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Persistence of the Larval Environment on Post-Metamorphic Performance and Population Dynamics in Amphibians

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Persistence of the larval environment on post-metamorphic performance and population dynamics in amphibians

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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LIST OF ABBREVIATIONS

AIC	Akaike Information Criterion
ANCOVA	analysis of covariance
ANOVA	analysis of variance
CAT	catalase
CI	confidence intervals
cm	centimeters
EWL	evaporative water loss
g	grams
GPx	glutathione peroxidase
GR	glutathione reductase
IACUC	Institutional Animal Care and Use Committee
K	number of parameters
kg	kilograms
$KMnO_4$	potassium permanganate
L	liters
ln	natural log
log	log10
Max	maximum
mg	milligrams
Min	minimum
ml	milliliters
NADPH	Nicotinamide adenine dinucleotide phosphate
nmol	nanomole
pH	potential of oxygen
RO	reverse osmosis
RSA	respiratory surface area
SD	standard deviation
SE	standard error
SOD	superoxide dismutase
SVL	snout vent length
TBARs	thiobarbituric acid reactive substances
U	enzyme unit
VA	Virginia
VCU	Virginia Commonwealth University
VWC	volumetric water content

ABSTRACT

PERSISTENCE OF THE LARVAL ENVIRONMENT ON POST-METAMORPHIC PERFORMANCE AND POPULATION DYNAMICS IN AMPHIBIANS

by
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Organisms with complex life cycles may experience diverse stressors during their development. Stressors experienced in early life stages may influence the quantity and quality of individuals in later life stages. However, it is unclear if these effects persist later in life and how they may influence population dynamics. This dissertation uses two amphibian species, the Western spadefoot toad (*Pelobates cultripes*) and the spotted salamander (*Ambystoma maculatum*) to explore how biotic and abiotic factors experienced in aquatic and terrestrial environments

influence phenotype and survival. We use a combination of field mesoscosm studies, laboratory studies and modeling to explore how early life history stressors persist in diverse environmental contexts. In Chapter 1, pond drying and larval density negatively influence multiple aspects of phenotype in the Western spadefoot toad. In Chapter 2, reduced body size due to larval stressors persisted in the first year of life in spotted salamanders in both high and low terrestrial resource environments. Additionally, there was no relationship between size at metamorphosis and post-metamorphic terrestrial survival. In Chapter 3, low terrestrial moisture levels reduced post-metamorphic growth rates by reducing food intake in juvenile spotted salamanders from both high and low larval density treatments. In Chapter 4, we scale up the effects of reduced body size in the Western spadefoot toad to explore how reduced body size due to pond drying may influence population densities using a stage specific matrix model. Reduced body size, by delaying maturity, may reduce adult densities in the Western spadefoot toad. This dissertation suggests that life stages are highly interconnected, as stressors experienced early in life stage may persist through their effects on phenotype in the absence of compensatory mechanisms. Variation in later life stages may also influence phenotype, but may not completely erase effects of early environments. Stressors experienced early in life may also translate to population level consequences, especially when organisms experience multiple stressors across life stages.

CHAPTER 1 EFFECTS OF DENSITY AND POND DRYING ON METAMORPHIC AND PHYSIOLOGICAL TRAITS IN SPADEFOOT TOADS

Julie F. Charbonnier, James R. Vonesh, Carmen Díaz-Paniagua, Ivan Gomez-Mestre

Introduction

Complex life cycles are predominant in nature (Werner & Gilliam, 1984). Most organisms pass through ecologically distinct stages and undergo dramatic development, physiological and behavioral changes as they shift from one life stage to the next (ontogenetic niche shift *sensu* Wilbur, 1980). Life stages can be decoupled to some extent (Ebenman, 1992; Moran, 1994). However, despite the dramatic development, physiological and behavioral changes experienced during ontogenetic switch points, life cycles are highly linked through carry-over effects (Mousseau & Fox, 1998; Podolsky & Moran, 2006; Pechenik, 2006; Marshall & Morgan, 2011). Carry-over effects are widespread across taxa and arise when environmental stressors experienced in one life stage influence survival and phenotype across life history switch points (Giménez, 2006; Pechenik, 2006), even after extended periods of growth and differentiation between them (Touchon et al., 2013).

Within life stages, organisms experience multiple biotic and abiotic environmental stressors simultaneously (Dunson & Travis, 1991). These stressors can interact to influence both the number of animals recruited to the next life stages (survival effects) and the quality of the animals recruited (phenotypic effects; McPeek & Peckarsky, 1998). Phenotypic effects not only include traditional measures of phenotype (time and size at metamorphosis) but other components such as morphology, locomotor performance, and physiological status. Fully understanding how stressors will influence populations requires quantifying their influence on both survival and phenotype. Simultaneous stressors may act either additively or non-additively. In the former, the cumulative impact of multiple stressors is the sum of their individual impacts (Darling & Côté,

2008). Emergent effects occur when two stressors interact to produce a greater (synergy) or lesser (antagonism) change than predicted by the sum of their individual effects (Folt et al., 1999; Relyea & Hoverman, 2008; Buck et al., 2012). Emergent interactions make predicting the magnitude and direction of carry-over effects challenging. Carry-over effects on phenotype are particularly difficult to predict because phenotypic responses are interdependent and highly integrated by growth and development processes (Gomez-Mestre et al., 2010). Shifts in one phenotypic trait may induce shifts in a suite of related traits, resulting in complex phenotypic responses (Álvarez & Nicieza, 2002).

Amphibians are ideal organisms for studying carry-over effects of biotic and abiotic stressors because they are sensitive to environmental variation, and their growth and developmental rates can be altered in response to different risks (Smith-Gill & Berven, 1979; Leips & Travis, 1994; Richter-Boix, Tejedo & Rezende, 2011). Several theoretical models predict the optimal timing and size at metamorphosis, which can help make predictions on the direction and magnitude of phenotypic carry-over effects (Wilbur & Collins, 1973; Werner, 1986). Larval amphibians are expected to both maximize growth and minimize mortality rates across life stages, and this optimization is bounded by size and development thresholds (Wilbur & Collins, 1973; Leips & Travis, 1994; Rose, 2005; Touchon et al., 2015).

One of the most important biotic stressors amphibians face is competition due to conspecific larval density. Most larval amphibians exhibit density dependent survival (Brockelman, 1969; Wilbur, 1977a; Alford & Harris, 1988; Skelly & Kiesecker, 2001). Because increasing larval density typically reduces growth rates, conspecific density can negatively influence some phenotypic characteristics (John-Alder & Morin, 1990; Goater, Semlitsch & Bernasconi, 1993; Newman, 1994; Relyea & Hoverman, 2003). However, such density dependent

effects do not occur in isolation, as abiotic factors occur concurrently and may exacerbate density dependent effects. Abiotic stressors may influence survival and phenotype consistently across densities (additive effect). Conversely, environmental stressors that intensify competition may exacerbate density effects (non-additive effect). Understanding how abiotic stressors result in carry-over effects on survival and phenotype also requires an understanding of how they interact with larval density (Gomez-Mestre & Tejedo, 2002).

Pond hydroperiod (duration of pond inundation) is a key abiotic regulator of pond communities (Wellborn, Skelly & Werner, 1996; Gómez-Rodríguez et al., 2012) that can influence survival and phenotype both independently and interactively with density. Many amphibian species accelerate development to escape drying ponds (Newman, 1998; Denver & Middlemis-Maher, 2010; Richter-Boix, Tejedo & Rezende, 2011). The density independent effects of pond drying on survival are well documented; if ponds dry out too quickly (exceeding an individual's ability to accelerate development), and/or before tadpoles can physiologically detect and respond to reduced water levels, then catastrophic die-offs can occur (Newman, 1992; Denver, Mirhadi & Phillips, 1998; Brady & Griffiths, 2000; Leips, McManus & Travis, 2000; Amburgey, Murphy & Funk, 2016). Less well understood are the potential density dependent effects of pond drying on survival. As density increases, the survival effects of pond drying are likely to intensify (Wilbur, 1987). Studies suggest that when resources are low, tadpoles may have insufficient resources to accelerate metamorphosis, resulting in lower survival in drying, low resource ponds than in drying, high resource ponds (Loman, 2002; Enriquez-Urzelai et al., 2013). Understanding the effects of pond drying may be increasingly important as global change alters historical rainfall regimes, resulting in increasingly shorter pond hydroperiods, especially in the Mediterranean (Winter, 2000; Walther et al., 2002; McMenamin, Hadly & Wright, 2008; IPCC, 2014; Greenberg et al., 2015).

Both pond drying and larval density may also independently and interactively influence post-metamorphic phenotype. Larval density primarily modulates growth rate, while pond drying primarily influences development rate, and these different processes interact to determine the magnitude of phenotypic carry-over effects (Leips & Travis, 1994; Gomez-Mestre et al., 2010). High growth rates due to low density (and high food availability) results in larger metamorphic size, larger fat reserves, longer hindlimbs, and higher jumping performance (Goater, Semlitsch & Bernasconi, 1993; Gomez-Mestre et al., 2010; Bouchard et al., 2016). Developmental acceleration due to pond drying may result in smaller metamorphic size and lower post-metamorphic fat reserves, as energy is allocated to development rather than growth (Kulkarni et al., 2011; Richter-Boix, Tejedo & Rezende, 2011). Developmental acceleration will also result in shorter absolute and relative hindlimb length, and lower jumping performance (Gomez-Mestre et al., 2010; Tejedo et al., 2010; Enriquez-Urzelai et al., 2013; Charbonnier & Vonesh, 2015). Additionally, as tadpoles increase metabolism and burn their fat reserves, they produce reactive oxygen species, and may increase the products of detoxifying antioxidant enzymes to buffer against oxidative damage (Glennemeier & Denver, 2002; Gomez-Mestre, Kulkarni & Buchholz, 2013). Oxidative stress is a proxy for cellular ageing and may be linked to poorer survival (Monaghan, Metcalfe & Torres, 2009). Both pond drying and competition increase the levels of the stress hormone corticosterone (Glennemeier & Denver, 2002; Gomez-Mestre, Kulkarni & Buchholz, 2013), linking the responses to both factors. The extent to which the multiple effects of pond drying and larval density on an organismal phenotype are independent of each other is unclear.

Pond drying has the potential to produce interactive effects on both survival and phenotype depending on larval density. At low larval densities, tadpoles may grow enough and accumulate sufficient fat bodies to buffer against the physiological consequences of developmental

acceleration (non - additive antagonistic effect). At high larval densities, intraspecific competition may limit growth rate to the extent of preventing tadpoles from accelerating metamorphosis, steeply increasing their risk of mortality from pond drying. To examine the potential interaction between density and pond drying (i.e. when growth rates become limiting), a range of densities must be used to capture both survival and phenotypic effects.

In this study, we used the Western spadefoot toad, *Pelobates cultripes*, a species which experiences a wide range of densities and develops in ponds with different durations (Díaz-Paniagua et al., 2005), to test how pond drying and larval density would interact to influence both survival and phenotype of emerging metamorphs. We conducted an outdoor mesocosm experiment where we manipulated larval density and pond duration to quantify the independent and interactive effects of these two factors. We quantified larval survival before the dry-down began and survival to metamorphosis. We focused on traits that are likely relevant to survival and have been previously shown to be influenced by pond drying and/or density including body size, fat reserves, morphology and locomotor performance (Goater, Semlitsch & Bernasconi, 1993; Richter-Boix, Llorente & Albert, 2006; Johansson, Lederer & Lind, 2010; Gomez-Mestre, Kulkarni & Buchholz, 2013). As a proxy for physiological stress, we quantified levels of antioxidant enzymes and a biochemical marker of oxidative damage. As with the other phenotypic variables, we expected higher levels of oxidative stress in dry-down toads which accelerated metamorphosis and such stress to be exacerbated as larval density increases. We hypothesized both antagonistic effects at low densities (due to high growth rates) and synergistic effects at high densities (due to low growth rates).

Methods

Study species and animal collection

This study occurred in the Biological Reserve at Doñana National Park, located in Southwest Spain (37.00° N, 6.50° W). The park includes a network of over 3,000 temporary ponds, a smaller number of permanent ponds and an extensive seasonal marsh (Díaz-Paniagua et al., 2010). Temporary ponds fill with autumn or winter rains, from September onwards and dry up during the spring and summer (Díaz-Paniagua et al., 2010). The Western spadefoot toad (*Pelobates cultripes*) colonizes ponds shortly after filling and utilizes both deep, permanent habitats and shallow, temporary habitats for reproduction (Díaz-Paniagua et al., 2005). Females lay 2500-4000 eggs and larvae hatch 6 to 12 days later (Díaz-Paniagua et al., 2005). Field densities are largely dependent on the amount of rainfall as pond size is highly variable, but field densities have been observed from 0.01 – 0.65 tadpole/ L (Díaz-Paniagua unpublished). Tadpole densities can be especially high in the late drying phase [400 tadpoles/m^2, (Cei & Crespo, 1971; Rodriguez-Jimenez & Prados, 1985). Fifteen egg clutches were collected from the park in November 2012. Clutches were maintained separately in 20 L buckets in a climatic chamber held at 14°C with aerated carbon-filtered tap water and a 12:12 light-dark cycle at the Estación Biológica de Doñana until Gosner stage 25 [free-feeding stage, (Gosner, 1960)]. When larvae reached Gosner stage 27 (approximately 2 weeks later), larvae were added to experimental mesocosms.

Mesocosm experiment

We performed a 5 x 2 factorial experiment to test the effects of larval density and pond drying on metamorphic survival and phenotype. Experimental treatments consisted of five density manipulations: 5, 15, 25, 35, and 50 tadpoles per mesocosm (0.09, 0.27, 0.46, 0.64, 0.91 tadpole/L) and two hydroperiod treatments: a constant water level treatment and a dry-down treatment. We chose these density treatments to explore the full range of responses. All treatments were replicated ten times except for the lowest density treatment (5 tadpoles/mesocosm), which was replicated

thirteen times (106 total tanks). Tanks were placed in an experimental array across five blocks and treatments were randomly assigned within blocks. All blocks had 2 replicates of each treatment (20 tanks total). The three extra lowest density treatment tanks were added to blocks four and five.

We used 55 L (53 x 53 x 60 cm) plastic, circular tubs filled with 50 L of well water to simulate experimental ponds. We added 0.850 kg of dry pond sediment and 5.00 kg of sand to seed aquatic communities and provide a substrate for plants. We planted two species of aquatic macrophytes: *Myriophyllum alterniflorum* and *Callitriche obtusangula* which commonly occur in natural ponds. Once tadpoles began consuming the plants, plants were replaced on a weekly basis (120 g of plant biomass per mesocosm). A wide mesh screen was placed across each block to prevent birds from falling into the tanks, but this net allowed large insects into the tanks. If insects were present, they were removed from the tank. As *Callitriche* became less common in the spring and summer, tadpoles were henceforth fed primarily *Myriophyllum*. To begin the experiment, tadpoles were haphazardly assigned to density treatments on November 26, 2012.

Prior to implementation of the dry-down manipulation, we assessed the number of surviving tadpoles. Tadpoles were dip-netted from their mesocosms, photographed and returned to their mesocosms within a 15 min period. The hydroperiod manipulation began on April 18, once tadpoles from the five density treatment were close to reaching Gosner stage 35, a development stage where they can physiologically respond to pond dry-down (Kulkarni et al., 2011). We simulated pond dry-down by manually removing water following the relationship $D_j = 1-(j/t)^a P$ (Wilbur, 1987), where D_j is the desired depth on day j, t is the target day for depth = 0 (40 days), a is a shape parameter (0.5 in our treatment) and P is the water depth at the start of the experiment. Water increments were removed from dry-down tanks every two days.

Post-metamorphic performance

As tadpoles reached metamorphic climax, metamorphs were collected daily at forelimb emergence [stage 42, (Gosner, 1960)] and weighed (± 0.01g). Metamorphs were maintained individually in 240 mL plastic cups in an ambient temperature laboratory until complete tail resorption [stage 46, (Gosner, 1960)]. Upon tail resorption (4 – 5 days), we measured snout-vent length (SVL) and tibia-fibula length using digital calipers and reweighed toads. To assess jumping performance, each toad was stimulated to jump in a circular array with 0.5 cm concentric markings. Jumping was initiated by lightly prodding the urostyle and estimated visually (Goater, Semlitsch & Bernasconi, 1993; Niehaus, Wilson & Franklin, 2006; Charbonnier & Vonesh, 2015). Toads were jumped three times per trial and three trials were conducted per toad (for a total of nine jumps), with a five min rest period in between trials.

Fat body dissections and oxidative stress analyses

A subset of randomly selected animals was euthanized and dissected for fat bodies (N = 74). No animals from the lowest density constant treatment were available for dissections, and only four animals from the 5 larval density dry-down treatment were available for this experiment (sample sizes listed in Supplementary Table 1.2). All analyses were performed in the Ecophysiology Laboratory at Doñana Biological Station in Seville.

Animals were euthanized individually by immersion in MS-222 and frozen in liquid nitrogen and stored at -80 °C. Animals were thawed and fat bodies were removed and weighed separately. Animals were eviscerated to prevent interference of gut contents with the assays. For all assays, individuals were homogenized using a Miccra (Miccra D-1) homonogenizer in a buffered solution (100mM PMSF, 1:4, w:v) to inhibit proteolysis. Samples were centrifuged at 4000 g for 30 min at 4ºC [for details on the assays see (Burraco, Duarte & Gomez-Mestre, 2013)].

The protein content in the supernatant fluid was determined following Bradford's procedure (Bradford, 1976).

We measured activity levels of four enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR). To quantify oxidative cellular damage, we measured thiobarbituric acid reactive substances (TBARS) formed during lipid peroxidation (Buege & Aust, 1978). We determined catalase (CAT) activity indirectly by measuring catalytic activity (Cohen & Somerson, 1969). Potassium permanganate ($KMnO_4$), an oxidizing and colored agent, acts on H_2O_2, the catalase substrate. $KMnO_4$ is thus reduced, producing a red product. Five minutes after adding $KMnO_4$, we determined the wavelength at 480 nm. Catalase activity was expressed as units per milligram of total proteins. We determined superoxide dismutase (SOD) activity indirectly by measuring the inhibition rate of cytochrome C reduction. Superoxide radicals (O_2^-) oxidize cytochrome C except in the presence of SOD, which competes for O_{2-} generated by hypoxanthine and xanthine oxidase action, reducing cytochrome C and producing hydrogen peroxide and oxygen. We monitored the increase in absorbance at 550 nm and defined one unit of SOD as the amount of enzyme that inhibited the rate of reduction of cytochrome C by 50% at 25ºC (McCord & Fridovich, 1969). We determined glutathione peroxidase (GPx) activity using an assay described by (Paglia & Valentine, 1967). Oxidized glutathione is reduced due to an excess of glutathione reductase (GR) and produces a constant level of reduced glutathione (GSH). Production of GSH from oxidized glutathione requires NADPH. To quantify GPx activity, we measured NADPH oxidation by reading absorbance at a wavelength of 340 nm. We quantified GR activity following (Cribb, Leeder & Spielberg, 1989) by measuring the change in absorbance at 340 nm due to NADPH oxidation, like in the GPx assay. We measured thiobarbituric acid reactive substances (TBARS) concentration following (Buege &

Aust, 1978). Lipid peroxidation produces malondialdehyde, which reacts with acid to produce TBARS, a red product that absorbs light at 535 nm. We measured the optical density values of the blank and the calibration curve. We recorded the absorbance value of each sample and subtract the absorbance value of the blank to obtain the TBARS concentration (nmolTBARS/ml). These values were compared to the calibration curve.

Statistics

Analyses were conducted using R version 3.3.1 (R Development Core Team, 2016) and packages nlme (Pinheiro et al., 2016) and multcomp (Hothorn, Bretz & Westfall, 2008). Early larval survival was defined as the number of tadpoles counted before the beginning of the dry-down divided by the initial larval density and was expressed as a percentage. Survival to metamorphosis was defined as the number of individuals successfully completing metamorphosis divided by the initial larval density and was expressed as a percent. Juvenile recruitment was defined as the number of juveniles successfully completing metamorphosis. Time to metamorphosis was calculated as the difference between date of hatching and date of metamorphosis [forelimb emergence, Gosner 42, Gosner 1960)]. Mass at metamorphosis was defined as mass at complete tail reabsorption 46 (Gosner, 1960). We examined data for normality and transformed data if necessary. Following these transformations, we employed the Breusch – Pagan test to evaluate assumptions of homoscedasticity (Breusch & Pagan, 1979). Analysis on survival, time to metamorphosis, size (mass and SVL), morphology and jumping performance at metamorphosis were performed on tank means.

Early larval survival before the dry-down began (survival before April 2013) was analyzed using a mixed generalized linear model with a binomial distribution, with initial density as a fixed effect and block as a random effect to test for differences in percent survival before the start of the

hydroperiod manipulation. Survival to metamorphosis was analyzed using a mixed generalized linear model with hydroperiod and density as factors and block as a random effect to test for differences in the percent of animals successfully completing metamorphosis. Juvenile recruitment was analyzed using a mixed generalized linear model with a Poisson distribution, with initial density as a fixed effect and block as a random effect to test for differences in the number of animals successfully completing metamorphosis.

We tested for the effects of initial larval density (five levels: 5, 15, 25, 35 and 50 tadpoles respectively) and hydroperiod manipulation (two levels: dry-down and constant water level) on the phenotypic response variables using a linear model with larval density and hydroperiod as fixed effects and block as a random effect. Tukey HSD post hoc test were conducted using TukeyHSD function (multcomp package). For analyses on locomotor performance, morphometric responses were log transformed. Previous work has shown that SVL may influence tibia-fibula length and we included log SVL as a covariate after testing for the main effects of density and pond dry-down (Phillips et al., 2006; Llewelyn et al., 2010). We analyzed tibia-fibula length with larval density and hydroperiod as fixed factors and log SVL as a covariate and block as a random factor. To analyze jumping performance, relative leg length was calculated by taking the residual scores from a linear regression of log tibia-fibula length against log SVL (Phillips et al., 2006; Llewelyn et al., 2010). We then analyzed maximum jump distance with hydroperiod and larval density as fixed factors, log SVL and relative leg length as covariates and block as a random factor. Models were fitted to the data and we then calculated the Akaike Information Criterion (AIC). We calculated the AIC score for models that included only main effects, then calculated AIC scores for models that included an interaction between covariates and main effects. Similarly, to determine whether the blocking variable should be included in the final model, we compared

models that included or excluded the blocking variable. We present results for the best of these models selected by the lowest AIC score (Johnson & Omland, 2004).

To estimate whether density or hydroperiod influenced fat body mass, we calculated arcsine square root transformed percent fat body mass (mass of fat bodies/total mass). Because of the missing 5 larval density constant hydroperiod data points, we conducted a one-way ANOVA (Analysis of Variance) to test whether density influenced percent fat body mass within dry-down mesocosm treatments. We then excluded the 5 larval density dry-down toads, and conducted a two-way ANOVA to test whether hydroperiod (two levels: dry-down and constant) and density (4 levels: 15, 25, 35 and 50 toads per mesocosm) influenced percent fat body mass.

We conducted parallel analyses on oxidative stress data as toads from the 5 larval density constant water level were not available for analyses. We conducted a one-way ANOVA to test whether density influenced CAT, SOD, GPx, GR activity and TBARs levels within dry-down treatments. We then excluded the 5 larval density dry-down toads and conducted two-way ANOVAs to test whether hydroperiod and density influenced CAT, SOD, GPx, GR activity and TBARs levels. These variables were log transformed to improve normality. For these analyses, block was not included as a random factor because of the smaller sample sizes.

Results

Four tanks were excluded from analyses due to an error during the experimental setup. (The incorrect number of tadpoles were placed in two tanks during setup. During the survival survey in April, tadpoles from one tank were accidently transferred to another tank, rendering both tanks unusable.) Before the dry-down portion of the experiment, early larval survival generally declined as larval density increased ($\chi^2 = 98.08$, $P < 0.0001$, Figure 1.1). Survival was 25.2 % higher in the 5 density level than the 50 density level [84.8% ± 2.9% (mean ± SE) and 59.6% ±

4.2%], respectively. However, larval survival did not differ between the 5 and 15 density levels and the 25 and 35 density levels.

Survival to metamorphosis also declined across larval densities (χ^2 = 261.3, P < 0.0001, Figure 1.2A) but was not influenced by hydroperiod treatment (Figure 1.2B, χ^2 = 1.50, P < 0.22). Survival to metamorphosis in the 5 and 15 density levels was on average 34.6% higher than in the 25 and 35 density levels and 42.9% higher than in the 50 density level. Survival to metamorphosis did not differ between the 5 and 15 density level or between the 25 and 35 density level. Survival to metamorphosis was on average 8.3% higher in the 25 and 35 density level than in the 50 density level. Survival ranged from the lowest in the 50 density level, constant treatment (13.6% ± 2.4) to the highest in the 5 density level dry-down treatment (65.7% ± 6.1).

Initial larval density influenced the number of metamorphs emerging (χ^2 = 44.42, P < 0.0001, variance $_{\text{Block}}$ = 0.0414, standard deviation $_{\text{Block}}$ = 0.2035, Figure 1.2C). On average, 2.32 times more metamorphs (~ 4 metamorphs) emerged from the 15, 25, 35 and 50 density levels than the 5 density level. There was no difference in the number of emerging metamorphs between the 15, 25, 35 and 50 density levels. There was no difference in the number of metamorphs emerging from constant and dry-down water levels (χ^2 = 0.718, P = 0.40, Figure 1.2D).

Both larval density and hydroperiod significantly influenced time to metamorphosis, but there was no interaction (Table 1.1). Across hydroperiod treatments, toads from the lowest density level emerged on average 9% earlier (~16 days) than toads from the remaining density levels (Figure 1.3A). Across density levels, toads from constant water level treatments emerged 7% later (~ 13 days, Figure 1.3B). The earliest metamorphs emerged from the 5 density, dry-down level [185.3 days ± 1.4, (mean ± SE)] and the latest metamorphs emerged from the 15 density, constant water level (216.9 days ± 2.2).

Both larval density and hydroperiod significantly affected mass at metamorphosis, but there was no interaction (Table 1.1). Across hydroperiod treatments, toads emerging from the 5 density level were on average 120% heavier than toads from the four other density levels (Figure 1.3C). Additionally, toads from the 15 density level were 30% larger than toads from the 50 density level. Across density levels, toads from constant water level treatments were on average 17% larger than toads from dry-down treatments (Figure 1.3D). The heaviest toads emerged from the 5 density, constant water level treatment, with an average mass of 2.67 g ± 0.08 (mean ± SE) while the lightest emerged from the 50 density, dry-down treatment at 0.86 g ± 0.08.

We found parallel results for SVL. Both larval density and hydroperiod significantly affected SVL, but there was no interaction (Table 1.1). Across both hydroperiod treatments, toads emerging from the 5 density level were on average 25% longer than toads from the four other density levels. Toads from the 15 density level were 9.8% longer than toads from the 50 density level. Across density levels, toads from constant water level treatments were on average 9% longer than toads from dry-down treatments. The longest metamorphs emerged from the 5 constant water level treatment (29.4 mm ± 0.40) and the smallest from the 50 dry-down treatments (20.9 mm ± 0.46).

