Ecology and Evolution

Reproductive colonization of land by frogs: embryos and larvae excrete urea to avoid ammonia toxicity

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ABSTRACT

Vertebrate colonization of land occurred multiple times, including over 50 origins of terrestrial eggs in frogs. Some environmental factors and phenotypic responses that facilitated these transitions are known, but responses to water constraints and risk of ammonia toxicity during early development are poorly understood. We tested if ammonia accumulation and dehydration risk induce a shift from ammonia to urea excretion during in early stages of four anurans, from three origins of terrestrial development. We quantified ammonia and urea concentrations during early development on land, under well-hydrated and dry conditions. Where we found urea excretion, we tested for a plastic increase under dry conditions and with ammonia accumulation in developmental environments. We assessed the potential adaptive role of urea excretion by comparing ammonia tolerance measured in 96h-LC_{50} tests with ammonia levels in developmental environments. Ammonia accumulated in foam nests and perivitelline fluid, increasing over development and reaching higher concentrations under dry conditions. All four species showed high ammonia tolerance, compared to fishes and aquatic-breeding frogs. Both nest-dwelling larvae of *Leptodactylus fragilis* and late embryos of *Hyalinobatrachium fleischmanni* excreted urea, showing a plastic increase under dry conditions. These two species can develop the longest on land and urea excretion appears adaptive, preventing their exposure to potentially lethal levels of ammonia. Neither late embryos of *Agalychnis callidryas* nor nest-dwelling larvae of *Engystomops pustulosus* risked toxic ammonia levels under dry conditions, and neither excreted urea. Our results suggests that an early onset of urea excretion, its increase under dry conditions, and elevated ammonia tolerance, can all help prevent ammonia toxicity during terrestrial development. High ammonia represents a general risk for development that can be exacerbated as climate change increases dehydration risk for terrestrial-breeding frogs. It may also be a cue that elicits adaptive physiological responses during early development.

Keywords: Centrolenidae, Developmental physiology, Dehydration risk, Leptodactylidae, Phenotypic plasticity, Phyllomedusidae

1.0 INTRODUCTION

Vertebrate colonization of land was a major evolutionary event that exposed animals to new ecological and physiological challenges (Bray, 1985). Most aquatic vertebrates excrete nitrogen in the form of cheap, water-soluble ammonia (Cragg et al., 1961; Wright & Fyhn, 2001). However, living on land increases the risk of dehydration and the need to conserve water creates a waste-disposal problem (Jørgensen, 1997; Shoemaker et al., 1969), increasing the risk of ammonia toxicity (Chew & Ip, 2014; Ip & Chew, 2010). The shift from excreting ammonia to the less toxic urea is hypothesized to be a key adaptation that facilitated the transition from water to land (Amemiya et al., 2013; Mommsen & Walsh, 1989). One successful approach to understand the environmental challenges and phenotypic responses involved in colonizing land focuses on extant species whose lives cross the water–land interface (Ashley-Ross et al., 2013; Graham & Lee, 2004; Martin & Carter, 2013; Wright & Turko, 2016). Although urea synthesis occurs in several vertebrate lineages and evolved in multiple environmental contexts (Anderson, 2001; Chew et al., 2004; Costanzo & Lee, 2005; Jørgensen, 1997; Randall et al., 1989; Saha & Ratha, 1989; Shoemaker & Nagy, 1977; Wright et al., 2004), its regulation during early development has not been addressed in the context of the colonization of land.

Developmental plasticity is hypothesized to facilitate colonization events and evolutionary change (Ghalambor et al., 2007; Lande, 2015; West-Eberhard, 2003) and may have played a role in transitions from aquatic to terrestrial environments. Environmentally induced traits may allow survival in altered developmental environments (Gomez-Mestre & Buchholz, 2006; Lande, 2009, 2014; Kulkarni et al., 2017), including early colonization of land by tetrapods (Standen et al., 2014). Assessing plasticity in physiological regulatory systems may help to clarify key developmental mechanisms that allow persistence in changing environments (Rundle & Spicer, 2016; Spicer & Burggren, 2003). For example, changes in physiological tolerance may enable habitat invasion and survival under stressful developmental conditions due to global climate change (Lee et al., 2011; Mueller et al., 2019; Tedeschi et al., 2016). Few studies have explored environmental conditions that may affect those physiological mechanisms underlying urea excretion in vertebrates, but some attempts have been made in aquatic organisms (Chadwick & Wright, 1999; Gomez-Mestre et al., 2004; Janssens, 1972; Wright et al., 1995; Wright & Wright, 1996). Understanding

the role developmental plasticity in urea excretion plays in the transition to terrestrial life requires assessing the environmental circumstances and physiological challenges facing vertebrates that move between aquatic and terrestrial environments during their development.

