 °C, followed by 14 °C and 21 °C, where they appeared to plateau around the second and third weeks to a maximum of approximately 14 mm and stage 47 (Fig. 2).

3.3. Carry-over effects of embryonic temperature on larvae at 7 °C

At larval temperature 7 °C, newts reared at all three embryonic temperatures grew to similar sizes (simulated-adjusted p-values for multiple comparisons, $p > 0.05$; Fig. 2A). However, larval development was dependent upon embryonic temperature. Larvae reared embryonically in 21 °C were more developed at hatching, and stayed more developed throughout the course of the experiment than newts reared embryonically at 7 °C (all $p < 0.02$) (Fig. 2B). Newts reared embryonically at 14 °C generally maintained developmental stages intermediate to the other treatments beginning at the second week post-hatching (Fig. 2B).

3.4. Carry-over effects of embryonic temperature on larvae at 14 °C

All newts initially reacted similarly to larval temperature 14 °C, regardless of embryonic rearing temperature in terms of growth (Fig. 2C; $p > 0.10$), but those reared embryonically at 21 °C were immediately more developed, and stayed that way throughout the experiment (all $p < 0.01$; Fig. 2D). By the second week, newts that were reared embryonically at 21 °C were also significantly larger than newts that were reared embryonically at 7 ($p < 0.0001$) and 14 °C ($p < 0.01$), and they were joined by newts reared embryonically at 14 °C by the third week to continue to be significantly larger than those larvae reared embryonically at 7 °C ($p < 0.0001$) (Fig. 2C). Newts reared embryonically at 14 °C were either intermediate in development, or more similar to newts that were reared embryonically at 7 °C, depending on the week (Fig. 2D). Larvae reared embryonically at 21 °C were the only animals to reach cut-off stage 46 in this larval temperature by the end of the experiment (Fig. 2D).

3.5. Carry-over effects of embryonic temperature on larvae at 21 °C

All newts, regardless of embryonic rearing temperature, started out at a similar size at larval temperature 21 °C (all $p > 0.50$), although larvae reared at 21 °C were more developed than those reared at 7 °C ($p < 0.01$). After one week of growth in 21 °C, however, those larvae that were reared embryonically at 14 and 21 °C were already significantly larger (Fig. 2E) and more developed (Fig. 2F) than those newts reared embryonically at 7 °C (all $p < 0.001$), and this trend persisted until the end of the experiment, with larvae reared embryonically at 7 °C never catching up in size or developmental stage to those reared at 14 or 21 °C (Fig. 2E and F). Larvae reared at 14 °C were statistically identical in size (Fig. 2E), but less developed (Fig. 2F), than larvae reared embryonically at 21 °C. Growth and development appeared to plateau during the third week, with larvae at 14 °C and 21 °C being statistically similar in size and development, ($p > 0.05$) reaching cut-off stage 46 (Fig. 2F).

4. Discussion

Temperature strongly influenced hatching timing and morphology as well as post-hatching growth and developmental trajectories. Higher temperatures caused newts to hatch earlier, and facilitated larger larval size and developmental stage, similar to studies on other amphibians (e.g., Álvarez and Nicieza, 2002; Arrighi et al., 2013; Brown, 1975; Harkey and Semlitsch, 1988; Kaplan, 1980a; Lillie and Knowlton, 1897; Moore, 1939). This may be due to increased rates of biochemical reactions necessary for cellular growth and differentiation during development (Gillooly et al., 2002; Smith-Gill and Berven, 1979; van der Have and de Jong, 1996). Increased rates of biochemical reactions at warmer temperatures can lead to increased metabolic rates for developing larvae (Gatten et al., 1992; Kaplan, 1980b; Lillie and Knowlton, 1897; Noland and Ultsch, 1981), and faster depletion of the residual larval yolk-sac (Kaplan, 1980b). This could explain the plateau of growth and development observed when larvae approached stage 46 at 21 °C (Fig. 2E and F), which Harrison (1969) defined as the period in which all remaining residual yolk in the larval intestine is absorbed.

Table 1
Overall effect of embryonic temperature, larval temperature, time, and their interactions on *Taricha granulosa* larval growth and development over three (A) or four (B) weeks (see Section 2).

	Length			Developmental stage		
Factor	*F*	df (n,d)	*p*	*F*	df (n,d)	*p*
A. 3 week analysis (7 °C, 14 °C, and 21 °C for both embryonic and larval temperature treatments)						
Embryonic temperature	21.18	2,106.9	< 0.0001	47.27	2,116.2	< 0.0001
Larval temperature	295.01	2,103.4	< 0.0001	534.42	2,109.8	< 0.0001
Embryonic × larval temperature	7.74	4,103.4	< 0.0001	2.75	4,109.8	0.0316
Time	1234.13	3,293.6	< 0.0001	1231.18	3,300.5	< 0.0001
Embryonic temperature × time	17.16	6,303.1	< 0.0001	16.43	6,308.4	< 0.0001
Larval temperature × time	121.69	6,303.1	< 0.0001	209.87	6,308.4	< 0.0001
Embryonic × larval temperature × time	2.16	12,308.9	0.0133	7.20	12,313.2	< 0.0001
B. 4 week analysis (7 °C, 14 °C, and 21 °C for embryonic; 7 °C and 14 °C for larval temperature treatment)						
Embryonic temperature	7.65	2,66.03	0.0010	26.93	2,72.72	< 0.0001
Larval temperature	377.02	1,62.5	< 0.0001	377.02	1,69.04	< 0.0001
Embryonic × larval temperature	6.99	2,61.24	0.0018	0.07	2,66.14	0.9293
Time	607.51	4,250.8	< 0.0001	531.18	4,257.9	< 0.0001
Embryonic temperature × time	7.75	8,259.1	< 0.0001	5.94	8,263.5	< 0.0001
Larval temperature × time	99.04	4,250.8	< 0.0001	123.97	4,257.9	< 0.0001
Embryonic × larval temperature×time	2.89	8,259.1	0.0042	7.21	8,263.5	< 0.0001

