Effects of larval foam-making and prolonged terrestriality on morphology, nitrogen excretion and development to metamorphosis in a Leptodactylid frog (#106621)

First submission

Guidance from your Editor

Please submit by **31 Oct 2024** for the benefit of the authors (and your token reward) .

Structure and Criteria
Please read the 'Structure and Criteria' page for guidance.

Custom checks
Make sure you include the custom checks shown below, in your review.

Raw data check
Review the raw data.

Image check
Check that figures and images have not been inappropriately manipulated.

If this article is published your review will be made public. You can choose whether to sign your review. If uploading a PDF please remove any identifiable information (if you want to remain anonymous).

Files

Download and review all files from the materials page.

6 Figure file(s)
3 Table file(s)
1 Video file(s)

Custom checks

Vertebrate animal usage checks

- Have you checked the authors ethical approval statement?
- Were the experiments necessary and ethical?
- Have you checked our animal research policies?

Field study

- Have you checked the authors field study permits?
- Are the field study permits appropriate?

Structure and Criteria

Structure your review

The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

When ready submit online.

Editorial Criteria

Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- **Impact and novelty is not assessed.** Meaningful replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult. I suggest you have a colleague who is proficient in English and familiar with the subject matter review your manuscript, or contact a professional editing service.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Effects of larval foam-making and prolonged terrestriality on morphology, nitrogen excretion and development to metamorphosis in a Leptodactylid frog

Javier Méndez Narváez [Corresp., 1, 2], **Karen M Warkentin** [2, 3]

[1] Calima, Fundación para la Investigación de la Biodiversidad y Conservación en el Trópico, Cali, Colombia

[2] Biology Department, Boston University, Boston, Massachusetts, United States

[3] Smithsonian Tropical Research Institute, Panama, Panama

Corresponding Author: Javier Méndez Narváez
Email address: jmendez@fundacioncalima.org

At ontogenetic transition points, animals often exhibit plastic variation in developmental trajectories, behavior and physiology in response to environmental conditions. In frogs, most terrestrial-breeding species have life cycles involving multiple transitions between aquatic and terrestrial environments. Some species can extend their initial terrestrial period, either as a plastic embryonic response to balance ecological trade-offs across environments, or as an enforced wait for rain that allows larvae to access aquatic habitats. Terrestrial larvae of the foam-nesting frog, *Leptodactylus fragilis*, can arrest development, make their own nest foam to prevent dehydration, and synthesize urea to avoid ammonia toxicity. These plastic responses enable survival during unpredictably long periods in underground nest chambers, waiting for floods to enable exit and continued development in water. However, such physiological and behavioral responses of early life stages may have immediate and long-term carry-over effects across subsequent ecological and developmental transitions. Here, we examined effects of prolonged terrestriality and the larval foam-making activity that supports it on larval physiology, development, and metamorphosis in *L. fragilis*. We tested for developmental changes in larval foam-making ability by measuring the size of nests produced following complete removal of the parental foam. We measured ammonia and urea levels in larval foam nests to assess nitrogen excretion patterns, testing for effects of larval age, soil hydration around parental nests, and repeated construction of larval nests. We also assessed immediate and long-term effects of larval foam-making and prolonged terrestriality on larval morphology at water entry and development to metamorphosis. We found that larvae arrested development during prolonged time on land and even young larvae were able to effectively produce multiple foam nests. We found high ammonia concentrations in larval nests, very high urea excretion by developmentally arrested older larvae, and faster growth of larvae in water

than while constructing nests. Nonetheless, sibling larvae had a similar aquatic larval period and age at metamorphosis, regardless of their nest-making activity and timing of water entry. Sibship size explained the size of larval foam nests and body size at metamorphosis, suggesting maternal effects in cooperative groups. Metamorph size also decreased with aquatic larval period. Our results highlight the extent of larval ability to maintain and construct a suitable developmental environment and excrete N-waste as urea, which are both crucial for survival during enforced extensions of terrestriality. Our results suggest that the energetic reserves in large eggs are sufficient to meet metabolic costs of urea synthesis and foam production during developmental arrest over an extended period on land, with no apparent carry-over effects on fitness-relevant traits at metamorphosis.

Effects of larval foam-making and prolonged terrestriality on morphology, nitrogen excretion and development to metamorphosis in a Leptodactylid frog

Javier Méndez-Narváez[1,2], Karen M Warkentin[1,3]

[1] Biology Department, Boston University, Boston, MA, USA
[2] Calima, Fundación para la Investigación de la Biodiversidad y Conservación en el Trópico, Cali, Colombia
[3] Smithsonian Tropical Research Institute, Panama, Republic of Panama

Corresponding Author:
Javier Méndez-Narváez[1,2]
Carrera 98 #48-30, Apt 610, Cali, Valle del Cauca, 760026, Colombia
Email address: jmendez@fundacioncalima.org

Abstract

At ontogenetic transition points, animals often exhibit plastic variation in developmental trajectories, behavior and physiology in response to environmental conditions. In frogs, most terrestrial-breeding species have life cycles involving multiple transitions between aquatic and terrestrial environments. Some species can extend their initial terrestrial period, either as a plastic embryonic response to balance ecological trade-offs across environments, or as an enforced wait for rain that allows larvae to access aquatic habitats. Terrestrial larvae of the foam-nesting frog, *Leptodactylus fragilis*, can arrest development, make their own nest foam to prevent dehydration, and synthesize urea to avoid ammonia toxicity. These plastic responses enable survival during unpredictably long periods in underground nest chambers, waiting for floods to enable exit and continued development in water. However, such physiological and behavioral responses of early life stages may have immediate and long-term carry-over effects across subsequent ecological and developmental transitions. Here, we examined effects of prolonged terrestriality and the larval foam-making activity that supports it on larval physiology, development, and metamorphosis in *L. fragilis*. We tested for developmental changes in larval foam-making ability by measuring the size of nests produced following complete removal of the parental foam. We measured ammonia and urea levels in larval foam nests to assess nitrogen excretion patterns, testing for effects of larval age, soil hydration around parental nests, and repeated construction of larval nests. We also assessed immediate and long-term effects of larval foam-making and prolonged terrestriality on larval morphology at water entry and development to metamorphosis. We found that larvae arrested development during prolonged time on land and even young larvae were able to effectively produce multiple foam nests. We found high ammonia concentrations in larval nests, very high urea excretion by developmentally arrested older larvae, and faster growth of larvae in water than while constructing nests. Nonetheless, sibling larvae had a similar aquatic larval period and age at metamorphosis, regardless of their nest-making activity and timing of water entry. Sibship size explained the size of larval foam nests and body size at metamorphosis, suggesting maternal effects in cooperative groups. Metamorph size also decreased with aquatic larval period. Our results highlight the extent of larval ability to maintain and construct a suitable developmental environment and excrete N-waste as urea, which are both crucial for survival during enforced extensions of terrestriality. Our results suggest that the energetic reserves in large eggs are sufficient to meet metabolic costs of urea synthesis and foam production during

47 developmental arrest over an extended period on land, with no apparent carry-over effects on
48 fitness-relevant traits at metamorphosis.
49
50 **Introduction**
51 Most animals with complex life cycles experience ontogenetic transitions that allow them to
52 exploit multiple environments (Rolff et al., 2019; Truman, 2019; Wassersug, 1975; Wilbur,
53 1980). ~~Their different life stages~~ may experience environment-specific selective factors that can
54 constrain or increase phenotypic diversity within each stage or between stages (Bardua et al.,
55 2021; Fabre et al., 2020; Phung et al., 2020). ~~Studying~~ how environmental conditions affect
56 phenotypic responses ~~across~~ development could clarify the role of developmental plasticity in
57 ~~ontogenetic switches in~~ ecology and morphology (Gilbert, 2012; Gilbert & Epel, 2015; West-
58 Eberhard, 2003; Moore & Martin, 2019). For instance, early life stages of vertebrates can
59 respond to multiple environmental conditions and cues (e.g., threats, resources) with
60 morphological, behavioral and physiological changes at life history transition points such as
61 hatching (Martin, 1999; Mueller et al., 2019; Warkentin, 2011) and metamorphosis (~~Wilbur &~~
62 ~~Collins 1973~~; Werner, 1986). These plastic responses at a particular life stage may have
63 immediate and long-term carry-over effects on expressed traits and survival in subsequent stages
64 or after transitions into a new environment (Gomez-Mestre & Buchholz, 2006; Touchon &
65 Warkentin, 2010; Cabrera-Guzmán et al., 2013; Morey & Reznick, 2001; Scott et al., 2007).
66
67 ~~Complex life cycles in anurans~~ are characterized by multiple ecological and physiological shifts
68 between aquatic and terrestrial environments (Crump, 2015; Duellman & Trueb, 1986; Elinson
69 & del Pino, 2012; Gomez-Mestre et al., 2012). Many studies have assessed how environmentally
70 induced responses in the aquatic larval environment (Gomez-Mestre et al., 2010; 2013; Gomez-
71 Mestre & Buchholz, 2006; Laurila & Kujasalo, 1999; Murillo-Rincón et al., 2017a; Rudolf &
72 Rödel, 2007; Touchon et al., 2013; Vonesh & Warkentin, 2006) influence traits in subsequent
73 life stages, including nutrition uptake efficiency and growth (Bonifas & Bouchard, 2021;
74 Bouchard et al., 2015; 2016; Zhu et al., 2019) with its underlying physiological pathways
75 (Burraco et al., 2017; 2020; Crespi & Warne, 2013; Murillo-Rincón et al., 2017b). ~~Thus~~
76 emerging metamorphs and juveniles carry effects of larval nutrition and physiology that
77 contribute to variation in locomotor performance, behavior, and survival on land (Bouchard et

al., 2016; Gomez-Mestre et al., 2010; Nicieza et al., 2006; Tarvin et al., 2015). While a large body of literature addresses effects of the aquatic larval environment on metamorphic and post-metamorphic phenotypes, much less is known about potential effects of terrestrial embryonic development on subsequent stages, including the aquatic larval period and metamorphosis (Touchon et al., 2013; Touchon & Warkentin, 2010).

Terrestrial and semi-terrestrial development are widespread and have evolved independently many times in amphibians (Duellman, 1985; Gomez-Mestre et al., 2012; Wells, 2007; Liedtke, et al., 2022). Embryos and larvae have evolved their own adaptations to conditions common during terrestrial development (Delia et al., 2013; 2014; Méndez-Narváez & Warkentin, 2022; Salica et al., 2017; Seymour & Bradford, 1995; Shoemaker & McClanahan, 1973; Warkentin, 1995; 2007), including the ability for extended or arrested embryonic development on land (Bradford & Seymour, 1985; Martin et al., 2011). In most frogs, terrestrial early development is followed by an aquatic larval period that ends in metamorphosis (Liedtke, et al., 2022). This generates life cycles with two habitat transitions separated by variable periods of growth and development within each habitat. The environmental conditions experienced in early life stages can affect hatchling size, morphology, and subsequent development and survival in the aquatic environment (Burraco et al., 2020; Delia et al., 2019; Kaplan & Phillips, 2006; Murillo-Rincón et al., 2017a; Touchon & Warkentin, 2010; Warkentin, 1995; Willink et al., 2014). Indeed carry-over effects of terrestrial embryonic environments may still be evident at metamorphosis and in post-metamorphic stages, after frogs re-emerge onto land (Touchon et al., 2013; Touchon & Warkentin, 2010; Vonesh & Bolker, 2005). Because adaptations that facilitate terrestrial development and survival under stressful conditions may involve changes in energetic demands (Méndez-Narváez & Warkentin 2022; Seymour et al., 1991), they are likely to alter some physiological costs and nutritional demands that affect aquatic larval development and metamorphosis (Burraco et al. 2021; 2022).

In organisms with complex life cycles, nutritional and neuroendocrine factors regulate and constrain transitions between life stages (Callier & Nijhout, 2011; Denver, 2021; Laudet, 2011; Mirth et al., 2014; Pfennig et al., 1991; Rolff et al., 2004). Indeed, during frog metamorphosis, when metabolic reorganization occurs, differentiation and growth of new structures depends on

energy reserves accumulated during larval development (e.g., fat bodies and liver) (Mueller et al., 2012; Zhu et al., 2020). Urea excretion is considered a key physiological adaptation that allowed tetrapod invasion of land (Amemiya et al., 2013 Mommsen & Walsh, 1989), but with a higher metabolic cost of synthesis in comparison with ammonia excretion (Shambaugh, 1977; Wright & Fyhn, 2001). In amphibians, it plays a key role in enabling the transition from aquatic to terrestrial environments, with an onset or upregulation at frog metamorphosis (Brown et al., 1959; Shambaugh, 1977; Wright & Wright, 1996; Wright and Fyhn, 2001; Zhu et al., 2020). However, long before metamorphosis, some terrestrial frog embryos and larvae also excrete urea (Alcocer et al., 1992; Grafe et al., 2005; Martin & Cooper, 1972; Shoemaker & McClanahan, 1973), particularly in response to dry conditions (Méndez-Narváez & Warkentin, 2022). This occurs via early onset of expression of the urea cycle enzymes arginase and CPSase (Méndez-Narváez & Warkentin 2023) and is clearly beneficial for terrestrial survival, by preventing ammonia toxicity (Méndez-Narváez & Warkentin, 2022). However, the benefits of urea excretion for terrestrial early life stages must be balanced against the metabolic cost of urea synthesis and related traits that enable facultative extensions of terrestriality, particularly for species that do not feed before their transition to water.

Frogs with early life stages that excrete urea during variable periods of prolonged terrestriality offer an excellent opportunity to study if and how the physiological challenges they experience during early development on land carry over to affect growth or morphology through aquatic larval stages and at metamorphosis. We studied key larval traits that facilitate prolonged terrestrial life and their carry-over effects to metamorphosis in the white-lipped frog, *Leptodactylis fragilis* (Brocchi, 1877). Early development occurs in a terrestrial foam nest, within a chamber that a male excavates near a temporary pond (Fig. 1A). Larvae must remain in the chamber until rainfall floods it, enabling them to swim out and reach nearby pools to continue development to metamorphosis (Méndez-Narváez & Warkentin, 2022), as in other as in other closely related foam-nesting frogs (Faggioni et al., 2017; Lucas et al., 2008; Oliveira Filho et al., 2005). However, the volume of parental foam typically decreases over time, especially in dry soil, and it can also be dissolved by rain that is insufficient to free larvae from their chamber (Caldwell & Lopez, 1989; Downie, 1984; Méndez-Narváez & Warkentin, 2022; Fig. 1A). In this context, terrestrial larvae of *L. fragilis* have evolved the ability to make foam, to supplement or

replace deteriorating parental foam or build an entirely new larval foam nest to extend their survival on land (Méndez-Narváez, 2022). In the absence of rain, nest-dwelling *L. fragilis* larvae appear to arrest development after eight days on land (N Belduque-Correa, K M Warkentin & J Méndez-Narváez 2019, unpublished data), which is hypothesized to reduce larval metabolism to conserve energy reserves (Downie, 1994). However, early larvae of *L. fragilis* can sustain periods of high activity during foam-making (Mendez-Narvaez 2022; Fig. 1C, Video S1). Moreover, even after the onset of apparent developmental arrest, during prolonged periods on land they excrete a large amount of urea and total nitrogen (ammonia + urea), presumably from breakdown of proteins in yolk reserves (Méndez-Narváez & Warkentin, 2022).

Variation in rainfall, affecting both soil hydration and when larvae can enter the water, as well as variation in the initial physical size of parental foam nests and number of larvae they contain, generates substantial variation in the need for larvae to make their own foam. ~~We mimicked this natural variation to assess~~ the short- and long-term consequences of prolonged terrestriality and larval foam-making activity on physiological and developmental traits. (1) Because foam-making depends on the development and function of foamy glands and performance of foam-making behavior, and because natural selection for foam-making may intensify as parental foam deteriorates over time, we hypothesized that foam-making ability increases developmentally. We therefore assessed larval foam-making ability at several ages. (2) Because foam-making appears energetically costly, and metabolic activity generates waste-products which would accumulate in an initially small amount of foam, especially after nest loss, we hypothesized that foam-making imposes an additional cost due to synthesis of less-toxic urea to avoid ammonia toxicity. To assess nitrogen excretion patterns at different ages and test if prior experience of wet vs. dry soil around the parental nest, or repeated construction of larval foam nests, affects nitrogen excretion in a new nest, we measured ammonia and urea levels in larval foam nests and tissue levels of urea and a key urea-synthesis enzyme. (3) Because yolk reserves are the sole, limited energy source for terrestrial larvae, we hypothesized that larval foam-making, especially repeated nest construction, may deplete their energy reserves or trade-off with growth to affect size or morphology when they enter the water with potential carry-over effects on traits at metamorphosis. We assessed immediate and long-term effects of larval foam-making and

prolonged terrestriality by comparing initial larval morphology, subsequent growth, and metamorphic timing across siblings that participated in making 0, 1, or 3 new nests.

Materials & Methods

Study site and experimental conditions

We conducted field work during the rainy season, from May to October of 2017 and 2018, in Gamboa, Panamá (9°07′14.8" N, 79°42′ 15.4" W) with permission from the Panamanian Ministry of the Environment (MiAmbiente permits SC/A-26-16, SE/A-56-17, SC/A-51-18, SE/A-25-19). We collected terrestrial foam nests of *L. fragilis* the morning after their oviposition, from subterranean chambers adjacent to ephemeral pools in males' territories (Fig. 1), and moved them to an open-air laboratory (~26°C, ~85% RH) at the Smithsonian Tropical Research Institute (STRI) in Gamboa, with approval from the STRI Animal Care and Use Committee (IACUC protocol # 2016-0520-2019A1–A3). We buried each nest in a plastic container filled with soil collected near breeding sites. For most sibships, we matched soil water content in the laboratory to that found in the field, spraying the soil with water once per day to maintain hydration (wet conditions). We kept a subset of nests under reduced soil water content (~ 50% lower, dry conditions) to simulate a period without rainfall (see methods in Méndez-Narváez & Warkentin 2022).

