Effect of temperature and type of diet on the metamorphosis of *Pleurodema thaul* (Lesson, 1826) in a population of south–central Chile

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Abstract

Effect of temperature and type of diet on the metamorphosis of Pleurodema thaul *(Lesson, 1826) in a population of south–central Chile*. The effect of the environment on the vital cycle of amphibians has been shown in diverse studies, indicating that diet and temperature affect the duration of the larval period, and the size of the newly metamorphosed. We analyzed the effect of temperature and quality of diet on the duration of the larval period and size reached by larvae up until metamorphosis in the species *Pleurodema thaul*. We used an experimental design with two temperatures (15 ºC and 25 ºC) and two types of diet, one rich in proteins (RP) and one low in proteins (LP). We evaluated body size (cm) and body mass (g), and staged the larval development according to Gosner (1960). Our results indicate that temperature is crucial for the larval development, affecting its duration, whereas diet has a secondary effect on size and mass of larvae, always depending on the temperature of development.

Key words: Anuran, Diet, Metamorphosis, Temperature

Resumen

Efecto de la temperatura y el tipo de alimentación en la metamorfosis de Pleurodema thaul *(Lesson, 1826) en una población del centro sur de Chile.* El efecto del ambiente en el ciclo vital de los anfibios ha sido puesto de manifiesto en diversos estudios. Estos han indicado que tanto la alimentación como la temperatura afectan a la duración de las etapas larvales y al tamaño de los recién metamorfoseados. En el presente estudio se analizó el efecto de la temperatura y la calidad de la alimentación en la duración del periodo larval y el tamaño alcanzado por las larvas hasta la metamorfosis en *Pleurodema thaul*. Para ello se utilizó un diseño experimental con dos temperaturas (15 ºC y 25 ºC) y dos tipos de alimentación, una con un alto contenido proteico (RP) y otra con bajo (LP). Se evaluó el tamaño (cm) y la masa corporal (g), y se identificó el estadio larval de acuerdo con Gosner (1960). Los resultados indican que el efecto de la temperatura resulta crucial para el desarrollo de las larvas, ya que afecta a la duración del mismo, mientras que la alimentación ejerce un efecto secundario en el tamaño y la masa de las larvas, dependiendo siempre de la temperatura de crianza.

Palabras clave: Anuro, Alimentación, Metamorfosis, Temperatura

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Introduction

Amphibians are the group of vertebrates whose complex life cycle involves sequential aquatic and terrestrial stages. To survive in these diverse environments they have particular physiological mechanisms (Hickman et al., 2002). In ecological terms, they undergo high mortality during their aquatic phase, basically due to predation and desiccation of the temporary ponds where they breed (Wilbur, 1980; Newman, 1988). Hence, fast development or shortening of the larval phase may be beneficial for their subsistence (Newman, 1989). Conditions of larval development thus create a critical period for survival, where the need to reach certain stages of development may be fundamental to the persistence of the newly metamorphosed (Arnold and Wassersug, 1978; Wilbur, 1980). However, a delay in metamorphosis may also have a positive effect, as it can result in a larger body size, giving advantages to individuals in the transition to land. It has been reported that large metamorphs may have a greater ability to resist starvation and desiccation, as well as better abilities to escape predators (Tracy et al., 1993; Semlitsch, 1993). This delay in metamorphosis, therefore, could impose a trade–off between pre– and post–metamorphic survival.

Environment and especially diet and temperature are vital factors for both development and metamorphosis of the larvae (Naya et al., 2008). Different studies have shown that larvae of anurans exhibit a high phenotypic plasticity in response to variations of temperature and food, which is expressed in aspects such as growth and rates of development (Álvarez and Nicieza, 2002a, 2002b; Benavides, 2003; Benavides et al., 2005).