Studies of reproductive biology in amphibians have helped to identify key ecological and environmental conditions that may have allowed evolutionary colonization of land (Haddad & Prado, 2005; Liedtke et al., 2017; Méndez-Narváez et al., 2015; Touchon & Worley, 2015; Zamudio et al., 2016). In frogs, evolutionary transitions from aquatic to terrestrial breeding have occurred many times, with at least 56 independent origins of terrestrial eggs (Goin, 1960; Gomez-Mestre et al., 2012). Moving anamniotic eggs to land creates a high risk of desiccation (Eads et al., 2012; Mitchell, 2002; Rudin-Bitterli et al., 2020). It also creates a potential problem of ammonia disposal; since the ammonia produced as embryos metabolize yolk proteins (Jorgensen et al., 2009) may not easily diffuse into the surrounding environment (Dhiyebi et al., 2013). Plastic extensions of terrestrial development, either *in ovo* after hatching competence or as terrestrial larvae, have also evolved in several lineages, sometimes facilitated by parental care (Delia et al., 2019; Warkentin 2011), favored by enhanced survival in the water (Warkentin, 1995; Willink et al., 2014), or enforced by the need for flooding to access or create an aquatic habitat (Bradford & Seymour, 1985; Downie, 1984; 1994). We hypothesize that the risk of lethal ammonia accumulation increases with (i) the use of terrestrial developmental environments, (ii) the dehydration risk on land and (iii) the extent of terrestrial development, including any voluntary or enforced plastic delay in the transition to water. If so, plastic physiological responses could allow terrestrial early life stages to avoid this risk and, thereby, increase survival until larvae transition to their aquatic environments.

We specifically hypothesize an early embryonic or larval onset of urea excretion may have facilitated development on land by preventing a likely common risk of ammonia toxicity under water constraints. In frogs, embryonic and larval adaptations have been described in response to biotic and abiotic environmental challenges on land (Alcocer et al., 1992; Bradford & Seymour, 1985; Downie, 1984; Seymour & Bradford, 1995; Warkentin, 2007),

including plasticity in hatching timing (Delia et al., 2014; Gomez-Mestre et al., 2008; Poo & Bickford, 2014; Touchon et al., 2011; Warkentin, 1995). In most frogs, a high capacity for urea synthesis seems to be acquired at metamorphosis, congruent with the developmental and physiological transition from water to land (Cohen, 1970; Munro, 1953). Urea excretion has been reported during terrestrial embryonic and larval development (Alcocer et al., 1992; Grafe et al., 2005; Martin & Cooper, 1972; Shoemaker & McClanahan, 1973; Schindelmeiser & Greven, 1981); however, when urea has been measured in parentally constructed developmental environments (Alcocer et al., 1992; Shoemaker & McClanahan, 1973), studies have not distinguished urea produced by parents versus that produced by the offspring. In addition, few studies have examined how environmental conditions during development, such as dehydration risk and ammonia toxicity, may directly induce or selectively favor urea excretion (Gomez-Mestre et al., 2004; Wright & Wright, 1996), which could elucidate its potential role in the repeated reproductive colonization of land by amphibians.

We studied four frog species, from three lineages that independently evolved terrestrial development, where variable extensions of development on land occur and dehydration risk is a common source of mortality (Delia et al., 2013; Méndez-Narváez et al., 2015; Salica et al., 2017; Zina, 2006). These included two species from the family Leptodactylidae, in which parents create foam nests — a non-aquatic environment for eggs —with variable degrees of terrestriality (Heyer, 1969; de Sá et al., 2014). In *Engystomops pustulosus*, embryos develop in foam nests floating on ephemeral pools and nest-dwelling hatchlings can tolerate a short period of extended terrestrial developmental if pools dry and refill (Dalgetty & Kennedy, 2010). In *Leptodactylus fragilis*, eggs and hatchlings develop in nests within subterranean chambers and require rainfall to flood their chamber before moving into an ephemeral pond. These larvae can arrest development and survive for extended periods on land, within larval-created foam nests (authors, unpublished). In both of these leptodactylids, extended terrestrial development is enforced by lack of access to an aquatic habitat.

We also studied two species whose embryos develop in arboreal gelatinous clutches that are parentally provisioned with water, with larvae that drop into water upon hatching. In *Agalychnis callidryas* (Phyllomedusidae), females hydrate their clutches at oviposition (Pyburn, 1970). Their embryos can extend terrestrial development *in ovo* by about 80% after reaching hatching competence, if rainfall and humidity maintain adequate egg hydration (Warkentin et al., 2017). In *Hyalinobatrachium fleischmanni* (Centrolenidae), males hydrate clutches throughout development (Delia et al., 2013), and hatching-competent embryos can extend their development *in ovo* by almost 200% under continued care (Delia et al., 2013). In both species, extended terrestrial development is opportunistic; under safe conditions embryos delay hatching to increase growth and development *in ovo*, which benefits the larvae (Delia et al., 2019; Warkentin, 1995; Willink et al., 2014), while adverse conditions induce earlier hatching (Delia et al., 2014; Salica et al., 2017; Warkentin, 1995).