Larval foam-making ability across ages

To assess ontogenetic changes in the ability of larvae of *L. fragilis* to construct new foam nests during prolonged time on land, we tested them before, at, and after the onset of developmental arrest (4.5, 8.5, and 12.5 d, respectively; N Belduque-Correa, K M Warkentin & J Méndez-Narváez 2019, unpublished data). For each sibship, we maintained embryos, then larvae, in their parental foam nest in wet soil until testing (Fig. 1C), then dissolved their original nest with aged tap water. We counted the larvae and used a plastic transfer pipette to move them to a small Petri dish (60 mm diameter x15 mm depth). We kept sibling larval groups (range 35–127 larvae, mean 77.39 ± 19.71 SD, N = 24) together in aged tap water for 2–4 hours before draining their dish. This simulates a limited flooding scenario, where rain dissolves the nest but is insufficient for larvae to move a larger pond. Because larvae trapped in foamless chambers in this context are typically surrounded by most or all of their siblings (Caldwell & Lopez 1989; Méndez-Narváez

personal observations), we did not split clutches to standardize group size. We left each sibling group of tadpoles in their drained dish for two days (N = 26), placing it vertically so that tadpoles remained together at the bottom, as in the cup of a nest chamber (Fig. 1C). Although larvae usually completed a new nest within 24 h of draining the Petri dish, then became inactive (J Méndez-Narváez, 2019 unpublished data), we allowed them a two full days to ensure nest completion; larvae can naturally spend prolonged periods in developmental arrest in their new foam. Then, to quantify the volume of foam that larvae produced, we photographed the flat, vertical face of the Petri dish with a Canon PowerShot SX40HS camera, including a scale in the frame (Fig. 1C). We used NIH ImageJ 2.0 (https://imagej.nih.gov/ij/index.html) to measure the area covered by the foam three times from each photograph, averaged these values, and multiplied by the depth of the dish (15 mm) to calculate foam volume. After photographing the nest, we repeated the nest dissolution and dish-draining procedure to induce groups of larvae to make a second (N = 21), then a third (N = 21) larval foam nest. We removed three or two larvae at each nest attempt to assess immediate effects on larval morphology, and reared others to metamorphosis to assess longer-term consequences. ~~Repeated nest construction may be a common challenge that larvae face in environments with high variation in temperature, humidity, and rainfall, increasing their metabolic requirements during a prolonged period of terrestriality.~~

Nitrogen excretion in larval nests during prolonged terrestriality

We used a different set of foam nests to measure nitrogen wastes from larval foam samples at three ages and collected and stored larval tissues that we later used for enzymatic analysis. We assessed the concentration of ammonia and urea in the first larval foam nest constructed after 4.5 and 12.5 d in parental nests on wet soil, and 12.5 d in parental nests on dry soil (N = 6, 12, and 9, respectively), following methods used to quantify nitrogenous wastes of terrestrial *L. fragilis* larvae in parental foam nests (Méndez-Narváez & Warkentin, 2022). For some 12.5 d larvae, we also collected foam from the third nest they made in Petri dishes (N = 4 sibships from wet soil, 2 from dry soil). We used 3 ml plastic transfer pipettes to collect foam samples and stored them in micro-centrifuge tubes at –20°C for enzymatic quantification of ammonia and urea with a commercial kit (Boehringer Mannheim Cat. No. 10542946035). We thawed samples and centrifuged foam for five minutes at 12000 rpm to obtain the liquid portion for analysis. We assessed ammonia and urea concentration simultaneously, using 0.2 ml of the sample in two

quartz cuvettes (0.1 ml each) and measuring changes in absorbance (at 340 nm) at room temperature with a UV-Visible Spectrophotometer (Thermo-Scientific Evolution 60S). We ran controls without samples to assess background absorbance of kit reagents for the ammonia and urea tests. Some samples produced values under the detection limit (zeros) or absorbances too high to quantify concentrations (NA). Assuming all excreted urea in the new foam nests was produced by conversion of ammonia, we also calculated a "predicted concentration" of ammonia to estimate what could have accumulated without urea excretion, for each sample (predicted ammonia = [ammonia]+2*[urea]). For first nests only, we estimated the amount (µmol) of ammonia, urea, and predicted ammonia excreted per larva into their new foam, from the recovered liquid portion of foam (waste mass = concentration × liquid volume, i.e., assuming no losses or other sources of these molecules) and compared their excretion following development in parental nests in wet vs. dry soil.

In addition, we collected tissues from larvae that had made one nest, after 12.5 d in parental nests in wet or dry soil, to measure tissue urea levels and activity of the enzyme arginase, which produces urea in the ornithine urea cycle (Brown et al., 1959; Mommsen and Walsh, 1989). We followed methods previously used for successful quantification of arginase activity and urea in tissues of terrestrial *L. fragilis* larvae (Méndez-Narváez & Warkentin, 2023). Briefly, we snap froze larvae in liquid nitrogen and stored them, intact, in micro-centrifuge tubes at –80°C until biochemical quantification of enzymatic activity. We quantified arginase activity (µmol min^{-1} mg^{-1} of wet mass) by a colorimetric method (Felskie et al., 1998) and urea in tissues (µmol mg^{-1} of wet mass) with the diacetyl-monoxime method (Rahmatullah & Boyde, 1980), using the biochemical conversion of arginine to urea. We ground frozen specimens to a fine powder in a mortar, using a pestle and liquid nitrogen and prepared extracts by homogenizing a sample of ~50 mg of tissue, pooled from sibling larvae, with 4 volumes of homogenization buffer (20 mM K_2HPO_4, 10 mM Hepes buffer, pH 7.5, 0.5 mM EDTA, 1 mM DTT). We determined protein concentration in each sample by the dye-binding method (Bradford, 1976) with the Thermo Scientific™ Coonassie Protein Assay kit. We performed all enzymatic assays at 26°C, from fresh homogenates stored at –80°C for not more than one month after homogenization. Enzymatic activities were standardized to specific activity using the protein concentration in

each sample (μmol min^{-1} mg^{-1} of protein). The estimated detection limit for arginase activity was 0.001 μmol min^{-1} mg^{-1} of protein.

Carry-over effects of prolonged terrestriality and larval foam-making

To assess short-term carry-over effects of larval foam-making activity, coupled with the extended terrestriality that necessitates it, we used a subset of seven larval sibships that began the foam-making experiment at age 12.5 days. For each sibship, we moved two or three 12.5 d larva to 150 ml of water in a plastic cup before draining their Petri dish to induce the remaining larvae to make foam. Like their siblings making a foam nest, the larvae in water were not fed, thus depended on yolk reserves. We collected two 14.5 d larvae after two days on land, constructing a larval nest, and compared their morphology with one sibling collected after two days in water (Fig. 1C). We also assessed the effects of repeated construction of larval foam nests on larval phenotypes by examining the morphology of siblings that had constructed one, two, and three nests (2 d per nest), collecting two additional larvae at each age (16.5, 18.5 d) from each nest. We collected larvae from the water treatment and following nest-construction by immersion in an overdose of the anesthetic MS-222 (tricaine methane sulfonate) at 250 g/L, buffered with sodium bicarbonate, and preserved them using buffered 10% formalin. Within three months of preservation, we staged each specimen under a dissecting microscope (Zeiss Stemi DV4 Stereo), following Gosner (1960), and took dorsal, lateral and ventral photographs with a Canon EOS 5D Mark III, including a scale. We used NIH ImageJ 2.0 to measure the nine linear dimensions most commonly used to compare tadpole morphology (McDiarmid & Altig, 1999) from these images. We measured total length (TL), tail length (TAL), tail muscle width (TMW), interorbital distance (IOD) and head width (HW) in dorsal view; tail muscle height (TMH) and tail height (TH) in lateral view; and yolk sac length (YSL) and yolk to mouth length (YML) in ventral view. We averaged measurements across siblings at each treatment level to conduct morphometric analysis. We assessed measurement repeatability by measuring photographs in triplicate for a random subset of 29 individuals, across sibships and treatment levels, and assessing the coefficient of variation for each measurement (mean CV = 1.76%, Table S1).

To assess long-term carry-over effects, we reared larvae to metamorphosis (Fig. 1D). These included one or two larva per sibship moved to water at 12.5 d (above, from 25 sibships) and

four more per sibship that were moved to water after they participated in making one or three larval foam nests (i.e., two each at age 14.5 d and 18.5 d, from 21 sibships at each age). Starting at 14.5 d, we fed aquatic larvae with rabbit chow *ad libitum*, replacing the food and about 80% of the water every second day. When larvae approached metamorphosis, we placed a small rock in their cup for metamorphs to climb, to avoid drowning, and began checking them daily for forelimb emergence (Gosner stage 42). At GS42, we weighed individuals to the nearest 0.1 mg with an electronic balance, photographed them in dorsal view with a scale, and measured their total length (TL) with ImageJ. Then we ceased providing food, reduced water level to about 1 cm to prevent drowning, and checked metamorphs daily for complete tail resorption (Gosner stage 46). At GS46, we measured their snout-vent length (SVL) to the nearest 0.1 mm with calipers and weighed them again. ~~Froglets were kept in their plastic cup, leaving a few millimeters of water to maintain hydration, until release at their original collection site that night or the following night.~~ We compared the periods from entry into the water until forelimb emergence (henceforth "aquatic larval period") and until complete tail resorption ("aquatic larval + metamorphic period"). We also compared size and age (measured from oviposition) at forelimb emergence and tail resorption.

Statistical analysis

We used linear mixed effects models (LMEM) (Bates et al., 2015; *lme4*) to test for differences in the volume of foam larvae produced after loss of their parental foam at three ages, with larval nest number (1, 2, 3) and size of larval group as covariables and sibship as a random factor. We used a linear model to compare ammonia and urea concentration in new larval foam nests made after 12.5 days in parental nests on wet vs. dry soil, and in the first vs. third larval foam nest. For first foam nests only, we assessed the effect of prior soil moisture on ammonia and urea concentration with the volume of liquid in the new nest as a covariable. We compared the amount of ammonia, urea, and predicted amount of ammonia excreted per larva in first larval nests across soil hydration treatments, and we tested if the amount of urea excreted per larva varied with the predicted ammonia concentration that could have accumulated in the new larval nest. We also tested for effects of soil hydration on subsequent proportion of N excreted as urea (i.e., urea-N/ammonia-N + urea-N) using a generalized linear mixed model (GLMMs) with an underlying Beta error distribution (Magnusson et al., 2019; *glmmTMB*) and likelihood ratio test

(LRT) to obtain p-values. We did not statistically analyze N-wastes for the first larval nest after 4.5 days in the parental nest, as it was measured in only a few sibships, all from wet soil (N=6); however, we include key descriptive statistics to compare with other ages in Table 1.

We tested for short-term effects of foam-making behavior and extended terrestriality on larval morphology in two ways: by comparison of 14.5 d larvae after 2 d making foam vs. 2 d in water, and by comparison of terrestrial larvae after making 1–3 nests (starting at age 12.5 d). We first used principal component analyses (PCA) to summarize the morphological variation across larvae measured for each comparison (Kassambara & Mund, 2017; *factoextra*). Then, we compared principal component scores (PC1 and PC2) for each analysis with LMEM, including sibship as a random factor. We tested for long-term effects of foam-making activity (0, 1, or 3 larval nests) on the age (from terrestrial oviposition) and aquatic larval period (from water entry) to forelimb emergence (GS 42), and the age and aquatic larval plus metamorphic period to tail resorption (GS 46). Then, we tested for effects of foam making, age, and aquatic time to these two transitions on measures of length (total length and SVL). We also tested for effects of foam-making, age, and aquatic larval (or larval + metamorphic) period on mass at each transition, including length (total or SVL) as a covariable. We used LMEM and used an AICc (corrected for small sample size) approach, using weighted AIC (AICcWt) and differences between best model all other models (deltaAICc), to choose the best models (Mazerolle, 2020; *AICcmodavg*). For the best model, we used ratio tests (LRT) to obtain p-values with a nested approach, removing some interactions between predictors when non-significant to estimate the main effects. We also calculated a conditional and marginal coefficient of determination, pseudo-R-squared, using *MuMIn* package (Barton 2023). We made pairwise comparisons (Tukey method), using the corresponding model structure in each case (Hothorn & Hothorn, 2009, *multcomp*). Model residuals were inspected for normality and homogeneity with the package DHARMa with 1000 simulations (Hartig, 2021). All statistical analyses were conducted in RStudio (version 1.1.463).

Results

Larval foam-making ability

Terrestrial larvae of *L. fragilis* were able to construct new foam nests at all tested ages (Fig. 2, Table S2). The total volume of foam produced by groups of sibling larvae increased with the number in a group ($X^2 = 22.39$, $p < 0.0001$), regardless of the age at transfer from their original

parental foam ($X^2 = 0.40$, Fig. 2A) and how many nests the larvae had made ($X^2 = 1.33$, $p = 0.51$, Fig. 2B). The foam volume per larva decreased with sibship size ($X^2 = 15.92$, $p < 0.0001$), with an interaction effect with age of transfer ($X^2 = 11.73$, $p = 0.003$, Fig. 2C) but no main age of transfer effect ($X^2 = 4.72$, $p = 0.094$). In smaller sibships, each larva made more foam, with the strongest effect for those that were oldest at transfer (12.5 days, Fig. 2C). However, the foam volume produced per larva (Fig. 2D) did not vary with how many nests the larvae had made ($X^2 = 2.12$, $p = 0.35$) or the age at transfer ($X^2 = 4.55$, $p = 0.10$).

Nitrogen excretion in larval foam nests

Ammonia was detected in larval foam nests at all tested ages, even shortly after hatching (age 4.5 d; Fig. 3A, Table 1), but urea was above the detection limit only after further development on land (by age 12.5 d; Table 1, Fig. 3B). In the first nest that larvae constructed after 12.5 d in parental foam, ammonia concentration was higher if the parental nest had been on dry soil ($F_{1,24} = 4.71$, $p = 0.04$, Fig. 3A). Ammonia concentration in larval foam was not affected by the number of larval nests constructed (first vs. third, $F_{1,24} = 1.37$, $p = 0.25$, Fig. 3A). Urea concentration in the new larval foam (first and third nests) was higher when individuals came from dry soil ($F_{1,21} = 6.99$, $p = 0.02$, Fig. 3B) and in the third vs. first larval nest ($F_{1,21} = 11.74$, $p = 0.002$, Fig. 3B), with no significant interaction effect.

Ammonia and urea concentrations decreased as the volume of water in first larval foam nests increased (ammonia, $t = -2.77$, $p = 0.01$; urea: $t = -2.41$, $p = 0.03$, Fig. 3C), with the volume being lower if the parental nest had been on dry soil ($t = -5.28$, $p < 0.0001$, Fig. 3C). Dry conditions experienced during development in parental nests on soil did not affect the estimated amount of ammonia accumulated in the larval foam nest (total: $t_{16.87} = -0.79$, $p = 0.40$; per larva: $t_{15.96} = -1.08$, $p = 0.295$, Fig. 3D, Table 1), nor did it significantly affect urea accumulation (total: $t_{10.10} = -1.85$, $p = 0.09$; per larva: $t_{10.31} = -1.64$, $p = 0.13$, Fig. 3D). However, the predicted ammonia concentration in the absence of the urea cycle explained urea excretion (total: $F_{1,15} = 140.08$, $p < 0.00001$; individual: $F_{1,14} = 133.43$, $p < 0.0001$), with no interaction effect with treatment. Dry conditions did not affect the predicted amount of ammonia accumulated (total: $t_{12.28} = -1.39$, $p = 0.18$; per larva: $t_{11.98} = -1.56$, $p = 0.14$, Fig. 3D), nor did it affect the proportion of total nitrogen excreted as urea (wet: 0.41 ± 0.20; dry: 0.49 ± 0.11, $X^2 = 1.87$, $p = 0.17$).

Arginase activity (μmol min^{-1} mg^{-1} of protein) in larval tissues was detected in all larval tissues and did not change between control and dry conditions ($t_{7.55}$ = -1.37, p = 0.21) nor did the urea concentration (μmol mg^{-1}) in tissues ($t_{7.55}$ = -0.69, p = 0.51, Table 1).

Short-term effects on larval size and morphology

Comparing the morphology of larvae at 14.5 d, after 2 d either in water or constructing a new larval foam nest, PC1, 2, and 3 accounted for 64.3%, 12.6% and 9.2% of variance, respectively (Fig. 4A, Table S3). Six measurements of body size made important contributions to PC1, all with positive loadings, with total length loading most heavily (Table S4). Tail muscle height and yolk sac length made the largest contributions to PC2, with positive loadings (Table S4). Tail muscle width and yolk sac length made the largest contributions to PC3, with positive and negative loadings, respectively (Table S4). Overall, there were morphometric differences between the larvae in these two groups (Manova, $F_{3,22}$ = 8.62, p = 0.0006, Pillai's $Trace_{1,24}$ = 0.54). At 14.5 d, after making a foam nest larvae had lower PC1 scores compared to their siblings that had been in water for two days (X^2 = 29.94, p < 0.0001, Fig. 4B), but similar PC2 scores (X^2 = 1.83, p = 0.175, Fig. 4C), and higher PC3 scores (X^2 = 4.97, p = 0.03).

Comparing across terrestrial larvae that had made 1 to 3 foam nests (age 14.5–18.5 d), PC1, 2 and 3 accounted for 64.45%, 14.09% and 8.93% of variance in morphology, respectively, (Fig. 4D, Table S3). Seven measurements of body size contributed strongly to PC1, all with positive loadings, with total length loading most heavily (Fig. 4D, Table S4). Tail muscle height and yolk sac length made the largest contributions to PC2, with positive loadings. Yolk sac length and tail height (positive loadings) and tail muscle height (negative loadings) contributed most to PC 3. Number of foam nests constructed did not affect scores on PC1 (X^2 = 3.98, p = 0.14, Fig. 4E), PC2 (X^2 = 0.80, p = 0.67, Fig. 4F) or PC3 (X^2 = 0.03, p = 0.98).