Temperature appears to be the environmental variable with the most pervasive effect on this taxon because it can be a substantial source of physiological stress on tadpoles and may cause selection pressure, favoring adaptive evolution in thermal tolerance and sensitivity (Tejedo et al., 2012). On the other hand, temperature has shown to affect developmental timing, reducing larval stage at higher temperatures (Atkinson, 1996). Diet quality also appears to play a role in this taxon. Álvarez and Nicieza (2002a, 2002b) reported that it affects both duration and size reached by newly metamorphosed individuals, establishing that diets rich in nutrients generate larger organisms.

Amphibians represent a small fraction of the endemic fauna in Chile. Interestingly, although they are the vertebrates with least diversity, they exhibit the highest degree of endemism (Spotorno, 1995; Vidal et al., 2009). One of the most abundant species of amphibians in Chile is *Pleurodema thaul* (Cei, 1962), a small from that lives in multiple climates (Iturra–Cid, 2007). Its life cycle has been described by Díaz–Páez and Ortiz (2001), who determined that the species breeds in winter–spring and that larvae remain in temporary pools until their metamorphosis. However, information on larval growth and development and the environmental variables affecting it are lacking. The present work thus aims to analyze the effect of temperature and the type of diet on larval growth and developmental rates in *P. thaul*.

Material and methods

In August 2014, we collected five egg clutches of *Pleurodema thaul* from a temporary pool in the Commune of Santa Barbara (37° 36' 42.1" S–72° 07' 41.6" W) in the Biobío Region, Chile. The clutches were taken to the Herpetological Ecophysiology Laboratory at the University of Concepción, Los Angeles Campus where they were separated in aquariums provided with 2 L of dechlorinated of water and equipped with an aeration pump. The aquariums were maintained at a temperature of 20ºC with a 12/12 hrs light/darkness photoperiod. Water was changed each week. After hatching, larvae were maintained in these conditions until they reached Gosner stage 25 (Gosner, 1960).

A 2 x 2 factorial design was used to analyze the effect of the type of food, with two treatments: low protein content (LP) and high protein content (RP). Similarly, the effect of the temperature was analyzed, selecting two treatments representing the temperature extremes of the optimal range for larval development, 15ºC (± 1ºC), and 25°C (± 1ºC) (Alvarez and Nicieza, 2002a, 2002b; Benavides, 2003; Benavides et al., 2005; Sanuy et al., 2008). In accordance with Benavides (2003), we used boiled lettuce in the diet of low protein content (1.3 % protein and 0.3 % fat per 100 g of lettuce, Granval and Gaviola, 1991); and for the rich diet, we used a commercial product of the Micron brand because of its high protein content (54.7 % protein and 2.6 % fat per 100 g of Micron). Larvae were fed *ad libitum* every two days and as their development progressed, the amount of food was increased in order to meet their growing demands. Excess food was removed along with water changes. A total of 180 larvae were randomly placed in each of the four experimental treatments (n = 15 per treatment) and three replicas per each treatment were established. Each recipient maintained two l of water in recirculation through an air pump, which helped to reduce thermal heterogeneity. To identify the different larval stages, we followed Gosner´s table of stages in Duellman and Trueb, 1986). Containers were checked every day to measure the larval stage, size (± 0.1 cm) and mass (± 0.1 mg) until full larval tail resorption, that is, Gosner stage 46. Four size classes were selected for further analyses: initial larval (stage 25–30), larval (stage 31–35), metamorphic (stage (36–40), and climax metamorphic (stage 41–46) (fig. 1). To estimate the averages of size, mass and stage per aquariums in the different treatments, we performed multiple variance analysis (Manova) and non–parametric tests using SPSS version 13.0 software (Blair and Taylor, 2008).