Both larval density and pond dry-down significantly affected absolute tibia-fibula length, but there was no interaction (Table 1.1). Across both hydroperiod treatments, toads emerging from the 5 density level had on average 32% longer tibia-fibulas than toads from the four other density levels (Figure 1.4A). Across density levels, toads from constant water level treatments had 9% longer tibia-fibulas than toads from dry-down treatments (Figure 1.4B). Toads from the 5 density constant treatment had the longest tibia-fibulas (11.3 ± 0.12 mm), while toads from 50 density dry-down treatments had the shortest tibia-fibulas (7.42 ± 0.19 mm). To test if the effects of

hydroperiod and density on tibia-fibula were driven by differences in SVL, we included log SVL as a covariate. Once we included SVL, the best model included a main effect of hydroperiod ($F_{1,95}$ = 9.24, P = 0.003), a main effect of initial larval density ($F_{4,95}$ = 6.88, P < 0.0001), and a main effect of log SVL ($F_{1,90}$ = 280.7, P < 0.0001; overall model: $F_{6,95}$ = 318.2, R^2= 0.95, P < 0.0001). Constant water level increased log tibia-fibula by 0.025 ± 0.008 across values of SVL and density levels (Figures 1.5A and Figure 1.5B).

Both larval density and pond drying influenced maximum jump distance (Table 1.1, Figures 1.4C and 1.4D). Increasing larval density and pond dry-down reduced jumping distance. Toads from 5 and 15 density levels had on average 12% longer maximum jump distance than toads from the 35 and 50 density levels. There were no differences in maximum jump distance between the 5, 15 and 25 density level. There was also no difference in maximum jump distance between the 25, 35 and 50 density level. Across density levels, toads from constant water level treatments jumped 17% farther than toads from dry-down treatment. The largest difference was between the 5 density, constant water level treatment and the 50 density, dry-down treatment (22.4 cm ± 0.8 versus 16.2 ± 0.3 cm respectively).

Because SVL and relative tibia-fibula length may also influence jumping performance, we included them in a subsequent analysis to determine if these differences were driving differences in jumping performance. We ran the full model which included SVL, relative tibia-fibula length, larval density, hydroperiod and all interactions. Blocking did not improve the fit of this analysis and was not retained. The best model retained main effects of hydroperiod ($F_{1,95}$ = 12.25, P = 0.0007), density ($F_{4,95}$ = 2.36, P = 0.059) and log SVL ($F_{1,95}$ = 8.64; P = 0.004; Overall model $F_{6,95}$ = 12.11, R^2= 0.40, P < 0.0001). A one unit increase in log SVL resulted in a 0.59 ± 0.20 cm increase

in log maximum jump. Across log SVL values, a constant water level increased log maximum jump distance by 0.10 ± 0.03 cm (Figures 1.5C and 1.5D).

Fat bodies and oxidative stress

Larval density had a significant effect on percent body fat in the dry-down treatment, ($F_{4,33}$ = 3.88, P = 0.011, Figure 1.6A). Toads from the 5 density levels had 4.80 times higher percent body fat than toads from the 15, 25 and 35 density levels. However, we did not detect a significant difference in percent body fat between the 5 and 50 density levels. Because we did not have 5 density constant water level toads for percent body fat assays, we excluded the 5 density toads to test how hydroperiod influenced fat bodies across the higher density levels. We found that hydroperiod significantly influenced percent body fat ($F_{1,62}$ = 9.40, P = 0.003), but not larval density ($F_{3,62}$ =0.17, P = 0.91, Figure 1.6B). Toads from constant water level had on average 1.25 times more percent body fat than toads from the dry-down treatments.

Within the dry-down treatment, toads from the 5 density levels had on average 73% lower CAT activity than toads from the 15, 25, 35 density levels ($F_{4,27}$ = 4.91, P= 0.001, Figure 1.6C). Once we excluded data from the 5 density levels, we found that only hydroperiod had a significant effect on CAT activity ($F_{1,48}$ = 11.25, P =0.002). Constant water level toads had 39% lower CAT activity than dry-down toads across the 15, 25, 35 and 50 density levels (Figure 1.6D). Within only the dry-down treatment, we found that density influenced TBARs levels ($F_{4,28}$ = 7.67, P = 0.0003). Toads from the 15 and 35 density levels had 64% lower TBARs levels than toads from 5 density level. Once we excluded data from the 5 density level, we found that there was no effect of larval density and hydroperiod on TBARs levels. Within the dry-down treatments, initial larval density did not influence activity of oxidative stress enzymes [SOD: 49.23 U/mg protein ± 14.86 (mean ± SD), $F_{4,28}$ = 1.00, P = 0.42, GPx: 22.93 U/mg protein ± 10.51, $F_{4,25}$ = 1.2 1, P = 0.33; GR: 39.97

U/mg protein ± 13.23, $F_{4,28} = 0.77$, $P = 0.56$]. Across the 15 through 50 density levels, there were no effects of larval density or hydroperiod on activity of three oxidative stress enzymes [SOD: 49.23 ± 14.86, larval density ($F_{3,52} = 0.26$, $P = 0.85$), hydroperiod ($F_{1,52} = 1.27$, $P = 0.26$), GPx = 22.96 U/mg protein ± 9.14, larval density ($F_{3,49} = 0.21$, $P = 0.89$), hydroperiod ($F_{1,49} = 0.52$, $P = 0.47$); GR = 38.74 U/mg protein ± 12.72, larval density ($F_{3,52} = 0.15$, $P = 0.93$), hydroperiod ($F_{1,52} = 0.84$, $P = 0.36$)].

Discussion

Multiple and simultaneous environmental stressors affect the timing and size at ontogenetic switch points in animals with complex life cycles, although different stressors may result in different carry-over effects (Pechenik, 2006; Gomez-Mestre et al., 2010). Here, we found that two common stressors to amphibian larvae, increasing conspecific density and pond drying, act additively rather than synergistically on tadpole development, causing large alterations in phenotype at metamorphosis.

Effects of larval density and pond drying on survival to metamorphosis

Although both factors induced stress and worsened body condition, increasing initial larval density reduced survival to metamorphosis whereas pond dry-down did not. Mortality intensified as tadpole density increased, as expected (Brockelman, 1969; Van Buskirk & Smith, 1991; Skelly & Kiesecker, 2001). Interestingly, the number of juveniles recruited was similar across the four highest density levels, suggesting a non-linear effect of density so that tadpoles reached carrying capacity even at relatively low densities (< 0.3 tadpoles/L).

We initially hypothesized that as density increased, reduced growth rates and insufficient fat bodies would impair tadpoles' capacity for developmental acceleration (Kulkarni et al., 2011), resulting in disproportionately lower survival in dry-down tanks. Other studies have reported such

non-additive reduced survival in dry-down treatments under high density or reduced resource availability but not under more benign conditions [*Ambystoma talpoideum* (Semlitsch, 1987), *Bufo calamita* (Tejedo & Reques, 1994), *Bufo terrestris* (Rogers & Chalcraft, 2008), *Discoglossus pictus* (Enriquez-Urzelai et al., 2013)]. Reduced survival in these experiments resulted from failure to accelerate metamorphosis in drying, highly competitive ponds. Intense competition at high density can preclude tadpoles from storing enough fat reserves to fuel the high metabolic cost of accelerating development in response to pond drying, which in *P. cultripes* represents a two-fold increase in metabolic rate (Kulkarni et al, in press). Here we focused on the phenotypic consequences of altered growth and development, and therefore we did not allow water in the tanks to dry up completely. Given that tadpoles at high density took considerably longer to metamorphose than at low density, mortality would have likely been catastrophic at high larval density had we allowed tanks to dry up completely, as would natural ponds.

In *P. cultripes*, pond dry-down may obviously be an important limitation to recruitment if ponds dry out too quickly or early in the season (density-independent effects on survival), and such events are predicted to increase in frequency under the current climatic change (IPCC, 2014). However, our experiment suggests that within our density range, spadefoot toad larvae were capable of effectively responding to pond dry-down by accelerating development, even though our density levels were within the upper range of observed field densities (Cei & Crespo, 1971; Rodriguez-Jimenez & Prados, 1985, Díaz-Paniagua, unpublished data).

Effects of larval density and pond drying on juvenile phenotype

Across density levels, dry-down toads emerged earlier but smaller, indicating that developmental acceleration resulted in reduced body size, as observed in other studies (Richter-Boix, Tejedo & Rezende, 2011; Gomez-Mestre, Kulkarni & Buchholz, 2013). Size at

metamorphosis was negatively density dependent. At low density, tadpoles had higher availability of food resources, and high resource levels result in high growth rates which result in large, early emerging metamorphs (Gomez-Mestre et al., 2010; Enriquez-Urzelai et al., 2013; Charbonnier & Vonesh, 2015).

The negative effects of higher density and pond dry-down on size at metamorphosis were additive rather than synergistic. Similarly, Semlitsch (1987), Crespi & Warne (2013), Searcy, Snaas & Shaffer (2014), Charbonnier & Vonesh (2015), and Mogali, Saidapur & Shanbhag (2016) found only additive effects of pond dry-down and density or resource levels. Tejedo & Reques (1994) found both independent effects and interactive effects of pond drying. Enriquez-Urzelai et al. (2013) found only an interactive effect with no main effect of pond drying, and Rogers & Chalcraft (2008) found no effect of pond drying on size at metamorphosis. These differences could be due to species specific responses, as many species show variation in their response to pond dry-down and larval density or due to differences in experimental procedures (Richter-Boix, Llorente & Montori, 2006; Edge et al., 2016).

In our experiment, differences in size generated by the density manipulation were greater than those generated by the hydroperiod manipulation, supporting previous work that suggests factors that influence growth rates rather than developmental rates are the main drivers of differences in size at metamorphosis (Gomez-Mestre et al., 2010). Larvae exposed to dry-down not only had shorter growing periods, but also burned accumulated fat reserves in order to accelerate metamorphosis, resulting in smaller toadlets with smaller fat reserves (see also Kulkarni et al., 2011). Additionally, toads from the 5 density level had greater relative fat content than toads from the higher density levels, indicating that competition also reduced their opportunity to accumulate fat reserves. Since larger size at metamorphosis is associated with higher post

metamorphic survival and fecundity (Smith, 1987; Berven, 1990; Scott, 1994; Gomez-Mestre & Tejedo, 2002; Earl & Whiteman, 2015) in the absence of compensatory growth, toads from dry-down environments therefore have lower survival odds and hence reduced fitness. The size of fat bodies influences the ability to compensate for small size at metamorphosis (Crespi & Warne, 2013), further suggesting that toads from dry-down environments may be unable to compensate for small size.

Both dry-down and density also influenced the morphology of emerging metamorphs. Absolute tibia-fibula length was reduced in dry-down treatments, consistent with a large body of research that shows how accelerated development shortens hindlimb length (Johansson, Lederer & Lind, 2010; Gomez-Mestre et al., 2010; Tejedo et al., 2010; Charbonnier & Vonesh, 2015). Toads from the lowest density had the longest legs, consistent with the hypothesis that high growth rates result in long legged, but early emerging frogs (Gomez-Mestre et al., 2010; Tejedo et al., 2010). We found a similar pattern for relative tibia-fibula length with toads from dry-down treatments having slightly shorter relative tibia-fibulas than toads from constant treatments (Gomez-Mestre, Kulkarni & Buchholz, 2013).

A central question is whether these carry-over effects on morphology can result in differences in ecologically relevant performance capacities (Emerson, 1978). In amphibians, jumping performance influences food acquisition, predator avoidance, and dispersal capacities (Arnold & Wassersug, 1978; Walton, 1988; Heinen & Hammond, 1997; Phillips et al., 2006; Patrick et al., 2008; Ward-Fear, Brown & Shine, 2010). We found that these differences in tibia-fibula length resulted in differences in jumping performance. Toads from constant water level treatments had longer maximum jump distances across density levels. Once we accounted for differences in SVL, toads from constant water level outperformed toads from dry-down treatments.

These findings are consistent with previous work that suggests that pond drying may negatively influence the jumping performance of juvenile frogs both by decreasing size at metamorphosis and shortening relative hindlimb length (Enriquez-Urzelai et al., 2013; Charbonnier & Vonesh, 2015). Density also influenced jumping performance, as larger toads from lower density levels jumped farther than toads from higher density levels (Goater, Semlitsch & Bernasconi, 1993; Tejedo, Semlitsch & Hotz, 2000). Although we do not have longer-term data on these effects, a recent meta-analysis found that carry-over effects on jumping performance have been observed for up to three months after metamorphosis (Charbonnier & Vonesh, 2015). In the short term, differences in jumping performance may be one of the factors explaining size-dependent survival after metamorphosis (Cabrera-Guzmán et al., 2013).

We also found evidence that toads from dry-down treatments experienced higher levels of oxidative stress, as toads from dry-down treatments had higher levels of catalase activity across the 15 through 50 density levels. However, there was no evidence of increased oxidative damage due to pond drying across density levels, as TBARs levels were similar, suggesting that higher levels of catalase may have neutralized oxidative damage. Within the dry-down levels, the 5 density toads had lower catalase activity but higher levels of oxidative damage, presumably caused by rapid growth (Burraco, Duarte & Gomez-Mestre, 2013). Our findings are consistent with previous work in this species and other amphibian species (Burraco et al., 2017, Burraco et al. in press).

Growth and differentiation are partially decoupled in anuran development (Smith-Gill & Berven, 1979; Smith-Gill, 1983), and environmental stressors acting more strongly on either of these factors are bound to affect the resulting size and shape of emerging juveniles differently (Gomez-Mestre et al., 2010). Here we show that both larval density and dry-down induced

developmental acceleration caused profound alterations on body size and relative tibia-fibula length, fat reserves and oxidative stress. Both factors affected tadpoles in the same direction but at different magnitudes, as larval density had a more severe impact on phenotypic variables than pond drying. Juveniles from high-density levels metamorphosed much smaller, with relatively shorter hindlimbs (which decreased their locomotor performance), reduced fat bodies and higher oxidative stress. Pond drying exacerbated these effects consistently across densities. Our results suggest that stressors experienced simultaneously may have additive (and thus predictable) impact on phenotype and survival. In this species, high larval density as ponds dry up may be an increasingly recurrent scenario under the ongoing global climate change, especially in Mediterranean areas (IPCC, 2014).

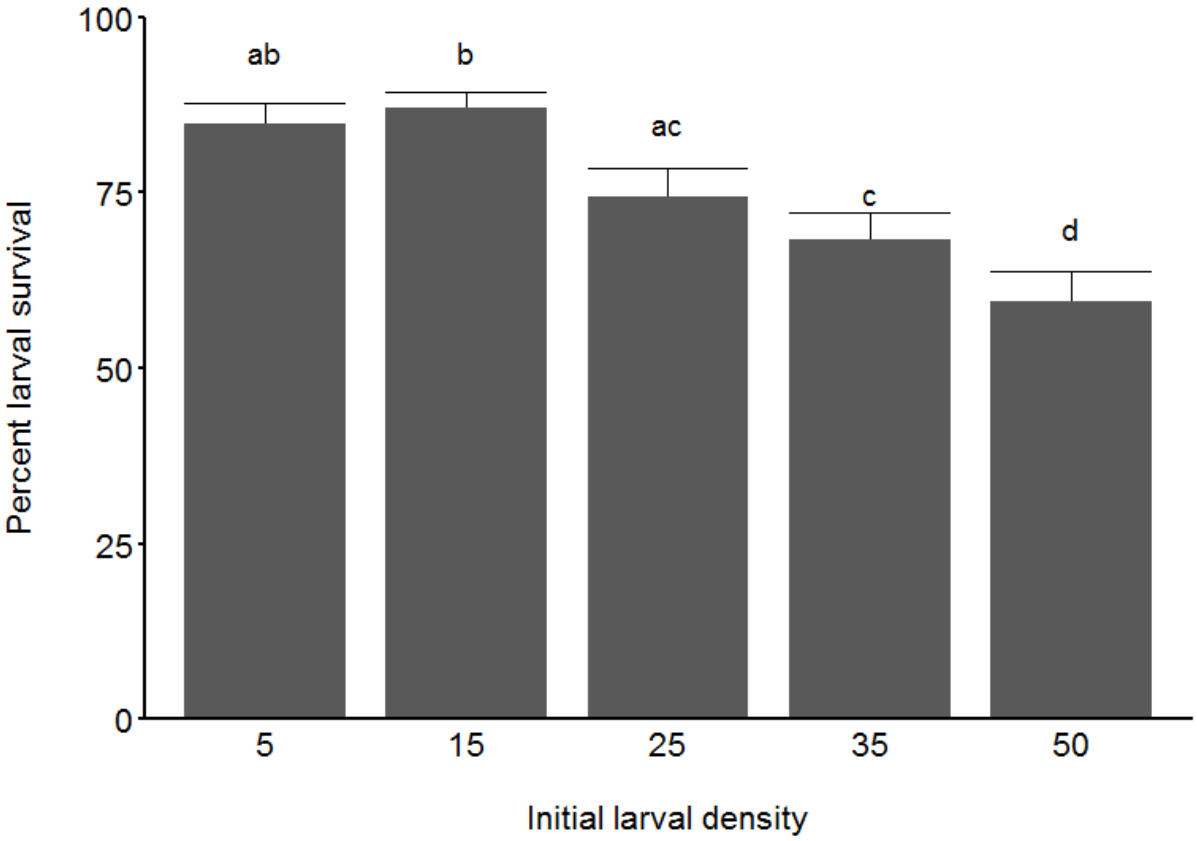

Figure 1.1 Effects of initial larval density on mean percent larval survival (before the dry-down portion of the experiment) in *P. cultripes*. Errors bars represent standard errors.

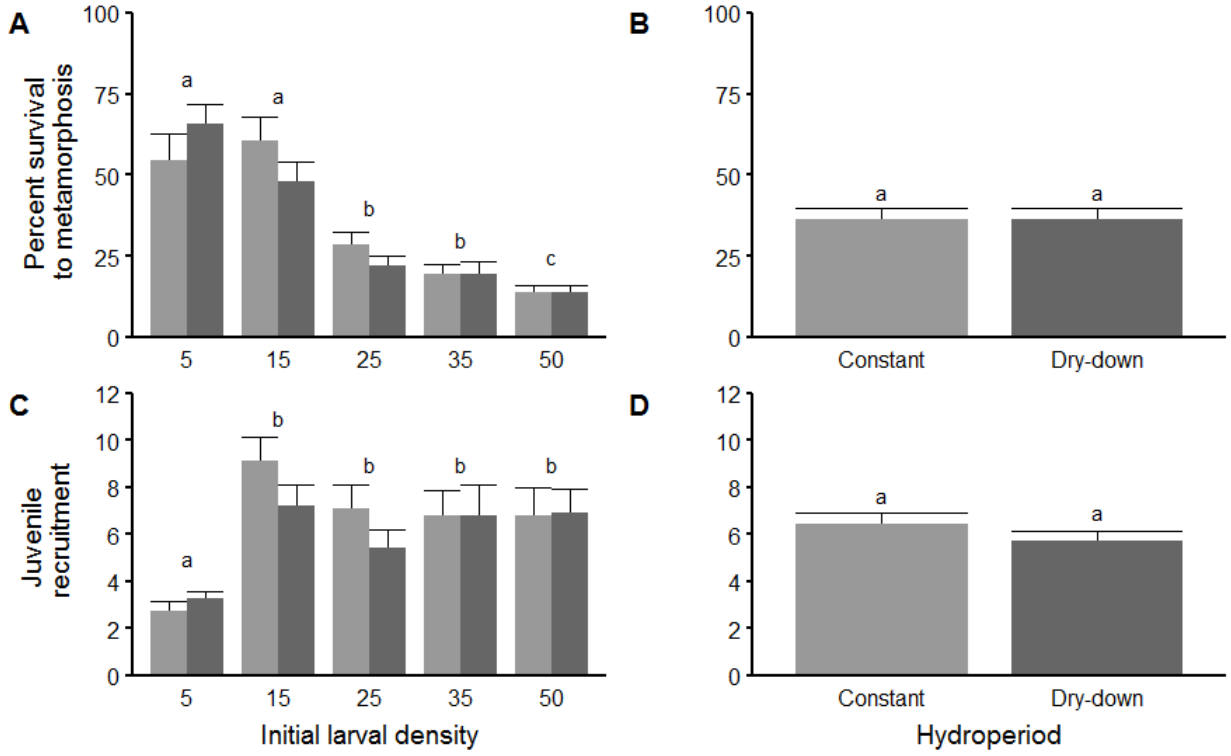

Figure 1.2 Effects of initial larval density and pond dry-down on mean percent survival to metamorphosis (A, B) and mean number of emerging metamorphs (C, D) in *P. cultripes.* Lighter colored bars represent constant water level treatments, darker bars represent dry-down treatments. Errors bars represent standard errors.

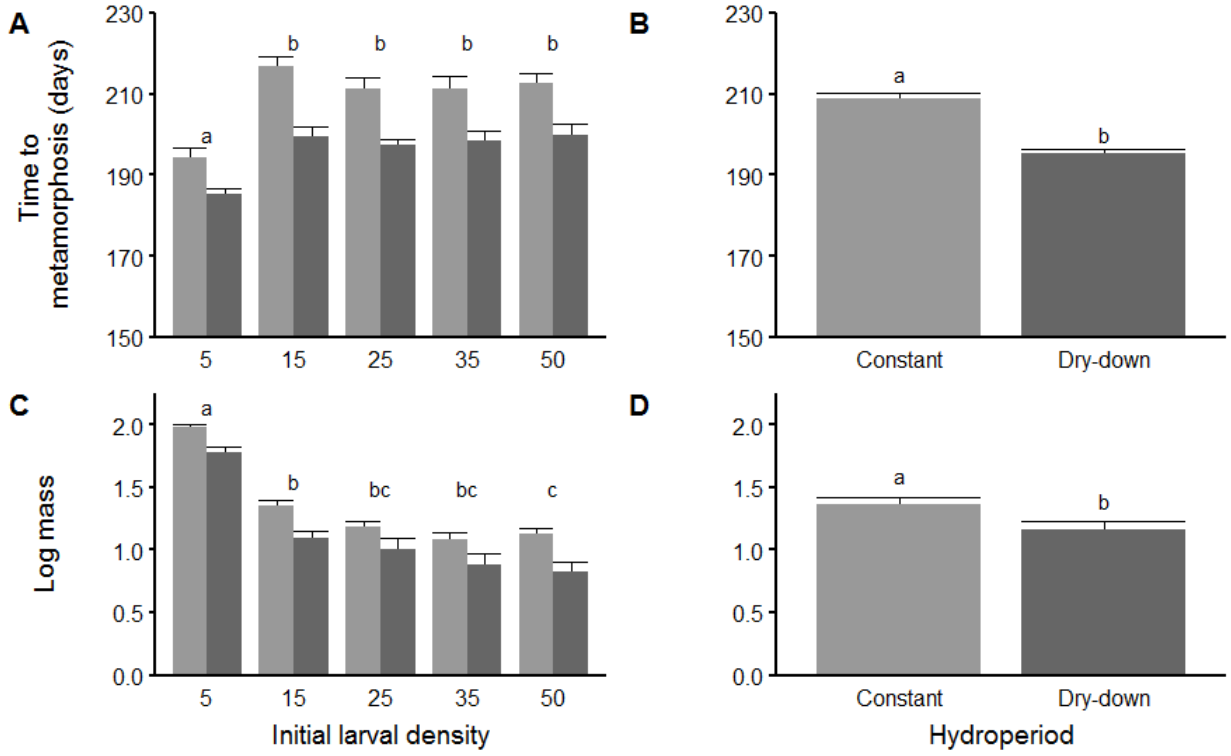

Figure 1.3 Effects of initial larval density and pond dry-down on mean time to metamorphosis (A, B) and mean size at metamorphosis (C, D) in *P. cultripes*. Lighter colored bars represent constant water level treatments, darker bars represent dry-down treatments. Errors bars represent standard errors.

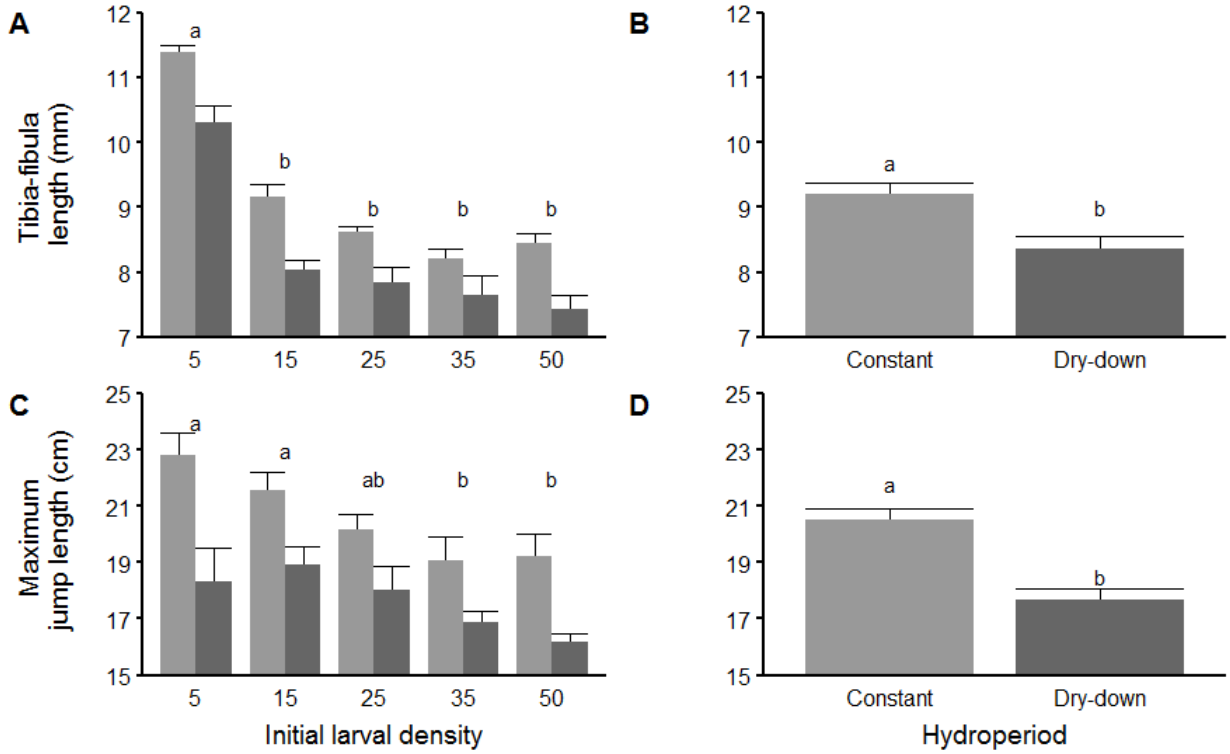

Figure 1.4 Effects of initial larval density and pond dry-down on mean tibia-fibula length (A, B) and mean maximum jump distance (C, D) in *P. cultripes*. Lighter colored bars represent constant water level treatments, darker bars represent dry-down treatments. Errors bars represent standard errors.