There might be an ecological explanation for some of the differences we observed in growth and development of newt larvae at the thermal limits of this study. For instance, embryos that were reared at 21 °C were significantly more developed upon hatching than those reared at cooler temperatures, but those reared at 7 °C hatched larger than those in the warmer treatments (Fig. 1), similar to results Gomez-Mestre et al. (2010), found in tadpoles. Accelerated embryonic development (but not growth) could allow the larvae to gain a "head-start" in gaining developmental morphologies necessary for terrestrial life (De Block et al., 2005), which might be vital considering that warmer temperatures can be indicative of drought conditions and the impending loss of aquatic habitat (Newman, 1989). Conversely, in cooler and more hydrologically-stable environments, it may be more beneficial to be larger at hatching (Semlitsch and Gibbons, 1990), and thus have a competitive advantage over conspecifics (Wilbur and Collins, 1973). Similar results have been observed due to drying environments, whereby external conditions activate the hypothalamic–pituitary–adrenal axis that in turn releases precursors to thyroid hormone, corticosteroids, or prolactin, insulin-like growth factor, or many other hormones that are important for growth and development (Denver, 1997; Krollos, 1961). Considering that these hormones are temperature-dependent (Ashley et al., 1968; Moriya, 1983), the interactions of hormones and temperature is an area of interest for future studies expanding upon this research.

Temperature experienced during embryonic development had significant carry-over effects on larval growth and development four weeks after hatching, interacting with the temperature in which larvae grew and developed. Larvae that experienced 7 °C during embryonic development did not grow as large or as developed as larvae in the other embryonic treatments, regardless of larval temperature. This inability to compensate for a "bad start" also occurs in amphibians exposed to osmotic stress during embryonic development (Hopkins et al., 2014; Wu et al., 2012). An exception to this pattern occurred at larval temperature 7 °C, where larval growth (but not development) was equally slow regardless of embryonic temperature (Fig. 2A). Although initially less developed than larvae that had been reared embryonically at 21 °C, the larvae that had been reared at 14 °C as embryos exhibited a surge in development by the second week and temporarily overtook larvae from the 21 °C embryonic environment (Fig. 2F). Interestingly, we did not see compensatory growth occur, despite its reported prevalence in the literature (e.g., Ali et al., 2003; De Block and Stoks, 2008; Metcalfe and Monaghan, 2001; Räsänen et al., 2002; Roussel, 2007), and our observations of compensatory development.

Our previous knowledge of carry-over effects in amphibians has been restricted to limited studies on anurans. For instance, predator avoidance and insecticide sensitivity during the larval stage has been shown to be affected by embryonic rearing temperature in an Australian frog (*Limnodynastes peronii*) (Broomhall, 2004). Similar to our results, Watkins and Vraspir (2006) found embryonic and larval developmental temperatures significantly affected larval wood frog (*Lithobates sylvaticus*) morphology. These effects resulted in altered swimming performance, which has important survival implications (Watkins and Vraspir, 2006). Considering that smaller, less developed larvae are more susceptible to pollution (Beebee, 1986; Cooke, 1979; Hopkins et al., 2014) and predators (Anderson et al., 2001; Boone et al., 2002; Gall et al., 2011; Touchon et al., 2013; Warkentin, 1995), the carry-over effects of embryonic temperature in salamanders and newts could have survival consequences in later life stages.

While it is clear that embryonic environmental conditions can have important and persistent effects post-hatching, the underlying mechanisms responsible for these carry-over effects are less clear. While there is a paucity of knowledge on amphibians, in fish, embryonic temperature is known to significantly affect early larval growth trajectories through modulating rates of muscle fiber recruitment, the number of muscle stem cells, and possibly other cells involved in larval growth regulation (Johnston et al., 1998; Nathanailides et al., 1995). Whether this embryonic "programming" of growth and developmental pathways occurs in a similar way in newts and other amphibians is, to the best of our knowledge, currently unknown. Investigating this programming, as well as hormonal cues that regulate development throughout the amphibian life-cycle will be necessary to more completely understand the mechanisms behind carry-over effects in this and other species.