Results

We found a significant effect of temperature on the mass of the larvae ($F_{1, 90} = 2.822$; $P < 0.001$) and on their body size ($F_{1, 35} = 4.410$; $P < 0.001$). Larvae exposed to high temperatures showed faster development and growth, reaching greater masses (fig. 2A) and sizes (fig. 2B) in less time. In contrast, larvae exposed

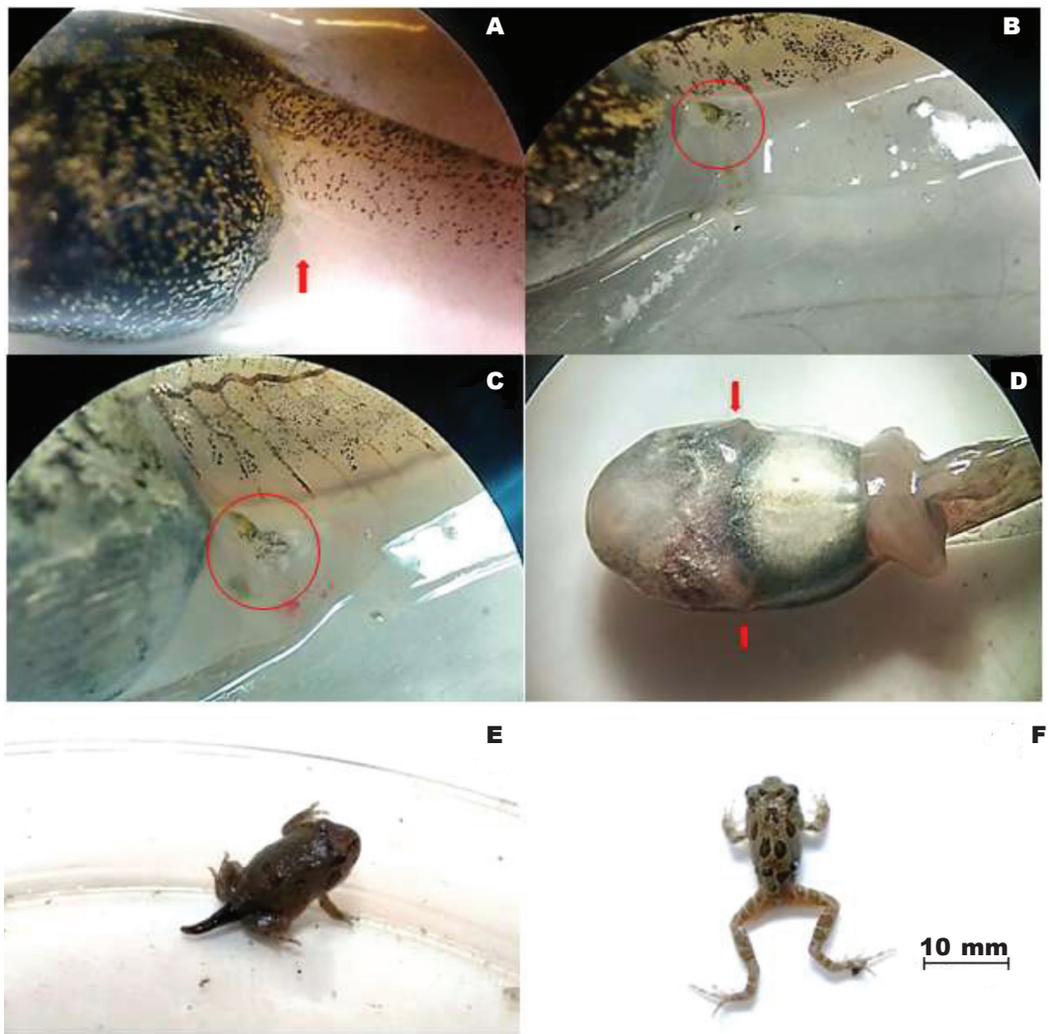

Fig. 1. Larval stages of development in *Pleurodema thaul*: A, stage 25, no budding of hind limbs; B, detail of stage 30 with the appearance of buds that give rise to the hind limbs; C, stage 34 with the appearance of the second and third fingers attached; D, stage 41 with the protuberances of the forelimbs and marked disappearance of anal fold; E, stage 45, almost complete metamorphosis with much of the tail reabsorbed; F, complete metamorphosis, stage 46.