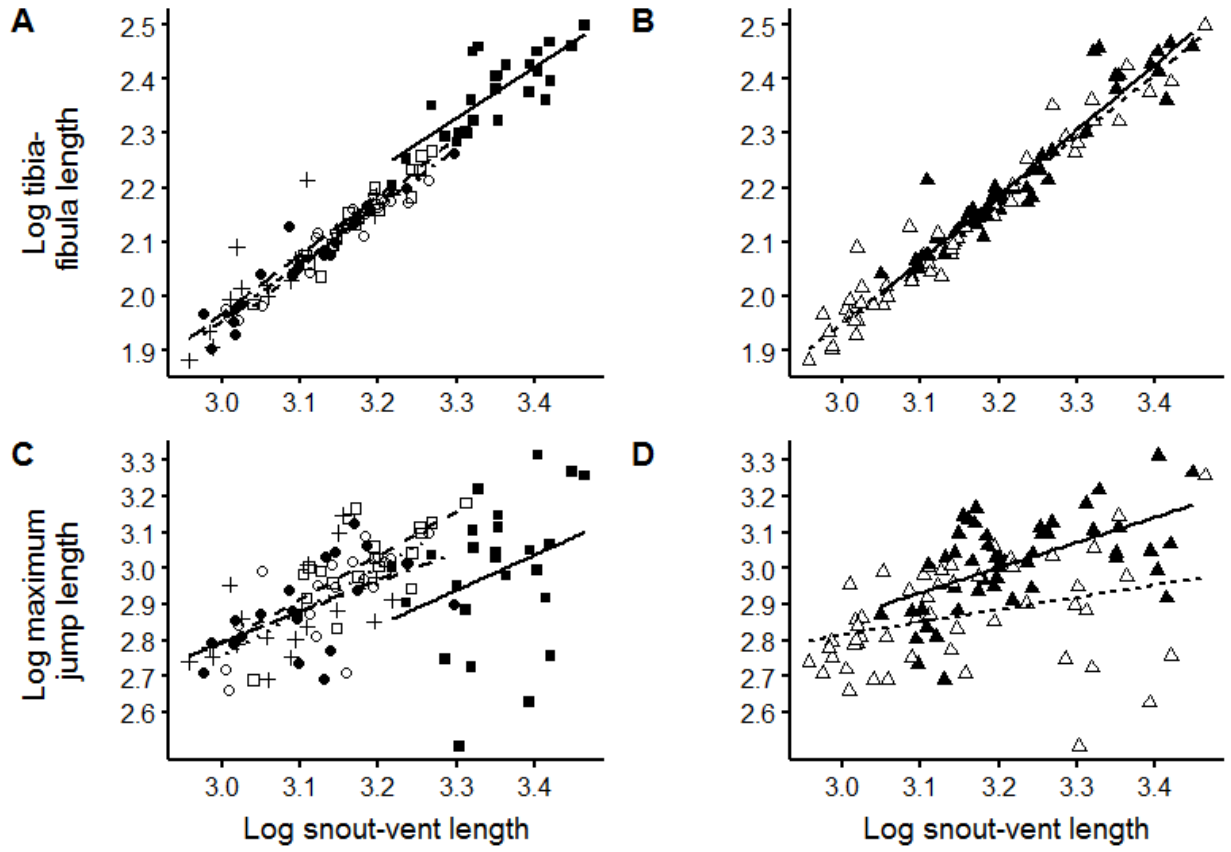

Figure 1.5 Relationship between log tibia-fibula length and log snout-vent length (A, B) and log maximum jump distance and log snout-vent length (C, D) as a function of initial larval density and pond dry-down in *P. cultripes*. Constant water level treatments (▲, solid line) and dry-down treatments (Δ, dotted line), 5 density treatment (■, solid line), 15 density treatment (□, dashed line), 25 density treatment (○, dotted line), 35 density treatment (●, dot-dashed line), 50 density treatment (+, long dashed line).

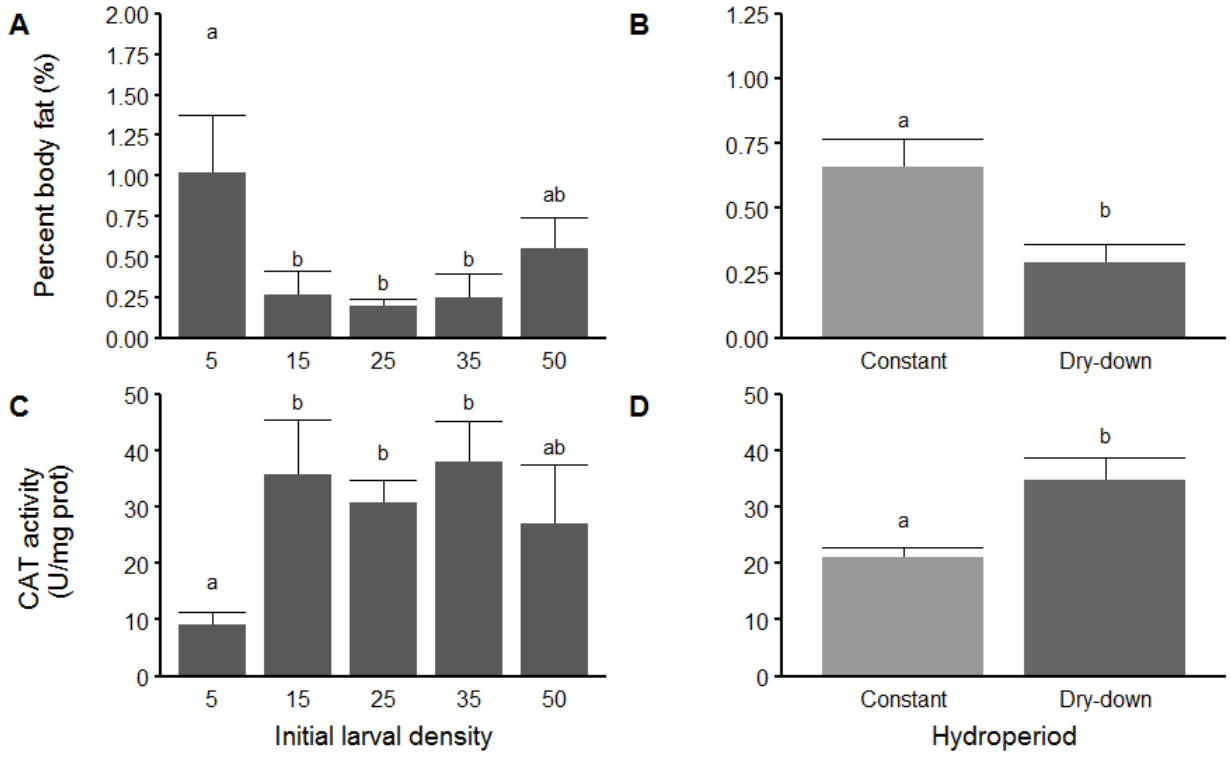

Figure 1.6 Effects of initial larval density in dry-down treatments on mean percent body fat (A) and mean catalase activity (U/mg protein) (C). Effects of hydroperiod manipulation across the 15 through 50 larval density treatments on the mean percent fat bodies (B) and on mean catalase activity (U/ mg protein) (D) in *P. cultripes*. Errors bars represent standard errors.

Table 1.1 Results of univariate tests that examined the impacts of initial larval density and hydroperiod on the phenotype of *P. cultripes.*

	Larval Density		Hydroperiod		Larval Density x Hydroperiod		Random effects Block		Residuals	
Response	*Chisq*	*P*	*Chisq*	*P*	*Chisq*	*P*	*Variance*	*Std. Dev*	*Variance*	*Std.Dev*
Time to metamorphosis	87.61	<0.0001	76.9	<0.0001	2.44	0.65	0.803	0.896	56.0	7.49
Log mass	410.0	<0.0001	46.5	<0.0001	1.65	0.80	0.005	0.069	0.03	0.176
Snout-vent length	328.2	<0.0001	53.1	<0.0001	2.14	0.71	0.298	0.546	1.94	1.39
Tibia-fibula length	367.7	<0.0001	55.1	<0.0001	1.93	0.75	0.022	0.148	0.37	0.60
Maximum jump distance	23.2	<0.0001	37.8	<0.0001	1.74	0.78	1.57e-15	3.89e-8	5.63	2.37

Table 1.2 Sample sizes for fat bodies and oxidative stress analyses.

Density Treatment	Hydroperiod Treatment	Number of individuals	Number of tanks
5	Dry-down	8	8
15	Constant	10	10
15	Dry-down	9	9
25	Constant	10	10
25	Dry-down	9	9
35	Constant	12	10
35	Dry-down	10	9
50	Constant	4	4
50	Dry-down	6	6
Total Number	Dry-down	42	
	Constant	36	
	Total	78	

CHAPTER 2 INFLUENCE OF LARVAL ENVIRONMENT AND TERRESTRIAL RESOURCE LEVELS ON GROWTH AND SURVIVAL IN A POND BREEDING SALAMANDER

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Introduction

Understanding the factors that shape linkages between life stages are a major focus of study in the organismal ecology literature (Mousseau & Fox, 1998; Pechenik, 2006; Marshall & Morgan, 2011). Carry-over effects are the historical constraints that past life stages and developmental histories exert on organisms (Mousseau & Fox, 1998; Podolsky & Moran, 2006; Pechenik, 2006; Marshall & Morgan, 2011). Across taxa with complex life histories (Wilbur, 1980; Moran, 1994), multiple studies have documented how early life stages ultimately influence multiple components of phenotype (Peckarsky et al., 2001; Tejedo et al., 2010; Barbasch & Benard, 2011; Crespi & Warne, 2013).

Because carry-over effects are widespread and diverse, understanding how they may influence demographic traits (e.g., survival, reproduction) is crucial for extrapolating their potential influence on populations. If carry-over effects on phenotype are transient, they are less likely to influence vital rates. The endpoint of most experimental studies is directly after life history switch points (e.g., hatching, metamorphosis) and it is unclear how carry-over effects persist over time (Altwegg & Reyer, 2003; Earl & Semlitsch, 2013). Relationships between previous life stages and organismal phenotype are expected to weaken with time, as current conditions continually influence the phenotype of the developing organism (Podolsky & Moran, 2006; Allen & Marshall, 2013). Subsequent life stages may negate or exacerbate initial differences in phenotype through subsequent compensatory growth and/or differential allocation (Lindström, 1999; Madsen & Shine, 2000; Metcalfe & Monaghan, 2001).

Larval amphibians are model organisms for studying complex life cycles and can modulate their growth and development rates, allowing them to respond to environmental stressors (Smith-Gill & Berven, 1979; Smith-Gill, 1983; Gomez-Mestre et al., 2010). Both field and laboratory studies have found that variation in embryonic and larval environment results in complex carry-over effects on phenotype in the subsequent life stage (Relyea & Hoverman, 2003; Richter-Boix, Llorente & Montori, 2006; Tejedo et al., 2010; Warkentin, 2011). However, whether these carry-over effects persist beyond metamorphosis will be dependent on their magnitude and environmental variation in subsequent environments (Podolsky & Moran, 2006; Earl & Semlitsch, 2013).

Size at life history switch points (e.g., metamorphosis) is the most commonly studied metric for approximating fitness later in life across taxa (Day & Rowe, 2002). However, in amphibians the relationship between size at metamorphosis and survival is highly variable. Earl & Whiteman (2015) suggest that the positive relationship between size at metamorphosis and later survival may increase with the proportion of the time to maturity reached at metamorphosis. Long lived species that spend a proportionally smaller amount of time as larvae, may have a weaker relationship between size at metamorphosis and survival, because they have more opportunities for compensatory growth later in life. Additionally, mark-recapture studies suggest that other traits besides size at metamorphosis may influence survival and lead to different life history strategies, where small, but early emerging metamorphs can still maintain high survival (Schmidt, Hödl & Schaub, 2012; Searcy et al., 2014).

The terrestrial environment may influence the relationship between size at metamorphosis and subsequent post-metamorphic survival in multiple ways. For example, in high quality terrestrial environments, compensatory growth may attenuate carry-over effects on size, and

enable small individuals to gain mass quickly and improve their chances of survival. In poor quality environments, the relationship between size and survival may be erased if survival is low across size classes. The relationship between size at metamorphosis and survival may also be dependent on experimental venue. Earl & Whiteman (2015) found stronger correlations between size at metamorphosis and survival in controlled laboratory experiments than field studies. Relationships between size and survival must be explored in field relevant conditions and across relevant size ranges experienced in nature.

Compensatory growth, and catch-up growth in subsequent life stages may attenuate size differences generated in early life stages (Vonesh and Bolker 2005, Nicieza & Álvarez, 2009; Hector & Nakagawa, 2012). In amphibians, the relationship between size at metamorphosis and later terrestrial growth is highly variable (Tarvin et al., 2015). In some cases, the relationship between size and later growth is negative, resulting in higher growth rates for smaller individuals. However, even if smaller individuals can maximize their growth rate, they may not be able to fully catch up to the mass of control treatments (Van Allen et al., 2010). Quantifying the relationship between size at metamorphosis and terrestrial growth in diverse contexts can improve our understanding of the benefits and limits of compensatory growth, and help us estimate potential impacts on demographic traits (e.g., size at maturity, number of reproductive efforts).

Here we present the results from two experiments that examine the relationship between larval environment effects on timing of a key life history switch point, metamorphosis, and subsequent growth and survival in varying terrestrial environments. The objective of our first experiment was to estimate how carry-over effects on size at metamorphosis due to larval density influence survival in the spotted salamander, *Ambystoma maculatum.* We hypothesized that larger salamanders from low density treatments would have both higher pre-and post-overwintering

survival. The objective of our second experiment was to test whether high resource levels in the terrestrial environment (before over-wintering) would increase survival by enabling salamanders to compensate for smaller size. We hypothesized that salamanders provided with higher food levels after metamorphosis would have a higher chance of surviving to winter. We hypothesized across both experiments that smaller individuals would have higher mass specific growth rates (a positive relationship between size at metamorphosis and terrestrial growth). We quantified growth trajectories to test how size at metamorphosis influenced mass specific growth rates over time. In the second experiment, we sampled natural variation in size at metamorphosis and manipulated the terrestrial environment. If smaller individuals within a terrestrial resource treatment experienced higher mass specific growth rates, this would suggest compensatory growth.

Methods

Study system and animal collection

This study was conducted at Virginia Commonwealth University Walter and Inger Rice Center for Environmental Life Sciences in Charles City County, Virginia (77°12'30.5" W, 37°19'56.32" N). The spotted salamander (*Ambystoma maculatum*, Family: Ambystomatidae) is found throughout the Eastern United States and is locally abundant. Egg masses are deposited on submerged stems in clumps during the first spring rain (February through March in this region) and hatch in 40 to 60 days (Whitford & Vinegar, 1966; Petranka, Rushlow & Hopey, 1998). Aquatic larvae develop in vernal pools for two to five months and emerge from the pond after metamorphosis (Wilbur & Collins, 1973). As juveniles and adults, salamanders are fossorial and utilize subterranean burrows (Madison, 1997; Faccio, 2003). Once they have reached sexual maturity, spotted salamanders may live up to 15 to 20 years (Wilbur, 1977b; Flageole & Leclair, 1992).

Experiment 1: Effects of larval density on survival and growth over the first year

Larval density manipulation

We conducted an outdoor mesocosm experiment where we manipulated larval density from March 2014 to September 2014. Aquatic mesocosms consisted of 1136 L Rubbermaid tanks (1.7 m diameter, 0.64 m depth) filled with James River water and inoculated with 2 L of concentrated zooplankton from nearby vernal pools (30 L poured through an 80 µm plankton net for each 10 L of pond water) and 2 kg of dry, local leaf litter. Mesocosms were arranged in three rows of seven tanks at the forest edge, covered with screen mesh and filled in March 2014. Spotted salamander larvae were collected from vernal pools with a dipnet at the U.S. Fish and Wildlife Service Harrison Lake National Fish Hatchery (77°11'17.85" W, 37°20'29.92" N) in Charles City County, VA under IACUC # 10000450 in May 2014.

Larvae were reared at two experimental densities: low density (six animals per tank, 0.006 larvae/L) and high density (18 animals per tank, 0.018 larvae/L). Experimental densities were based upon field estimates of densities reported in the literature to simulate low and high density environments (0. 012 larvae/L; Boone & James, 2003; 0.012 - 0.036 larvae/L;Metts, Hopkins & Nestor, 2005) and within the range of what larvae would experience in natural field settings (0.002-.08 larvae/L; Figiel & Semlitsch, 1990). High density treatments were replicated eight times and low density treatments were replicated 12 times for a total of 216 animals distributed among 20 tanks. Density treatments and animals were randomly assigned to aquatic tanks.

Aquatic tanks were monitored weekly and 1 L of concentrated zooplankton was added to the tanks every other week (Metts, Hopkins & Nestor, 2005). Tanks were checked daily, once we observed animals with receding gills. Animals were collected every other day and housed in 1.89 L plastic containers with a wet paper towel in an outdoor shaded and screened facility. Animals were temporarily held in these containers until they absorbed their gills (one to two weeks). Before

entering the terrestrial portion of the experiment, individuals were weighed and photographed and we recorded larval duration (date entered tank - day of metamorphosis).

Post-metamorphic mesocosm experiment

To determine how aquatic larval density influences survival and growth in the terrestrial juvenile stage, animals were transferred to individual pens in the forest (Figure 2.1). Pens consisted of 10 L rectangular Rubbermaid tubs (33 cm x 55 cm) buried 30 cm into the ground. Sixty pens were arranged in a 10 by 6 grid (8 x 5.5-meter grid). Pens were filled with soil to a depth of 20 cm, 5 cm depth of leaf litter (~600 grams), and a terrestrial burrow at least five days before they received a salamander. Pens were covered by a plastic top with a mesh lid to allow natural sunlight but prevent animal escape. Each pen received roughly 15 earthworms to promote soil aeration. The order in which pens received salamanders and treatment assignments (low versus high density) was randomized. Because metamorphosis spanned multiple weeks, we added salamanders to pens in blocks as they emerged, so that two to five salamanders per treatment (four to ten salamanders total) entered the terrestrial portion of the experiment at the same time. Salamanders were placed in pens at night near the burrow entrance. Three small *Blaptica dubia* roaches (0.5 cm – 1 cm in length) were added to pens weekly as salamander prey. From December 2014 through February 2015, salamanders were not fed to simulate winter conditions.

Terrestrial pens were surveyed both fully and partially to monitor growth (Figure 2.2). Full surveys consisted of removing all soil from the pens and sifting through the dirt until we found the salamander. If the salamander was not found, we sifted through the dirt for at least 30 minutes. After being fully surveyed, pens were refilled with their respective dirt and the burrow and leaf litter were replaced. Partial surveyed consisted of checking the burrows, surface, and leaf litter twice with flashlights and a plumbing camera endoscope. During partial surveys, if a salamander

was detected, it was removed, weighed and photographed and returned to its pen within six hours. These partial surveys allowed us to monitor survival and growth without completely disturbing the pen. We also conducted surveys to assess survival only, where pens were partially searched but the salamander not removed from the enclosure. At the end of the first experiment, five days after emptying the pens, we searched the pens again to ensure we captured all survivors and that the pens were empty for the next experiment.

The cohort monitored for growth (no replacements made) was censored from August 30, 2014 through May 14, 2015 (257 days total). The first batch of salamanders entered July 1st and were in the pens a total of 316 days. To ensure terrestrial pens were adequate for salamander growth, 12 pens (six per treatment) were randomly fully searched for salamanders on July 25th. Salamanders that were not found were replaced with salamanders from the appropriate treatment. On August 22 through 24, we fully surveyed all pens. To ensure all living salamanders had been found, we searched pens again on August 27 and 28 and no additional salamanders were found. Salamanders were weighed and added back to the experiment on August 29. Salamanders that had died were replaced with salamanders from the appropriate treatment on those two dates.

Terrestrial pens were searched in October and November 2014, January 2015, and May 2015. During the latter, all pens were emptied of their soil and fully searched for salamanders. Salamanders were weighed and photographed and euthanized using MS-222.

Experiment 2: Terrestrial resource manipulation

We conducted a second experiment in the terrestrial pens to test whether natural variation in body size would influence post-metamorphic growth and survival under different resource conditions (Figure 2.1). We utilized 53 terrestrial pens (described above). The experiment lasted from June 28, 2015 to November 22, 2015 (147 days total).

We collected 53 larval salamanders that were close in development stage and had begun metamorphosis (retraction of the gills) from a local pond at the VCU Rice Center. Salamanders were maintained in 1.89 L containers with a wet paper towel until completed gill reabsorption (three to seven days). Once metamorphosis was complete, salamanders were randomly assigned to terrestrial pens. Animals were added in two batches on June 28th and June 30th, 2015. Half of terrestrial pens were randomly assigned to high food treatments (three *B. dubia* roaches/week) and half were assigned to low food treatments (one *B. dubia* roach/week). Salamanders were fed weekly based on their assigned resource treatments. Pens were searched in August, September, October and November (Figure 2.2). Salamanders were weighed and photographed a final time before being euthanized in MS-222. As a measure of body size, salamanders from both experiments were measured digitally with ImageJ from snout to the tip of the tail (total length) using ImageJ (Schneider, Rasband & Eliceiri, 2012).

Adult growth rates and body size

To link initial size at metamorphosis to age at reproductive maturity, we surveyed the literature for minimum and maximum body size at maturity. We then used adult growth rates to project when both low and high density salamanders from our experiment would reach size at maturity. We surveyed the literature for field studies that measured male and female body sizes during reproduction. We extracted the minimum, maximum and average body sizes of reproducing adults for each study.

To project adult growth rates, we used growth rates and maximum sizes estimated by Blackwell et al. (2003), where (L_2= a-[(a)-L_1]*$e^{[(-k)]}$) , and $L_1$is SVL at time 1, and L_2 is SVL at time 2, k is the growth rate, a is the maximum body size (a_{male} = 110.7 mm, a_{female} = 131.35 mm,

k_{male} = 0.384, k_{female} = 0.087). We set L_1 to the mean values for size at metamorphosis from our density manipulation experiment.

To compare our measurements of total length with other studies, we converted total length measurement to snout-vent length (SVL), SVL= 0.41 (total length) + 0.88). We obtained this relationship from a second experiment where we repeated the low and high density treatments and measured both SVL and total length (Charbonnier et al. unpublished data).

Experiment 1 & 2: Statistical analyses

Statistical analyses were conducted in R version 3.3.1 (R Development Core Team, 2016). For the first experiment, analyses on survival to metamorphosis, time to metamorphosis, and mass at metamorphosis were conducted on tank means. Survival to metamorphosis was analyzed using a general linear model with a binomial distribution. Juvenile recruitment was defined as the number of salamanders that emerged from each tank. For the first experiment, given that we did not have information on hatching date, time to metamorphosis, rather than being an absolute measure of larval duration, is a relative measure of how long salamanders spent in the aquatic habitat.

For both experiments, mass at metamorphosis was log transformed to fit assumptions of normality. For analyses on terrestrial growth, the experimental unit was the individual salamander. We used repeated measures mixed linear models to test for the effects of size at metamorphosis and larval treatment on terrestrial growth. Size at metamorphosis and larval treatment were treated as fixed effects and individual as a random effect (Pinheiro et al., 2016). Parameters were estimated using restricted maximum likelihood. Statistical significance was set at $\alpha = 0.05$. We also calculated mass specific growth rates ($\ln mass_{time2}$ - $\ln mass_{time1}/time_2$ - $time_1$) to qualitatively compare results from both experiments. We calculated the instantaneous mortality rates by taking

the ln (number surviving/total number) for each experiment to correct for the different length of the experiments and compare between experiments.

For the first experiment, because salamanders were added in multiple batches from July through August 2014, we recorded the number of salamanders that had died by August 22, 2014 and defined this number as summer mortality. Once the final cohort had been added and no additional salamanders were added (after August 22, 2014), terrestrial survival was estimated using the Cormack-Jolly-Seber model with Program MARK 8.2 (White & Burnham, 1999) to account for imperfect detection in partial surveys. We constructed models where survival and recapture probability were constant, time dependent, and treatment dependent (experiment 1: larval density, experiment 2: resource treatment). For the second experiment, we repeated these analyses but included initial mass at metamorphosis as a covariate for survival. We assessed model fit using Akaike's Information Criterion (AIC) values. Detection probability at the end of both experiments was set to one since animals were either found alive or presumed dead because terrestrial pens were fully searched. We computed model averaged parameter estimates to obtain estimates of survival and detection (Burnham & Anderson, 2002). To estimate the proportion alive through time, we multiplied the survival probability estimates raised to their respective time interval [$(\varphi , \text{time}_{x+2}) = (\varphi \text{ time}_{x})^{\text{time}} \text{ x } (\varphi, \text{time}_{x+1})^{\text{time } x+1}$]. We included mass at metamorphosis as a covariate and re-ran the top five models to determine if mass improved the fit of the models.

Results

Experiment 1: Effects of larval density on survival and growth over the first year

Larval density manipulation

A total of 84 animals emerged from aquatic mesocosms: 52 high density animals and 32 low density animals. Two tanks did not produce any salamanders (one tank per treatment) and

were excluded from analyses. Mean survival to metamorphosis was similar across larval density treatments (low density: 48.4% ± 7.9%, high density: 41.3% ± 6.4 (mean ± SE). There were no differences in survival to metamorphosis among larval treatments (χ^2= 0.91, P = 0.34). Larval treatment did not influence time to metamorphosis ($F_{1,16}$ = 0.76, P = 0.09, average = 56.5 days ± 9.3 days (SD) but did impact size at metamorphosis ($F_{1,\ 16}$ = 12.7, P = 0.003). Low density salamanders were on average 61% heavier (0.67 grams) than salamanders from high density treatments.

Post-metamorphic mesocosm experiment

On July 25, 2014 we randomly fully searched 12 terrestrial pens to ensure animals were surviving. We found five out of six low density salamanders alive, and two out of six high density salamanders alive. On August 29, we determined that 4 salamanders (1 low density tank, 3 high density tanks) had died. In total, ten animals — eight high density animals and two low density animals — died before August 24 (73% and 93% survival respectively) and were replaced on August 29.

Across salamanders in both larval density treatments, mass increased by 0.01 g per week (0.003 log10 g ± 0.0006 (SE), P < 0.0001). Low density salamanders maintained their size advantage and were 0.72 g heavier than high density salamanders (0.21 log10 g ± 0.05, P < 0.0001). Total length of salamanders increased by 0.004 ± 0.007 cm per week (P < 0.0001). Salamanders from low larval density treatments were 0.214 ± 0.041 cm longer than high density salamanders (P < 0.001).

The top - ranked model estimated that juvenile survival and detection probabilities were dependent on time. The next highest model suggested survival probability was constant and detection to be time dependent (Table 2.1). There was no evidence that larval density treatment

influenced either juvenile survival or detection probability (Table 2.2, Figure 2.3A). We found a total of 15 salamanders (nine low density, six high density) in May 2015 (25% overall survival).