In this study we have shown that temperature has significant and long-lasting effects at, and across, early life history stages of a caudate amphibian. In particular, our results suggest that early larval growth and development may become programmed by temperature during embryonic development. Although we do not yet understand the mechanism behind such programming, our results provide an interesting insight into how early growth and developmental pathways can be shaped in amphibians. Understanding how these carry-over effects persist, and how they interact in an ecological context, will be

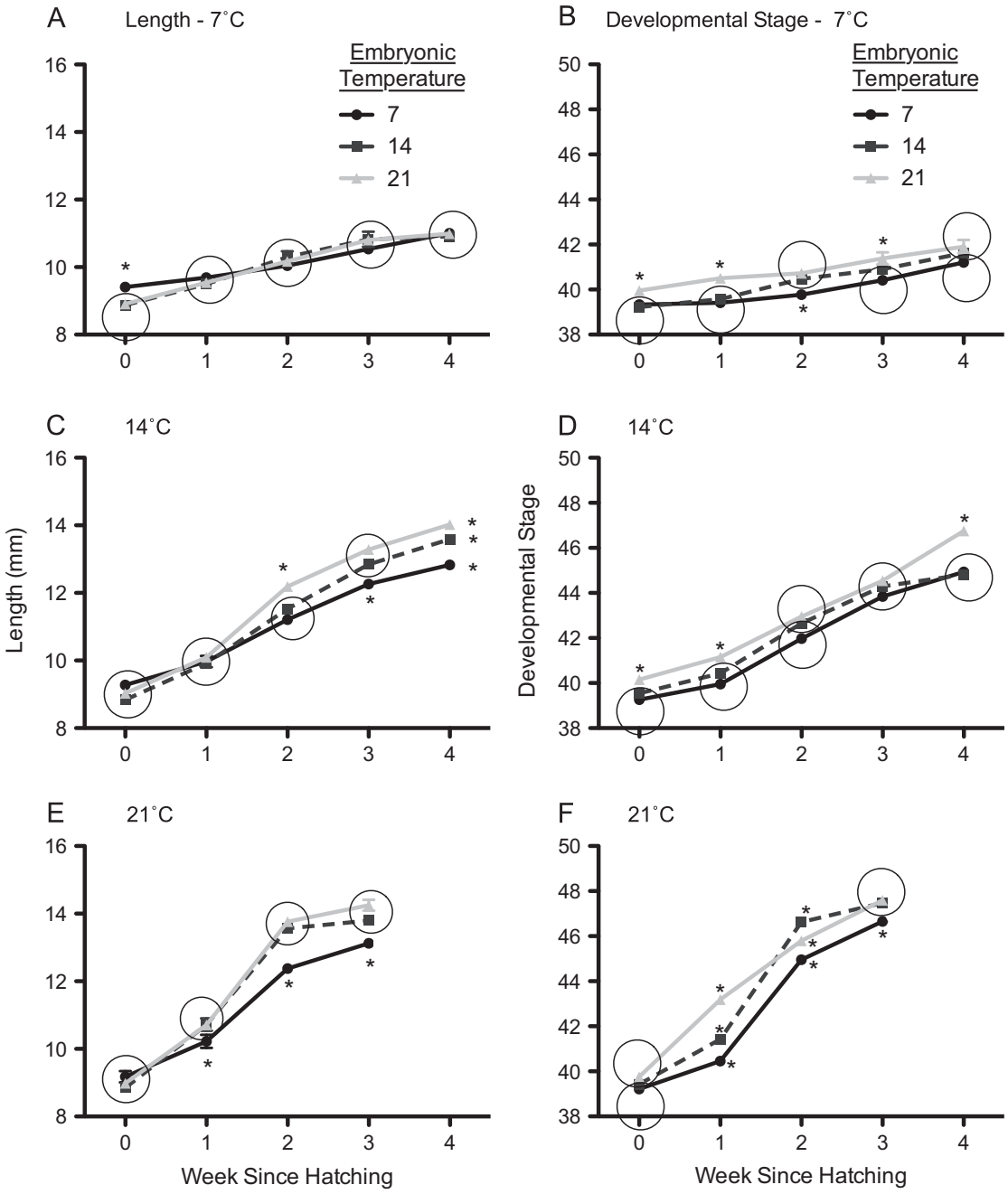

Fig. 2. Larval newt growth (total body length) and developmental stage at hatching (week 0) and three to four weeks post-hatching in three different larval temperatures: (A and B, for growth and development, respectively) 7 °C, (C and D) 14 °C, and (E and F) 21 °C. Different shades/patterns of lines indicate different embryonic rearing temperatures. Asterisks indicate significant ($p < 0.05$) carry-over effects of embryonic temperature post-hatching at each week. Those larvae from different embryonic temperatures that were statistically similar at any given point in time are grouped together in a circle.

necessary to fully understand how temperature affects growth, development, and, ultimately, survival.

Acknowledgments

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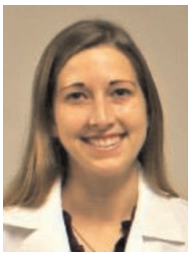

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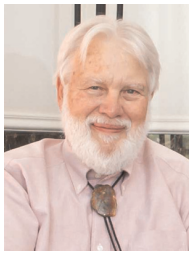

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