Fig. 1. Estados de desarrollo larval en Pleuroderma thaul*: A, estadio 25 sin brotes de extremidades posteriores; B, detalle del estadio 30 con la aparición de los brotes que darán origen a las extremidades posteriores; C, estadio 34 con la aparición del segundo y tercer dedo unidos; D, estadio 41 con las protuberancias de los miembros anteriores y la marcada desaparición del pliegue anal; E, estadio 45, metamorfosis casi completa con gran parte de la cola reabsorbida; F, metamorfosis completa, estadio 46.*

to low temperatures showed prolonged duration in development (fig. 2). We observed no effect of diet on the mass of the larvae ($F_{1, 90}$ = 1.210; P = 0.095). However, we found that diet had a noticeable effect on size ($F_{1, 35}$ = 1.634; P = 0.012), so that larvae fed RP reached larger sizes than those fed LP (table 1).

Similarly, temperature was the variable that most affected larvae mass and body size, with a significant effect on both variables ($F_{1, 380}$ = 1.429; P < 0.001). This indicates that temperature is a crucial factor on size and mass of *P. thaul* in its larval development, minimizing the impact of diet on these parameters in the development of this species *P. thaul* ($F_{1, 380}$ = 0.808; P = 0.993) (table 2).

Larvae increased in both size and mass in all treatments (fig. 2). Temperature had a strong effect on the duration of the larval stage ($F_{4, 1201}$ = 29.47; P = 0.003), causing larvae exposed to 25 ºC to have