Experiment 2: Terrestrial resource manipulation

The mean initial mass of salamanders was 0.625 g ± 0.12 (SD) and the mean initial total length of salamanders collected was 5.03 cm ± 0.359 (SD). The 2014 low density salamanders were 1.97 times the size of the salamanders we collected in 2015 and high density salamanders were 0.83 times larger than salamanders collected in 2015.

Mass increased by 0.03 g per week (0.013 log10 g ± 0.0006 (SE), $P < 0.0001$). In high terrestrial resources treatments, salamanders gained an additional 0.0192 g per week than low terrestrial resource treatments (0.0084 log10 g ± 0.0009 (SE), $P < 0.0001$, time x terrestrial treatment). Total length of salamanders increased by 0.016 ± 0.01cm per week ($P < 0.0001$). High terrestrial resource salamanders gained an additional 0.11 ± 0.015 cm per week than salamanders in low terrestrial resource treatments ($P < 0.001$, time x terrestrial treatment).

The top - ranked model found juvenile survival to be constant and detection probabilities to be time dependent. The next highest model found both juvenile survival and detection probability to be constant (Table 2.1). There was no evidence that terrestrial resources influenced survival or detection probability (Table 2.1, Figure 2.3B). We found a total of 15 salamanders (9 low resources, 6 high resources) in November 2015 (28.3% overall survival).

Comparing qualitatively across both experiments, mass specific growth rates were higher in the 2015 than in the 2014 experiment (Figures 2.4A and 2.4B).

Adult growth rate and body sizes

We found 13 studies from 14 populations of spotted salamanders (Supplementary Table 2.4). Across these studies, the minimum SVL ever reported for a female breeding salamander was

58 mm. The average minimum SVL and mean average SVL for females were 74.1 mm and 91.3 mm respectively. The minimum SVL ever reported for a male breeding salamander was 54 mm (Husting, 1965). The average minimum and mean average SVL for males were 66.8 mm and 82.3 mm respectively. To estimate years to reproductive maturity when projecting growth rates, we used the minimum sizes ever reported and average minimum size for both males and females.

We estimated that low density females from experiment 1 will take two to five years to reach maturity, while high density females will take three to six years to reach maturity (Figure 2.5A). Our projections indicate that because males have higher initial growth rates, high and low density male animals will reach reproductive maturity at similar times (one to two years, Figure 2.5B). These estimates are within the range observed in past studies (Windmiller, 1996; Rothermel & Semlitsch, 2006).

Discussion

Across both experiments, we did not find evidence of compensatory growth in the terrestrial stage. We also did not find evidence of a positive relationship between size at metamorphosis and pre-wintering and post-wintering survival. Higher resources in the terrestrial environment enabled salamanders to grow quickly, but this effect was independent of size at metamorphosis. Across experiments, salamanders that emerged smaller were unable to catch up to the size of their larger counterparts. Our results demonstrate how in the absence of some form of compensation, effects of the larval environment may persist beyond metamorphosis and across terrestrial environments.

In amphibians, the relationship between size at metamorphosis and survival is assumed to be positive, but this assumption is rarely tested, especially in field relevant settings (but see (Altwegg & Reyer, 2003; Earl & Semlitsch, 2013; Searcy et al., 2014; Earl & Whiteman, 2015).

If the relationship between size and survival is positive, smaller juveniles should maximize growth rates if it possible to do so, in order to reduce the negative effects of small size at metamorphosis such as decreased jumping performance and higher desiccation rate (Schmid, 1965; Spight, 1968; Spotila, 1972; Goater, Semlitsch & Bernasconi, 1993; Harper & Semlitsch, 2007; Cabrera-Guzmán et al., 2013; Charbonnier & Vonesh, 2015). In the first experiment, larval density manipulation generated large differences in size at metamorphosis consistent with previous work in amphibians (Wilbur, 1977a; Walls, 1998; Skelly & Kiesecker, 2001) and this species (Metts, Hopkins & Nestor, 2005; Davis & Maerz, 2009). However, larger emerging salamanders from low density treatments had similar chances of survival than small salamanders from high density treatments. The lack of a relationship between size and survival was also observed in the second experiment, where we sampled natural variation in size at metamorphosis.

Previous work in this species has not found consistent results on the relationship between size at metamorphosis and survival (Table 2.3). Todd et al. (2014) found a positive correlation between size at metamorphosis in spotted salamanders and survival to the first fall in a Northeast population but not in the Midwest, but did not find a correlation between size and survival to the next spring. Rothermel & Semlitsch (2006) found that salamanders that survived to the first summer were 9.3% larger at metamorphosis than salamanders who did not survive. These results showcase that the relationship between size at metamorphosis and later survival may be highly variable, even within species. Because Ambystomatid salamanders reach a large portion of their final adult size in the larval environment, they are expected to have stronger, positive correlation between size and survival than other amphibian species [e.g., Bufonids (Earl & Whiteman, 2015)]. However, within the Ambystomatids, *A. maculatum* is more likely to maximize growth rates in the terrestrial rather than larval environment (Wilbur, 1977a). Within family variation may also

contribute to differences in the size- survival relationship.

Size differences generated by larval environments persisted for nine months (experiment 1) and five months (experiment 2) following metamorphosis. Because size at metamorphosis was not positively correlated with growth rate in either experiment, smaller emerging individuals remained small and were unable to catch-up to larger individuals. Morey & Reznick (2001) found that differences in size at metamorphosis persisted six months after metamorphosis in the spadefoot toad (*Spea hammodii*), except for male toads in high terrestrial resource treatment, which compensated for their small size. In our second experiment, we found that salamanders in high resource terrestrial environments had higher growth rates then animals in low resource environments. However, this result was independent of size at metamorphosis, and salamanders that emerged small remained smaller within a given terrestrial resource treatment. This result highlights that the terrestrial environment may influence growth rates, but may not completely erase the effects of early environments. Higher resources in terrestrial environments did not improve survival to the first fall. This highlights that high terrestrial habitat quality may enable juveniles to maximize growth, but may not necessarily result in higher survival.

Recent syntheses indicate that the relationship between size and survival, and size and post-metamorphic growth are highly variable and may be species dependent (Earl & Whiteman, 2015; Tarvin et al., 2015). We did not find a positive relationship between size and survival in *A. maculatum*. Although carry-over effects of size persisted, they did not result in survival differences. Similarly, larger juveniles *A. talpoideum* were larger at first reproduction, but did not have higher chances of survival (Semlitsch & Wilbur, 1988). Low density *A. opacum* were also larger at first reproduction, and a larger proportion returned to breed, suggesting the low density animals have higher survival (Scott, 1994). Thus, while the relationship between size and survival

may vary between species, there is evidence that carry-over effects of size may persist beyond metamorphosis in multiple Ambystomatids.

Understanding when juvenile mortality occurs may be important for understanding the mechanisms that constrain survivorship, especially in highly fossorial species that cannot be easily found as juveniles. Rothermel & Semlitsch (2006) and Todd et al. (2014) found that mortality was highest in the first summer after metamorphosis (Table 2.4). Even including animals that died during the summer, our survival rates for the first fall were relatively high. Rothermel & Semlitsch (2006) suggests that high temperature and dry conditions during release may have caused initial decreases in survival. The most critical survival period in this species may be when they migrate and must find a suitable burrow and avoid desiccation (Shoop, 1974; Osbourn, Connette & Semlitsch, 2014). In our experiments, salamanders did not have to find suitable terrestrial habitat, which may have inflated summer survival. We still found relatively low survival by November in our second experiment, and only 25% survival by May in our first experiment. Thus, while summer may indeed be a highly critical period, salamanders that successfully survive the summer may still face further reductions in survival in the winter. Our experiments are one of the few outdoor terrestrial studies that quantify amphibian survival and growth after manipulating the larval environment, and thus contributes to our understanding of how the effects of early environments may persist.

Although our experiments monitored individuals five and nine months after metamorphosis, they obviously cannot capture the lifelong effects of smaller metamorphic size on survival in reproduction in a species that may live up 15+ year. However, because life history parameters are well studied for this species, we can make predictions about the potential effects of smaller metamorphic size in the absence of compensatory growth for male and female

salamanders. Once reproductive maturity is reached, adults *A. maculatum* are thought to have relatively high survival (Husting, 1965; Whitford & Vinegar, 1966). Using growth rates from the literature, we showed there may be different consequences to emerging smaller for male and female spotted salamanders. We show that male *A. maculatum* are estimated to have faster growth rates, suggesting that differences in size due to larval density may disappear before reproductive maturity. However, in females, differences in size are maintained, and low density salamanders reach reproductive maturity one year earlier. High density environments, or small size at metamorphosis, may lengthen time to reproductive maturity for females but not males, allowing low density females to breed earlier than their high density counterparts.

An additional consequence of reduced body size for females may be reduced lifelong egg production. In *A. maculatum*, the relationship between female body size and clutch size is thought to be positive (Wilbur, 1977b; Kaplan & Stanley, 1979; Glorioso, Waddle & Heffner, 2012) but see (Shoop, 1974; Woodward, 1982). Glorioso, Waddle & Heffner (2012) estimates that a one mm increase in SVL results in 3.5 additional eggs in female *A maculatum*. Assuming salamanders breed every year, low density salamander females would produce roughly 158 (8%) more eggs than high density salamanders over their lifetime. However, these results will be dependent on the minimum body size for each sex, and individual growth rates [which may be highly variable, see (Blackwell et al., 2003)], and terrestrial growth rates. Additionally, spotted salamander females frequently skip breeding opportunities, but this it is unclear whether this is related to size (Husting, 1965; Phillips & Sexton, 1989). Our results suggest that in the absence of compensatory growth, female salamanders may have reduced lifelong reproductive output.

Our results suggest that environmental stressors experienced in early life stages may have long term effects through their effect on body size if smaller individuals are unable to exhibit

compensatory growth. Even in high terrestrial resource environments, smaller individuals were unable to make up for differences in size generated by the larval environment. While reduced body size was not related to terrestrial survival in this species, smaller females may have lower reproductive output through both delayed maturation and smaller clutch size. Our work suggests that early and late life stages can be linked through phenotypic differences between individuals that persist later in life.

Table 2.1 A) Top five candidate models for experiment 1. Apparent survival (φ) and recapture probabilities (*p*) of juvenile *A. maculatum*. Number of parameters (K) and wi (Akaike weight) for each model. B) Top five candidate models set for experiment 2.

Experiment 1

Model	AICc	Δ AICc	w_i	Model Likelihood	K	Deviance
φ_{t}, p_{t}	247.7278	0	0.60769	1	7	12.3345
$\varphi_{.}, p_{t}$	250.0207	2.2929	0.1931	0.3178	4	21.1158
$\varphi_{density}, p_{t}$	251.6083	3.8805	0.08731	0.1437	5	20.5693
$\varphi_{t}, p_{t \times density}$	252.8762	5.1484	0.04631	0.0762	10	10.7277
$\varphi_{t \times density}, p_{t}$	253.5547	5.8269	0.03299	0.0543	11	9.0923

Experiment 2

Model	AICc	Δ AICc	w_i	Model Likelihood	K	Deviance
$\varphi_{t}, p_{.}$	218.2262	0	0.29258	1	5	31.3207
$\varphi_{.}, p_{.}$	219.3124	1.0862	0.16997	0.5809	2	38.9446
$\varphi_{.}, p_{t}$	220.1848	1.9586	0.10988	0.3756	4	35.5064
$\varphi_{t}, p_{resource}$	220.5025	2.2763	0.09375	0.3204	6	31.3198
$\varphi_{resource}, p_{.}$	220.506	2.2798	0.09358	0.3198	3	38.0063

Table 2.2 Estimates of apparent survival (φ) and recapture probabilities (*p*) for experiment 1 and experiment 2.

Experiment 1	High density	SE	95% CI	Low density	SE	95% CI
φ 1	0.907	0.032	0.702 - 0.976	0.909	0.032	0.724 - 0.974
φ 2	0.808	0.079	0.581 - 0.928	0.811	0.078	0.583 - 0.929
φ 3	0.720	0.078	0.455 - 0.888	0.833	0.078	0.466 - 0.900
φ 4	0.862	0.050	0.707 - 0.941	0.863	0.490	0.717 - 0.940
p 1	0.951	0.034	0.801 - 0.989	0.950	0.035	0.798 - 0.989
p 2	0.746	0.095	0.509 - 0.893	0.733	0.095	0.507 - 0.879
p 3	0.430	0.121	0.201 - 0.693	0.438	0.119	0.213 - 0.692
Experiment 2	High resources	SE	95% CI	Low resources	SE	95% CI
φ 1	0.920	0.015	0.865 - 0.953	0.915	0.016	0.865 - 0.948
φ 2	0.964	0.007	0.791 - 0.995	0.960	0.007	0.769 - 0.994
φ 3	0.963	0.020	0.753 - 0.996	0.958	0.021	0.738 - 0.995
φ 4	0.920	0.023	0.840 - 0.962	0.920	0.024	0.840 - 0.960
p 1	0.513	0.076	0.326 - 0.700	0.520	0.080	0.332 - 0.701
p 2	0.557	0.077	0.395 - 0.701	0.555	0.081	0.390 - 0.711
p 3	0.572	0.079	0.385 - 0.741	0.573	0.084	0.377 - 0.750

Table 2.3 Mean mass at metamorphosis and survival of *A. maculatum* in our experiments and Rothermel & Semlitsch (2006) and Todd et al. (2014).

Study	Mass (g)	Region	Survival fall	Survival spring
Rothermel & Semlitsch (2006)	0.844	Midwest	0.333	0.17
Todd et al. (2014)	0.52	Northeast	0.49	0.05
Todd et al. (2014)	1.08	Midwest	0.17	0.11
Experiment 1- Low density	1.8	Northeast	0.73	0.30
Experiment 1- High density	1.00	Northeast	0.53	0.20
Experiment 2	0.625	Northeast	0.39	-

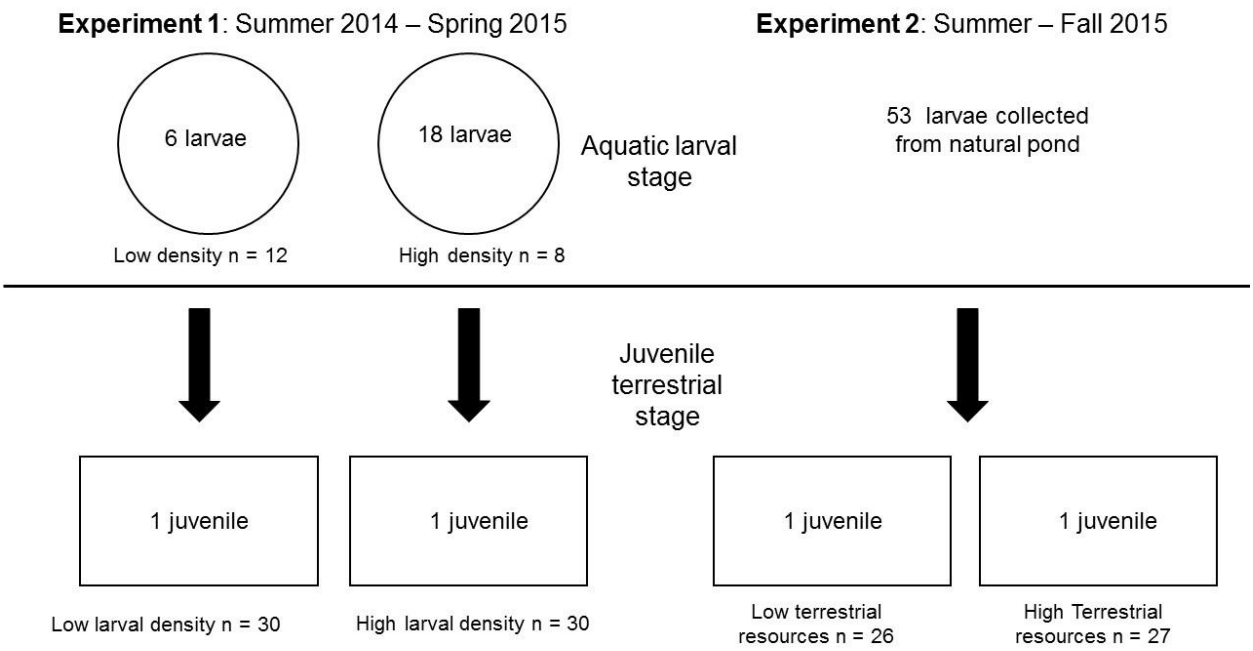

Figure 2.1 Experimental design for both experiments.

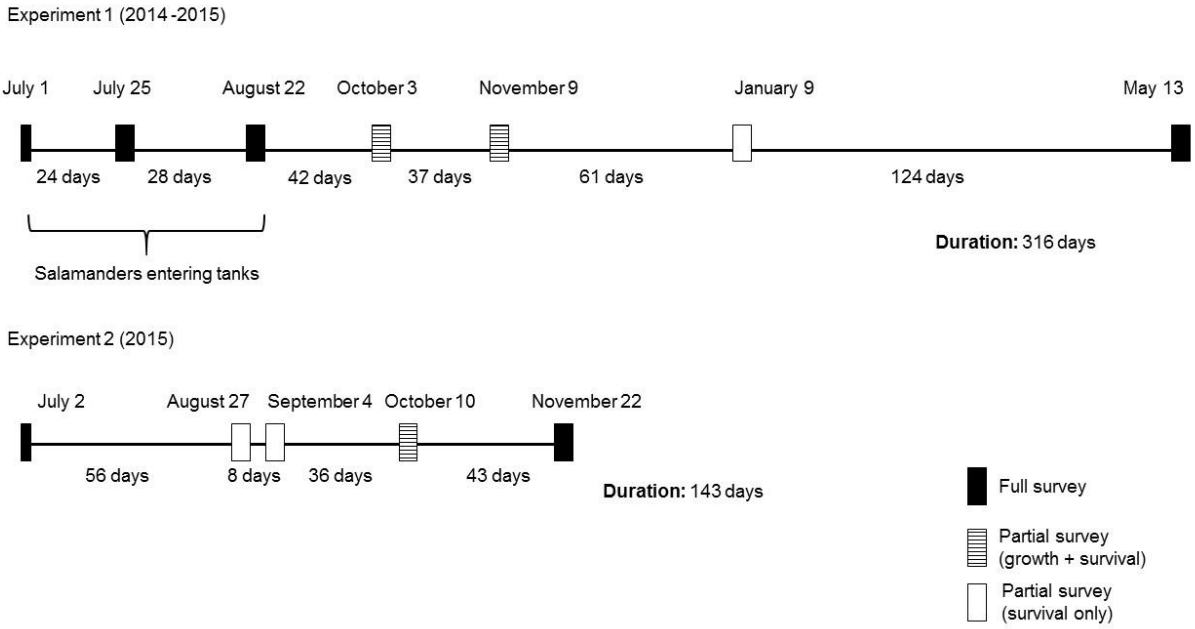

Figure 2.2 Sampling intervals and survey types for both experiments.

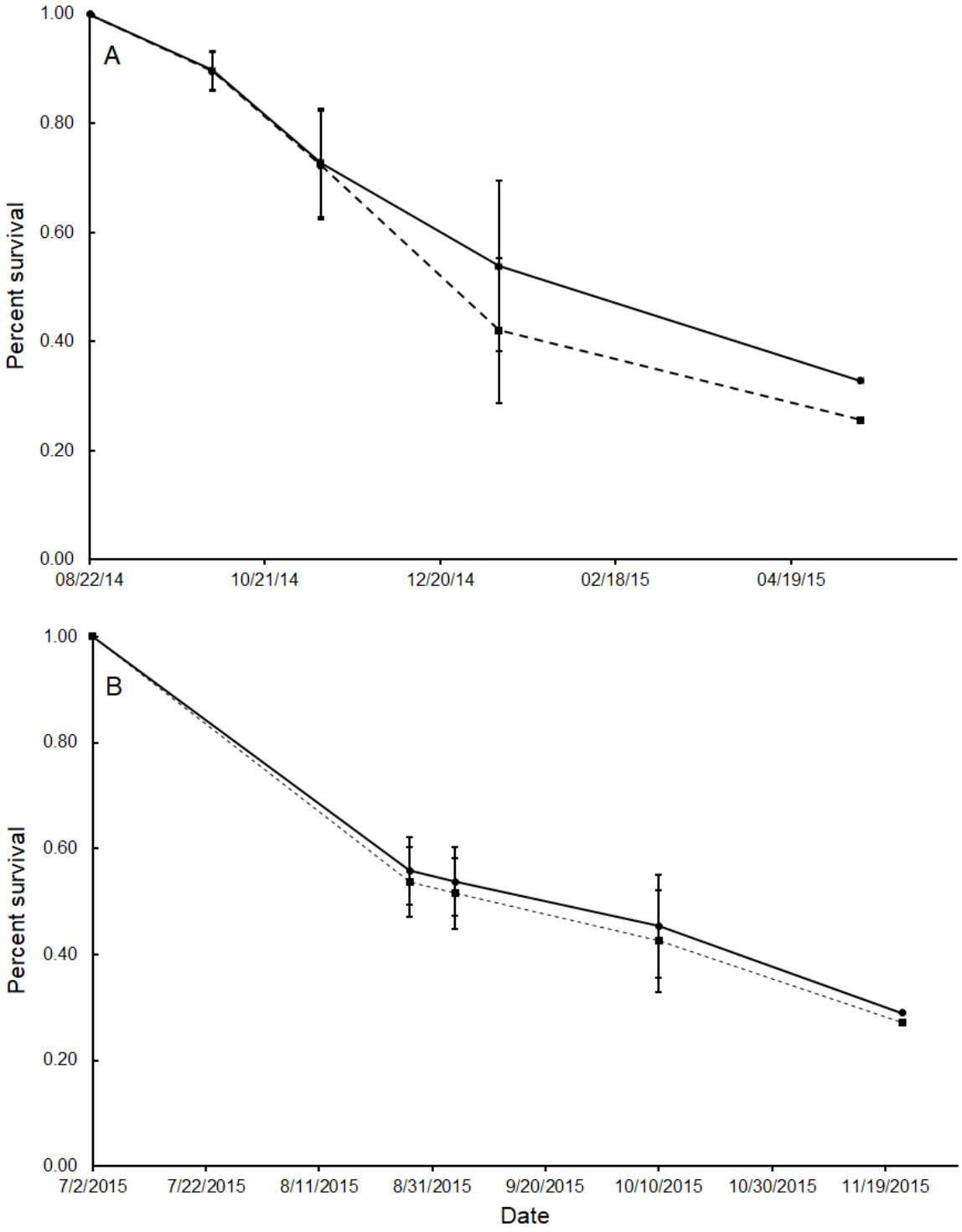

Figure 2.3 A) Cumulative estimated percent survival for experiment 1. Solid line represents survival for low density *A. maculatum*, dashed line represents survival for high density of *A. maculatum*. B) Cumulative estimated percent survival for experiment two. Solid line represents survival for *A. maculatum* in high resource treatments, dashed line represents survival for *A. maculatum* in low resource treatments. Error bars represent standard errors.

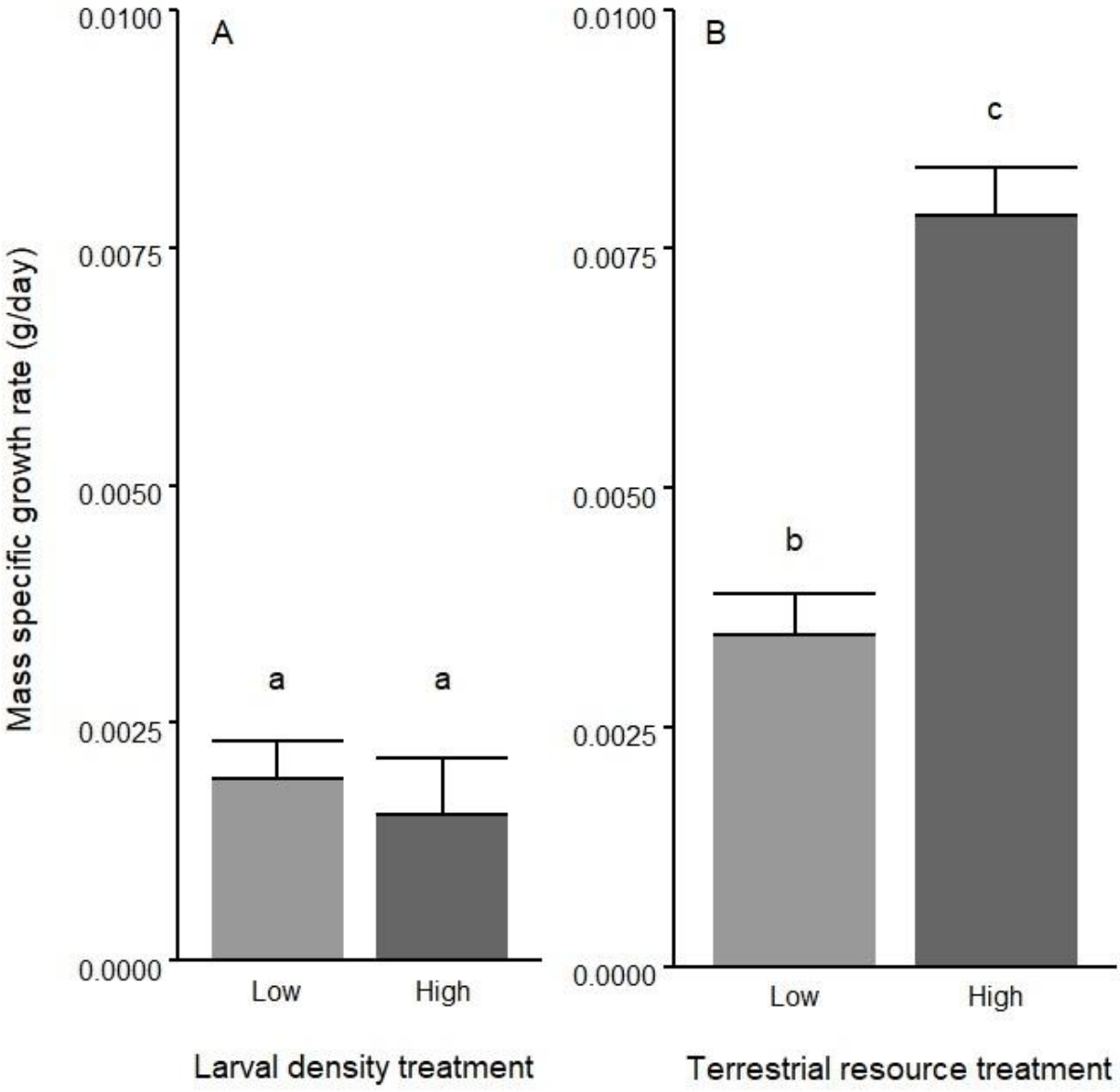

Figure 2.4 Mean mass specific growth rates of juvenile *A. maculatum* for both experiments. Error bars represent standard errors.