Carry-over effects of extended terrestriality and foam-making on larval development to metamorphosis

Measured from oviposition, age at forelimb emergence was explained (R^2m = 0.57; R^2c = 0.71) by larval nest-construction (X^2 = 105.61, p < 0.0001), but not by sibship size (X^2 = 1.87, p = 0.17). It was marginally different for larvae that made zero or one foam nest (Fig. 5A; p = 0.04),

but longer for those that made three nests (Fig. 5A; 0 vs. 3, $p < 0.001$; 1 vs 3, $p < 0.001$). The aquatic larval period (water entry to forelimb emergence) was between 15 and 28 days (Table S5). Variation in this period was not explained ($R^2m = 0.07$; $R^2c = 0.36$) by larval foam-making ($X^2 = 5.20$, $p = 0.07$) or sibship size ($X^2 = 1.87$, $p = 0.17$). Tadpoles that made one or three nests had an aquatic larval period similar to siblings that made zero nests (Fig. 5A; 0 vs 1, $p = 0.20$; 0 vs 3, $p = 0.91$; 1 vs 3, $p = 0.07$).

Measured from oviposition, age at tail resorption was explained ($R^2m = 0.47$; $R^2c = 0.65$) by larval nest construction ($X^2 = 64.22$, $p < 0.0001$), but not by sibship size ($X^2 = 2.31$, $p = 0.12$). At tail resorption, individuals that made one nest were similar to siblings that made zero nests (Fig. 5B; $p = 0.88$), but younger than those than made three nests (Figure 5B; 0 vs 3, $p < 0.001$; 1 vs 3, $p < 0.001$). The period from water entry to tail resorption was 20 to 33 days (Table S5). Variation in this time was not explained ($R^2m = 0.04$; $R^2c = 0.36$) by larval nest-making (Fig. 5B; $X^2 = 0.99$, $p = 0.61$) and only marginally by sibship size ($X^2 = 2.31$, $p = 0.05$).

Total length of larvae at forelimb emergence was explained by a model that included a negative effect of the aquatic larval period ($R^2m = 0.12$; $R^2c = 0.38$; X2 =4.41, $p = 0.04$, Fig. 5E), but not age or larval foam-making (Fig. 5C). This model also included sibship size with a positive effect on total length ($X^2 = 4.19$, $p = 0.04$; Fig. 5G). Snout–vent length (SVL) at tail resorption was best explained by a model that included a negative effect of either age or aquatic period ($R^2m = 0.15$; $R^2c = 0.47$; $X^2 = 6.68$, $p = 0.01$; $R^2m = 0.12$; $R^2c = 0.48$; $X^2 = 3.92$, $p = 0.05$, respectively; Fig. 5D, F), but neither model included an effect of larval foam-making. In these models, number of siblings in the group had only a marginal positive effect on SVL at tail resorption (age: $X^2 = 3.78$, $p = 0.05$; aq. + metamorphic period: $X^2 = 3.36$, $p = 0.07$; Fig. 5H), with no interaction effects.

Mass at forelimb emergence was best explained ($R^2m = 0.68$; $R^2c = 0.74$) by a model including a positive effect of total length at forelimb emergence ($X^2 = 91.62$, $p < 0.0001$; Fig. 6A), a negative effect of either aquatic period (Fig. 6C) or age ($X^2 = 5.43$, $p = 0.02$; $X^2 = 8.02$, $p = 0.005$, respectively), and a marginal positive effect of sibship size ($X^2 = 3.05$, $p = 0.08$, Fig. 6E). Mass at tail resorption was best explained by a model ($R^2m = 0.76$; $R^2c = 0.83$) including a

positive effect of SVL at tail resorption ($X^2 = 94.30$, $p < 0.0001$; Fig. 6B) and a marginal interaction with sibship size ($X^2 = 3.52$, $p = 0.06$). The number of siblings in the foam, the time from water entry to tail resorption or age at tail resorption did not affect mass at tail resorption once SVL was included (Fig. 6D, F). Larval nest construction did not affect mass at forelimb emergence or tail resorption.

Discussion

We found that terrestrial larvae of *L. fragilis* are highly capable of constructing and maintaining their own foam nests. They can start making nest foam within a day of hatching and retain this ability for at least two weeks longer. Throughout this period, larvae have sufficient energy reserves to construct multiple entirely new nests, with no evidence of developmental changes in this ability (Fig. 2). Moreover, foam-making appears metabolically expensive, as we found very high levels of ammonia and, for older larvae, urea in newly constructed larval nests (Fig. 3). We found that larvae grew more after entering the water, compared to their siblings on land who were constructing foam nests. Larval size and morphology remained similar throughout a prolonged terrestrial period and the construction of multiple nests, suggesting their growth and development was arrested (Fig. 4). While substantial research has examined parental strategies to construct or modify developmental environments for their offspring, with ecological and evolutionary consequences (Campos-Cerda & Bohannan, 2020; Laland et al., 2017), our results highlight the importance of developmental environments constructed by early life stages themselves. The ecological and physiological importance of such larvae-constructed environments are recognized in insects (e.g. Tonelli et al., 2018; Williams & Simon, 1995; Baer & Marquis, 2020; Oliveira et al., 2016) but less studied in vertebrates. However, they could play a crucial role for survival of young in unpredictable environments, for instance in the vertebrate colonization of land. We are not aware of studies addressing the ability of early larvae to modify their developmental environment in other frog lineages, or their potential consequences during development; indeed, such larval behavior has been previously described in just a few groups of leptodactylids, all with initial or complete larval development in subterranean foam nests (Caldwell & Lopez, 1989; Downie, 1984; Giaretta et al., 2011; Kokubum & Giaretta, 2005).

Environmental conditions during both terrestrial embryonic development and the aquatic larval period of frogs have been documented to affect fitness-related traits at subsequent life-history

transitions (see introduction). In contrast, the potential carry-over effects of environmental conditions for terrestrial frog larvae, with associated behavioral and metabolic demands, have been relatively unexplored, although delayed physiological costs may occur (Burraco et al., 2017; Murillo-Rincón et al., 2017b). We hypothesized that the energetic costs of repeated nest construction and urea excretion during an extended non-feeding period on land would have carry-over effects on the aquatic larval period or size at metamorphosis; however such effects were not apparent. Larvae that made three nests were older at at forelimb emergence (GS 42) and tail resorption (GS 46), but they spent a similar period in the water as their non-nest-making siblings (Fig. 5A) and emerged at a similar size (Fig. 5B). We found that both older individuals and those with longer aquatic larval periods were shorter and lighter at metamorphosis (Fig. 5C—F; Fig. 6), regardless of their foam-making history, and those from larger families tended to be larger, suggesting a maternal effect (Fig. 5G, H).

Foam-making ability and prolonged larval survival on land

Foam nests provide a critical, parentally constructed microhabitat that enables embryos, then larvae, of Leptodactylid frogs to survive for prolonged periods on land (Downie, 1984; Heyer, 1969; Méndez-Narváez et al., 2015). However, over time and with drying, the nests that parents provide deteriorate, and they can dissolve in rain without freeing larvae into pools (Caldwell & Lopez, 1989; Downie, 1984). Our results suggest the importance of larval foam-making ability in facilitating survival through a prolonged period on land in *L. fragilis*. After hatching, larvae remain in the parental foam nest waiting for rain (Fig. 1B), as in other terrestrial-breeding leptodactylids (Downie, 1984). However, the parental foam loses its integrity over time and changes in nest structure (Fig. 1C) reflect the ability of larvae to create new foam. In many cases, larvae may gradually add foam to maintain their nest as parental foam deteriorates but, if necessary, they can construct an entirely new replacement nest (Fig. 1C; Fig. 2). Contrary to our prediction, we found no evidence that larval foam-making ability increases with age, as the likelihood and extent of parental foam loss or deterioration increases. It appears to be similar from age 4.5–18.5 days, based on the foam volume produced at our three tested ages (Fig. 2A) and across multiple nest-construction events (Fig. 2B). This suggests that through this entire period the larvae are fully competent to reconstruct the microhabitat they need to survive in soil.

Nonetheless, our experimental protocol may not have captured more subtle differences in the speed with which larvae replaced their nests.

Although foam-making appears metabolically expensive, it may also be highly adaptive, particularly when incomplete flooding occurs and larvae must remain in the soil awaiting another rainfall (Fig. 1B). Out of the nest, well-grown tadpoles (GS30–35) of the closely related *L. fuscus* survive better in damp mud than do heterospecific aquatic tadpoles (e.g. *Engystomops pustulosus* and *Rhinella beebei*), but they still suffer 35% mortality within 48 h and survivors show a loss of wet mass (Downie & Smith, 2003). We do not know how long new larval nests can last, but considering their small size it seems likely that larvae may need to continually or repeatedly produce foam, either to maintain their nests or to replace them if another flooding event fails to release them from their chamber.

The high variability of rainfall may have selected for this high foam-nesting capacity across early larval development and through a period of developmental arrest. Even short periods without rain have been associated with increased risk of mortality by dehydration for terrestrial embryos in three Neotropical frog lineages: *Dendropsophus* (Touchon & Warkentin, 2009), Centrolenidae (Delia et al., 2013), and Phyllomedusinae (González et al., 2021; Salica et al., 2017). In these lineages, previous studies described parental strategies, such as oviposition site plasticity and extended male care, to prevent embryo mortality by dehydration (Delia et al., 2019; 2020; Touchon et al., 2011; Touchon & Warkentin, 2008). However, in these lineages the embryos hatch and enter the water earlier when faced with dry conditions (Salica et al., 2017; Touchon & Warkentin, 2010) high temperature (Guevara-Molina et al., 2022) or high ammonia levels (Lisondro et al., 2024), and only extend their development on land under good hydration (Delia et al., 2019). In other amphibians and fishes with terrestrial eggs, developmental arrest and low embryonic metabolism have been associated with prolonged time on land while waiting for a flooding cue to hatch (Bradford & Seymour, 1985; Martin, 1999; Petranka & Petranka, 1981). In such species, metabolic costs have been reported in the context of soil dehydration, and their effect on growth rate and development was reported during embryonic development (Seymour et al., 1991), but not assessed at later stages. The foam-making ability of *L. fragilis* functions as an

adaptive behavioral response of larvae to variability in their terrestrial environmental and the loss of extended benefits conferred by a parentally provided structure.

Parental foam-nesting behavior is hypothesized to have facilitated the transition from aquatic to terrestrial breeding in the family Leptodactylidae (Heyer, 1969; Méndez-Narváez et al., 2015). For instance, foam may delay water loss, decreasing dehydration risk (Zina, 2006), and provide a thermal buffer (Méndez-Narváez et al., 2015). We do not know to what extent larval foam fulfills equivalent functions as those hypothesized or demonstrated for parental foam nests, particularly during prolonged larval development on land. However, the bubbles trapped in foam may facilitate oxygen uptake to sustain metabolism (Seymour & Loveridge, 1994; Seymour & Roberts, 1991), and larval foam may decrease the tight, gravity-mediated packing of siblings into the bottom of their chamber, spreading them out to improve oxygen uptake (Seymour, 1999). It would be worth assessing the selective factors and fitness consequences of foam-making from the perspective of both parents and offspring in terrestrial breeding Leptodactylids, in particular within an extended phenotype and niche construction framework, given this apparent parent-offspring convergence in ecology and behavior (Badyaev & Uller, 2009; Laland et al., 2017; Uller, 2008). Both parental and larval foam nests may facilitate niche exploitation, by increasing fitness (both stages) and survival (offspring), and have facilitated aquatic to terrestrial breeding transitions in some members of the family Leptodactylidae (Heyer, 1969; Méndez-Narváez et al., 2015; Méndez-Narváez & Warkentin, 2022). Currently, the increasing frequency of short periods without rainfall during the rainy season (Touchon & Warkentin, 2009) is likely increasing both foam nest dehydration and the need for larval foam-making, increasing the metabolic costs associated with preventing ammonia toxicity (Méndez-Narváez & Warkentin, 2022).

Nitrogen excretion in larval foam nests

Terrestrial embryos and larvae can face a waste-disposal problem, as nest dehydration increases ammonia concentration in their developmental environments, increasing the risk of toxicity (Méndez-Narváez & Warkentin, 2022). At 12.5 days, nest-dwelling larvae of *L. fragilis* increase their urea excretion under dry conditions in soil (Méndez-Narváez & Warkentin, 2022) and their tissues exhibit high activity levels of two key urea cycle enzymes, CPSase 1 and arginase (Méndez-Narváez & Warkentin, 2023). Here we found that, over just 2 days, very high

572 concentrations of ammonia and urea accumulated in new larval foam nests (Table 1, Fig. 3). For
573 instance, measurements of parental nests in dry soil at 12.5 d found 53.5 ± 48.9 mmol/L
574 ammonia and 59.2 ± 71.0 mmol/L urea-N (See Méndez-Narváez & Warkentin, 2022; Table A1;
575 Fig. 3C, D). Here, larvae from such conditions produced new nests containing 195.8 ± 150.1
576 mmol/L ammonia and 461.6 ± 510.4 mmol/L urea-N (Table 1), over 3-fold and 8-fold higher,
577 respectively. Although larval nests are smaller, these high concentrations are largely due to
578 higher amounts of ammonia and especially urea accumulated per larva (parental nests, Méndez-
579 Narváez & Warkentin, 2022: 0.01 ± 0.01 µmol and 0.01 ± 0.01 µmol; larval nests, this study,
580 Table 1: 0.03 ± 0.02 µmol and 0.06 ± 0.06 µmol; ammonia and urea respectively). This suggests
581 a high metabolic cost of foam production, particularly since these terrestrial larvae have already
582 entered developmental arrest (N Belduque-Correa, K M Warkentin and J Méndez-Narváez 2019,
583 unpublished data). Because these terrestrial larvae have no access to external food, their nitrogen
584 wastes are byproducts of protein catabolism from the yolk reserves that provide energy for
585 differentiation, growth, and activity (Dworkin & Dworkin-Rastl, 1991; Finn et al., 1995;
586 Jorgensen et al., 2009). We found no urea, only ammonia, accumulated in foam nests produced
587 by 4.5 d larvae, suggesting that these early larvae lack the enzymatic mechanisms to synthesize
588 urea. This is consistent with our previous findings of no urea in parental foam nests at age 8.5
589 days (Méndez-Narváez & Warkentin, 2022). Nonetheless, ammonia levels in new nests produced
590 by 4.5 d larvae were over twice as high as levels accumulated in parental nests after 12.5 d in dry
591 soil, and half of them were well into the range where mortality occurs for larvae of *L. fragilis* in
592 water (Méndez-Narváez & Warkentin, 2022), again emphasizing the metabolic cost of foam-
593 making. These ammonia levels did not increase further, for older larvae (12.5 d) or with
594 sequential construction of multiple larval nests; rather, larvae began to excrete urea.
595
596 Urea excretion can prevent the accumulation of high ammonia levels in developmental
597 environments during prolonged periods of embryonic or larval life on land (Méndez-Narváez &
598 Warkentin, 2022). Nonetheless, the ammonia levels that accumulated over 2 days as larvae
599 constructed new foam nests, after 12.5 d in parental nests on soil (Fig. 3A, Table 1), were often
600 within the range that caused 50% mortality of 12.5 d larvae in aquatic ammonia solutions (95%
601 CI for 48 h-LC_{50}: 108–122 mmol/L; Méndez-Narváez & Warkentin, 2022), and concentrations
602 were especially high for larvae that came from dry soil (Table 1), probably because they had less

603 water for foam hydration (water in larval nests: 0.03 vs. 0.02 ml, from wet and dry soils
604 respectively, Fig. 3C). Despite this, we observed no larval mortality during our nest-construction
605 trials. This may be a result of the arrested development and potentially lower metabolism of nest-
606 dwelling vs. aquatic larvae. However, ammonia concentrations predicted to have accumulated
607 without the urea cycle, assuming all urea came from conversion of ammonia (Table 1), are
608 several orders of magnitude higher than predicted levels in parental nests in soil and, at least for
609 larvae that came from dry soils, above the concentration that would lead to complete mortality of
610 aquatic larvae (Méndez-Narváez & Warkentin, 2022). By the third larval nest, foam made by
611 larvae from dry vs. wet soil had similar ammonia levels and, in both cases, their predicted
612 ammonia level would be lethal for aquatic larvae (Fig. 3, Table 1).
613
614 The total amount of accumulated N-waste per larva (and thus potential ammonia level) was
615 higher in parental nests in dry vs. wet soil, suggesting that metabolic demands vary with
616 hydration during prolonged time on land (Méndez-Narváez & Warkentin, 2022). Thus, elevated
617 metabolism may be a direct consequence of higher larval foam-making activity to supplement
618 the parental foam, which deteriorates faster in dry soil, as well as a cost of ammonia
619 detoxification under the higher levels of accumulated ammonia (Méndez-Narváez & Warkentin,
620 2022). Here, we did not find significantly more ammonia or urea in nests produced by larvae
621 from dry soils; rather, their higher concentrations were correlated with lower water volumes (Fig.
622 3C). Nonetheless, we found that urea excretion was explained by the predicted ammonia levels
623 in the new larval nest, as it was in parental nests in wet and dry soils (Méndez-Narváez &
624 Warkentin, 2022). The higher metabolic cost of urea synthesis through the urea cycle, compared
625 to ammonia excretion (Shambaugh, 1977; Wright & Wright, 1996), may favor plasticity in N-
626 waste excretion with time on land, moisture availability, and foam-making efforts, to reduce this
627 cost when possible. Especially for larvae from dry soil, we found arginase activity in larval
628 tissues to be higher for 14 d larvae that had made new nests compared to earlier measurements
629 for 12 d larvae maintained in their parental nests (on wet soil: 0.08 vs. 0.07, on dry soil: 0.11 vs.
630 0.06 μmol min^{-1} mg^{-1}, respectively; Table 1 and Méndez-Narváez & Warkentin, 2023). Dry
631 conditions on land may favor earlier and/or greater urea excretion, as ammonia concentrates in
632 nests losing water and the metabolic cost of larval foam-making, to replace deteriorating parental
633 foam, is met by protein catabolism that increases N-wastes. A similar scenario may occur if early

flooding fails to release larvae into ponds, so that wastes produced as trapped, nestless larvae make new foam accumulate in a small volume of foam. Thus, larval foam-making may be a key trait that both enables survival on land and necessitates a high capacity for ammonia detoxification. Moreover, it would be worth studying other physiological consequences of high activity during foam-nest construction or exposure to high ammonia that may affect long-term survival, such as oxidative stress and antioxidant activity, where the relationship with the energy budget is complex (Costantini, 2019; Guo et al., 2023; Zamora-Camacho et al., 2023).