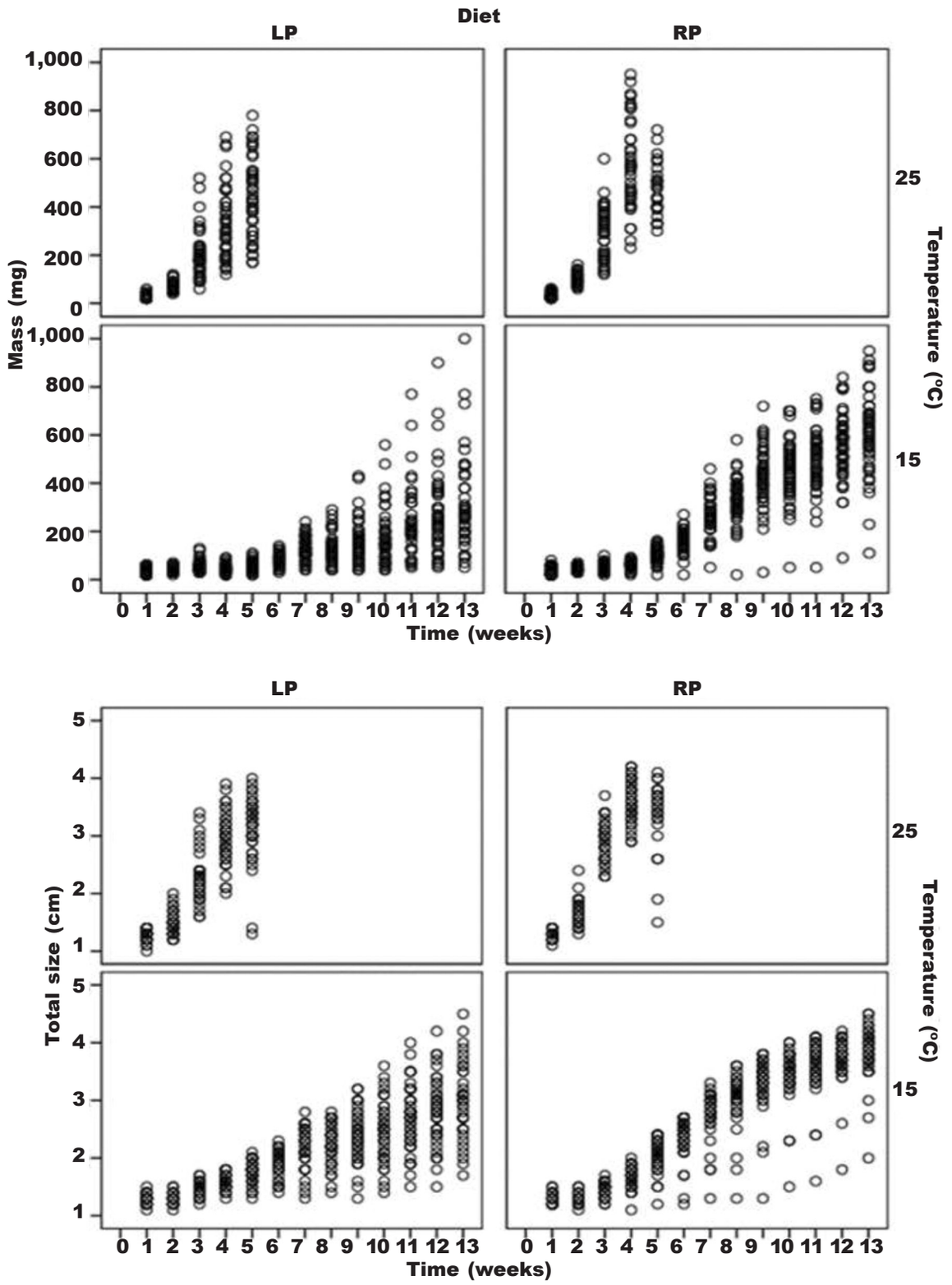

Fig. 2. Effect of temperature and type of diet on: A, larval size; and B, larval mass.

Fig. 2. Efecto de la temperatura y el tipo de alimentación en: A, el tamaño larval; y B, la masa larval.

Table 1. Data for mass (M, in mg) and body size (Ts, total size, in cm) in tadpoles of *Pleurodema thaul* for each stage grouping (Li, larval initial; L, larval; Mt, metamorphic; Cm, climax metamorphic), in two temperature (15 ºC and 25 ºC) and two diet (LP, low protein content; RP, high protein content) treatments (values are given as mean ± SE). Analyses from Mann–Whitney test of differences between the diet treatments at each stage group are given (*Z*: NS, not significant; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$). Analyses from Kruskal–Wallis H tests comparing the different stage group within treatment are presented as the χ^2–value.

Tabla 1. Datos relativos a la masa y (M, en mg) y el tamaño corporal (Ts, tamaño total, en cm) de los renacuajos de Pleurodema thaul *agrupados por estado de desarrollo (Li, larval inicial; L, larval; Mt, metamórfico; Cm, clímax metamórfico) en dos tratamientos de temperatura (15ºC y 25ºC) y dos de alimentación (LP, contenido porteico bajo; RP, contenido proteico alto) (los valores son indicados como media ± EE). Se utiliza el test de Mann–Whitney para analizar las diferencias entre los tratamientos de alimentación en cada grupo de estados de desarrollo (Z: NS, no significativo; * P < 0,05; ** P < 0,005; *** P < 0,001). Se utiliza el test de Kruskal–Wallis H para comparar los diferentes grupos de estado dentro de los tratamientos, los valores se representan como valor de* χ^2.

			Development stage group				
Treatment (ºC)			Li (25–30)	L (31–35)	Mt (36–40)	Cm (41–46)	χ^2
Ts	15	LP	1.89 ± 0.48	3.11 ± 0.39	4.20 ± 0.30	–	270.00***
		RP	1.99 ± 0.65	3.53 ± 0.42	3.98 ± 0.22	–	470.30***
	Z		–1.292 NS	–8.355***	–1.355 NS	–	
M	15	LP	93.47 ± 65.40	339.04 ± 144.90	833.33 ± 145.72	–	195.77***
		RP	128.16 ± 106.50	479.45 ± 117.36	667.86 ± 130.28	–	465.88***
	Z		13.49***	–8.29***	–2.09 NS	–	
Ts	25	LP	1.67 ± 0.53	2.86 ± 0.33	3.38 ± 0.28	2.62 ± 0.99	122.96***
		RP	1.77 ± 0.58	2.88 ± 0.31	3.51 ± 0.33	3.11 ± 0.78	146.08***
	Z		–2.70*	–0.84 NS	–3.57***	–1.93*	
M	25	LP	107.86 ± 95.50	288.85 ± 82.33	516.75 ± 124.64	383.33 ± 115.00	131.15***
		RP	77.19 ± 66.69	296.57 ± 95.42	563.18 ± 185.26	493.00 ± 114.53	146.58***
	Z		–2.01*	–0.44 NS	–1.08 NS	3.13 NS	