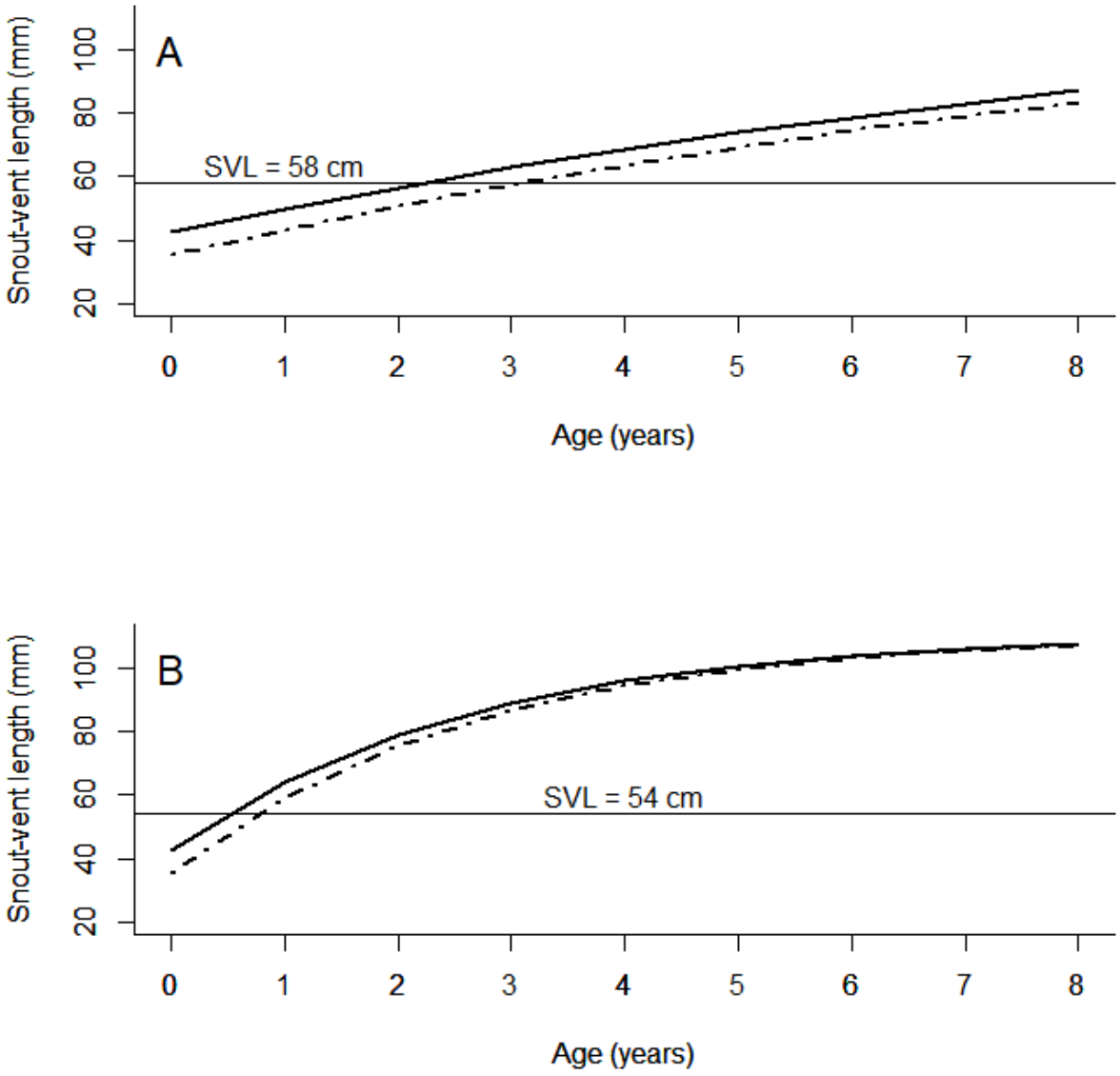

Figure 2.5 A) Age - specific snout-vent length (SVL) for low density (solid line) and high density (dashed line) female and B) male *A. maculatum* using adult growth rates from Blackwell et al. (2003).

Table 2.4 Female and male mean, minimum and maximum snout-vent length (mm) at reproduction for *A. maculatum* from the literature.

Paper	Location	Female			Male		
		Mean	Min	Max	Mean	Min	Max
Blackwell et al. (2003)	Alabama	110.7	95	125	98.1	66.5	111.8
Flageole & Leclair (1992)	Quebec	90.3	74	103	82.9	63	100
Glorioso, Waddle & Hefner (2015)	Louisiana	76.1	69	84	NA	NA	NA
Glorioso, Waddle & Hefner (2015)	Mississippi	82.9	64	96	NA	NA	NA
Hillis (1977)	Maryland	90.2	70	106	86.3	70	105
Husting (1965)	New York	84.2	58	103.2	74.3	54	99
Morgan et al. (2014)	North Carolina	99.7	NA	NA	91.5	NA	NA
Myers (2003)	West Virginia	98	84	117	70	110	91.9
Semlitsch & Anderson (2016)	Missouri	93.9	75	112	85.5	56	101
Sexton et al. (1986)	Missouri	94	74	111	76	60	89
Wacasey (1961)	Michigan	93.2	84	106	85.3	72	93
Wilson (1976)	New York	89.3	68	110	80.5	60	100
Windmiller (1996)	Massachusetts	84.1	65.3	102	74.4	56.5	95
Woodward (1982)	Connecticut	NA	82.9	109.1	NA	NA	NA

CHAPTER 3 INFLUENCE OF LARVAL DENSITY AND TERRESTRIAL SOIL MOISTURE ON GROWTH AND FOOD INTAKE OF A TERRESTRIAL SALAMANDER, AMBYSTOMA MACULATUM

Julie F. Charbonnier, Jacquelyn Pearlmutter, James R. Vonesh, and Kristine Grayson

Introduction

Organisms with complex life cycles pass through ecologically distinct stages during ontogeny and often relocate to new habitats during life history switch points (Moran, 1994). Early ecological theory predicted that life history switch points allow modularity and independence across life stages by fundamentally remodeling organisms (Ebenman, 1992; Moran, 1994). However, there is overwhelming evidence that life stages are interdependent and that stressors experienced early in life can have a lasting impact on the phenotype of organisms through carry-over effects (Mousseau & Fox, 1998; Podolsky & Moran, 2006; Pechenik, 2006; Marshall & Morgan, 2011).

While numerous studies have documented carry-over effects across taxa, fewer have addressed how these effects may interact with conditions experienced in later life stages (Pechenik, 2006; Allen & Marshall, 2013). The impacts of carry-over effects on subsequent vital rates may be dependent on the quality of later environments (Auer, Sheldon & McPeek, 2010). Environmental variation in later life stages may erase or exacerbate carry-over effects on phenotype through subsequent compensatory growth and/or differential allocation (Lindström, 1999; Madsen & Shine, 2000; Metcalfe & Monaghan, 2001). However, early life history conditions may constrain an organism's ability to exhibit such compensatory mechanisms. Organisms that have experienced stressors early in life may be more susceptible to subsequent stressors. Quantifying how conditions in early and later life stages interact to shape phenotype is critical for understanding the importance of carry-over effects in diverse environmental contexts.

Although most metazoans have complex life cycles, amphibians are ideal model organisms for studying the impacts of both early and late stressors. They are highly sensitive to environmental change and their growth and development rates are flexible, allowing them to respond to both abiotic and biotic stressors (Wilbur & Collins, 1973; Smith-Gill & Berven, 1979; Leips & Travis, 1994; Gomez-Mestre et al., 2010). One of the most well studied biotic stressors is intraspecific competition in larval amphibians (Brockelman, 1969; Wilbur, 1977a; Skelly, 2002). Size is dependent on density in most species of amphibians and is hypothesized to be positively relate to survival because smaller metamorphs may have reduced locomotive performance, reduced fat bodies and be more susceptible to desiccation (Spight, 1968; Scott et al., 2007; Cabrera-Guzmán et al., 2013; Charbonnier & Vonesh, 2015).

Smaller body size may be particularly disadvantageous in dry conditions where the risk of desiccation is higher. This is especially true for terrestrial salamanders, because they have an elongated shape resulting in a higher surface area ratio and can desiccate very quickly (Etheridge, 1990). Interestingly, field and observational studies show that activity levels and feeding success are higher in moist environments and after rainfall events and decrease during dry events (Spotila, 1972; Jørgensen, 1997). However, these studies do not account for differences in prey type or prey abundance in moist and dry environments. Additionally, a large body of literature suggests that reduced survival and growth of multiple amphibians species in clear cut forests are driven by low moisture levels, especially when suitable refugia are missing (Rothermel & Semlitsch, 2002; Semlitsch et al., 2009). However, clear cutting also influences the number of suitable refugia and prey items available. In low moisture environments, salamanders may spend more time in their refuge or burrows to avoid desiccation, thus reducing their opportunity to feed, especially for small salamanders which may desiccate more quickly (Etheridge, 1990). One hypothesis is that reduced

moisture levels, by forcing animals to stay in their burrows, leads organisms to drain their current fat reserves, reducing the energy available for potential growth. If small salamanders desiccate more quickly, and are forced to forgo feeding due to dry conditions, they may be more adversely affected by reductions in soil moisture. Thus, organismal responses to terrestrial moisture stress may be dependent on early life history stressors, specifically those which reduce initial size at metamorphosis.

Our objectives were to test how the effects of a larval stressor (higher conspecific density) would influence the response to a terrestrial stressor (reduced terrestrial moisture). Specifically, we tested how larval density and juvenile terrestrial moisture influence growth, food consumption, evaporative water loss and rehydration rates five months after metamorphosis. We hypothesized that low moisture levels would reduce growth rates across density treatments because salamanders would consume less food in dry conditions. We hypothesized that smaller salamanders from high larval density treatments would be more adversely affected by dry conditions because of their higher rates of water loss. We used the spotted salamander, *Ambystoma maculatum* a species that utilizes terrestrial burrows during the juvenile phase.

Methods

Study system and animal collection

The larval density manipulation portion of this study was conducted at Virginia Commonwealth University (VCU) Walter and Inger Rice Center for Environmental Life Sciences in Charles City County, Virginia (77°12’30.5” W, 37°19’56.32” N). Our study species, the spotted salamander (*Ambystoma maculatum*, Family: Ambystomatidae) is found throughout the East Coast of the United States and is locally abundant at our study site. Egg masses are deposited on submerged stems in clumps during the first spring rain and hatch in 40 to 60 days (Whitford & Vinegar, 1966;

Petranka, Rushlow & Hopey, 1998). Aquatic larvae develop in vernal pools for two to five months and emerge from the pond after metamorphosis (Wilbur & Collins, 1973). As juveniles and adults, salamanders are fossorial and utilize subterranean burrows (Madison, 1997; Faccio, 2003).

Larval density manipulation

Between March 2016 and July 2016 we conducted an outdoor mesocosm experiment where we manipulated larval density. Aquatic mesocosms consisted of 1136 L Rubbermaid tanks (1.7 m diameter, 0.64 m depth) filled with James River water and inoculated with 2 L of concentrated zooplankton from nearby vernal pools and 2 kg of dry, local leaf litter. Mesocosms were arranged in three rows of seven tanks at the forest edge, covered with screen mesh and filled on March 30, 2016. Ten egg mases were collect from vernal pools in the VCU Rice Center under IACUC # 10000450 on March 23, 2016. Eggs masses were kept in 10 L buckets with pond water and allowed to hatch. After hatching, larvae from all clutches were mixed and haphazardly added to tanks on April 27, 2016.

Larvae were reared in two experimental densities: low density (six larvae per tank, 0.006 larvae/L) and high density (18 larvae per tank, 0.018 larvae/L). Experimental densities were based upon field estimates of densities reported in the literature to simulate low and high density environments (0. 012 larvae/L; Boone & James, 2003; 0.012 - 0.036 larvae/L;Metts, Hopkins & Nestor, 2005) and within the range of what larvae would experience in natural field settings (0.002-.08 larvae/L; Figiel & Semlitsch, 1990). High density treatments were replicated ten times and low density treatments were replicated 20 times for a total for a total of 420 larvae distributed across 30 tanks. Density treatments and larvae were randomly assigned to aquatic tanks.

Aquatic tanks were monitored weekly and 1 L of concentrated zooplankton (30 L poured through an 80 µm plankton net for each 10 L of pond water) was added to the tanks every other

week (Metts, Hopkins & Nestor, 2005). Tanks were checked daily once we observed animals with receding gills. Larvae were collected every other day and housed in 1.89 L plastic containers with a wet paper towel and transferred to the University of Richmond laboratory. Individuals were weighed (± 0.001 g) and photographed and we recorded larval duration (date entered tank - date of metamorphosis). Salamanders from both experiments were measured digitally with ImageJ from snout to the tip of the tail (total length) using ImageJ (Schneider, Rasband & Eliceiri, 2012).

Terrestrial moisture manipulation

The post-metamorphic portion of this study took place at the University of Richmond (IACUC 16-05-001) in an ambient temperature laboratory (mean temperature = 21.46 °C ± 2.12 (SD) in small experimental terrariums. Metamorphs were held in 1.89 L containers with a wet paper towel and fed three *Blaptica dubia* roaches once a week until the beginning of the experiment. Containers were cleaned three times per week. The interim period took place until August 9, 2016, until we had sufficient number of metamorphs from both density treatments to begin the experiment. All metamorphs were fed at least once before entering the experiment.

Experimental terrariums consisted of 5.68 L rectangular Sterilite® clear boxes (35.9 cm x 20 cm x 12.4 cm) with a 26 cm x 10 cm top cut out and covered with mesh for air flow. Organic top soil (Black Kow, pH = 7) was dried for 48 hours at 100°C and 2.3 kg of dried dirt (~ 5.7 cm deep) was added to the container. On one end of the container, we created a tubular burrow (7.62 cm long). To prevent the burrow from collapsing, we constructed a tubular mesh support (7.6 x 2.3 cm mesh). Terrariums also contained a plastic feeding dish (44 ml) in which we added *B. dubia* roaches (1 – 3 mm in size) as prey. *B. dubia* roaches were raised in the laboratory and fed JK's Dubia Diet.

To determine the effects of larval density and terrestrial moisture on salamander growth,

we conducted a 2 x 2 factorial experiment where we assigned high and low density metamorphs to two terrestrial moisture levels replicated 16 times for total of 64 containers. Metamorphs were randomly assigned to treatments on August 9, 2016. Water was added every three days (40 ml for low moisture treatments and 100 ml for high moisture treatments) throughout the tanks but concentrated on the burrow. In the high moisture treatment, 100 ml allowed the soil to be saturated without standing water. In the low moisture treatments, 40 ml represented moderate desiccation stress. Moisture manipulation began on August 12, 2016. The experiment took place for a total of 16 weeks (131 days) from August 9 through November 30, 2016. Salamanders were fed weekly (three *B. dubia* roaches until October 10 and six roaches thereafter. Roaches were placed in the plastic container and could not escape. Because the small container was placed at the opposite side of the burrow, salamanders had to leave the burrow to feed. We recorded the number of uneaten roaches weekly. Volumetric water content (VWC) (the ratio of volume of water in a given volume of soil to the total soil volume) was measured weekly using TDR 100 Fieldscout ® Soil Moisture Meter. Laboratory conditions were a 12:12-h photoperiod.

Dehydration and rehydration trials

To test how larval density and terrestrial moisture influenced salamander rates of water loss and water gain, we conducted dehydration and rehydration trials at the end of the experiment. Trials were performed in a Percival® model I22 VL. Before starting the experiment, we hydrated salamanders for 2 h by placing them inside plastic petri dishes with 2 cm of RO water. Salamanders were patted dry with paper towels, and their hydrated mass was recorded (± 0.001 g). Salamanders were added to the chamber in 4 batches (16 individuals per trial, 4 individuals per treatment) spread over two shelves within the chamber. Rehydration trials began immediately after the dehydration trials. For rehydration trials, we placed salamander in a petri dished filled with 2 cm of RO water.

After 20 minutes, the salamander was removed, blotted dried, and reweighed (Winters & Gifford 2013).

Statistical analyses

Statistical analyses were conducted in R version 3.3.1 (R Development Core Team, 2016). Analyses on survival to metamorphosis, time to metamorphosis, and mass at metamorphosis were conducted on tank means. Survival to metamorphosis was analyzed using a general linear model with a binomial distribution. Mass specific growth rate was calculated as [ln $mass_{week16}$ – ln $mass_{week1}$] /113 days and length specific growth rate [ln total $length_{week16}$ – ln total $length_{week1}$] /113 days (Sinervo & Adolph, 1989; Crespi & Warne, 2013). Percent roaches consumed was calculated as number of roaches eaten/ total roaches. Number of roaches consumed was analyzed using a general linear model with a binomial distribution with initial mass, moisture treatment and density treatment as explanatory variables. We also tested how growth rates (mass and length specific) and number of roaches eaten were related using linear regression. We first analyzed whether larval density, terrestrial moisture and their interaction influenced mass and length specific growth rates using a two-way ANOVA. We then reran the analysis with size at metamorphosis as a covariate and larval density and terrestrial moisture as factors and all possible interactions.

Dehydration and rehydration rates were estimated using the equation: EWL (evaporative water loss) = $(M_1–M_2)/[RSA \times (T_2-T_1)]$ where M_1, M_2, T_1, T_2, represent fully hydrated mass (g), final body mass, and stop and end time (Liu & Hou, 2012). RSA represents the respiratory surface area (cm^2) and was estimated using (Whitford & Hutchison, 1967) for *A. maculatum*, where $RSA = 8.34(M)^{0.684}$. We also estimated the rehydration rate using the same equation but where M_1 represented desiccated mass and M_2 rehydrated mass. We analyzed dehydration and rehydration rates using a two-way analysis of covariance with moisture treatment, density treatment and initial

mass as fixed factors and trial as a random factor. Because we had multiple measurements per box, soil moisture was analyzed using a repeated measures mixed linear model with moisture treatment and density treatment as fixed factors and individual box as a random factor. This analysis was used using the nlme statistical package and parameters estimated using the restricted maximum likelihood (Pinheiro et al., 2017). Statistical significance was assessed at the $\alpha = 0.05$.

Results

Aquatic tank survival and size at metamorphosis

A total of 96 metamorphs emerged: 42 high density animals and 54 low density animals. Three low density tanks did not produce any salamanders and were excluded from analyses. Mean survival to metamorphosis was twice as high in low density treatments [low density: 52.9% ± 4.8% (mean ± SE), high density: 23.3% ± 4.3; $\chi^2 = 25.08$, $P < 0.0001$]. Juvenile recruitment did not differ between larval treatments ($\chi^2 = 1.82$, $P = 0.18$) with 3.2 ± 0.29 (SE) salamanders from low density tanks and 4.2 ± 0.77 salamanders emerging from high density tanks.

Larval density treatment did not influence time to metamorphosis [low density = 89.5 days ± 1.29 (SE), high density= 93.2 days ± 1.09 (SE), $F_{1,25} = 3.83$, $P = 0.06$], however animals from the low density treatment were on average 30% heavier (low density = 1.48 ± 0.06, high density = 1.12 ± 0.03, $F_{1, 25} = 9.16$, $P = 0.006$) than salamanders from high density treatments.

Moisture manipulation in terrariums

Across the whole experiment, on average high moisture treatments had 56.2% VWC ± 4.63 (SD) and low moisture treatments had 26.5% ± 6.87 (SD). On average, VWC was 19.7% ± 1.58 (SE) lower in low moisture treatment (t-value = - 20.4, $P < 0.001$). Moisture decreased by 0.37% ± 0.06 (SE) per week (t-value = 6.09, $P < 0.0001$) and by 1.45% ± 0.09 (SE) per week in low moisture treatments (t-value = -16.9, $P < 0.0001$).

Juvenile survival and growth in terrariums

One salamander died before the end of the experiment (high density, low moisture). Two additional salamanders survived to November 30, 2016 but did not exhibit a righting response and were not included in the dehydration and rehydration trials.

From the subset of salamanders randomly chosen for the experiment (64 total), at the beginning of the experiment, salamanders from low density tanks had on average 9% (0.575 cm) longer total length ($F_{1,62}$ = 17.4, P < 0.0001) and were on average 31% heavier than high density salamanders ($F_{1,62}$ = 24.8, P < 0.0001). Terrestrial moisture influenced mass specific growth rate over sixteen weeks ($F_{1,57}$ = 11.2, P = 0.0015, Figure 3.1), but larval density ($F_{1,57}$ = 1.65, P = 0.20) and the interaction between moisture and density treatment ($F_{1,57}$ = 0.71, P = 0.40) had no effect. When initial mass at metamorphosis was incorporated, mass specific growth rates were dependent on moisture treatment ($F_{1,56}$ = 11.7, P = 0.001) and initial mass ($F_{1,56}$ = 4.51, P = 0.04, Figure 1b). High moisture salamanders across both larval density treatments gained mass twice as fast as salamanders from low density treatments (Figure 3.1), a one-unit increase in ln initial mass resulted in a 0.0035 ± 0.0016 decrease in mass specific growth rate (t-value = -2.277, P = 0.027, Figure 3.2). Total length specific growth rate was not influenced by density treatment ($F_{1,57}$ = 3.0, P = 0.10), moisture treatment ($F_{1,57}$ = 2.5, P = 0.12), or their interaction ($F_{1,57}$ = 1.32, P = 0.26). When we included initial length, length specific growth rates over sixteen weeks was only dependent on initial total length ($F_{1,56}$ = 10.1, P = 0.002). A one-unit increase in ln total length resulted in a 0.003 ± 0.009 decrease in length specific growth rate (t- value = -3.185, P = 0.002, Figure 3.3). Terrestrial moisture treatment influenced the percentage of roaches eaten (χ^2= 246.0, P < 0.0001), but density treatment (χ^2= 0.08, P = 0.78) and the interaction between moisture and density had no effect (χ^2= 1.1, P = 0.30, Figure 3.4). When initial body mass was incorporated as a covariate, salamanders

from high moisture treatments consumed a greater percentage of roaches (χ^2= 220.0, P < 0.0001). There was also a moisture treatment by initial size interaction (χ^2= 18.2, P < 0.0001), a density treatment by initial size interaction (χ^2= 5.8, P < 0.02) and a three-way interaction of moisture, density and initial size (χ^2= 36.6, P < 0.0001). The total number of roaches eaten was a positive predictor of mass specific growth rate (R^2 = 0.80, $F_{1,60}$ = 244.1, P < 0.0001) and length specific growth rate (R^2 = 0.35, $F_{1,60}$ = 31.1, P < 0.0001).

Dehydration and rehydration trials

The blocking variable did not improve the fit of this analysis and was not retained. Our estimate of evaporative water loss was not influenced by mass, moisture treatment, or density treatment (Table 3.1, Figure 3.5A). For rehydration rates, two animals were excluded because they were statistical outliers as defined by Hoaglin & Welsch (1978) and Fox (1997).

On average, salamanders from high moisture had 30.3% higher rehydration rate. Salamanders which experienced higher EWL regained a greater percentage of mass (slope= 0.50 ± 0.18 (SE), t-value = 2.85, P = 0.006, Figure 3.5B, Table 3.2).

Discussion

Our results suggest that low moisture may limit terrestrial growth in the spotted salamander primarily by reducing food intake. Although we did not find that larval density influenced juvenile growth, size at metamorphosis (which was largely determined by larval density) was negatively correlated with terrestrial growth. Smaller salamanders exhibited compensatory growth, allowing them to gain more mass for their size across terrestrial environments.

In amphibians, larger body size may be positively related to survival and reproductive output (Wells, 2007; Earl & Whiteman, 2015). Early life history stressors that influence body size can potentially influence lifetime fitness if these size differences persist. In our experiment, we

found that high conspecific density resulted in smaller size at metamorphosis, consistent with previous studies across amphibians and this species (Skelly & Kiesecker, 2001; Metts, Hopkins & Nestor, 2005). Five months after metamorphosis, smaller individuals had gained a greater percentage of their body mass, allowing them to partly make-up for their size disadvantage. Such compensatory growth has been demonstrated in other species, and suggests that under certain conditions, carry-over effects of early environments may be partially mitigated (Goater, 1994; Boone, 2005).

Terrestrial growth was also dependent on the terrestrial environment. Animals in low moisture treatments ate fewer roaches, which resulted in lower mass specific growth rates. Compensatory growth is often a result of higher foraging or food intake (or early onset of feeding) (Morey & Reznick, 2001; Benard & Middlemis Maher, 2011), thus dry environments, by limiting food intake, may have inhibited compensatory growth. This work is consistent with past field studies that suggests reduced foraging rates during periods of low moisture and in low moisture environments in *Desmognathus ochrophaeus* (Feder & Londos, 1984). Although we did not directly measure foraging in our experiment, salamanders had to leave the burrow to reach the roaches, suggesting that salamanders in low moisture environment spent less time foraging. Additionally, Rohr & Palmer (2013) found decreased foraging rate in dry moisture treatments in *Amybystoma barbouri* during a one week laboratory experiment. However, it is possible that salamanders in dry treatments spent an equal time out of the burrow foraging, but chose not to feed, although we believe this is unlikely. Gomez-Mestre & Tejedo (2005) found that *Bufo calamita* in dry environments were also less efficient at catching prey. The prey in our experiment was confined to a small container, so we assume that salamanders in both low and high moisture treatments were equally efficient at catching the prey.

We did not find differences in evaporative water loss, suggesting that small and large salamanders lost water at a similar rate. This is contrary to what we predicted, as smaller salamanders are expected to have higher rates of water loss per unit area (Spight, 1968). It is probable that our water loss trial did not capture small differences in water loss between salamanders. Although the relationship between dehydration and rehydration has been predicted to be reciprocal (Spotila, 1972), we found that salamanders from the low moisture treatment had slightly higher rates of water uptake. Because of their higher osmotic concentration, desiccated animals should rehydrate more quickly. Indeed, we found that salamanders that experienced higher evaporative water loss gained water more quickly. It is possible that animals from low moisture treatments, which were chronically dry, reabsorbed water more quickly. If water reuptake is maximized, then salamanders in dry environments should be able to forage for longer periods of time (Winters & Gifford, 2013).

Our results suggests that the persistence of carry-over effects may be context dependent (Podolsky & Moran, 2006). In high moisture environments, small salamanders may be more likely to catch up to larger individuals. In low moisture environments, although small salamanders had higher growth rates than large salamanders, depressed growth rates suggest small salamanders will take longer to make up for initial size differences. In dry environments, carry-over effects on size would be predicted to persist for a longer period of time than in moist environments.

Although early life stages may influence phenotype, whether these effects persist beyond life history switch point is unclear. Persistence of carry-over effects on phenotype will depend on whether individuals can exhibit compensatory growth in diverse environments. In our experiment, we show that low moisture levels may depress growth rates, inhibiting the ability of smaller individuals to make up for initial size differences.