We found arginase activity in nest-makers from dry soil close to that in aquatic tadpoles after 4 days in high environmental ammonia (Méndez-Narváez & Warkentin, 2023: 0.13 μmol min^{-1} mg^{-1}, measured at 12.5 d). Although arginase, which produces urea from arginine, catalyzes the last step in the urea cycle (Mommsen & Walsh, 1989), this enzyme has other metabolic roles (Srivastava & Ratha, 2010; Yina et al., 2016). Thus, its activity is necessary, but not sufficient, to demonstrate a functional ornithine urea cycle (OUC). For instance, arginase was always detected in the tissues of *A. callidryas* and *H. fleischmanni* embryos from drying eggs but carbamoyl phosphate synthetase (CPSase), whose expression often limits rates of urea synthesis (CPSase 1; Brown et al., 1959; Wright et al., 1995), was sometimes undetectable (Méndez-Narváez & Warkentin, 2023). Although a more sensitive test for CPSase 1 might reveal some activity, its absence may instead reflect the facultative nature of extended terrestriality in these species, as embryos can hatch to escape dehydration (Delia et al., 2014; Salica et al., 2017). Although we did not test for CPSase activity in this study, in the same *L. fragilis* population larvae in parental foam nests or high-ammonia water show elevated CPSase (1 + 2) activity, compared to larvae in low-ammonia water (Méndez-Narváez and Warkentin, 2023). This suggests these larvae constitutively express a high capacity for urea excretion on land, which may be key to enable larval survival through an unpredictably prolonged period waiting for rains (Méndez-Narváez & Warkentin, 2023). If OUC capacity is already sufficient to meet detoxification needs, enzyme activity need not change with environmental challenge (Chew et al., 2004; Loong et al., 2005; Méndez-Narváez & Warkentin, 2023; Steele et al., 2001; Weng et al. 2004;Wright & Wright, 1996). Nonetheless, even without enzyme up-regulation, urea synthesis itself consumes energy, as must the bubble-blowing behavior and mucus secretion required for nest construction. These

664 energetic costs would presumably accumulate as larvae remain on land longer and engage in
665 more foam-making activity, drawing on their yolk reserves.
666
667 **Short-term consequences of extended terrestriality and foam-making**
668 We found that prolonging larval life on land has immediate effects on growth and morphology;
669 particularly, unfed larvae were larger after two days in water compared with age-matched
670 siblings that remained on land making a new foam nest (Fig. 4A, B). Environmentally cued
671 changes in the timing of hatching and transition to water for terrestrial frog embryos have
672 immediate consequences for morphology and development after hatching (Delia et al., 2019;
673 Touchon & Warkentin, 2010; Warkentin, 1999). Previous studies have found higher mortality of
674 smaller larvae when exposed to aquatic predators (Gomez-Mestre et al., 2008; Vonesh, 2005;
675 Warkentin, 1995), although larval mortality also depends on the types of predators in a pond,
676 which may differentially consume different prey sizes (Willink et al., 2014). The size difference
677 we found between age-matched aquatic and terrestrial larvae resembles that between the aquatic
678 larvae vs. terrestrial embryos of the grunion fish, *Leurethes tenuis*, which largely arrest
679 development to prolong survival while awaiting flooding (Moravek & Martin, 2011). Moreover,
680 terrestrial egg clutches may impose physiological constraints on growth and development, as
681 suggested to explain the accelerating effect of hatching on growth and differentiation in *A.*
682 *callidryas* (Warkentin, 1999; 2007). It is possible that terrestrial foam nests may similarly
683 impose such constraints, including metabolic depression due to accumulation of urea in tissues
684 (Muir et al., 2008; Yancey & Somero, 1979). Yolk reserves were similar between age-matched
685 aquatic and terrestrial siblings in *L. fragilis*, suggesting that larvae deplete yolk similarly in both
686 environments, but for different functions (Fig. 3C). Large yolk reserves are hypothesized to
687 extend embryonic survival times on land in *L. tenuis* and the frog *Pseudoprhyne bibronii*
688 (Bradford & Seymour, 1985; Moravek & Martin, 2011). Terrestrial leptodactylid embryos also
689 have large yolk reserves, compared to aquatic-nesting frogs in the same family (Méndez-
690 Narváez, 2012; Pereira et al., 2015). However, rapid consumption of these reserves could explain
691 the rapid growth observed for initially unfed *L. fragilis* larvae in water (Fig. 4A, B), as in *A.*
692 *callidryas* and *L. tenuis* (Martin et al., 2011; Moravek & Martin, 2011; Warkentin, 1999).
693

Extended development in terrestrial eggs has been associated with morphological changes that benefit larvae when they transition to water, such as larger, stronger tails that improve swimming and gut development that reduces time to external feeding (Delia et al., 2019; Warkentin, 1999). We found no such differences in larval size or form with prolonged time on land constructing foam, e.g., comparing 14.5 to 18.5 d siblings that made one to three nests (Fig. 4D—F), suggesting the delay confers no benefit upon entry into water. However, our design cannot separate effects of time on land from those of foam-making activity *per se*, thus the effects of prolonged terrestriality in a persistent parental nest may differ. There is evidence that continued terrestrial development during the first five days after hatching at about 3.5 days may benefit larvae. During this period, nest-dwelling larvae of *L. fragilis* increase in size and show substantial morphological change; then their growth and development seem to stop (N Belduque-Correa, K M Warkentin & J Méndez-Narváez 2019, unpublished data). Their terrestrial persistence after 8.5 days could be considered a period of developmental arrest, as described for nest-dwelling larvae of *L. fuscus* (Downie, 1994), although older larvae of *L. fragilis* are clearly still behaviorally and metabolically active, as evidenced by their foam-making capacity (Fig. 2) and excretion of nitrogen wastes (Méndez-Narváez & Warkentin, 2022; 2023). Grunion fish embryos show a linear increase in metabolism during development to hatching competence, then can remain in a steady high metabolic state for several weeks, using oil from yolk reserves (Darken et al., 1998). In contrast, a 90% decrease in oxygen consumption can occur with developmental arrest of somite proliferation and DNA content in the annual killifish *Austrofundulus limnaeus* (Podrabsky & Hand, 1999). As suggested for *L. tenuis* embryos, it may be advantageous for *L. fragilis* larvae to retain metabolic activity in an unpredictable terrestrial environment, both to make new foam if needed and to take advantage of brief flooding opportunities to escape from their chambers.

Carry-over effects of extended terrestriality and foam-making on development to metamorphosis

Size advantages evident when larvae enter the water do not necessarily persist; compensatory growth can occur during aquatic development of initially smaller larvae, allowing them catch up, either rapidly or at some point before metamorphosis (Touchon et al., 2013; Touchon & Warkentin, 2010). On the contrary, reduced food availability and high larval density can both

delay metamorphosis and result in emergence of smaller, shorter-legged frogs (Bouchard et al., 2016; Gomez-Mestre et al., 2010). We found no evidence for compensatory growth in those larvae that spent two or six extra days (from age 12.5 to 14.5 or 18.5 d) on land, making new foam nests, nor did these conditions affect morphology at metamorphosis. Neither did we find any long-term delay. These larvae had a similar aquatic larval period (Fig. 5A) and metamorph size and weight as their siblings that made no new nest and entered the water at age 12.5 days (Figs 5C–F; Fig. 6). Our results suggest that well-adapted terrestrial *L. fragilis* larvae cope with an unpredictably prolonged period on land and associated metabolic costs without long-term consequences for fitness-relevant phenotypic traits and development to metamorphosis. They seem to avoid potential carry-over effects on growth and development, and physiological consequences that in some contexts reduce survival, which have been documented in other species (Burraco et al., 2017; 2020).

Larval adaptations and potential parental effects of egg size may mitigate costs of extended terrestrial development on subsequent aquatic development to metamorphosis in *L. fragilis*. These terrestrial endotrophic larvae can use their large yolk reserves for energy to construct new foam nests and synthesize urea to prevent ammonia toxicity (Fig. 2; Fig. 3). By arresting development after 8 days, larvae enter water at a similar size and shape even after building multiple foam nests (Fig. 4E, F); alternatively, if young larvae enter the water they experience an initial period of rapid growth (Fig. 4B, C), which may also be enabled by their large yolk reserves. However, as we did not test the youngest hatchling larvae, with the largest yolk reserves at their earliest possible time of water entry, we do not know if our observed metamorphic phenotypes are already carrying a cost of prolonged terrestrial life or, alternatively, may represent a form of canalized phenotype.

We found variation in metamorph size that was not explained by larval foam-making but was correlated with aquatic period and, in some cases, sibship size (Fig. 5). Some sibships grow faster and metamorphose early and large, whereas others grow slower and metamorphose later and smaller (Fig. 5C, F). In anurans, larger body size at metamorphosis is associated with higher fitness in post-metamorphic stages (Cabrera-Guzmán et al., 2013; Gomez-Mestre et al., 2010; Nicieza et al., 2006; Scott et al., 2007). However, a negative correlation of size with age, as in

our study, can occur when larvae experience growth-constraining conditions before the onset of feeding (Bouchard et al., 2016; Gomez-Mestre et al., 2010). It is worth assessing benefits of both larger size and younger age at metamorphosis, as slower-growing larvae may sacrifice size to limit their larval period. Moreover, exposing aquatic larvae to harsh environmental conditions (e.g. pond drying) can trigger plastic changes in size and time to metamorphosis (Gomez-Mestre et al., 2010; 2013); these would not be evident under our experimental conditions. As *L. fragilis* larvae develop in temporary ponds with high risk of drying, short larval periods may sometimes be crucial for survival, as in other frogs that must either arrive early or leave ponds quickly to escape pond drying (Laurila & Kujasalo, 1999; Murillo-Rincón et al., 2017a). This contrasts with species that use longer-lasting pools and, without such time constraints, may have longer and more broadly variable larval periods (Bouchard et al., 2015; Touchon et al., 2013b).

Our results suggest that the initial clutch size, a maternal effect, may have consequences at multiple levels during development. Evolutionary and intraspecific changes in clutch characteristics, such as the number and size of eggs, have been studied in the context of allocation of reproductive effort, parent-offspring co-evolution and evolutionary transitions in reproductive modes and parental care behavior (Delia et al., 2020; Gomez-Mestre et al., 2012; Kasimatis & Riginos, 2016; Pupin et al., 2010). Scaling relationships between individual traits and body size affect morphology and development, and are also relevant in physiological and ecological contexts (Gould, 1966; Shrimpton et al., 2021; White et al., 2019). We found the relationship between sibship size and the amount of foam produced was not isometric (Fig. 2A, B). Rather, there was a negative correlation between sibship size and foam volume per larva; larvae in large families produced less foam per capita, suggesting the possibility of energy savings in larger groups. This might explain the trend toward positive effects of sibship size on size at metamorphosis (Fig. 5 G, H). This trend is opposite to more commonly studied group size or density effects, such as those mediated by intraspecific competition for food in aquatic environments. For instance, high larval density or low per capita food availability can affect larval nutritional traits (fat bodies and gut morphology) with lasting effects on metamorph or juvenile body size (Gomez-Mestre et al., 2010; Bouchard et al., 2015).

Conclusions

Early life stages that are compelled, by unpredictable external factors, to spend an extended time on land do not only sit and wait for better developmental conditions or the opportunity to move to water, but actively respond to environmental challenges with adaptive phenotypes (Alcocer et al., 1992; Mitchell & Seymour, 2000; Mueller et al., 2012; Méndez-Narváez & Warkentin, 2022; 2023; Caldwell & Lopez, 1989; Downie, 1984). We hypothesized that plastic larval responses that enable extended terrestrial survival in *L. fragilis* involve energetic costs that carry over to affect the aquatic larval period and size at metamorphosis. We found that larvae of *L. fragilis* are well-adapted to respond to terrestrial challenges such as dehydration risk, accumulation of nitrogen wastes, and loss of the parental foam nest. These larvae experience enforced fasting and rely on yolk reserve while awaiting flooding of their nest chambers. Although they enter developmental arrest, ceasing morphological change, larvae are metabolically and behaviorally active. Sibling groups can quickly construct a new foam nest, multiple times if needed, starting soon after hatching, with no evidence for changes in their ability to do so over a two-week period. Moreover older larvae show a high capacity for urea synthesis that functions to prevent ammonia toxicity. Compared to siblings that remain on land making foam nests, aquatic larvae rapidly increase size, even while fasting, presumably by a shift in yolk utilization. However, carry-over effects of extended terrestriality and nest-making were not apparent at metamorphosis. Large yolk reserves and temporary developmental arrest seem to allow unfed larvae to meet high energy demands during active periods on land and may explain this apparent lack of lasting carry-over effects on development. Nonetheless, we found some effects on metamorph size that could be mediated by maternal effects in cooperative groups, when siblings face challenges during early development on land.

Our work emphasizes the value of studying changes in metabolism and behavior, in addition to morphological traits not captured by standard staging tables (Moravek & Martin, 2011; Warkentin, 1999), in anamniotic vertebrates that have evolved plastic extensions of terrestriality in early life. Our results also suggest that terrestrial early life stages can construct and modify their microenvironment and alter their physiology to survive harsh conditions or take advantage of more benign ones, without necessarily paying long-term costs. These performance traits of early life stages could be just as important as parental strategies for enabling evolutionary colonization of new environments.We suggest that further study of the behavioral and

physiological ecology of early stages of semi-terrestrial vertebrates across habitat transitions would be valuable. As well as performance traits facilitating terrestrial survival, further studies could assess costs and potential carry-over effects in the context of subsequent challenges for aquatic larvae, such as predation risk, nutritional challenges, and pond drying. Elucidating the adaptive plastic responses of early life stages to their variable environments across terrestrial-to-aquatic transitions, and the context-dependent longer-term fitness consequences of those responses, would improve our understanding of the evolutionary importance of changes in early life stages for the reproductive colonization of land. It would also be useful for predicting responses and understanding vulnerability in the context of climate and habitat change that are altering developmental environments for amphibians.

Acknowledgements

We thank Carolina Amorocho for field assistance, Team Treefrog for sharing experiences during field work and the Gamboa Frog Group and allies for their intellectual support in Panama. We thank Jack Friend for his assistance measuring larval morphology. We also thank Rachel Page and Roberto Ibañez for enabling this research in Panamá. Nitrogen quantifications were conducted in the STRI Biogeochemistry lab, which was led at the time by Dr. Ben Turner. We also thank Richard Wilkie and Oana Birceanu for training JMN in enzymatic analysis at Wilfrid Laurier University and Jennifer Bhatnagar and Corrine Vietorisz for facilitating laboratory work at Boston University. We thank Javier's PhD committee—Chris Schneider, Jennifer Bhatnagar, Sean Mullen, Ivan Gomez-Mestre, and Peter Buston—for their intellectual support.

841 **References**

842 Alcocer, I., Santacruz, X., Steinbeisser, H., Thierauch K. H., & del Pino E. M. (1992).
843 Ureotelism as the prevailing mode of nitrogen excretion in larvae of the marsupial frog
844 *Gastrotheca riobambae* (Fowler) (Anura, Hylidae). *Comparative Biochemistry and*
845 *Physiology Part A: Physiology 101*(2): 229–231. https://doi.org/10.1016/0300-
846 9629(92)90527-W
847 Amemiya, C. T., Alföldi, J., Lee, A. P., Fan, S., Philippe, H., MacCallum, I., Braasch,
848 I., Manousaki, T., Schneider, I., Rohner, N., Organ, C., Chalopin, D., Smith, J.
849 J., Robinson, M., Dorrington, R. A., Gerdol, M., Aken, B., Biscotti, M. A., Barucca, M.,
850 … Lindblad-Toh, K. (2013). The African coelacanth genome provides insights into
851 tetrapod evolution. *Nature*, 496(7445), 311–316. https://doi.org/10.1038/nature12027
852 Badyaev, A. V., & Uller, T. (2009). Parental effects in ecology and evolution: Mechanisms,
853 processes and implications. *Philosophical Transactions of the Royal Society B:*
854 *Biological Sciences*, *364*(1520), 1169–1177. https://doi.org/10.1098/rstb.2008.0302
855 Baer, C. S., & Marquis, R. J. (2020). Between predators and parasitoids: Complex interactions
856 among shelter traits, predation and parasitism in a shelter-building caterpillar community.
857 *Functional Ecology*, *34*(10), 2186–2198. https://doi.org/10.1111/1365-2435.13641
858 Bardua, C., Fabre, A. C., Clavel, J., Bon, M., Das, K., Stanley, E. L., Blackburn, D. C., &
859 Goswami, A. (2021). Size, microhabitat, and loss of larval feeding drive cranial
860 diversification in frogs. *Nature Communications*, *12*(1), 2503.
861 https://doi.org/10.1038/s41467-021-22792-y
862 Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models
863 using lme4. *Journal of Statistical Software, 67*(1), 1–48.
864 https://doi.org/10.18637/jss.v067.i01
865 Bonifas, S., & Bouchard, S. (2021). Competition induces silver spoon effects in developing
866 anuran larvae. *Hydrobiologia*, *848*, 1219–1230. https://doi.org/10.1007/s10750-020-
867 04492-8
868 Bouchard, S. S., O'Leary, C. J., Wargelin, L. J., Charbonnier, J. F., & Warkentin, K. M. (2016).
869 Post-metamorphic carry-over effects of larval digestive plasticity. *Functional Ecology*,
870 *30*(3), 379–388. https://doi.org/10.1111/1365-2435.12501