shorter times of development than those exposed to 15 ºC. Complete tail reabsorption concluded in five weeks (fig. 1) in larvae exposed to 25 ºC but at 13 weeks in larvae exposed to 15 ºC (fig. 3). Additionally, mortality was higher at the low temperature (90%), and a high percentage of larvae (X = 6.83 ± 3.06) in this 15 ºC treatment group did not complete metamorphosis.

To better understanding the treatment variation with larval age we grouped data into four age groups or developmental stage. The results showed that at the lower temperature, development was not affected by diet (table 1), so that the larvae did not increase in size due to diet. The effect was concentrated in the masses during Gosner stages 25 to 35, demonstrating a notable increase in the mass for the larvae fed RP. On the other hand, at higher temperatures (25 ºC), the effects were reverted, so that RP generated significantly larger larvae than those fed LP, while the mass variation was not significant (table 1).

Finally, we found that larvae in the RP/15 ºC treatment reached the largest masses and body sizes (table 1) during all stages of development. Conversely, larvae in the LP/25 ºC group reached the smallest mass and body mass (table 1).

Discussion

Analysis of ecological physiology in amphibians is essential to understand many aspects of the biology of these organisms. This includes the conditions that define their fundamental niches, geographical distribution and evolutionary dynamics which in turn allow to determine their vulnerability to climate change (Gutiérrez–Pesquera et al., 2016). At the same time, the thermal environment exerts a strong effect on aspects of life history in ectotherms, playing an important role in their growth rate and body size (Angilletta et al., 2004; Angilletta, 2009).

Table 2. Two–way ANOVA for effect of temperature and diet on larval development: M, mass of tadpole; Ts, body size of tadpole; Diet, diet; Temp, temperature of water in aquarium; Type III MS, type III of sum of squares: [(a)] R^2 = 0.386 (adjusted R^2 = 0.115); [(b)] R^2 = 0.467 (adjusted R^2 = 0.232); Df, degrees of freedom. (NS, not significant; * *P* < 0.05; ** *P* < 0.005; *** *P* < 0.001).

Tabla 2. ANOVA bidireccional para el efecto de la temperatura y la alimentación en las larvas: M, masa del renacuajo; Ts, tamaño corporal de los renacuajos; Diet, alimentación; Temp, temperatura del agua en el acuario; Type III MS, tipo III de suma de cuadrados; [(a)] R^2 = 0,386 (R^2 ajustada = 0,115); [(b)] R^2 = 0,467 (R^2 ajustada = 0,232); Df, grados de libertad. *(NS, no significativo;* * P < *0,05;* ** P < *0,005;* *** P < *0,001).*

Trait	Source	Type III MS	Df	Type III MS	*F*	*P*
Model correct	Diet	159,482 [(a)]	505	0.316	1.430	0.000
	Temp	20,644.427 [(b)]	505	40.880	2.011	0.000
Intercept	Diet	900,868	1	900.868	4,079.880	0.000
	Temp	105,359.122	1	105,359.122	5,182.925	0.000
M	Diet	24,051	90	0.267	1.210	0.095 [NS]
	Temp	5,163.739	90	57.375	2.822	0.000 ***
Ts	Diet	12,624	35	0.361	1.634	0.012 *
	Temp	3,137.374	35	89.639	4.410	0.000 ***
M * Ts	Diet	67,838	380	0.179	0.808	0.993 [NS]
	Temp	11,037.085	380	29.045	1.429	0.000 ***
Error	Diet	254,591	1153	0.221		
	Temp	23,438.322	1153	20.328		
Total	Diet	4,248.000	1659			
	Temp	465,051.000	1659			
Total corrected	Diet	414,074	1658			
	Temp	44,082.749	1658			