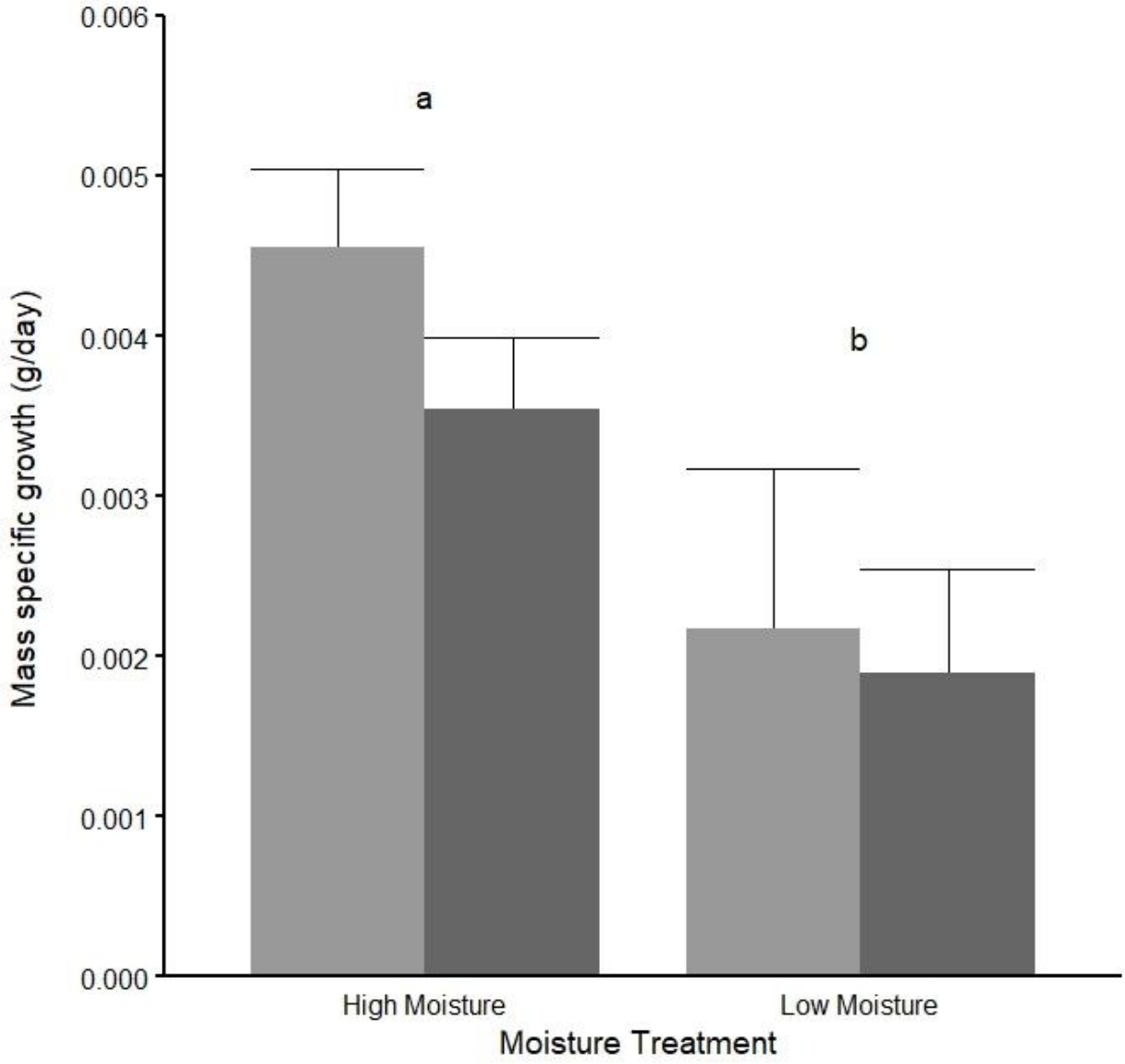

Figure 3.1 Mean mass specific growth rates (g/day) of juvenile *A.maculatum* across high and low moisture treatments and high larval density (light bars) and low larval density (dark bars). Errors bars represent standard errors.

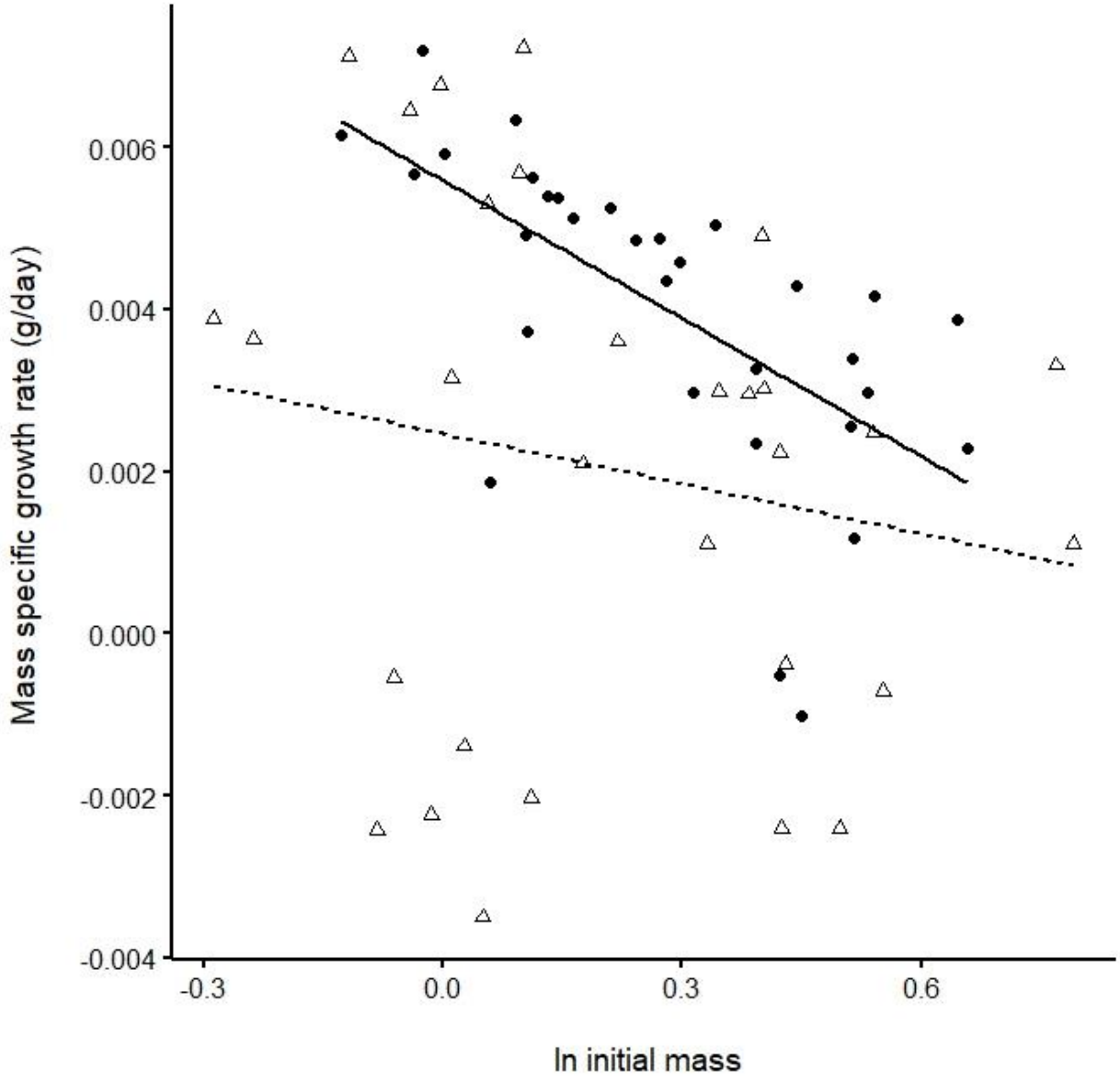

Figure 3.2 Mean mass specific growth rate (g/day) of *A. maculatum* as a function of ln mass at metamorphosis in high moisture (— solid line, •) and low moisture (---- dashed line, Δ).

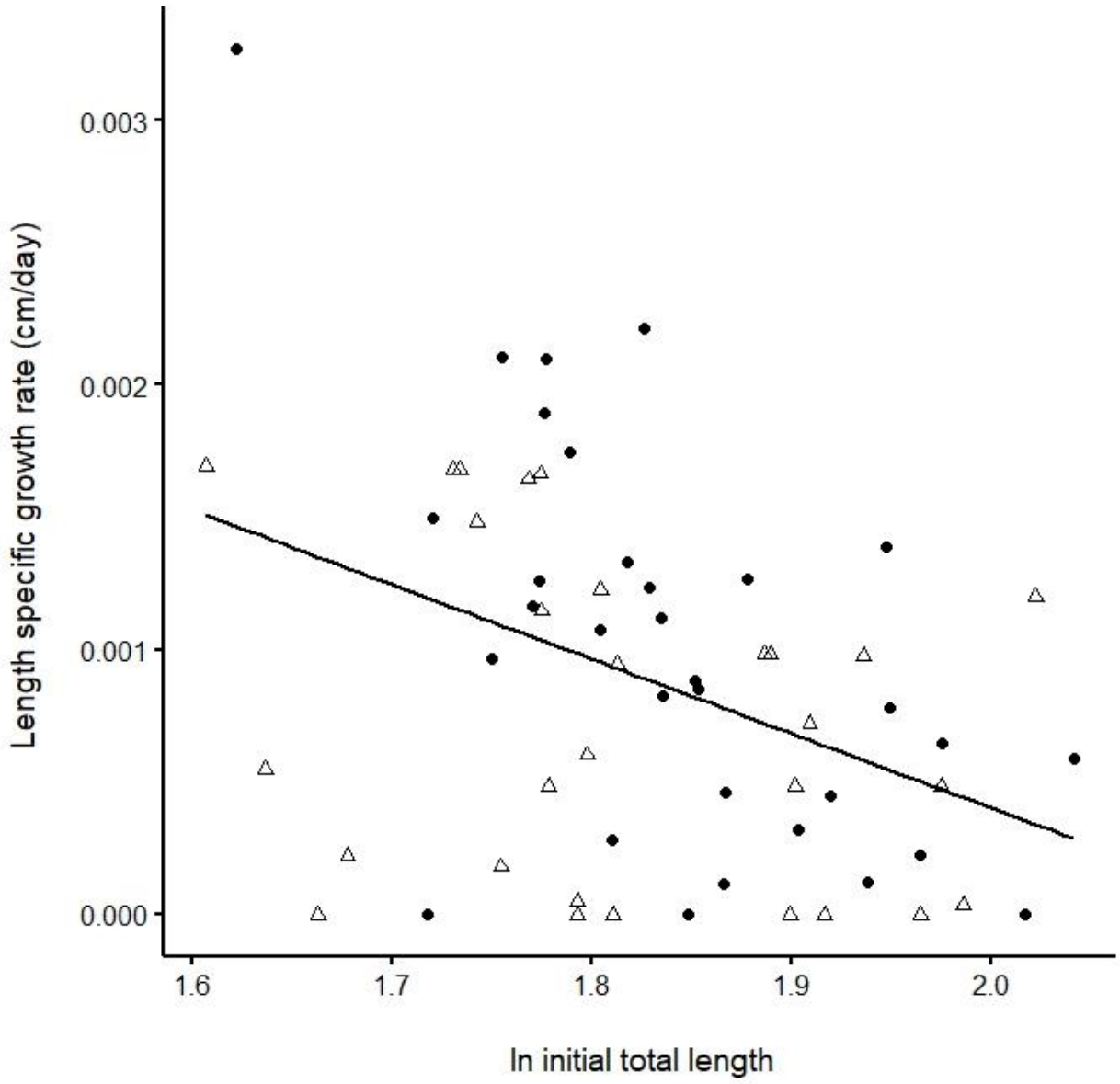

Figure 3.3 Relationship between length specific growth rates (cm/day) of *A. maculatum* and ln initial length across both treatments (— solid line). High moisture treatments (•) and low moisture (Δ).

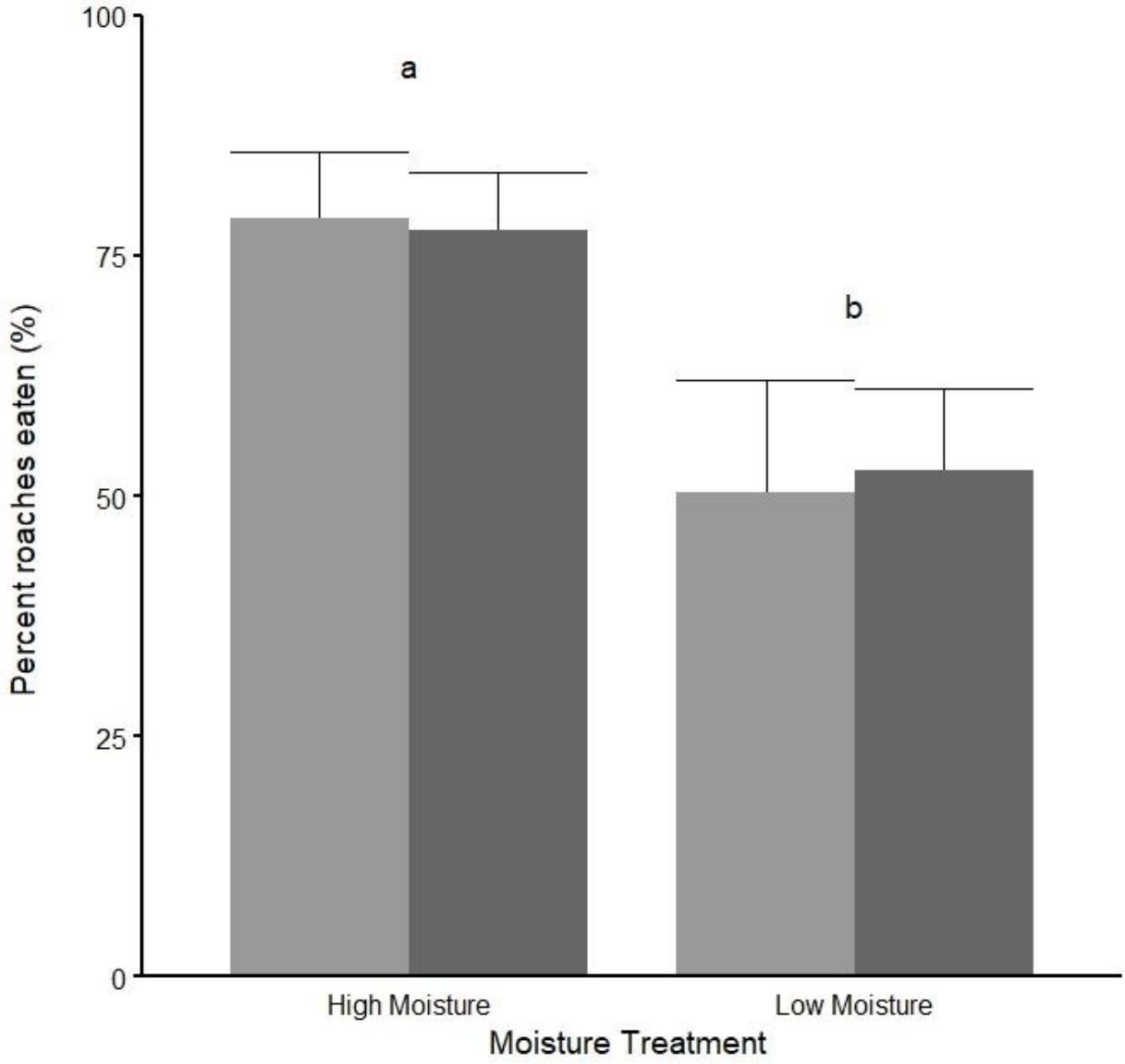

Figure 3.4 Mean percent roaches eaten by juvenile of *A. maculatum* across high and low moisture treatments and high larval density (light bars) and low density (dark bars). Errors bars represent standard errors.

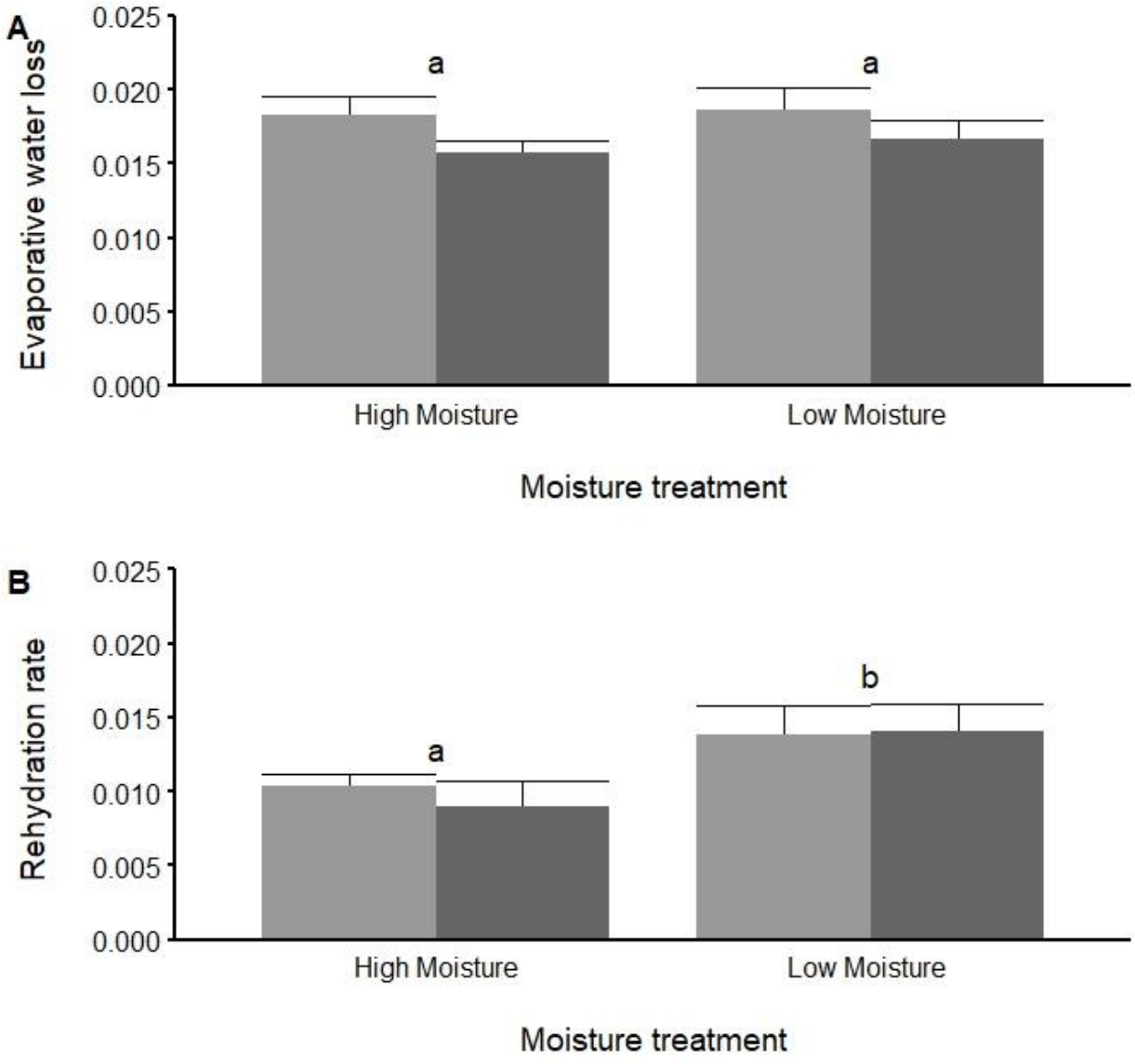

Figure 3.5 A) Evaporative water loss (g/cm^2h) and B) rehydration rate (g/cm^2h) of *A. maculatum* across high and low moisture treatments and high larval density (light bars) and low larval density (dark bars). Errors bars represent standard errors.

Table 3.1 Results from ANCOVA to examine the effects of terrestrial moisture (high, low), and larval density (high and low) and initial mass on evaporative water loss rate of *A. maculatum*.

Factor	df	F	P
Mass	1	0.55	0.46
Moisture treatment	1	0.001	0.97
Density treatment	1	0.78	0.38
Mass x moisture treatment	1	0.04	0.84
Mass x density treatment	1	0.58	0.45
Moisture treatment x density treatment	1	0.0025	0.63
Mass x moisture treatment x density treatment	1	0.05	0.83

Table 3.2 Results from ANCOVA to examine the effects of terrestrial moisture (high, low), larval density (high and low), initial mass, and evaporative water loss on rates of water reuptake in *A. maculatum*. Statistically significant results are shown in bold.

Factor	df	F	P
Mass	**1**	**4.3**	**0.04**
Moisture treatment	**1**	**6.94**	**0.02**
Density treatment	1	0.0003	0.99
EWL	**1**	**8.79**	**0.005**
Mass x moisture treatment	1	2.35	0.13
Mass x density treatment	1	1.35	0.25
Moisture treatment x density treatment	1	0.05	0.82
Mass x EWL	1	1.42	0.24
Moisture treatment x EWL	1	0.5	0.48
Density treatment x EWL	1	3.28	0.08
Mass x moisture treatment x density treatment	1	0.69	0.41
Mass x moisture treatment x EWL	1	0.79	0.38
Mass x density treatment x EWL	**1**	**7.59**	**0.009**
Moisture treatment x density treatment x EWL	1	1.31	0.26
Mass x moisture treatment x density treatment	1	0.95	0.33

CHAPTER 4 POND DESICCATION AND POPULATION DYNAMICS OF PELOBATES CULTRIPES IN DOÑANA NATIONAL PARK – CONTRIBUTIONS OF EARLY MORTALITY AND DEVELOPMENTAL PLASTICITY

Julie F. Charbonnier, Derek Johnson, Carmen Díaz-Paniagua, Ivan Gomez-Mestre, James R. Vonesh

Introduction

Quantifying the influence of biotic and abiotic stressors on organisms and their populations is a central objective in ecology. In animals with complex life cycles, stressors experienced early in life can influence the survival and phenotype of organisms across life stages (Beckerman et al., 2002; Pechenik, 2006; Touchon et al., 2013). Because of global change, organisms may experience novel or magnified biotic and abiotic stressors across life stages resulting in carry-over effects on survival and phenotype. Understanding how these carry-over effects scale up to influence population growth is crucial for management and conservation.

Stressors experienced in previous life stages may scale up to influence population dynamics through two main pathways (McPeek & Peckarsky, 1998). The first is direct changes to the number of individuals that are recruited from one life stage to the next. The second is changes to the quality or condition of individuals: past environmental stressors may induce changes in maturation, stage specific survival, or fecundity of individuals (Wilson & Nussey, 2009). To capture the full effects of past environmental stressors on population growth rates, demographic models must incorporate and account for shifts in both the number and quality of individuals.

Past environmental stressors may influence the number of individuals that successfully transition to the next life stage in complex ways because of density dependence. In the simplest form, in a stage structured population, the number of juveniles can be proportionally related to the number of larvae. However, survival across life stages is often non-linear and a function of population density. The shape of density dependence can mitigate the effects of earlier

environmental stressors (Grant, 1998; Moe, Stenseth & Smith, 2002; Goodwin et al., 2006). In compensatory density dependence, recruitment in later stages asymptotes as early stage density increases. When density dependence is over-compensatory, recruitment of later stages is a hump-shaped function of early stage density, with highest recruitment at intermediate early stage densities. These non-linear relationships can result in counterintuitive population effects: high survival in early stages may not necessarily result in a higher number of recruits in the next life stage (Vonesh & De la Cruz, 2002). Several studies have experimentally demonstrated that larval density dependence serves as a buffer to early environmental stress (Moe, Stenseth & Smith, 2002). Failure to consider density dependence may lead to inaccurate predictions on the demographic impacts of early life stressors (Forbes et al., 2010).

Carry-over effects on phenotype may influence population dynamics. For instance, smaller individuals may take longer to reach sexual maturity or have lower stage specific survival. Such carry-over effects on phenotype may interact with density dependence to unexpectedly influence population growth. For example, Willson et al. (2012) found that delayed maturity due to larval mercury exposure had a positive effect on population growth in American toads, as it reduced the number of eggs produced each year, reducing competition in the larval stage. Conversely, Taylor & Scott (2006) suggest that a three year delay in maturation due to high larval densities has negative effects on the demography of the marbled salamander. Demographic consequences of carry-over effects on fecundity and maturity may be dependent on the population structure and the density-dependent relationships between early life stages. Ultimately, both phenotypic effects and survival effects of early life history stressors must be combined while accounting for density dependence to understand the population-level consequences of environmental change (McPeek & Peckarsky, 1998).

Amphibians are particularly vulnerable to global change because of their dependence on aquatic and terrestrial habitats and permeable skin (Carey & Alexander, 2003; Blaustein et al., 2010). While global change will likely alter multiple aspects of amphibian environments (Blaustein et al., 2010), changes in water availability are thought to be the most important because many amphibians rely on aquatic habitats to complete their life cycle. Changes in temperature, precipitation, evapotranspiration, and ground water pumping may contribute to shortening of pond inundation (Araújo, Thuiller & Pearson, 2006). As ponds dry, many larval amphibians have the capacity to accelerate development to some extent to escape desiccation. This response typically results in early emergence but smaller metamorphic size (Kulkarni et al., 2011; Gomez-Mestre, Kulkarni & Buchholz, 2013). However, these plastic responses may be insufficient to prevent mortality if ponds desiccate faster than tadpoles can respond (Amburgey, Murphy & Funk, 2016). Furthermore, for amphibian species that utilize ponds for embryonic and larval development, shallower, warmer and faster drying ponds may increase egg and larval mortality (Kiesecker, Blaustein & Belden, 2001; Blaustein et al., 2010). While the physiological mechanisms and phenotypic consequences of pond drying have been well studied (Richter-Boix, Llorente & Montori, 2007; Denver, 2009), the potential population level consequences have not been addressed.

We sought to explore how pond drying may influence population dynamics in the Western spadefoot toad, *Pelobates cultripes* in Doñana National Park. Although Doñana National Park has protected status (Natural World Heritage site), groundwater abstraction from surrounding villages has further reduced the low water table and changed the chemical composition of soils (Serrano et al., 2016). Additionally, climate models predict increasingly dry conditions in this region due to climate change (Araújo, Thuiller & Pearson, 2006). *P. cultripes* may be particularly susceptible to

changes in hydroperiod because it breeds early in the season and has a long larval period. Serrano et al. (2016) report high levels of egg mortality in drying ponds, as pond drying may alter the capacity of soil to buffer water pH when the ponds flood after summer desiccation. *P. cultripes* tadpoles can respond to pond drying by accelerating development and emerging at smaller metamorphic size (Chapter 1). However, rapidly drying environments are often stressful and may result in higher mortality in dry-down ponds. In a laboratory experiment, Gomez-Mestre, Kulkarni & Buchholz (2013) found reduced survival to metamorphosis in tadpoles raised in low water volume treatments compared to high water volume treatments. Thus, *P. cultripes* may be particularly vulnerable to pond drying because of potential reductions in egg and tadpole survival and delayed maturity due to smaller metamorphic size. However, to our knowledge these sequential reductions in egg and tadpole survival and reduced size at metamorphosis have not been addressed in a demographic framework.

We explore how sequential reductions in egg, larval survival and delayed maturity due to pond drying may influence *P. cultripes* population dynamics. Because larval density dependent can have important effects on population size, we quantified the relationship between larval density and survival in both constant water level and drying ponds. Additionally, because pond drying and larval density can reduce metamorphic size, we used published adult growth rates to link size at metamorphosis to age at maturity. We predicted that toads emerging from drying ponds would take longer to reach reproductive maturity than toads emerging from constant water level ponds.