871 Bouchard, S. S., O'Leary, C. J., Wargelin, L. J., Rodriguez, W. B., Jennings, K. X., &
872 Warkentin, K. M. (2015). Alternative competition-induced digestive strategies yield
873 equal growth, but constrain compensatory growth in Red-eyed treefrog larvae. *Journal of*
874 *Experimental Zoology Part A: Ecological Genetics and Physiology*, *323*(10), 778–788.
875 https://doi.org/10.1002/jez.1991
876 Bradford, D. F., & Seymour, R. S. (1985). Energy conservation during the delayed-hatching
877 period in the frog *Pseudophryne bibroni*. *Physiological Zoology*, *58*(5), 491–496.
878 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram
879 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*,248
880 254.https://doi.org/10.1006/abio.1976.9999
881 Brocchi, P. (1877). Sur quelques batraciens raniformes et bufoniformes de l'Amérique Centrale.
882 Bulletin de la Société Philomathique de Paris. 7,175–197.
883 Brown, G. P., & Shine, R. (2009). Beyond size–number trade-offs: Clutch size as a maternal
884 effect. *Philosophical Transactions of the Royal Society B: Biological Sciences*,
885 *364*(1520), 1097–1106. https://doi.org/10.1098/rstb.2008.0247
886 Brown, G. W., Brown, W. R., & Cohen, P. P. (1959). Comparative biochemistry of urea
887 synthesis. II. Levels of urea cycle enzymes in metamorphosing *Rana catesbeiana*
888 tadpoles. *The Journal of Biological Chemistry*, *234*(7), 1775–1780.
889 Burraco, P., Díaz-Paniagua, C., & Gomez-Mestre, I. (2017). Different effects of accelerated
890 development and enhanced growth on oxidative stress and telomere shortening in
891 amphibian larvae. *Scientific Reports*, *7*(1), 7494. https://doi.org/10.1038/s41598-017-
892 07201-z
893 Burraco, P., Valdés, A. E., & Orizaola, G. (2020). Metabolic costs of altered growth trajectories
894 across life transitions in amphibians. *Journal of Animal Ecology*, *89*(3), 855–866.
895 https://doi.org/10.1111/1365-2656.13138
896 Cabrera-Guzmán, E., Crossland, M. R., Brown, G. P., & Shine, R. (2013). Larger body Size at
897 metamorphosis enhances survival, growth and performance of young cane toads
898 (*Rhinella marina)*. *Plos One*, *8*(7), e70121. https://doi.org/10.1371/journal.pone.0070121
899 Caldwell, J. P., & Lopez, P. T. (1989). Foam-generating behavior in tadpoles of *Leptodactylus*
900 *mystaceus*. *Copeia*, *1989*(2), 498–502. https://doi.org/10.2307/1445453

Callier, V., & Nijhout, H. F. (2011). Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. *Proceedings of the National Academy of Sciences*, *108*(35), 14664–14669. https://doi.org/10.1073/pnas.1106556108

Campos-Cerda, F., & Bohannan, B. J. M. (2020). The Nidobiome: A framework for understanding microbiome assembly in neonates. *Trends in Ecology & Evolution*, *35*(7), 573–582. https://doi.org/10.1016/j.tree.2020.03.007

Chew, S. F., Chan, N. K., Loong, A. M., Hiong, K. C., Tam, W. L., & Ip, Y. K. (2004) Nitrogen metabolism in the African lungfish (*Protopterus dolloi*) aestivating in a mucus cocoon on land. *Journal of Experimental Biology 207,* 777–786. https://doi.org/10.1242/jeb.00813

Costantini, D. (2019). Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. *Journal of Experimental Biology*, *222*(13), jeb194688. https://doi.org/10.1242/jeb.194688

Crespi, E. J., & Warne, R. W. (2013). Environmental conditions experienced during the tadpole stage alter post-metamorphic glucocorticoid response to stress in an amphibian. *Integrative and Comparative Biology*, *53*(6), 989–1001. https://doi.org/10.1093/icb/ict087

Crump, M. L. (2015). Anuran reproductive modes: Evolving perspectives. *Journal of Herpetology*, *49*(1), 1–16. https://doi.org/10.1670/14-097

Darken, R. S., Martin, K. L., & Fisher, M. C. (1998). Metabolism during delayed hatching in terrestrial eggs of a marine fish, the grunion *Leuresthes tenuis*. *Physiological Zoology*, *71*(4), 400–406. https://doi.org/10.1086/515423

Delia, J., Bravo-Valencia, L., & Warkentin, K. M. (2017). Patterns of parental care in Neotropical glassfrogs: Fieldwork alters hypotheses of sex-role evolution. *Journal of Evolutionary Biology*, *30*(5), 898–914. https://doi.org/10.1111/jeb.13059

Delia, J., Bravo-Valencia, L., & Warkentin, K. M. (2020). The evolution of extended parental care in glassfrogs: Do egg-clutch phenotypes mediate coevolution between the sexes? *Ecological Monographs*, *90*(3), e01411. https://doi.org/10.1002/ecm.1411

Delia, J., Rivera-Ordonez, J. M., Salazar-Nicholls, M. J., & Warkentin, K. M. (2019). Hatching plasticity and the adaptive benefits of extended embryonic development in glassfrogs. *Evolutionary Ecology*, *33*(1), 37–53. https://doi.org/10.1007/s10682-018-9963-2

Delia, J., Ramírez-Bautista, A., & Summers, K. (2013). Parents adjust care in response to weather conditions and egg dehydration in a Neotropical glassfrog. *Behavioral Ecology and Sociobiology*, *67*(4), 557–569. https://doi.org/10.1007/s00265-013-1475-z

Delia, J., Ramírez-Bautista, A., & Summers, K. (2014). Glassfrog embryos hatch early after parental desertion. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1785), 20133237. https://doi.org/10.1098/rspb.2013.3237

Denver, R. J. (2021). Stress hormones mediate developmental plasticity in vertebrates with complex life cycles. *Neurobiology of Stress*, *14*, 100301. https://doi.org/10.1016/j.ynstr.2021.100301

Downie, J. R. (1994). Developmental arrest in *Leptodactylus fascus* tadpoles (Anura Leptodactylidae) II Does a foam borne factor block development. *The Herpetological Journal*, *4*(2), 39–45.

Downie, J. R. (1984). How *Leptodactylus fuscus* tadpoles make foam, and why. *Copeia*, *1984*(3), 778–780. https://doi.org/10.2307/1445168

Downie, J. R., & Smith, J. (2003). Survival of larval *Leptodactylus fuscus* (Anura: Leptodactylidae) out of water: Developmental differences and interspecific comparisons. *Journal of Herpetology*, *37*(1), 107–115.

Duellman, W. E. (1985). Reproductive modes in anuran amphibians: Phylogenetic significance of adaptive strategies. *South African Journal of Science, 81*:174–178.

Duellman, W. E., & Trueb, L. (1986). Biology of Amphibians. New York, U.S.A.: McGraw-Hill Book Company.

Dworkin, M. B., & Dworkin-Rastl, E. (1991). Carbon metabolism in early amphibian embryos. *Trends in Biochemical Sciences*, *16*, 229–234. https://doi.org/10.1016/0968-0004(91)90091-9

Earl, J. E., & Whiteman, H. H. (2015). Are commonly used fitness predictors accurate? A meta-analysis of amphibian size and age at metamorphosis. *Copeia*, *103*(2), 297–309. https://doi.org/10.1643/CH-14-128

Elinson, R. P., & del Pino, E. M. (2012). Developmental diversity of amphibians. *Wiley Interdisciplinary Reviews. Developmental Biology*, *1*(3), 345–369. https://doi.org/10.1002/wdev.23

962 Fabre, A. C., Bardua, C., Bon, M., Clavel, J., Felice, R. N., Streicher, J. W., Bonnel, J., Stanley,
963 E. L., Blackburn, D. C., & Goswami, A. (2020). Metamorphosis shapes cranial diversity
964 and rate of evolution in salamanders. *Nature Ecology & Evolution*, *4*(8), 1129–1140.
965 https://doi.org/10.1038/s41559-020-1225-3
966 Faggioni, G., Souza, F., Uetanabaro, M., Landgref-Filho, P., Furman, J., & Prado, C. (2017).
967 Reproductive biology of the nest building vizcacheras frog *Leptodactylus bufonius*
968 (Amphibia, Anura, Leptodactylidae), including a description of unusual courtship
969 behaviour. *Herpetological Journal*, *27*(1), 73–80.
970 Felskie, A. K., Anderson, P. M., & Wright, P. A. (1998). Expression and activity of carbamoyl
971 phosphate synthetase III and ornithine urea cycle enzymes in various tissues of four fish
972 species. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular*
973 *Biology*, *119*(2), 355–364. https://doi.org/10.1016/S0305-0491(97)00361-1
974 Finn, R. N., Fyhn, H. J., & Evjen, M. S. (1995). Physiological energetics of developing embryos
975 and yolk-sac larvae of Atlantic cod (*Gadus morhua*). I. Respiration and nitrogen
976 metabolism. *Marine Biology*, *124*(3), 355–369. https://doi.org/10.1007/BF00363909
977 Giaretta, A. A., Freitas, F. G. D., Antoniazzi, M. M., & Jared, C. (2011). Tadpole buccal
978 secretory glands as new support for a Neotropical clade of frogs. *Zootaxa*, *3011*(1), 38–
979 44. https://doi.org/10.11646/zootaxa.3011.1.4
980 Gilbert, S. F. (2012). Ecological developmental biology: Environmental signals for normal
981 animal development. *Evolution & Development*, *14*(1), 20–28.
982 https://doi.org/10.1111/j.1525-142X.2011.00519.x
983 Gilbert, S. F., & Epel, D. (2015). Ecological Developmental Biology (Second).
984 //global.oup.com/ushe/product/ecological-developmental-biology-9781605353449
985 Goin C. J. (1960). Amphibians, pioneers of terrestrial breeding habits. *Annual Reports*
986 *Smithsonian Institute*. 1959:427–455.
987 Gomez-Mestre, I., & Buchholz, D. R. (2006). Developmental plasticity mirrors differences
988 among taxa in spadefoot toads linking plasticity and diversity. *Proceedings of the*
989 *National Academy of Sciences*, *103*(50), 19021–19026.
990 https://doi.org/10.1073/pnas.0603562103

991 Gomez-Mestre, I., Kulkarni, S., & Buchholz, D. R. (2013). Mechanisms and consequences of
992 developmental acceleration in tadpoles responding to pond drying. *Plos One*, *8*(12),
993 e84266. https://doi.org/10.1371/journal.pone.0084266
994 Gomez-Mestre, I., Pyron, R. A., & Wiens, J. J. (2012). Phylogenetic analyses reveal unexpected
995 patterns in the evolution of reproductive modes in frogs. *Evolution*, *66*(12), 3687–3700.
996 https://doi.org/10.1111/j.1558-5646.2012.01715.x
997 Gomez-Mestre, I., Saccoccio, V. L., Iijima, T., Collins, E. M., Rosenthal, G. G., & Warkentin,
998 K. M. (2010). The shape of things to come: Linking developmental plasticity to post-
999 metamorphic morphology in anurans. *Journal of Evolutionary Biology*, *23*(7), 1364–
1000 1373. https://doi.org/10.1111/j.1420-9101.2010.02016.x
1001 Gomez-Mestre, I., Wiens, J. J., & Warkentin, K. M. (2008). Evolution of adaptive plasticity:
1002 risk- sensitive hatching in neotropical leaf-breeding treefrogs (*Agalychnis*: Hylidae).
1003 *Ecological Monographs*, *78*(2), 20.
1004 Gonzalez, K., Warkentin, K., & Güell, B. (2021). Dehydration-induced mortality and premature
1005 hatching in Gliding treefrogs with even small reductions in humidity. *Ichthyology &*
1006 *Herpetology*, *109*. https://doi.org/10.1643/h2020085
1007 Gould, S. J. (1966). Allometry and size in ontogeny and phylogeny. *Biological Reviews*, *41*(4),
1008 587–638. https://doi.org/10.1111/j.1469-185X.1966.tb01624.x
1009 Grafe, T. U., Kaminsky, S. K., & Linsenmair, K. E. (2005). Terrestrial larval development and
1010 nitrogen excretion in the Afro-tropical Pig-nosed frog, *Hemisus marmoratus*. *Journal of*
1011 *Tropical Ecology*, *21*(2), 219–222. JSTOR.
1012 Guevara-Molina, E. C., Gomes, F. R., & Warkentin, K. M. (2022). Heat-induced hatching of
1013 Red-eyed treefrog embryos: Hydration and clutch structure increase behavioral thermal
1014 tolerance. *Integrative Organismal Biology*, *4*(1), obac041.
1015 https://doi.org/10.1093/iob/obac041
1016 Guo, M., Xu, Z., Zhang, H., Mei, J., & Xie, J. (2023). The effects of acute exposure to ammonia
1017 on oxidative stress, hematological parameters, flesh quality, and gill morphological
1018 changes of the large yellow croaker (*Larimichthys crocea*). *Animals*, *13*(15), 2534.
1019 https://doi.org/10.3390/ani13152534
1020 Hartig, F. (2017). Package 'DHARMa'. The R Project for Statistical Computing.

1021 Hector, K. L., & Nakagawa, S. (2012). Quantitative analysis of compensatory and catch-up
1022 growth in diverse taxa. *The Journal of Animal Ecology*, *81*(3), 583–593.
1023 https://doi.org/10.1111/j.1365-2656.2011.01942.x
1024 Heyer, W. R. (1969). The adaptive ecology of the species groups of the genus *Leptodactylus*
1025 (amphibia, Leptodactylidae). *Evolution*, *23*(3), 421–428. https://doi.org/10.1111/j.1558-
1026 5646.1969.tb03525.x
1027 Hothorn, T., Bretz, F., & Hothorn, M. T. (2009). The multcomp package. The R Project for
1028 Statistical Computing.
1029 Jorgensen, P., Steen, J. A. J., Steen, H., & Kirschner, M. W. (2009). The mechanism and pattern
1030 of yolk consumption provide insight into embryonic nutrition in *Xenopus*. *Development*,
1031 *136*(9), 1539–1548. https://doi.org/10.1242/dev.032425
1032 Kaplan, R. H., & Phillips, P. C. (2006). Ecological and developmental context of natural
1033 selection: Maternal effects and thermally induced plasticity in the frog *Bombina*
1034 *Orientalis*. *Evolution*, *60*(1), 142–156. https://doi.org/10.1111/j.0014-
1035 3820.2006.tb01089.x
1036 Kassambara, A., & Mundt, F. (2017). Package 'factoextra'. Extract and visualize the results of
1037 multivariate data analyses. The R Project for Statistical Computing.
1038 Kasimatis, K., & Riginos, C. (2016). A phylogenetic analysis of egg size, clutch size, spawning
1039 mode, adult body size, and latitude in reef fishes. *Coral Reefs*, *35*(2), 387–397.
1040 https://doi.org/10.1007/s00338-015-1380-1
1041 Kokubum, M. N. de C., & Giaretta, A. A. (2005). Reproductive ecology and behaviour of a
1042 species of *Adenomera* (Anura, Leptodactylinae) with endotrophic tadpoles: Systematic
1043 implications. *Journal of Natural History*, *39*(20), 1745–1758.
1044 https://doi.org/10.1080/00222930400021515
1045 Kulkarni, S. S., Denver, R. J., Gomez-Mestre, I., & Buchholz, D. R. (2017). Genetic
1046 accommodation via modified endocrine signalling explains phenotypic divergence among
1047 spadefoot toad species. *Nature Communications*, *8*(1), 993.
1048 https://doi.org/10.1038/s41467-017-00996-5
1049 Laland, K., Odling-Smee, J., & Endler, J. (2017). Niche construction, sources of selection and
1050 trait coevolution. *Interface Focus*, *7*(5), 20160147. https://doi.org/10.1098/rsfs.2016.0147

1051 Laudet, V. (2011). The origins and evolution of vertebrate metamorphosis. *Current Biology: CB*,
1052 *21*(18), R726-737. https://doi.org/10.1016/j.cub.2011.07.030
1053 Laurila, A., & Kujasalo, J. (1999). Habitat duration, predation risk and phenotypic plasticity in
1054 common frog (*Rana temporaria*) tadpoles. *Journal of Animal Ecology*, *68*(6), 1123–1132.
1055 https://doi.org/10.1046/j.1365-2656.1999.00354.x
1056 Liedtke, H. C., Wiens, J. J., & Gomez-Mestre, I. (2022). The evolution of reproductive modes
1057 and life cycles in amphibians. *Nature communications*, *13*(1), 7039.
1058 https://doi.org/10.1038/s41467-022-34474-4
1059 Lisondro-Arosemena, A. K., Salazar-Nicholls, M. J., & Warkentin, K. M. (2024). Elevated
1060 ammonia cues hatching in Red-eyed treefrogs: A mechanism for escape from drying
1061 eggs. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution,*
1062 *342*(5), 406–411. https://doi.org/10.1002/jez.b.23253
1063 Loong, A. M., Hiong, K. C., Min Lin, L. S., Wong, W. P., Chew, S. F., & Ip, Y. K. (2005)
1064 Ornithine-urea cycle and urea synthesis in African lungfishes, *Protopterus aethiopicus*
1065 and *Protopterus annectens*, exposed to terrestrial conditions for six days. *Journal of*
1066 *Experimental Zoology A: Comparative Experimental Biology, 303,* 354–365.
1067 https://doi.org/10.1002/jez.a.147
1068 Lucas, E. M., Brasileiro, C. A., Oyamaguchi, H. M., & Martins, M. (2008). The reproductive
1069 ecology of *Leptodactylus fuscus* (Anura, Leptodactylidae): New data from natural
1070 temporary ponds in the Brazilian Cerrado and a review throughout its distribution.
1071 *Journal of Natural History*, *42*(35–36), 2305–2320.
1072 https://doi.org/10.1080/00222930802254698
1073 Magnusson, A., Skaug, H., Nielsen, A., Berg, C., Kristensen, K., Maechler, M., & Brooks, M.
1074 M. (2017). Package 'glmmTMB'. The R Project for Statistical Computing.
1075 Martin, A. A., & Cooper, A. K. (1972). The Ecology of Terrestrial Anuran Eggs, Genus *Crinia*
1076 (Leptodactylidae). *Copeia*, *1972*(1), 163–168. JSTOR. https://doi.org/10.2307/1442793
1077 Martin, K. L. M. (1999). Ready and waiting: Delayed hatching and extended incubation of
1078 anamniotic vertebrate terrestrial eggs. *American Zoologist*, *39*(2), 279–288.
1079 https://doi.org/10.1093/icb/39.2.279
1080 Martin, K., Moravek, C., & Walker, A. (2011). Waiting for a sign: Extended incubation
1081 postpones larval stage in the beach spawning California Grunion *Leuresthes tenuis*

(Ayres). *Environmental Biology of Fishes*, *91*, 63–70. https://doi.org/10.1007/s10641-010-9760-4

Mazerolle, M. J., & Mazerolle, M. M. J. (2017). Package 'AICcmodavg'. The R Project for Statistical Computing.