Larvae of aquatic amphibians are an ideal model to analyze thermal adaptations (Gutiérrez–Pesquera et al., 2016) due to their high dependence on temperature in the surrounding environment. These larvae have a low ability to regulate body temperature (Hutchison and Dupré, 1992), making them poikilothermic organisms (Balogová and Gvozdík, 2015). Their search for favorable microhabitats is therefore limited as the temperature of the pool affects their rate of development and growth and the duration of the metamorphosis.

Most studies on larvae have shown that development responds to temperature, following Bergmann's Rule (1847) and resulting in large sizes at low temperatures (Ashton, 2002; Álvarez and Nicieza, 2002a; Laugen et al., 2005; Walsh, 2008; Walsh et al., 2008). Álvarez and Nicieza (2002b) reported that hatching temperature had no effect on mass loss during metamorphosis in *Discoglossus galganoi*, confining its main effect to the size and larval duration at metamorphosis. Our results show that temperature affects the development of larvae of *Pleurodema thaul*, causing those exposed to higher temperatures to reach metamorphosis in less time than those exposed to low temperatures. This is because of the direct dependence between development and metabolic rate which it is increased with temperature, causing a faster development at higher temperatures (Barja de Quiroga, 1993). This is not surprising, since the hormones that regulate metamorphosis also control development of limbs and are highly sensitive to temperature (Ryan and Winne, 2000; Álvarez and Nicieza, 2002a, 2002b).

Kehr (1998) indicated that the tadpole's increase in the rate of growth and development is generally regarded as a mechanism to decrease the risk of mortality through predation or desiccation of the habitat (Goldberg et al., 2012). This is why when individuals develop in an environment with high temperatures, the duration of larval stages are shorter, causing newly metamorphosed individuals to have both smaller body and mass than those organisms that develop at lower temperatures (Benavides, 2003; Benavides et al., 2005). We found a similar trend in *P. thaul*, where temperature had a direct effect on both mass and body size of the newly metamorphosed