We incorporated the relationships between metamorphic survival and phenotype and larval density and pond hydroperiod in a stage-structured matrix model. With this model, we tested how reductions in embryonic and larval survival and delayed maturity due to pond dry-down would

independently influence population densities. Because pond drying is likely to influence these parameters simultaneously, we also tested how concurrent reductions in embryonic and larval survival and delayed maturity would influence population densities.

Methods

Modeling spadefoot toad population dynamics

We constructed a discrete-time, stage-structured, matrix population projection model (Caswell, 2001) of female toads based on a general amphibian population model developed by Lampo & Deleo (1998) and Vonesh & De la Cruz (2002) (Figure 1). Each year spadefoot toads mate (sex ratio: ρ) and lay eggs (clutch size: ϕ). Eggs survive to become tadpoles at probability σ_t, which may depend on pond hydroperiod. Tadpole survival is density dependent (Equation 1) and is a function of maximal tadpole survival in the absence of larval competition (σ_{tmax}), the strength of larval stage density dependence (γ; see *Estimating the density dependent exponent and maximum tadpole survival*), d is a scaling parameter (Vonesh & De la Cruz, 2002) and T is the number of tadpoles. Thus, tadpole survival is defined as

$$\sigma_t = \frac{\sigma_{tmax}}{(1+dT)^{\gamma}} \qquad \text{Equation 1}$$

New metamorphs survive to the following spring at probability σ_m. Tadpole and new metamorph survival all occurs within one time step of the model and is captured in Equation 2, where F(A) is defined as

$$F(A) = \rho\phi\sigma_e \frac{\sigma_{tmax}}{(1+d\rho\phi\sigma_e A)^{\gamma}} \sigma_m \qquad \text{Equation 2}$$

Surviving metamorphs recruit into two juvenile stages, J_1 and J_2. The proportion of metamorphs entering each juvenile stage is determined by size at metamorphosis, which is dependent on pond hydroperiod and larval density dependence (*Modeling ω as function of density and hydroperiod*). Larger metamorphs may recruit directly into the second juvenile stage (J_2) at a rate of ω which survive (σ_{j2}) to become adults in year 3, while smaller metamorphs recruit into the first (J_1) stage at rate (1-ω) and must survive (σ_{j1}) and mature to become larger juveniles (J_2), at probability P, before reaching sexual maturity. Modeling two juvenile age classes enable us to incorporate how changes in size at metamorphosis would influence population dynamics. Transitions between juveniles and adults are linked by survival rates of juveniles and adults (σ_{j2}, σ_a). The transition matrix was defined as:

$$\begin{bmatrix} J1 \\ J2 \\ A \end{bmatrix}_{t+1} = \begin{bmatrix} \sigma_{j1}(1\text{-}P) & 0 & (1\text{-}\omega)F(A) \\ \sigma_{j1}(P) & 0 & \omega\, F(A) \\ 0 & \sigma_{j2} & \sigma_a \end{bmatrix} \qquad \begin{bmatrix} J1 \\ J2 \\ A \end{bmatrix}_t$$

This model enables us to examine both changes in stage specific survival parameters (e.g., σ_{tmax}) and delays in maturation (1-ω) that arise from emerging earlier and smaller to escape drying ponds for a range of density dependent scenarios ranging from weak (or even no) larval stage density dependence to very strong density dependence (*Estimating density dependent tadpole survival*).

The model was parametrized from published studies on *P. cultripes*, unpublished data and our field mesocosm data (Table 4.1). Whenever possible, we used data specific to Doñana National Park but report parameter estimates from other locations. If data were not available, we used parameter values from closely related species.

Estimating density dependent tadpole survival

Several studies have already incorporated a density dependent component in deterministic, stage structured, matrix models in amphibians (Vonesh & De la Cruz, 2002; Karraker, Gibbs &

Vonesh, 2008). If larval density dependence is defined by Equation 1, then when $\gamma = 0$, recruitment is linear (i.e., there is no density dependence); when $0<\gamma<1$ recruitment is compensatory, and when $\gamma<1$ survival is over-compensatory. Abiotic stressors experienced in early environments result in density-independent effects and thus only influence σ_{tmax}. For instance, a sudden temperature spike may kill a proportion of individuals independent of density (lowering σ_{tmax}). In this case, the functional form of density dependence (γ) may be similar across environments. Per capita effects may alter the strength of density dependence, and may have a larger influence at certain densities (e.g., predator satiation may induce larger effects at low densities). We were interested in determining whether pond drying influences either σ_{tmax} or γ because pond drying may influence survival to metamorphosis in complex ways. For example, pond drying may result in decreased survival across densities if ponds become too hot (lowering σ_{tmax}), or may intensify density dependent competition as tadpoles become more crowded (raising γ).

We first estimated the maximum tadpole survival (σ_{tmax}) and the density dependent exponent (γ) in constant water level treatments (Equation 1) using survival data from a factorial experiment outdoor mesocosm experiment (Chapter 1) where we manipulated both larval density (five levels, 0.09 – 0.91 tadpole/L) and pond hydroperiod (two levels, drying and constant water level). We then tested whether pond drying influenced either tadpole survive or the density dependent exponent. We calculated separate fits of the model for each hydroperiod (drying, constant) to identify which coefficients were constant or varied between hydroperiods.

Modeling ω as a function of larval density and hydroperiod

To estimate the proportion of juveniles transitioning directly to the second juvenile class as a function of larval density and pond hydroperiod, we combined initial size at metamorphosis,

adult growth rates, and minimum size at maturity. We used data on size at metamorphosis from the outdoor mesocosm experiment described above (Chapter 1).

To link size at metamorphosis to size at maturity, we used adult growth rates to determine at what age spadefoot toads from each density and pond hydroperiod combination would reach the minimum size at first reproduction [40 mm, estimate from (Leclair, Leclair & Gallant (2005)]. We used the growth equation from (Leclair, Leclair & Gallant, 2005), where (L_2= a-[(a)-L_1]*$e^{[(-k)]}$) , and L_1 is SVL at time 1, and L_2 is SVL at time 2, k is the growth rate, a is the maximum body size [a_{female}= 106.3 mm, k_{female} = 0.094, (Leclair, Leclair & Gallant (2005)]. The values of L_1 were based on size at metamorphosis in our field mesocosm experiment (Chapter 1, Figure 1.3C).

We determined that for toads to reach 40 mm (minimum size at metamorphosis) by the second year, the minimum size at metamorphosis (SVL) would need to be ≥ 26.3 mm. We calculated the percent of metamorphs that were above this threshold size across our treatment combinations. We modeled the percentage of individuals above this threshold size as a function of density and hydroperiod. Individuals equal or above this threshold size were assumed to directly transition to J_2, while individuals below this threshold size were assumed to transition to J_1.

For modeling ω (juvenile transition probability) as a function of density, we used

$$\omega = \frac{1}{(1+gT)} \qquad \text{Equation 3}$$

where T is the number of tadpoles. We tested whether the parameter g was dependent on hydroperiod (drying or constant water level). We also calculated the value of ω across drying and constant water level ponds (independent of density). This approach allowed us to model how changes in size at metamorphosis due to developmental plasticity would scale up and influence population dynamics.

Statistical analyses

To fit both larval percent survival (Equation 1) and juvenile transition probability (Equation 3) we used non-linear models. We tested whether hydroperiod influenced either σ_{tmax} or γ. We calculated separate fits of the model for each water level (drying, constant) to identify which coefficients were common or varied between the hydroperiod. We used model selection with AIC (Akaike Information Criterion) (Burnham & Anderson, 2002) to compare the relative fit of the model with and without hydroperiod influencing the parameters. All analyses were performed using R (R Development Core Team, 2016).

Estimating how hydroperiod effects on survival and phenotype influence adult equilibrium densities

We first explored general model behavior (no effects of pond drying) to determine how changing γ, $\sigma_{tmax,}$ $\sigma_{egg,}$ and ω would influence equilibrium adult densities ($\hat{A}$) (see Table 4.1 for range explored). We quantified the relationships between adult densities and early life history parameters. We examined the effect of large changes in a demographic parameter on equilibrium adult density (Schmitt, Holbrook & Osenberg, 1999; Vonesh & De la Cruz, 2002). This allowed us to compare how similar proportional changes in $\sigma_{egg,}$ σ_{tmax} and ω influenced adult equilibrium densities (e.g., compare the effects of a 10% reduction in egg survival versus the effects of a 10% reduction in tadpole survival).

Because pond drying influences these parameters to different degrees, we used a scenario based approach to estimate the independent and simultaneous effects of reported changes in $\sigma_{egg,}$ σ_{tmax} and ω due to pond dry-down. We incorporated reported percent reductions to these parameters from previous studies and from our outdoor mesocosm experiment (Table 4.1). We set $\sigma_{egg\ -\ dry\text{-}down} = 0.23$ based on data from Serrano et al. (2016), which represented a 57% decrease in egg survival compared to constant hydroperiod. We set $\sigma_{tmax\ -\ dry\text{-}down} = 0.73$ based on laboratory data

which found a 15% reduction in tadpole survival (Gomez-Mestre, Kulkarni & Buchholz, 2013). Finally, we incorporated a 9.4% reduction in ω ($\omega_{constant} = 0.329$ versus $\omega_{dry\text{-}down} = 0.234$). Estimates of $\sigma_{egg\ -\ constant}$ Charbonnier et al., unpublished), and estimates of $\sigma_{tmax\ -\ dry\text{-}down}$ and $\omega_{dry\text{-}down}$ were based on our field mesocosm experiment (see Results).

We tested how reductions in these parameters would influence adult equilibrium densities both independently and simultaneously. We calculated the baseline adult equilibrium density under a constant hydroperiod with no dry-down effects on survival and phenotype (Table 4.2). For each subsequent scenario, we then reduced parameters by the predicted amount and compared $\Delta\hat{A} = (\hat{A}_{constant} - \hat{A}_{drydown}) / \hat{A}_{constant}$ (Vonesh & De la Cruz, 2002). For scenario 2 through 4, we calculated $\Delta\hat{A}$, when dry-down influenced only one parameter value. For scenario 5 through 7, we calculated $\Delta\hat{A}$ assuming dry-down effects on two of the parameters, while scenario 8 calculated $\Delta\hat{A}$ when all three parameters were reduced due to pond dry-down. This approach allowed us to quantitatively compare the independent and simultaneous effects of reduced survival (σ_{egg}, σ_{tmax}) and delayed maturity (ω) due to pond drying.

Results

Modeling ω as function of density and hydroperiod

Using growth rates from the literature, the average female *P. cultripes* from the lowest density treatment (36.9 tadpole/L) from both constant water level and drying ponds would reach sexual maturity after year two. The average female emerging from the 110.5 – 368.5 tadpole/L density treatment would reach maturity between year three and four, and thus would breed in year three. Within each larval density tested, females from drying ponds would reach metamorphic size later, but within the same year as their constant hydroperiod counterparts.

Across all densities, constant hydroperiod treatments had 32.8% of animals emerging above the threshold size and thus reaching maturity after two years (ω, proportion mature age three), compared to 23.4% of animals emerging from dry-down treatments. We also modeled percent above threshold size (ω, proportion mature age three) as a function of density (see Methods). Although we found the most support for the model where ω was influenced by hydroperiod (ω = 1/(1+g[hydroperiod](T)), Table 4.3), however, the model over-estimated values of ω for higher densities (Figure 4.2).

Effects of hydroperiod on γ and σ_{tmax}

We did not find that hydroperiod influenced either maximum tadpole survival (σ_{tmax}) or the density dependent exponent (γ) (ΔAIC<1). We estimated maximum tadpole survival to be 0.88 (± 0.22, standard error) and the scaling parameter, d = 0.010 (± 0.018 SE) and γ = 1.04 (± 0.065 SE) (Figure 4.3). We used these values as our fixed (baseline values) constant hydroperiod, Table 4.2). Although we did not find that hydroperiod influenced these parameters, we explored possible hypothetical scenarios where pond dry-down would influence the maximum tadpole density and/or the density dependent exponent. The relationship between σ_{tmax} and Â was linear (Â= 4.5614(σ_{tmax}) – 0.0546, Figure 4.4). Because of this linear relationship, a 10% decrease in σ_{tmax} resulted in a 0.45 reduction in Â. The relationship between Â and γ was best described by the power function $\hat{A} = 468.15\,\gamma^4 - 2095.5\,\gamma^3 + 3531.1\,\gamma^2 - 2664.3\,\gamma + 764.56$ (Figure 4.5). The relationship between σ_e and Â was best described by a polynomial equation, $\hat{A} = -28.827\,\sigma_e^6 + 99.684\,\sigma_e^5 - 139.95\,\sigma_e^4 + 102.47\,\sigma_e^3 - 41.777\,\sigma_e^2 + 9.3489\,\sigma_e + 2.9705$ (Figure 4.6). The relationship between egg survival and Â was asymptotic. For example, reducing egg survival from 20% to 10% resulted in 0.21 reduction in Â, while reducing egg survival from 90% to 80% resulted in 0.00607 change in Â.

Modeling spadefoot toad population dynamics

The relationship between adult densities (Â) and ω was Â = 9.2 ω + 3.03 (Figure 4.7). Because of this linear relationship, a 10% decrease in ω resulted in a 0.92 decrease in Â across all values of ω. When ω was modeled as a function of density, we found the most support for ω = 1/(1+g[hydroperiod]*density tadpole/m^2) where $g_{drydown}$= 0.025 and $g_{constant}$ = 0.0148 (Table 4.3). When we incorporated the two values of g (0.025 and 0.0148 respectively), the result was a 1.8% reduction in Â (when $g_{drydown}$ = 0.025, Â = 3.12, while when and $g_{constant}$ = 0.0148, Â = 3.18).

We modeled eight scenarios which incorporated reported decreases in $\sigma_{e,}$ $\sigma_{tmax,}$ and ω due to pond dry-down individually and simultaneously to explore the combined effects of survival and phenotypic effects. Expected reductions (57%) in σ_e resulted in the smallest proportional change in Â (2.1%). A 15% decrease in σ_{tmax} resulted in a 17.3% reduction in Â. A 9.4% decrease in ω resulted in a 14.3% decrease in Â. When both ω and σ_{tmax} were reduced simultaneously, Â was reduced by 29.1%. The greatest reduction in Â occurred when egg survival, tadpole survival and ω were simultaneously reduced (31.3% decrease in Â) (Figure 4.8).

Discussion

Pond drying may influence survival and phenotype of amphibian species(Blaustein et al., 2010; Richter-Boix, Tejedo & Rezende, 2011), however it is unclear how hydroperiod may scale up to influence population dynamics. Smaller metamorphic size due to developmental acceleration was incorporated in our demographic model by assuming smaller size at metamorphosis would result in delays in reproductive maturity. Reductions in animals transitioning directly to the larger, early maturing age class resulted in greater decreases in adult populations densities than proportional reductions in tadpole and egg survival. A combination of phenotypic and survival effects of pond dry-down across life stages resulted in the greatest reduction in adult densities.

The proportion of animals that reached reproductive maturity at age three (ω) was dependent on hydroperiod and density. Across densities, in constant hydroperiod treatments, a greater proportion of animals transitioned directly to the larger size class. When we included density-dependent ω as a function of hydroperiod, changes in adult equilibrium densities were small (less than 2% change in population densities). This suggests that at the equilibrium densities observed in our model, a very small proportion of animals will transition directly to the second age class. Indeed, at high densities, a similar, small percentage of individuals attain a larger size regardless of hydroperiod. At lower densities, a greater percentage of animals emerge at a large size in constant hydroperiod ponds. This is likely because there are both minimum and maximum sizes at metamorphosis (Wilbur & Collins, 1973; Rose, 2005): At very high densities, further reductions in size are not possible, and small tadpoles that have not reached the size threshold die. At high densities, we observe fewer phenotypic effects, because mortality from desiccation is high.

We also tested how changes in ω due solely to pond drying would influence adult equilibrium densities. Although this approach may over-estimate the number of animals that transition directly to the second class of juveniles at high densities, it provides a framework for understanding how phenotypic effects of reduced hydroperiod may scale up. In this species, some individuals can attain a large size regardless of density, so it is not infeasible to conclude large individuals may emerge from high density ponds. A roughly 15% reduction in adult equilibrium densities was observed when pond dry-down was estimated to cause a nearly10% decrease in ω. This phenotypic effect resulted in a greater percent change in population density than a 57% reduction in egg survival. This is consistent with previous work, which suggests early life stage mortality may have minimal impacts on population size due to larval density dependence (Vonesh & De la Cruz, 2002; Willson et al., 2012). However, Serrano et al.(2016) observed complete egg

mortality in at least one year, and our model does not account for such stochastic catastrophic mortality events. Repeated, catastrophic egg mortality may obviously have important consequences for population size if no new juveniles are produced in one year. Future work should incorporate how catastrophic egg mortality over multiple years may influence population densities. Our work suggests that reductions in phenotype due to pond dry-down can have greater influence on population size than egg mortality in this species.

Our model did not incorporate how reductions in female body size may influence clutch size. The relationship between female body size and clutch size is positive in this species (Morangoni et al. 2008). While reduction in ω may thus result in smaller clutch sizes, this would be equivalent to reducing egg survival, and would thus have minimal effects on equilibrium densities.

Reducing the maximum tadpole survival by 15% had greater influence on adult equilibrium densities than the combined reductions in ω and egg survival reductions. However, we did not find support for reduced tadpole survival in drying ponds in our study (Chapter 1). We include changes in tadpole survival because other studies have found reduced survival in low water volume environments (Gomez-Mestre, Kulkarni & Buchholz, 2013, Burraco et al. in press). It is possible that our dry-down manipulation was not rapid enough to generate survival effects, and instead generated primarily phenotypic effects. Faster dry-down, where fewer tadpoles can escape desiccation, may lead to reduced recruitment in drying ponds (Amburgey, Murphy & Funk, 2016).

Our model does not incorporate size specific growth rates. If small metamorphs (J_1) can catch up to the size of their J_2 due to higher growth rates, than a higher proportion of individuals would breed in the third year of life. Our model also does not include higher survival the larger juvenile class. The relationships between size at metamorphosis, post-metamorphic growth and

survival may change the relationship between hydroperiod and ω. However, our model provides a starting point until further data is available, and suggests that the phenotypic effects may result in changes in population density even when survival of large individuals is not enhanced. Furthermore, our model suggests developmental plasticity due to pond drying, by changing the number of individuals in each size class, may result in changes in equilibrium densities. Many species exhibit this plastic response, and future work should attempt to link how phenotypic shifts influence population size in species with different life histories.

In this species, pond dry-down may result in sequential reductions in egg survival, tadpole survival and reduced size at metamorphosis. Our work suggests that these stressors, when combined may, result in significant reduction in adult equilibrium in this species. In this system, if density dependence were to become over-compensatory due to pond dry-down, we would expect small decreases in adult equilibrium densities. This result highlights the importance of quantifying relationships between density and survival to metamorphosis, as the shape of density dependence may determine how early life history stressors influence adult densities.

Because pond dry-down can influence survival across multiple life stages, we quantified how multiple, sequential reductions in egg, maximum tadpole survival and ω influence adult densities based on previous estimates for constant and dry-down ponds. Predicted reductions in maximum tadpole density, egg survival and ω resulted in relative changes in adult equilibrium densities of nearly 35 %. Our results suggest that the effects of reduced hydroperiod may influence population size in this species. While plasticity may enable organisms to escape mortality in drying ponds, the phenotypic effects of reduced body size may scale up and influence population size.

1

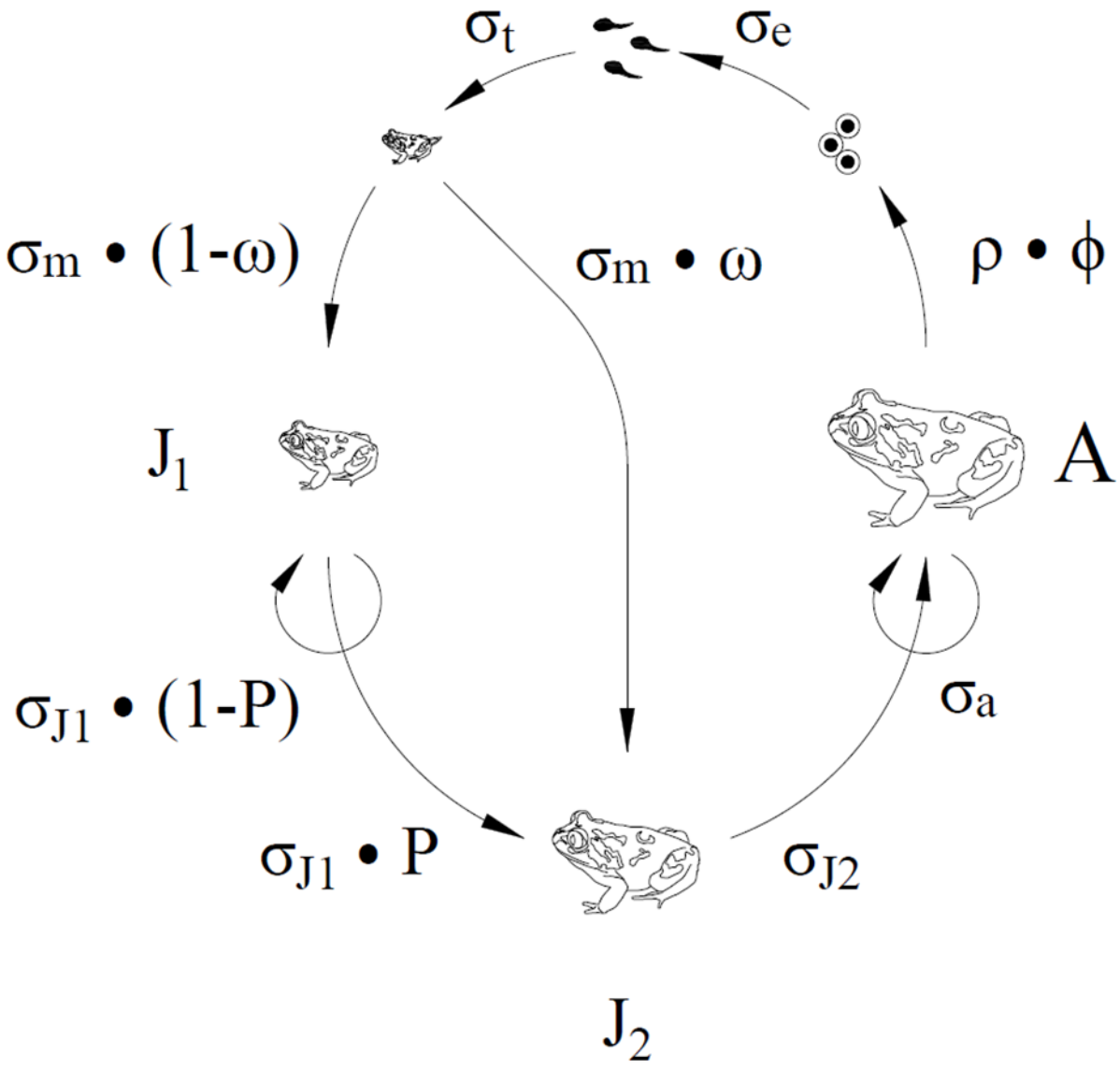

Figure 4.1 Life cycle for *P. cultripes* based on the Vonesh and De la Cruz (2002) life cycle, modified to include one additional juvenile age class.