McDiarmid, R. W., & Altig, R. (1999). Tadpoles: the biology of anuran larvae. University of Chicago Press.

Méndez-Narváez, J., & Warkentin, K. M. (2023). Early onset of urea synthesis and ammonia detoxification pathways in three terrestrially developing frogs. *Journal of Comparative Physiology B*, *193*(5), 523-543. https://doi.org/10.1007/s00360-023-01506-4

Méndez-Narváez, J., & Warkentin, K. M. (2022). Reproductive colonization of land by frogs: Embryos and larvae excrete urea to avoid ammonia toxicity. *Ecology and Evolution*, *12*(2), e8570. https://doi.org/10.1002/ece3.8570

Méndez-Narváez, J., Flechas, S. V., & Amézquita, A. (2015). Foam nests provide context-dependent thermal insulation to embryos of three Leptodactylid Frogs. *Physiological and Biochemical Zoology*, *88*(3), 246–253. https://doi.org/10.1086/680383

Méndez-Narváez, J. (2022). The role of phenotypic plasticity in reproductive colonization of land by frogs: Urea excretion and mechanisms to prevent ammonia toxicity during terrestrial development. Ph.D. Dissertation, Boston University, Boston, MA. https://hdl.handle.net/2144/44809

Méndez-Narváez, J. (2012). Recolonizando la tierra: Clinas de terrestrialidad y función termorregulatoria de los nidos de espuma en ranas de la familia Leptodactylidae. Ms.C. Dissertation, Universidad de los Andes, Bogota. http://hdl.handle.net/1992/11691

Metcalfe, N. B., & Monaghan, P. (2001). Compensation for a bad start: Grow now, pay later? *Trends in Ecology & Evolution*, *16*(5), 254–260. https://doi.org/10.1016/s0169-5347(01)02124-3

Mirth, C. K., Tang, H. Y., Makohon-Moore, S. C., Salhadar, S., Gokhale, R. H., Warner, R. D., Koyama, T., Riddiford, L. M., & Shingleton, A. W. (2014). Juvenile hormone regulates body size and perturbs insulin signaling in *Drosophila. Proceedings of the National Academy of Sciences*, *111*(19), 7018–7023. https://doi.org/10.1073/pnas.1313058111

Mitchell, N. J., & Seymour, R. S. (2000). Effects of temperature on energy cost and timing of embryonic and larval development of the terrestrially breeding moss frog, *Bryobatrachus*

nimbus. *Physiological and Biochemical Zoology*, *73*(6), 829–840. https://doi.org/10.1086/318097

Mommsen, T. P., & Walsh, P. J. (1989). Evolution of urea synthesis in vertebrates: The piscine connection. *Science*, *243*(4887), 72–75. https://doi.org/10.1126/science.2563172

Moore, M. P., & Martin, R. A. (2019). On the evolution of carry-over effects. *Journal of Animal Ecology*, *88*(12), 1832-1844. https://doi.org/10.1111/1365-2656.13081

Moravek, C. L., & Martin, K. L. (2011). Life goes on: Delayed hatching, extended incubation, and heterokairy in development of embryonic California Grunion, *Leuresthes tenuis*. *Copeia*, *2011*(2), 308–314. https://doi.org/10.1643/CG-10-164

Morey, S., & Reznick, D. (2001). Effects of larval density on postmetamorphic Spadefoot toads (S*pea hammondii*). *Ecology*, *82*(2), 510–522. https://doi.org 10.1890/0012-9658(2001)082[0510:EOLDOP]2.0.CO;2

Mueller, C. A., Augustine, S., Kooijman, S. A. L. M., Kearney, M. R., & Seymour, R. S. (2012). The trade-off between maturation and growth during accelerated development in frogs. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *163*(1), 95–102. https://doi.org/10.1016/j.cbpa.2012.05.190

Mueller, C. A., Bucsky, J., Korito, L., & Manzanares, S. (2019). Immediate and persistent effects of temperature on oxygen consumption and thermal tolerance in embryos and larvae of the Baja California chorus frog, *Pseudacris hypochondriaca*. *Frontiers in Physiology*, *10*. https://doi.org/10.3389/fphys.2019.00754

Muir, T. J., Costanzo, J. P., & Lee, R. E. (2008). Metabolic depression induced by urea in organs of the wood frog, *Rana sylvatica*: Effects of season and temperature. *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology*, *309*(2), 111–116. https://doi.org/10.1002/jez.436

Murillo-Rincón, A. P., Kolter, N. A., Laurila, A., & Orizaola, G. (2017a). Intraspecific priority effects modify compensatory responses to changes in hatching phenology in an amphibian. *Journal of Animal Ecology*, *86*(1), 128–135. https://doi.org/10.1111/1365-2656.12605

Murillo-Rincón, A. P., Laurila, A., & Orizaola, G. (2017b). Compensating for delayed hatching reduces offspring immune response and increases life-history costs. *Oikos*, *126*(4), 565–571. https://doi.org/10.1111/oik.04014

1144 Nicieza, A. G., Álvarez, D., & Atienza, E. M. S. (2006). Delayed effects of larval predation risk
1145 and food quality on anuran juvenile performance. *Journal of Evolutionary Biology*, *19*(4),
1146 1092–1103. https://doi.org/10.1111/j.1420-9101.2006.01100.x
1147 Oliveira, D. C., Isaias, R. M. S., Fernandes, G. W., Ferreira, B. G., Carneiro, R. G. S., & Fuzaro,
1148 L. (2016). Manipulation of host plant cells and tissues by gall-inducing insects and
1149 adaptive strategies used by different feeding guilds. *Journal of Insect Physiology*, *84*,
1150 103–113. https://doi.org/10.1016/j.jinsphys.2015.11.012
1151 Oliveira Filho, J. C. de, Costa, H. C. de M., & Braga, Ú. M. L. (2005). Egg-laying and foam-
1152 beating in *Leptodactylus fuscus* (Anura, Leptodactylidae). *Biota Neotropica*, *5*, 319–320.
1153 https://doi.org/10.1590/S1676-06032005000300022
1154 Orizaola, G., Dahl, E., & Laurila, A. (2010). Compensating for delayed hatching across
1155 consecutive life-history stages in an amphibian. *Oikos*, *119*(6), 980–987.
1156 Pereira, E. B., Collevatti, R. G., Kokubum, M. N. de C., Miranda, N. E. de O., & Maciel, N. M.
1157 (2015). Ancestral reconstruction of reproductive traits shows no tendency toward
1158 terrestriality in leptodactyline frogs. *BMC Evolutionary Biology*, *15*(1), 91.
1159 https://doi.org/10.1186/s12862-015-0365-6
1160 Petranka, J. W., & Petranka, J. G. (1981). On the evolution of nest site selection in the Marbled
1161 salamander, *Ambystoma opacum*. *Copeia*, *1981*(2), 387–391.
1162 https://doi.org/10.2307/1444227
1163 Pfennig, D. W., Mabry, A., & Orange, D. (1991). Environmental causes of correlations between
1164 age and size at metamorphosis in *Scaphiopus multiplicatus*. *Ecology*, *72*(6), 2240–2248.
1165 https://doi.org/10.2307/1941574
1166 Phung, T. X., Nascimento, J. C. S., Novarro, A. J., & Wiens, J. J. (2020). Correlated and
1167 decoupled evolution of adult and larval body size in frogs. *Proceedings of the Royal*
1168 *Society B: Biological Sciences*, *287*(1933), 20201474.
1169 https://doi.org/10.1098/rspb.2020.1474
1170 Podrabsky, J. E., & Hand, S. C. (1999). The bioenergetics of embryonic diapause in an annual
1171 killifish, *Austrofundulus limnaeus*. *The Journal of Experimental Biology*, *202*, 2567–
1172 2580.

Podrabsky, J. E., & Hand, S. C. (2015). Physiological strategies during animal diapause: Lessons from brine shrimp and annual killifish. *Journal of Experimental Biology*, *218*(12), 1897–1906. https://doi.org/10.1242/jeb.116194

Pupin, N., Gasparini, J., Bastos, R., Haddad, C., & Prado, C. (2010). Reproductive biology of an endemic *Physalaemus* of the Brazilian Atlantic Forest, and the trade-of between clutch and egg size in terrestrial breeders of the *P. signifer* group. *Herpetological Journal*, *20*, 147–156.

Rahmatullah, M., & Boyde, T. R. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *107*(1–2), 3–9. https://doi.org/10.1016/0009-8981(80)90407-6

Rolff, J., Johnston, P. R., & Reynolds, S. (2019). Complete metamorphosis of insects. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *374*(1783), 20190063. https://doi.org/10.1098/rstb.2019.0063

Rolff, J., Van de Meutter, F., & Stoks, R. (2004). Time constraints decouple age and size at maturity and physiological traits. *The American Naturalist*, *164*(4), 559–565. https://doi.org/10.1086/423715

Rudolf, V. H. W., & Rödel, M.-O. (2007). Phenotypic plasticity and optimal timing of metamorphosis under uncertain time constraints. *Evolutionary Ecology*, *21*(1), 121–142. https://doi.org/10.1007/s10682-006-0017-9

Salica, M. J., Vonesh, J. R., & Warkentin, K. M. (2017). Egg clutch dehydration induces early hatching in Red-eyed treefrogs, *Agalychnis callidryas*. *PeerJ*, *5*. https://doi.org/10.7717/peerj.3549

Scott, D. E., Casey, E. D., Donovan, M. F., & Lynch, T. K. (2007). Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. *Oecologia*, *153*(3), 521–532. https://doi.org/10.1007/s00442-007-0755-6

Seymour, R. S. (1999). Respiration of aquatic and terrestrial amphibian embryos. *American Zoologist*, *39*(2), 261–270.

Seymour, R. S., & Bradford, D. F. (1995). Respiration of amphibian eggs. *Physiological Zoology*, *68*(1), 1–25.

1203 Seymour, R. S., & Loveridge, J. P. (1994). Embryonic and larval respiration in the arboreal foam
1204 nests of the African frog *Chiromantis xerampelina*. *Journal of Experimental Biology*,
1205 *197*(1), 31–46.
1206 Seymour, R. S., Geiser, F., & Bradford, D. F. (1991). Metabolic cost of development in
1207 terrestrial frog eggs (*Pseudophryne bibronii*). *Physiological Zoology*, *64*(3), 688–696.
1208
1209 Seymour, R. S., & Roberts, J. D. (1991). Embryonic respiration and oxygen distribution in
1210 foamy and nonfoamy egg masses of the frog *Limnodynastes tasmaniensis. Physiological*
1211 *Zoology*, *64*(5), 1322–1340. https://doi.org/10.1086/physzool.64.5.30156248
1212 Shambaugh, G. E., III. (1977). Urea biosynthesis I. The urea cycle and relationships to the citric
1213 acid cycle. *The American Journal of Clinical Nutrition*, *30*(12), 2083–2087.
1214 https://doi.org/10.1093/ajcn/30.12.2083
1215 Shoemaker, V. H., & McClanahan, L. L. (1973). Nitrogen excretion in the larvae of a land-
1216 nesting frog (*Leptodactylus bufonius*). *Comparative Biochemistry and Physiology. A,*
1217 *Comparative Physiology*, *44*(4), 1149–1156. https://doi.org/10.1016/0300-
1218 9629(73)90253-3
1219 Shrimpton, S. J., Streicher, J. W., Gower, D. J., Bell, R. C., Fujita, M. K., Schott, R. K., &
1220 Thomas, K. N. (2021). Eye-body allometry across biphasic ontogeny in anuran
1221 amphibians. *Evolutionary Ecology*, *35*(2), 337–359. https://doi.org/10.1007/s10682-021-
1222 10102-3
1223 Srivastava, S., & Ratha, B.K. (2010) Does fish represent an intermediate stage in the evolution of
1224 ureotelic cytosolic arginase I? *Biochemical and biophysical research*
1225 *communications*, *391*(1),1–5. https://doi.org/10.1016/j.bbrc.2009.11.018
1226 Steele, S. L., Chadwick, T. D., Wright, P. A. (2001) Ammonia detoxification and localization of
1227 urea cycle enzyme activity in embryos of the Rainbow trout (*Oncorhynchus mykiss*) in
1228 relation to early tolerance to high environmental ammonia levels. *Journal of*
1229 *Experimental Biology 204,* 2145–2154
1230 Tarvin, R. D., Bermúdez, C. S., Briggs, V. S., & Warkentin, K. M. (2015). Carry-over effects of
1231 size at metamorphosis in Red-eyed treefrogs: Higher survival but slower growth of larger
1232 metamorphs. *Biotropica*, *47*(2), 218–226.

1233 Tonelli, M., Gomes, G., Silva, W. D., Magri, N. T. C., Vieira, D. M., Aguiar, C. L., & Bento, J.
1234 M. S. (2018). Spittlebugs produce foam as a thermoregulatory adaptation. *Scientific*
1235 *Reports*, *8*(1), 4729. https://doi.org/10.1038/s41598-018-23031-z
1236 Touchon, J. C., McCoy, M. W., Vonesh, J. R., & Warkentin, K. M. (2013). Effects of plastic
1237 hatching timing carry over through metamorphosis in Red-eyed treefrogs. *Ecology*, *94*(4),
1238 850–860. https://doi.org/10.1890/12-0194.1
1239 Touchon, J. C., Urbina, J., & Warkentin, K. M. (2011). Habitat-specific constraints on induced
1240 hatching in a treefrog with reproductive mode plasticity. *Behavioral Ecology*, *22*(1), 169–
1241 175. https://doi.org/10.1093/beheco/arq192
1242 Touchon, J. C., & Warkentin, K. M. (2008). Reproductive mode plasticity: Aquatic and
1243 terrestrial oviposition in a treefrog. *Proceedings of the National Academy of Sciences*,
1244 *105*(21), 7495–7499. https://doi.org/10.1073/pnas.0711579105
1245 Touchon, J. C., & Warkentin, K. M. (2009). Negative synergism of rainfall patterns and
1246 predators affects frog egg survival. *The Journal of Animal Ecology*, *78*(4), 715–723.
1247 https://doi.org/10.1111/j.1365-2656.2009.01548.x
1248 Touchon, J. C., & Warkentin, K. M. (2010). Short- and long-term effects of the abiotic egg
1249 environment on viability, development and vulnerability to predators of a Neotropical
1250 anuran. *Functional Ecology*, *24*(3), 566–575. https://doi.org/10.1111/j.1365-
1251 2435.2009.01650.x
1252 Truman, J. W. (2019). The evolution of insect metamorphosis. *Current Biology*, *29*(23), R1252–
1253 R1268. https://doi.org/10.1016/j.cub.2019.10.009
1254 Uller, T. (2008). Developmental plasticity and the evolution of parental effects. *Trends in*
1255 *Ecology & Evolution*, *23*(8), 432–438. https://doi.org/10.1016/j.tree.2008.04.005
1256 Vonesh, J. R. (2005). Egg predation and predator-induced hatching plasticity in the African reed
1257 frog, *Hyperolius spinigularis*. *Oikos*, *110*(2), 241–252. https://doi.org/10.1111/j.0030-
1258 1299.2005.13759.x
1259 Vonesh, J. R., & Bolker, B. M. (2005). Compensatory larval responses shift trade-offs associated
1260 with predator-induced hatching plasticity. *Ecology*, *86*(6), 1580–1591.
1261 https://doi.org/10.1890/04-0535

1262 Vonesh, J. R., & Warkentin, K. M. (2006). Opposite shifts in size at metamorphosis in response
1263 to larval and metamorph predators. *Ecology*, *87*(3), 556–562. https://doi.org/10.1890/05-
1264 0930
1265 Warkentin, K. M. (1995). Adaptive plasticity in hatching age: A response to predation risk trade-
1266 offs. *Proceedings of the National Academy of Sciences of the United States of America*,
1267 *92*(8), 3507–3510. https://doi.org/10.1073/pnas.92.8.3507
1268 Warkentin, K. M. (1999). Effects of hatching age on development and hatchling morphology in
1269 the Red-eyed treefrog, *Agalychnis callidryas*. *Biological Journal of the Linnean Society*,
1270 *68*(3), 443–470. https://doi.org/10.1111/j.1095-8312.1999.tb01180.x
1271 Warkentin, K. M. (2007). Oxygen, gills, and embryo behavior: Mechanisms of adaptive
1272 plasticity in hatching. *Comparative Biochemistry and Physiology. Part A, Molecular &*
1273 *Integrative Physiology*, *148*(4), 720–731. https://doi.org/10.1016/j.cbpa.2007.02.009
1274 Warkentin, K. M. (2011). Plasticity of hatching in amphibians: Evolution, trade-offs, cues and
1275 mechanisms. *Integrative and Comparative Biology*, *51*(1), 111–127.
1276 https://doi.org/10.1093/icb/icr046
1277 Wassersug, R. J. (1975). The adaptive significance of the tadpole stage with comments on the
1278 maintenance of complex life cycles in anurans. *American Zoologist*, *15*(2), 405–417.
1279 https://doi.org/10.1093/icb/15.2.405
1280 Wells K.D. 2007. The ecology and behavior of amphibians. University of Chicago Press,
1281 Chicago.
1282 Werner, E. E. (1986). Amphibian metamorphosis: Growth rate, predation risk, and the optimal
1283 size at transformation. *The American Naturalist*, *128*(3), 319–341.
1284 West-Eberhard, M. J. (2003). Developmental Plasticity and Evolution. OUP USA.
1285 White, C. R., Marshall, D. J., Alton, L. A., Arnold, P. A., Beaman, J. E., Bywater, C. L.,
1286 Condon, C., Crispin, T. S., Janetzki, A., Pirtle, E., Winwood-Smith, H. S., Angilletta, M.
1287 J., Chenoweth, S. F., Franklin, C. E., Halsey, L. G., Kearney, M. R., Portugal, S. J., &
1288 Ortiz-Barrientos, D. (2019). The origin and maintenance of metabolic allometry in
1289 animals. *Nature Ecology & Evolution*, *3*(4), 598–603. https://doi.org/10.1038/s41559-
1290 019-0839-9
1291 Yina, S., Chenghua, L., Weiwei, Z., Zhenhui, W., & Zhimeng, L. (2016). The first description of
1292 complete invertebrate arginine metabolism pathways implies dose-dependent pathogen

regulation in *Apostichopus japonicus*. *Scientfic Reports 6* (1), 23783. https://doi.org/10.1038/srep23783