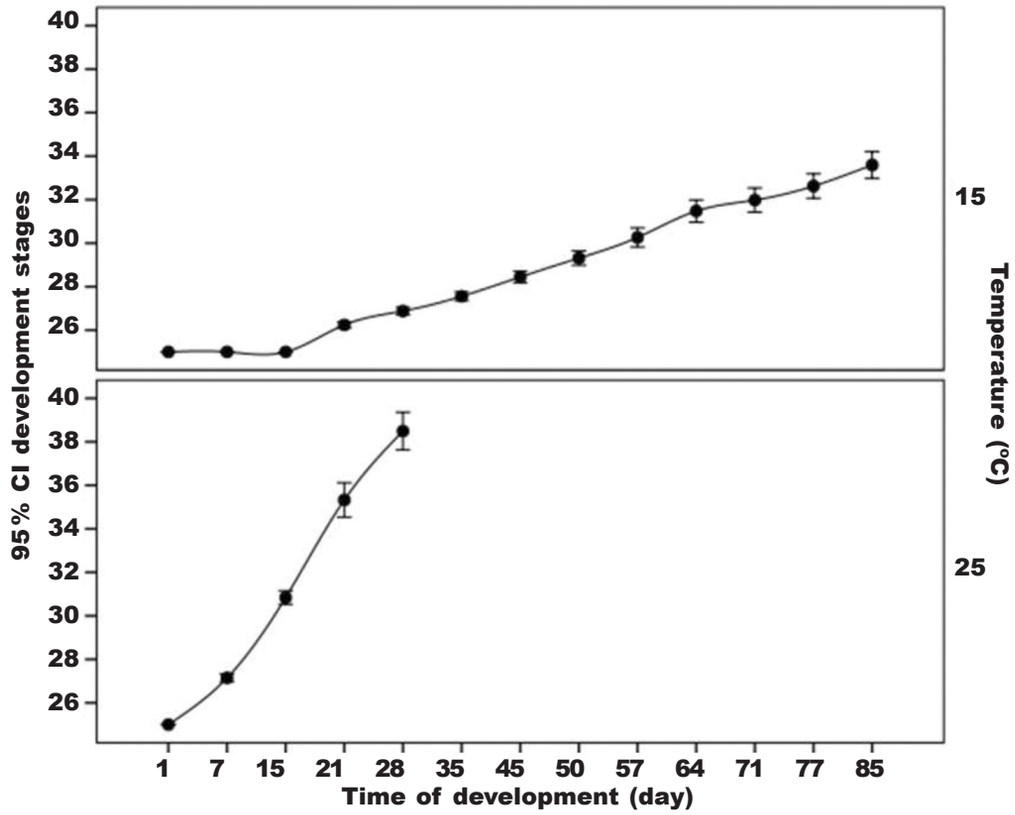

Fig. 3. Results of larval development according to Gosner's table (1960) from stage 25 to 46. This indicates the percentage of larvae in each of the stages during the weeks of the experiment for each treatment. The results are presented separately for treatment at 15ºC and treatment at 25ºC.

Fig. 3. Resultados del desarrollo larval de acuerdo con la tabla de Gosner (1960), desde el estadio 25 al 46. En ella se indica el porcentajes de larvas en cada estadio para cada tratamiento durante las semanas que duró el experimento. Los resultados se presentan separados por tratamiento de temperatura a 15 ºC y tratamiento a 25 ºC.

individuals. This has also been reported by Blouin and Brown (2000) in *Rana cascadae*. On the other hand, studies by Sanuy et al. (2008) on *Epidalea calamita* (sin. *Bufo calamita*; Frost, 2014) suggest that this species requires a minimum size and a larval body mass to complete its metamorphosis; an effect not evidenced in *P. thaul* because individuals completed their metamorphosis at different sizes and body masses.

The study of phenotypic plasticity in amphibian larval development has long been of interest to ecologists. Our results show that temperature is the main factor affecting larval development, whereas the quality of diet presents a secondary effect on larval size and body mass. This is only significant in the initial larval stage (Gosner 25 to 30), probably coincident with the beginning of the metamorphic transformation. By observing the effect produced by the type of diet on larvae, it can be affirmed that there is a relationship between mass and body size of the individual, because those larvae fed a diet rich in protein (RP) reach a greater mass and body size than those fed a diet poor in protein (LP). This is because the presence of proteins in the diet is a determining factor for growth as they correspond to the main component of organs cell structures of tissues (Pelegrín et al., 2004). In addition, the lack of nutrients in diet constitutes an important selective factor since it influences the functioning of the thyroid gland, which would directly affect the rate of growth and differentiation of organisms (Álvarez and Nicieza, 2002a).

We can conclude that both diet and temperature affect the development of larvae of *P. thaul*, where those larvae exposed to higher temperatures reach lower sizes and body mass than those found at lower temperatures. On the other hand, larvae fed with RP have greater mass and size than those fed with LP. When both factors are combined, diet and temperature, individuals exposed at higher temperatures and fed with LP reach metamorphosis more slowly than

those fed an RP diet. These results relegate the effect of diet over that of temperature, confirming the main effect of temperature over diet. It should be noted that the temperature of 25ºC is considered optimum for larval growth in this species, which leads to the fact that larvae can maintain a similar development with lower amounts of proteins (Kupferberg et al., 2011). It would also be expected (similarly to findings in studies on metamorphosis of insects; Stevens, 2004) that temperature has high importance on the rates of development (Gillooly et al., 2002), influencing the duration and size of the amphibians at the end of the metamorphic climax (Walsh, 2008). Thus, the influence of temperature on the development of larvae makes it a selective agent, promoting thermal adaptations (Angilletta, 2009; Bozinovic et al., 2011).

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