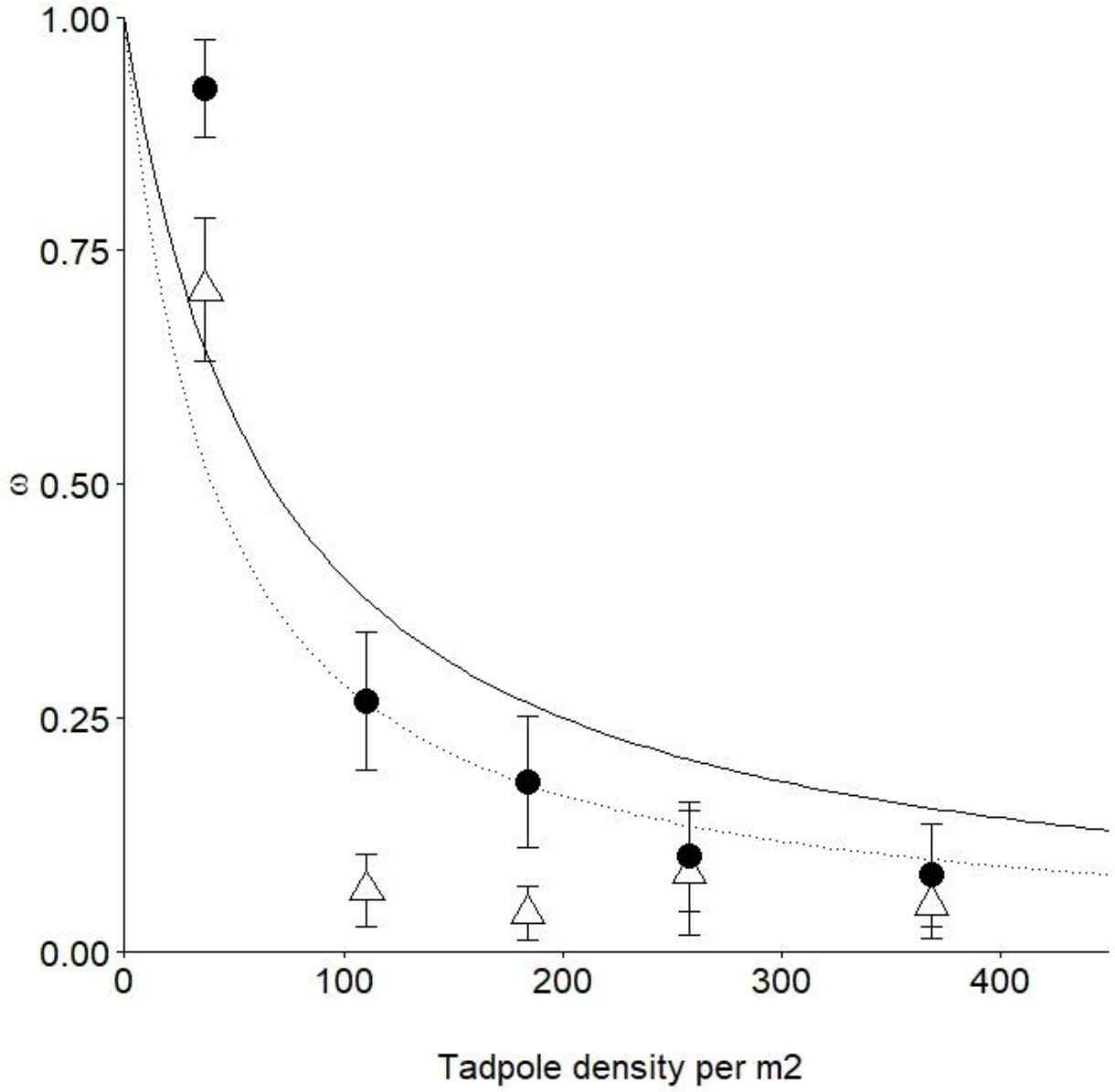

Figure 4.2 Relationship between ω (percent juveniles *P. cultripes* emerging larger than SVL = 26.3 mm) and tadpole density across constant hydroperiod (•,— solid line) and dry-down (Δ,⋯ dotted line) using Equation 3. The model with the most support had the parameter g dependent on hydroperiod ($g_{drydown}$ = 0.025 and $g_{constant}$ = 0.015, Table 4.3)

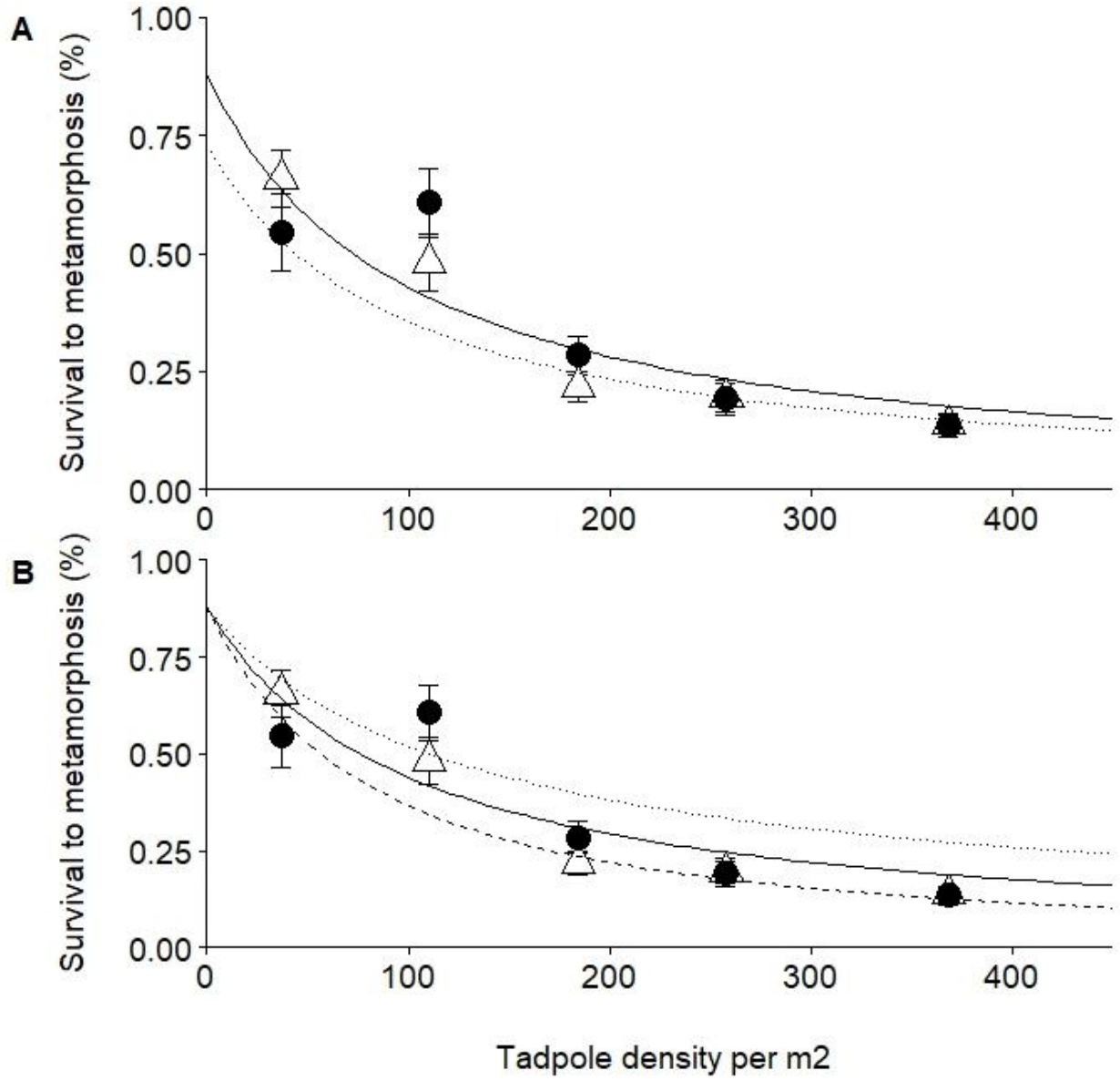

Figure 4.3 A) Relationship between *P. cultripes* survival to metamorphosis (σ_t) and tadpole density from Chapter 1 experiment using the function form using Equation 1. The best fitting model where $\sigma_{tmax} = 0.88$ and $\gamma = 1.04$, $d = 0.010$ (— solid line) and $\sigma_{tmax} = 0.73$ (…. dotted line) and B) where $\gamma = 1.04$, $d = 0.010$ (— solid line), $\gamma = 1.04$ (…. dotted line) and $\gamma = 1.26$ (--- dashed line).

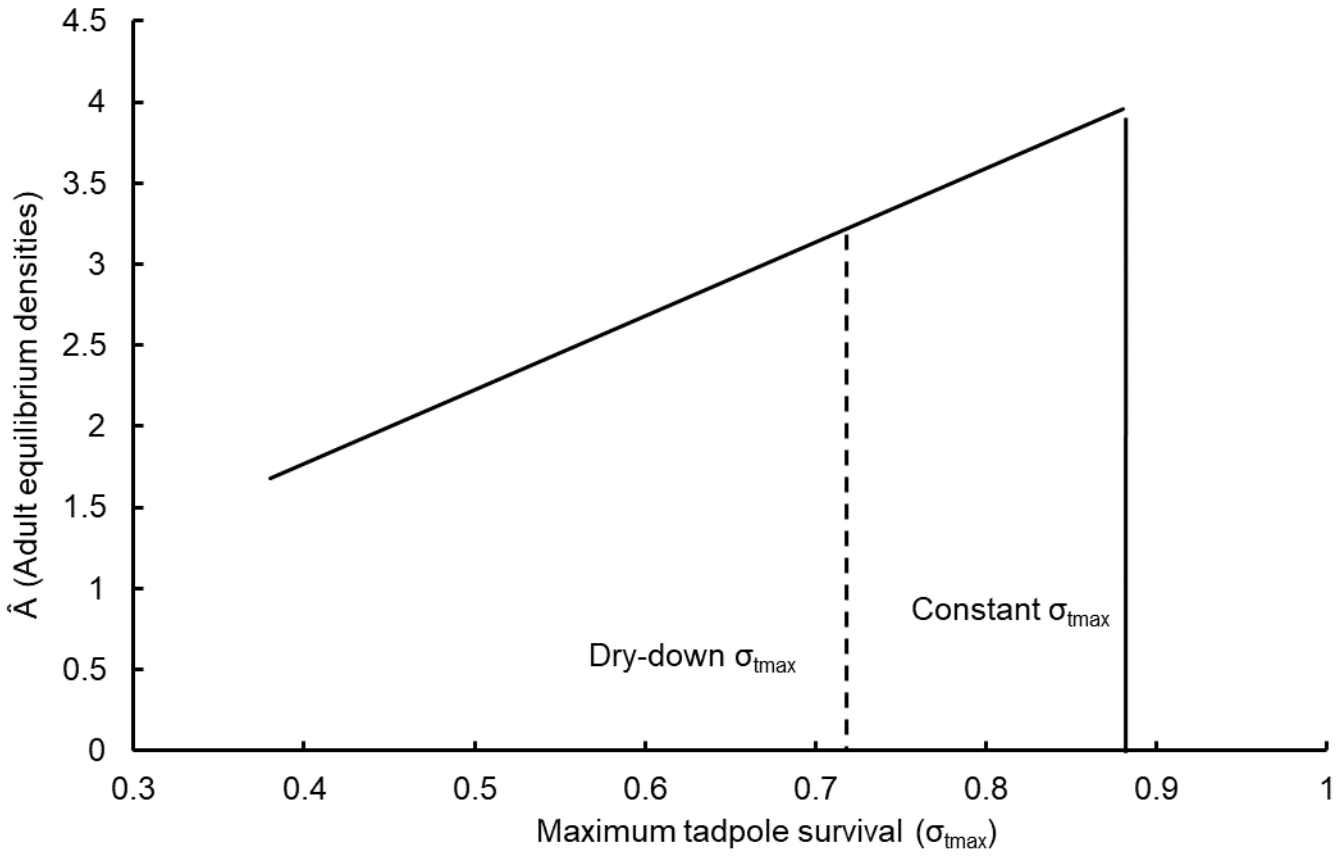

Figure 4.4 Relationship between maximum tadpole survival and adult equilibrium density of *P. cultripes*. All other parameters held constant at fixed values (Table 4.1).

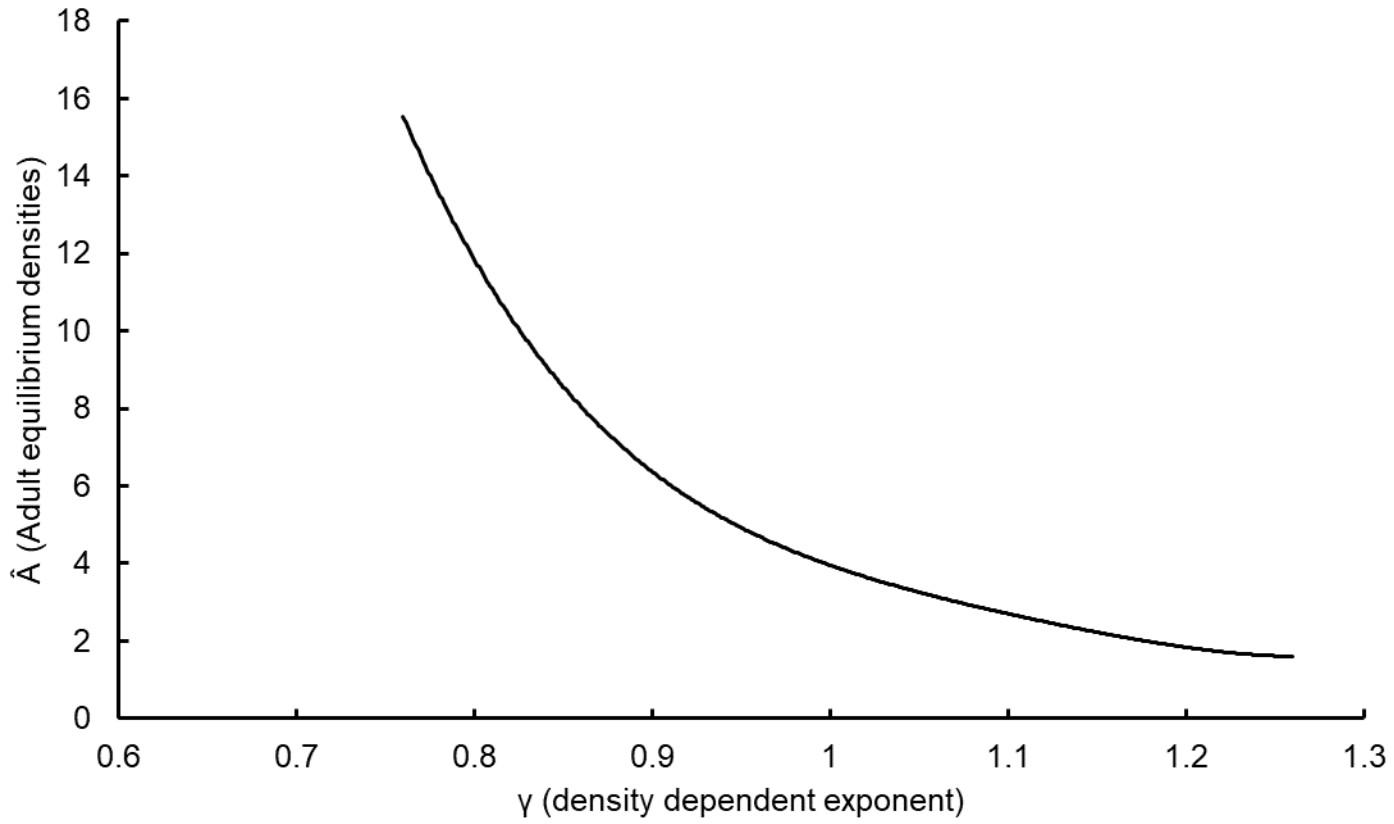

Figure 4.5 Relationship between density dependent exponent and adult equilibrium density of *P. cultripes*. All other parameters held constant at fixed values (Table 4.1).

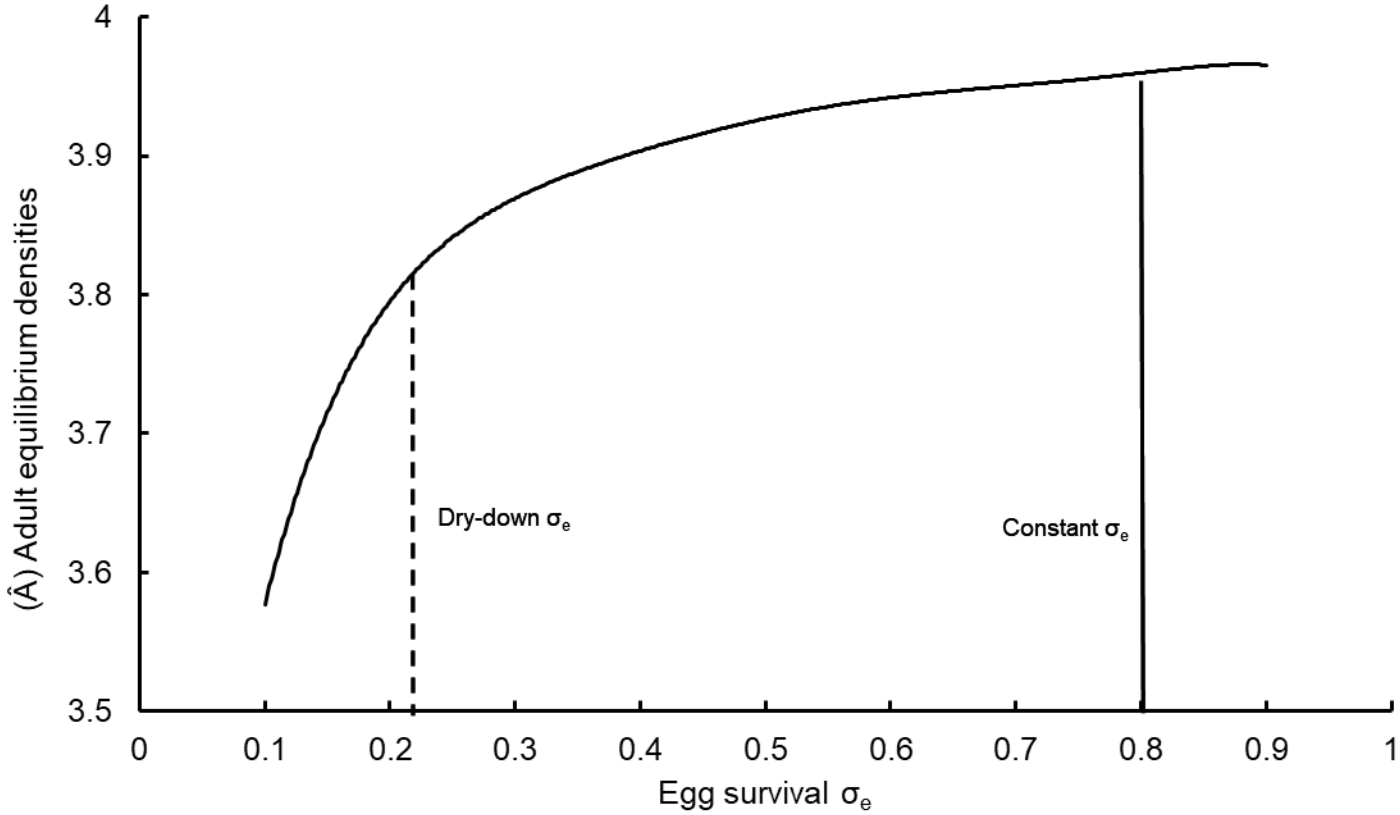

Figure 4.6 Relationship between egg survival and adult equilibrium density of *P. cultripes*. All other parameters held constant (Table 4.1).

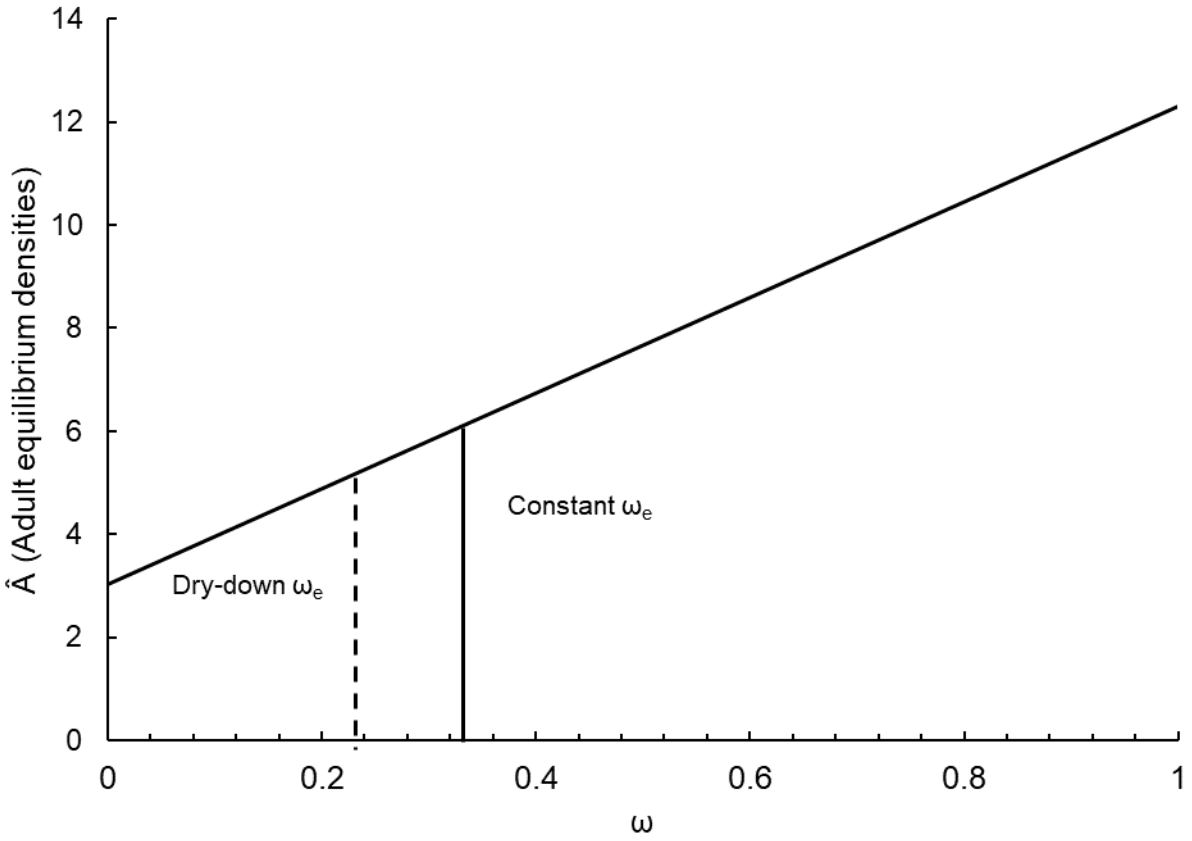

Figure 4.7 Relationship between ω (proportion mature at age 3) and adult equilibrium density of *P. cultripes*, other parameters held constant at fixed values (Table 4.1)

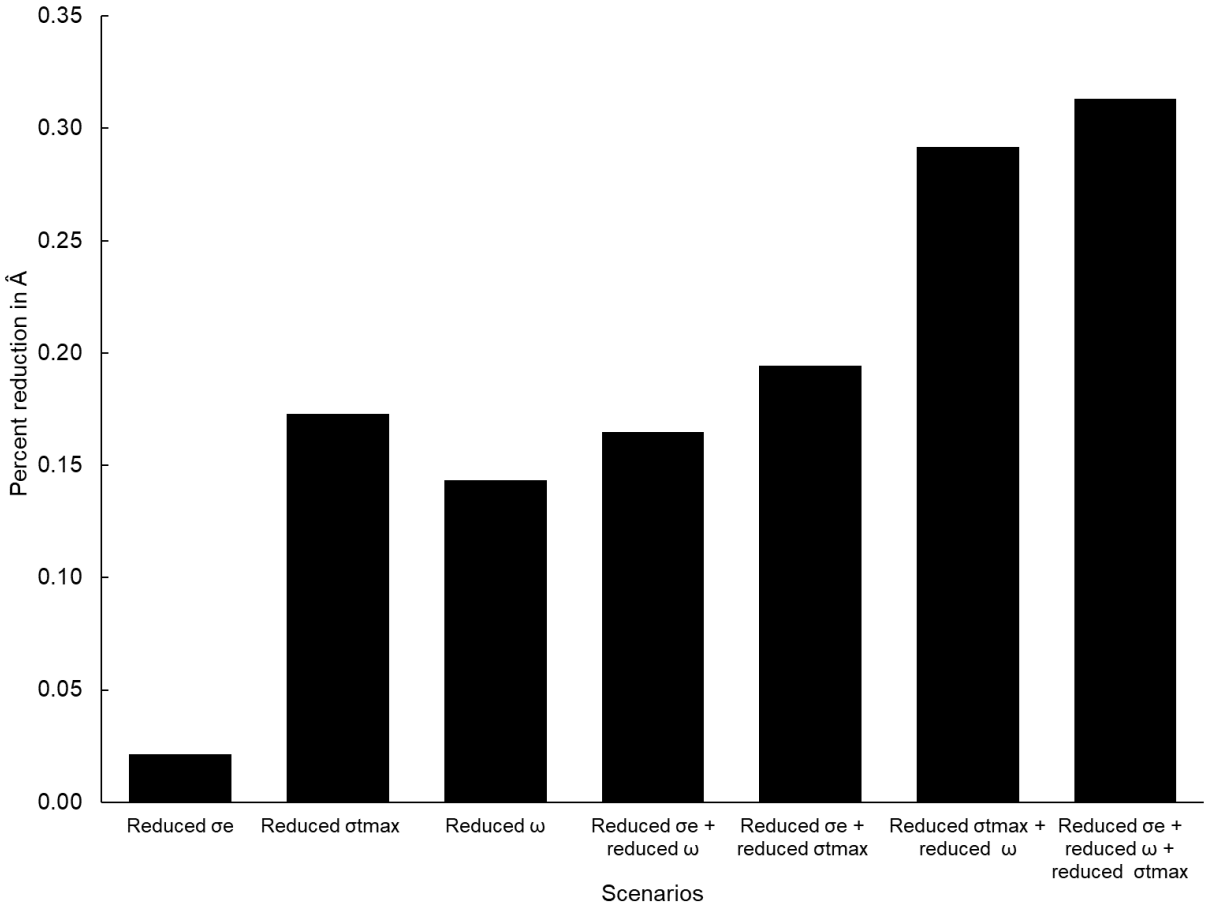

Figure 4.8 Changes in relative adult equilibrium of *P. cultripes* ($\hat{A}_{constant} - \hat{A}_{drydown}$) /$\hat{A}_{constant}$ under different scenarios (parameter values in Table 4.2).

Table 4.1 Model parameters for *P. cultripes*.

Parameter	Symbol	Range in literature	Fixed baseline value	Values explored	Source(s)
Average Clutch size	Φ	1610 - 2538	2288	Fixed	Lizana, Marquez & Martin-Sanchez, (1994); Marangoni & Tejedo, (2008)
Sex ratio	ρ	0.71 - 2.97 :1	0.71	Fixed	Lizana, Marquez & Martin-Sanchez (1994)
Egg survival	σ_e	0.8	0.8	0.23 & 0.80	Charbonnier et al. (unpublished),
Metamorph survival	σ_m	0.2	0.2	Fixed	Bayliss (1994) (*B. marinus*)
Juvenile 1 survival	σ_{j1}	0.2 - 0.4	0.2	Fixed	Schmidt, Hödl & Schaub (2012) (*P. fuscus*)
Juvenile 2 survival	σ_{j2}	0.2 - 0.4	0.4	Fixed	Schmidt, Hödl & Schaub (2012) (*P. fuscus*)
Probably of Maturing	P	0.5	0.5	Fixed	Schmidt, Hödl & Schaub (2012) (*P. fuscus*)
Adult survival	σ_a	0.43	0.43	Fixed	Schmidt, Hödl & Schaub (2012) (*P. fuscus*)
Density-dependent exponent	γ	0.76 - 1.26	1	0.76 - 1.26	Vonesh & De la Cruz (2002)
Density-dependent coefficient	d	0.004 - 0.05	0.01	Fixed	Chapter 1
Maximum tadpole survival	σ_{tmax}	0 - 1.0	0.88	0.73 - 0.88	Chapter 1, Gomez-Mestre, Kulkarni & Buchholz (2013)
Proportion mature age 2	ω	NA	0.1	0 - 1	Chapter 1

Table 4.2 : Parameter values explored to assess relative change in adult equilibrium densities in *P. cultripes* due to reduced hydroperiod. Estimates for σ_{egg} – dry-down was based on Serrano et al. (2016), σ_{tmax} – dry-down.was based on Gomez-Mestre et al. (2013).

Scenario	Dry-down effects	Number parameters modified	σ_e	σ_{tmax}	ω	(Â)	ΔÂ
1	None		0.8	0.88	0.328	6.07	
2	Survival	1	0.23	0.88	0.328	5.94	0.13
3	Survival	1	0.8	0.73	0.328	5.02	1.05
4	Phenotype	1	0.8	0.88	0.234	5.2	0.87
5	Survival	2	0.23	0.73	0.328	4.89	1.18
6	Combination	2	0.23	0.88	0.234	5.07	1.00
7	Combination	2	0.8	0.73	0.234	4.3	1.77
8	Combination	3	0.23	0.73	0.234	4.17	1.90

Table 4.3 AIC values and parameter estimates for models tested that describe the relationship between ω and larval density.

Model	AIC	$b_{constant}$	SE	$b_{drydown}$	SE	$a_{constant}$	SE	$a_{drydown}$	SE
ω = a[hydroperiod]/(1+b[hydroperiod]*density)	6.77	0.015	0.008	0.025	0.02	1	0.27	1	0.45
	AIC	$b_{constant}$	SE	$b_{drydown}$	SE	$a_{constant=drydown}$	SE		
ω = a/(1+b[hydroperiod]*density)	4.77	0.0148	0.007	0.025	0.01	1	0.23		
ω = 1/(1+b[hydroperiod]*density	2.77	0.0148	0.003	0.025	0.005	1	NA		
	AIC	$b_{constant=drydown}$	SE	$a_{constant}$	SE	$a_{drydown}$	SE		
ω = a[hydroperiod]/(1+b*density)	9.28	0.018	0.008	1	0.25	0.91	0.22		
	AIC	$b_{constant=drydown}$	SE	$a_{constant=drydown}$	SE				
ω = 1/(1+b*density)	5.85	0.019	0.0025	1	NA				

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