Zamora-Camacho, F. J., Burraco, P., Zambrano-Fernández, S., & Aragón, P. (2023). Ammonium effects on oxidative stress, telomere length, and locomotion across life stages of an anuran from habitats with contrasting land-use histories. *Science of The Total Environment*, *862*, 160924. https://doi.org/10.1016/j.scitotenv.2022.160924

Zhu, W., Chang, L., Zhao, T., Wang, B., & Jiang, J. (2020). Remarkable metabolic reorganization and altered metabolic requirements in frog metamorphic climax. *Frontiers in Zoology*, *17*(1), 30. https://doi.org/10.1186/s12983-020-00378-6

Zhu, W., Zhang, M., Chang, L., Zhu, W., Li, C., Xie, F., Zhang, H., Zhao, T., & Jiang, J. (2019). Characterizing the composition, metabolism and physiological functions of the fatty liver in *Rana omeimontis* tadpoles. *Frontiers in Zoology*, *16*, 42. https://doi.org/10.1186/s12983-019-0341-x

Zina, J. (2006). Communal nests in *Physalaemus pustulosus* (Amphibia: Leptodactylidae): experimental evidence for female oviposition preferences and protection against desiccation. *Amphibia-Reptilia*, *27*(1), 148–150. https://doi.org/10.1163/156853806776052092

Figure 1

Larval foam-making in *Leptodactylus fragilis*: natural context and methods to assess effects on development to metamorphosis

Adults of *L. fragilis* construct a foam nest in a subterranean chamber (A), where nest-dwelling larvae may spend a prolonged period waiting for rain and constructing new larval foam (B). We removed larvae from the parental nest before, at, and after the onset of developmental arrest (~~C~~: 4.5 d, 8.5 d and 12.5 d, respectively) to assess their foam-making ability. We transferred sibling groups to small Petri dishes where we gave the larvae two days to create a new foam nest (larval nest # 1), then repeated this procedure twice more (larval nests # 2 and 3). To assess carry-over effects of extended terrestriality and larval foam-making, we transferred two individuals to water at 12.5 d when we moved their siblings to the nest-making treatment. We collected siblings from the water and first nest at 14.5 d, and from the third nest at 18.5 d and photographed them in three views to assess larval morphology (D). We left a non-nesting sibling in water (since 12.5 d) and transferred two from larval nests 1 (at 14.5 d) and 3 (at 18. 5 d) to water to rear to metamorphosis, then assessed time and size at forelimb emergence (GS42) and tail resorption (GS 46).

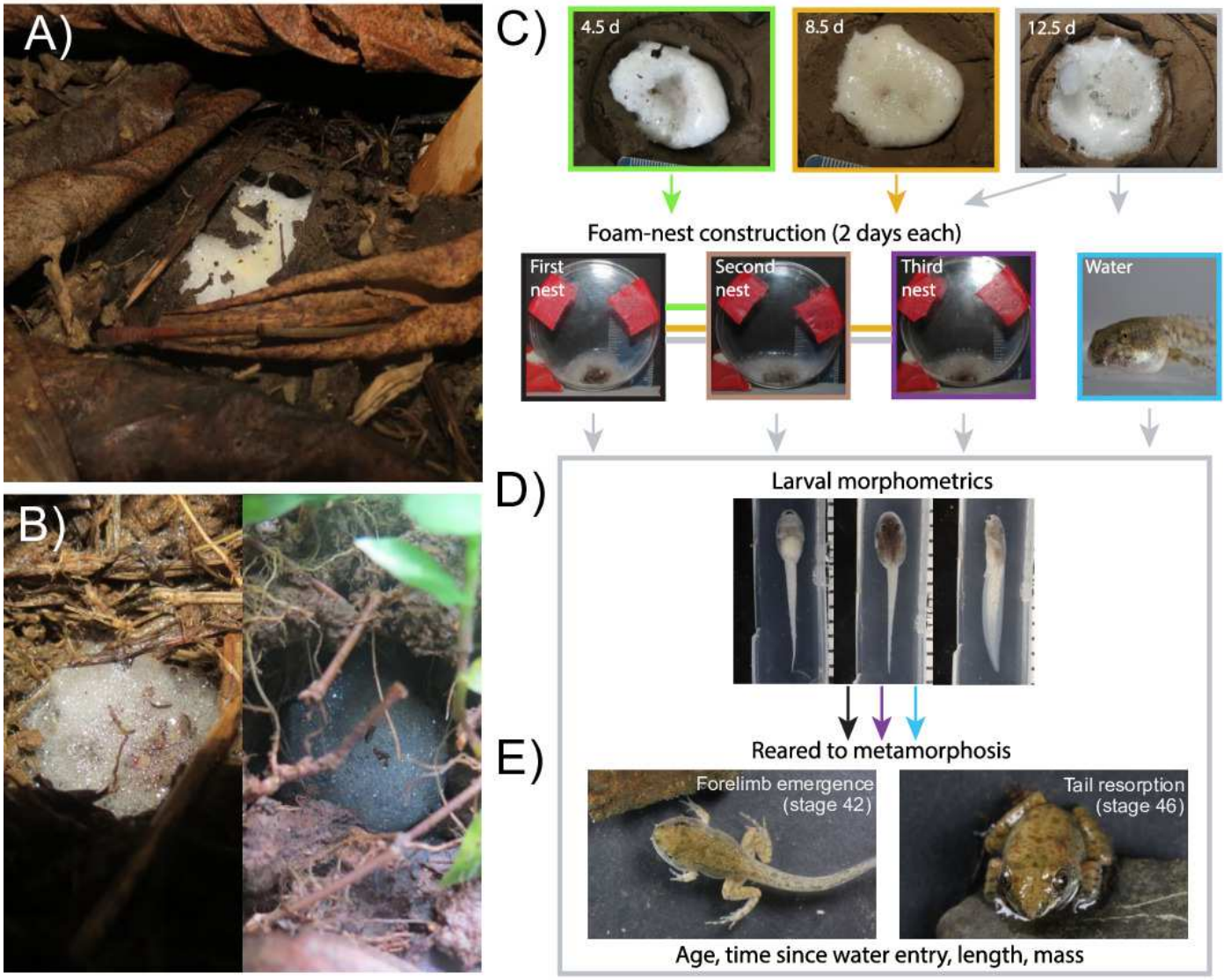
A)
B)
C)
4.5 d
8.5 d
12.5 d
Foam-nest construction (2 days each)
First nest
Second nest
Third nest
Water
D)
Larval morphometrics
E)
Reared to metamorphosis
Forelimb emergence (stage 42)
Tail resorption (stage 46)
Age, time since water entry, length, mass

Figure 2

Foam-nesting performance of *Leptodactylus fragilis* larvae in relation to age, sibship size, and repeated nest-making.

Foam volume produced by sibling groups over two days in a Petri dish, after removal of their prior nest. Total volume of foam per nest produced after larvae developed for 4.5 d (green), 8.5 d (orange) and 12.5 d (gray) in parental foam nests in soil (A), pooled across larval nests 1–3, and in the first (black), second (brown) and third (purple) foam nest that larvae made (B), pooled across age at transfer. Volume of foam produced per larva in relation to sibship size at the three transferred ages, pooled across larval nest number (C). Volume of foam produced per larva after removal from the parental nest at three ages and after construction of different numbers of larval nests (D). Box plots show median, first and third quartiles, and extent of data to 1.5 X IQR; data points are also shown. Sample sizes (N) are indicated.

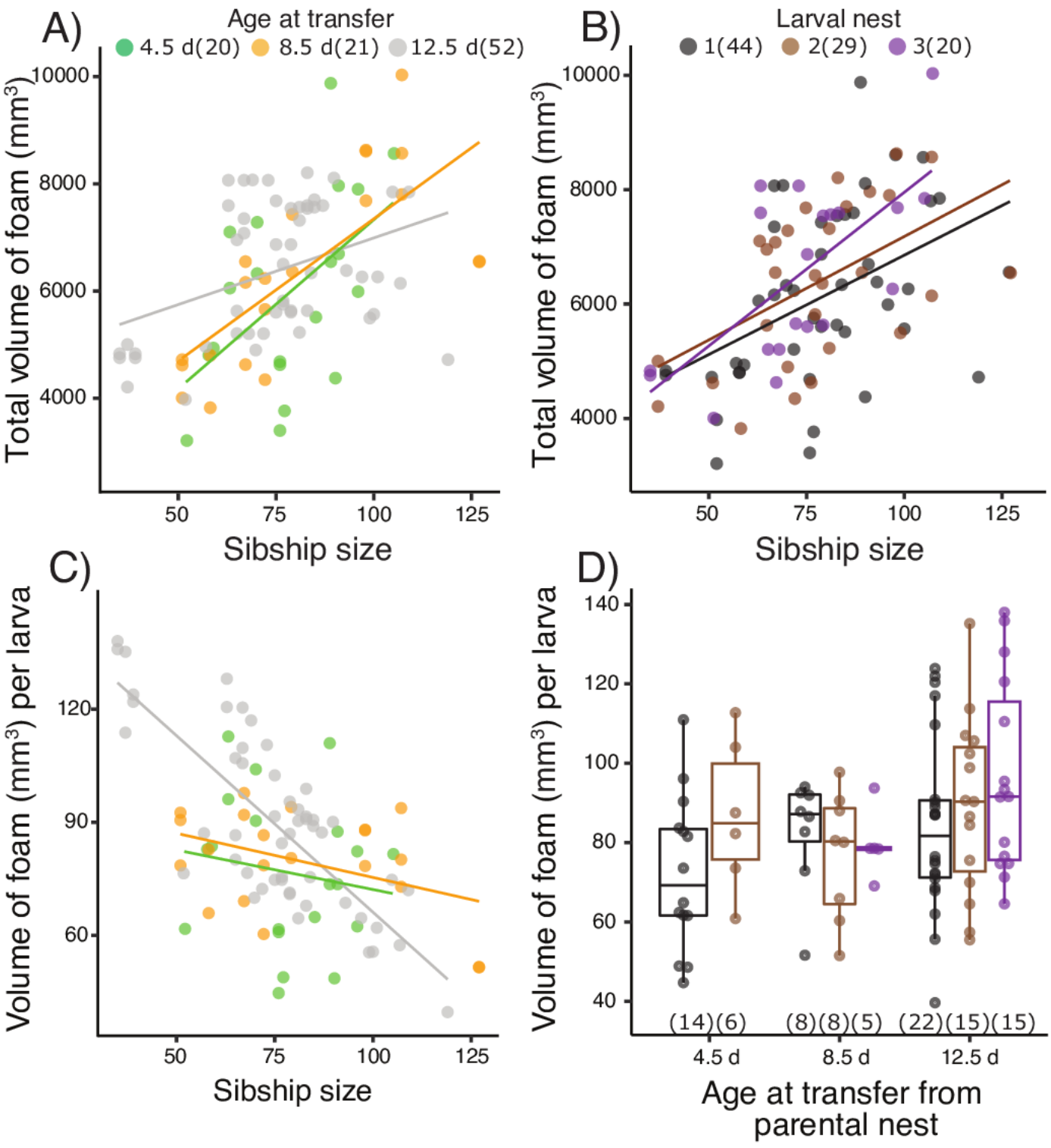
A)
Age at transfer
4.5 d(20)
8.5 d(21)
12.5 d(52)
Total volume of foam (mm3)
Sibship size
B)
Larval nest
1(44)
2(29)
3(20)
Total volume of foam (mm3)
Sibship size
C)
Volume of foam (mm3) per larva
Sibship size
D)
Volume of foam (mm3) per larva
(14)(6)
(8)(8)(5)
(22)(15)(15)
4.5 d
8.5 d
12.5 d
Age at transfer from parental nest

Figure 3

Nitrogen waste accumulation in foam nests constructed by *Leptodactylus fragilis* larvae.

(A) Ammonia and (B) urea concentration in larval foam nests constructed after transfer from parental nests at two ages (4.5 d and 12.5 days). For older larvae, parental nests were maintained in wet (dark blue) or dry (dark brown) soil, and larvae made one or three foam nests. (C) Concentration of nitrogen wastes in relation to water volume in the larval nest and (D) the amount of ammonia and urea accumulated per larva, as well as the ammonia calculated to accumulate if none were converted to urea, is shown for first nests made by 12.5 d larvae from each soil hydration history. Box plots show median, first and third quartiles, and extent of data to 1.5 X IQR; data points are also shown. Horizontal dashed gray line shows the detection limit for urea. Sample sizes (N) are indicated.

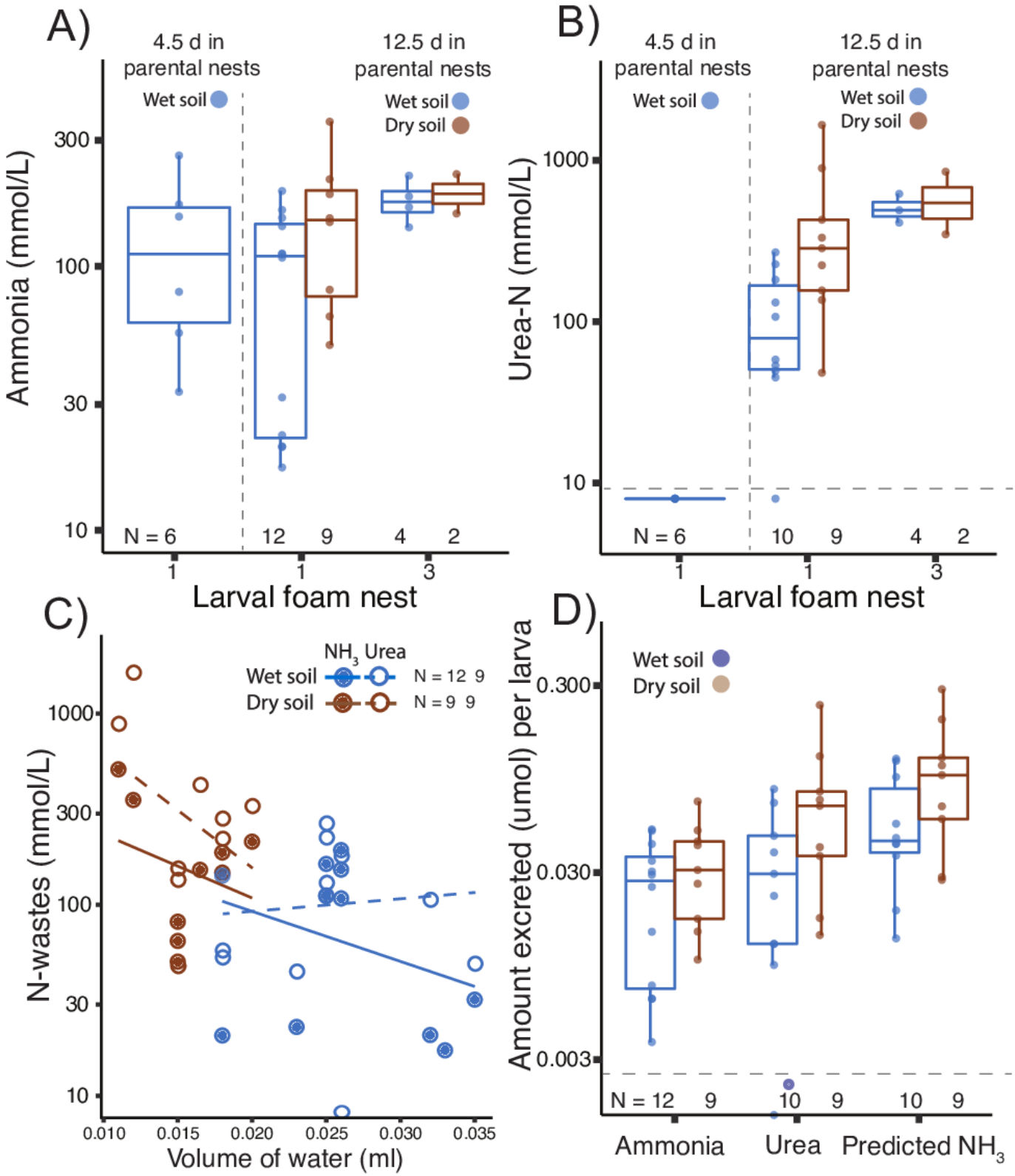
A)
4.5 d in parental nests
12.5 d in parental nests
Wet soil
Dry soil
Ammonia (mmol/L)
300
100
30
10
N = 6
12
9
4
2
1
1
3
Larval foam nest
B)
4.5 d in parental nests
12.5 d in parental nests
Wet soil
Dry soil
Urea-N (mmol/L)
1000
100
10
N = 6
10
9
4
2
1
1
3
Larval foam nest
C)
NH3 Urea
Wet soil
Dry soil
N = 12 9
N = 9 9
N-wastes (mmol/L)
1000
300
100
30
10
0.010
0.015
0.020
0.025
0.030
0.035
Volume of water (ml)
D)
Wet soil
Dry soil
Amount excreted (umol) per larva
0.300
0.030
0.003
N = 12 9
10 9
10 9
Ammonia
Urea
Predicted NH3

Figure 4

Short-term effects of extended terrestriality and foam-making on the morphology of *Leptodactylus fragilis* larvae after 12.5 d in the parental foam nest on soil.

(A) PCA biplot showing the first two PC which encompass 76.87% of the variation in morphology among 14.5 d larvae after 2 d in water (blue centroid) or constructing a larval foam nest (black centroid). (B, C) Comparison of PC1 and PC2 scores between siblings that created one foam nest (black) or were transferred to water without making a foam nest (blue). (D) PCA biplot showing the first two PC which encompass 78.54% of the variation in morphology among larvae that made one (black centroid), two (brown centroid), and three (purple centroid) new foam nests. (E, F) Comparison of PC1 and PC2 scores among siblings across number of nests constructed. Contributions of original morphometric variables to each PC are displayed by arrows in biplots. Box plots show median, first and third quartiles, and extent of data to 1.5 X IQR; data points are also shown. Sample sizes (N) are indicated in first row panels.

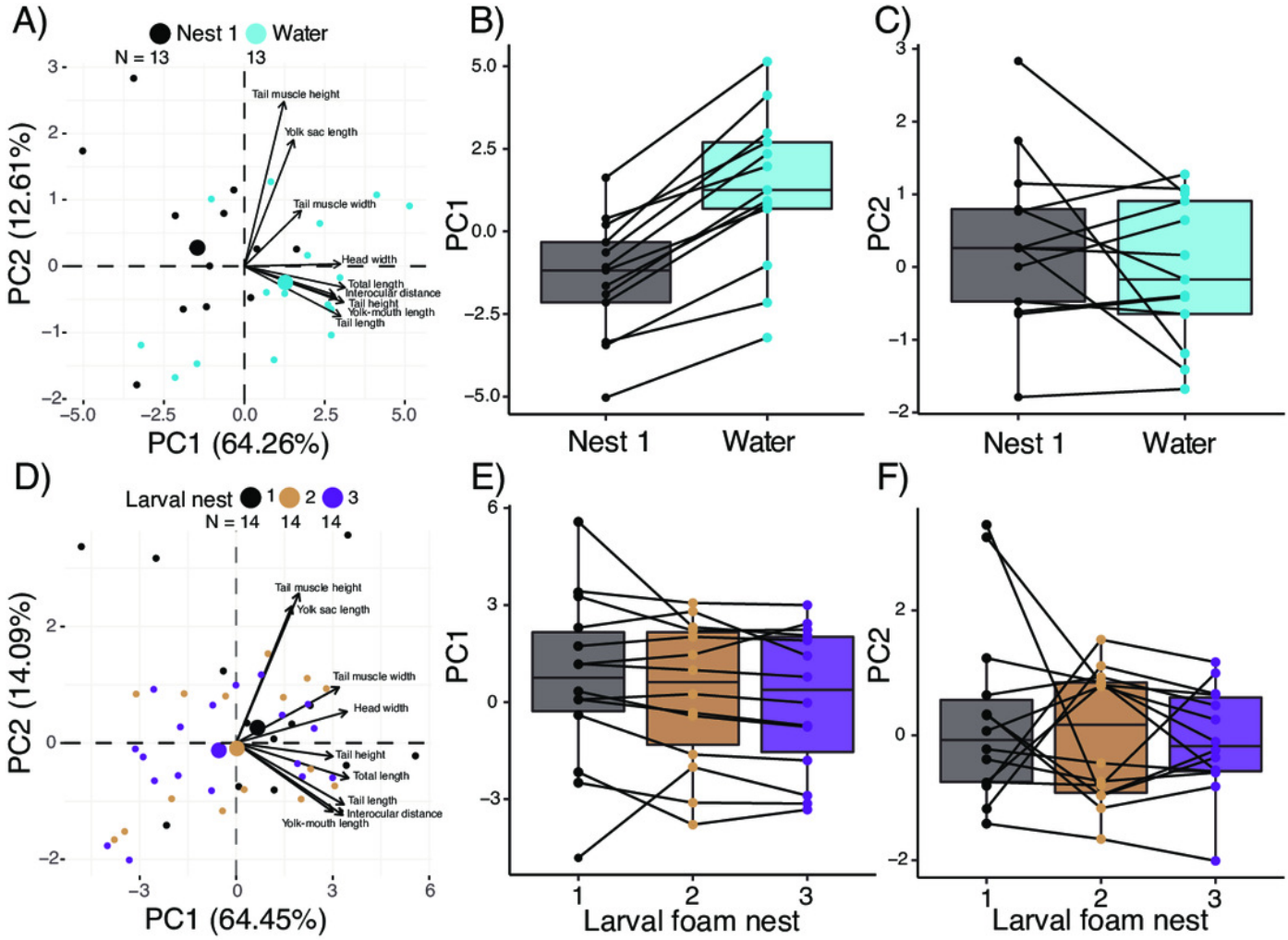
A)
Nest 1
Water
N = 13
13
Tail muscle height
Yolk sac length
Tail muscle width
Head width
Total length
Interocular distance
Tail height
Yolk-mouth length
Tail length
PC2 (12.61%)
PC1 (64.26%)
B)
PC1
Nest 1
Water
C)
PC2
Nest 1
Water
D)
Larval nest
1
2
3
N = 14
14
14
Tail muscle height
Yolk sac length
Tail muscle width
Head width
Tail height
Total length
Tail length
Interocular distance
Yolk-mouth length
PC2 (14.09%)
PC1 (64.45%)
E)
PC1
Larval foam nest
F)
PC2
Larval foam nest

Figure 5

Carry-over effects of extended terrestriality and foam-making on time to and size at metamorphosis for *Leptodactylus fragilis*.

Terrestrial larvae entered the water after making zero (blue), one (black), or three (purple) new foam nests. Times from oviposition (age) and water entry (aquatic period) to forelimb emergence (A) and to tail resorption (B). Box plots show median, first and third quartiles, and extent of data to 1.5 X IQR; data points are also shown. Total length at forelimb emergence in relation to age (C), aquatic period (E), and number of siblings in the nest (G). Snout–vent length at tail resorption in relation to age (D), aquatic larval + metamorphic period (F), and sibship size (H). Scatter plots show regression lines for each number of larval nests constructed. Sample sizes (N) are indicated for time and size data.

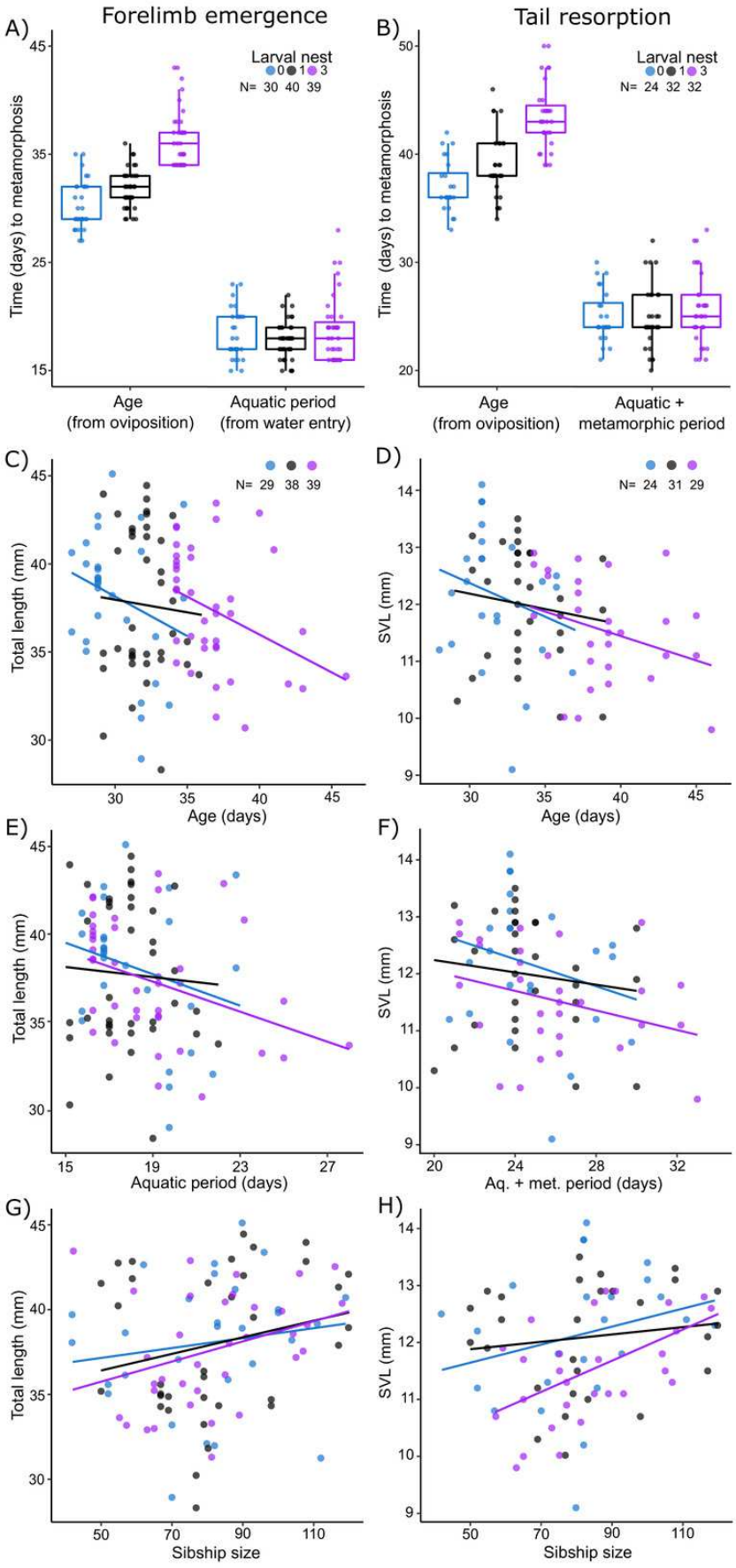
A)
Forelimb emergence
Larval nest
0 1 3
N= 30 40 39
Time (days) to metamorphosis
Age (from oviposition)
Aquatic period (from water entry)
B)
Tail resorption
Larval nest
0 1 3
N= 24 32 32
Time (days) to metamorphosis
Age (from oviposition)
Aquatic + metamorphic period
C)
N= 29 38 39
Total length (mm)
Age (days)
D)
N= 24 31 29
SVL (mm)
Age (days)
E)
Total length (mm)
Aquatic period (days)
F)
SVL (mm)
Aq. + met. period (days)
G)
Total length (mm)
Sibship size
H)
SVL (mm)
Sibship size

Figure 6

Carry-over effects of extended terrestriality and foam-making on mass at metamorphosis for *Leptodactylus fragilis*.

Terrestrial larvae entered the water after making zero (blue), one (black), or three (purple) new foam nests. Mass at forelimb emergence is plotted in relation to total length (A) and sibship size (B). Mass at tail resorption is plotted in relation to snout–vent length (C) and sibship size (D). Data points represent individuals, under each nest-making treatment, across sibship ID and lines are regression fits for each nest number. Sample sizes (N) are indicated.

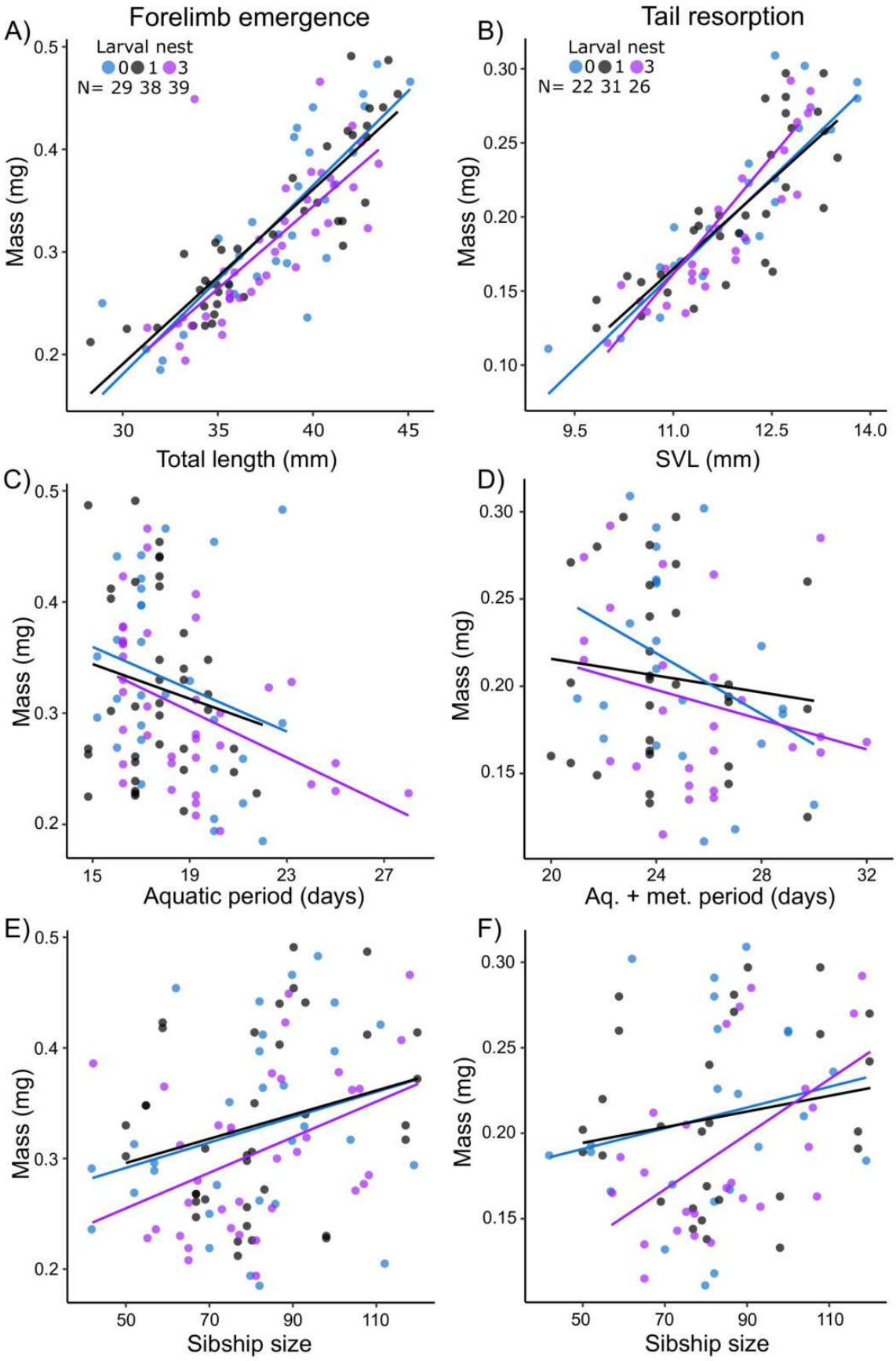
A)
Forelimb emergence
Larval nest
0 1 3
N= 29 38 39
Mass (mg)
Total length (mm)
B)
Tail resorption
Larval nest
0 1 3
N= 22 31 26
Mass (mg)
SVL (mm)
C)
Mass (mg)
Aquatic period (days)
D)
Mass (mg)
Aq. + met. period (days)
E)
Mass (mg)
Sibship size
F)
Mass (mg)
Sibship size

Table 1(on next page)

Nitrogen excretion in larval foam nests of *Leptodactylus fragilis* and arginase activity in larval tissues.

(A) Ammonia and urea accumulated in foam nests made by *Leptodactylus fragilis* larvae after 4.5 and 12.5 d in parental foam nests on wet soil or 12.5 d in parental nests on dry soil and, for older larvae, in the first and third larval nests. (B) N-waste accumulated per larvae over 2 days constructing their first new nest. (C) Activity of the urea cycle enzyme arginase and concentration of urea in larval tissues. Data are mean, SD, and N for values above the detection limit, plus number of “zero” values for samples below the detection limit and “NA” for samples where high absorbances prevented estimation. “–” indicates no samples were in that category

Table 1. **Nitrogen excretion in larval foam nests of *Leptodactylus fragilis* and arginase activity in larval tissues.** (A) Ammonia and urea accumulated in foam nests made by *Leptodactylus fragilis* larvae after 4.5 and 12.5 d in parental foam nests on wet soil or 12.5 d in parental nests on dry soil and, for older larvae, in the first and third larval nests. (B) N-waste accumulated per larvae over 2 days constructing their first new nest. (C) Activity of the urea cycle enzyme arginase and concentration of urea in larval tissues. Data are mean, SD, and N for values above the detection limit, plus number of "zero" values for samples below the detection limit and "NA" for samples where high absorbances prevented estimation. "–" indicates no samples were in that category

Time in parental nest on soil (days)	Larval nest	Sampling age (days)	**A.** Concentration of nitrogen wastes (mmol/L) in new larval foam mean ± SD, **N**					
			Ammonia		Urea		Predicted ammonia	
			Wet	Dry	Wet	Dry	Wet	Dry
4.5	First	6.5	126.43 ± 86.30, **6**	–	0, **5** NA, **1**	–	126.43 ± 86.30, **5** **NA, 1**	–
12.5	First	14.5	91.31± 65.12, **12**	195.76 ±150.12 , **9**	124.65± 83.85, **9** NA, **2** 0, **1**	461.55 ± 510.40, **9**	209.24 ± 131.15, **10, NA, 2**	657.31 ± 633.01, **9**
12.5	Third	18.5	178.22 ± 33.47, **4**	191.08± 45.93, **2**	506.37 ± 105.41, **3** NA, **1**	598.20 ± 354.63, **2**	682.65 ± 120.48, **3** NA**, 1**	789.28 ± 400.56, **2**
			B. Amount (umol) of nitrogen waste per individual larva in new larval foam					

12.5	First	14.5	0.02 ± 0.02, **12**	0.03 ± 0.02, **9**	0.03 ± 0.03, **9** NA, **2** 0, **1**	0.08 ± 0.07, **9**	0.06 ± 0.04, **10** NA**, 2**	0.11 ± 0.09, **9**

C. Arginase activity and urea accumulated in larval tissues

			Arginase (μmol min^{-1} mg^{-1} protein)		Urea (μmol mg^{-1} wet mass)	
			Wet	Dry	Wet	Dry
12.5	First	14.5	0.08 ± 0.03, **7**	0.11 ± 0.04, **5**	0.22 ± 0.03, **7**	0.22 ± 01, **5**

10