Adaptive plastic responses of embryos and larvae to common environmental threats and cues (e.g., hypoxia, dehydration, predation, diet) are phylogenetically widespread in amphibians, suggesting convergent evolution and potentially repeated use of shared underlying mechanisms (Gomez-Mestre & Buchholz, 2006; Ledón-Rettig et al., 2010; Warkentin, 2011). Here, we take advantage of the repeated evolution of terrestrial development to test the hypothesis that facultative urea excretion during early development occurs in response to dry conditions, preventing ammonia accumulation to toxic levels. We further hypothesize that species with longer periods of terrestrial development – whether enforced or opportunistic – are more likely to exhibit urea excretion. To test these hypotheses, we measured the accumulation of ammonia and urea within developmental environments (i.e., eggs and foam nests) at embryonic and early larval stages under ecologically relevant control (well-hydrated) and dry conditions for four species. We estimated the amount of ammonia and urea excreted and tested their plasticity in response to dry conditions. Finally, we estimated LC_{50} values for late embryos and early larvae and compared them with ammonia concentrations measured in developmental environments and predicted to occur in the absence of urea excretion.

2.0 METHODS

2.1 Study site

We conducted field work in Gamboa Panamá, at and near the Experimental Pond (9°07′14.8” N, 79°42′ 15.4” W) and along streams crossing Pipeline Road in Soberanía National Park (9°04′ 33.7” N, 79°39′33.2” W). We collected foam nests and gelatinous clutches from breeding sites during the 2016–2019 rainy seasons 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 conducted experiments in an open-air laboratory, at ambient temperature and humidity (~26.5°C, ~85%), at the Smithsonian Tropical Research Institute (STRI) in Gamboa. We performed laboratory analysis at STRI facilities in Gamboa and Panama City.

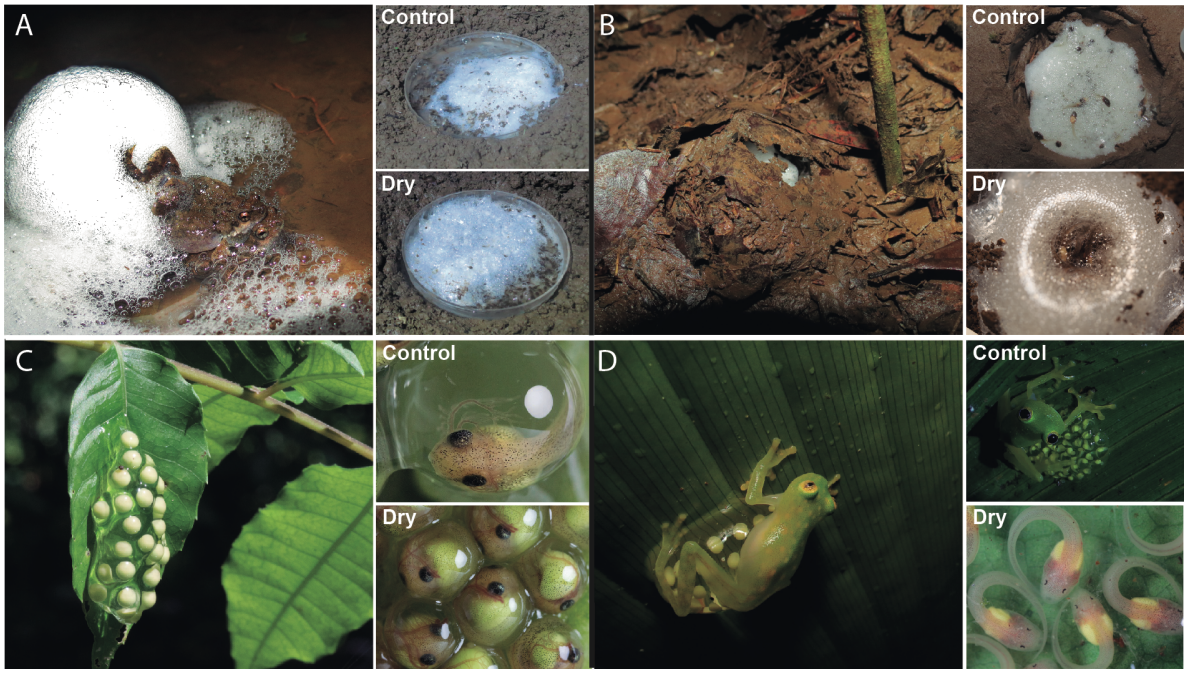


Fig. 1. Study species represent four reproductive modes and three origins of terrestrial eggs, and all commonly face the risk of dehydration. We exposed aquatic foam nests of *Engystomops pustulosus* (A), terrestrial foam nests of *Leptodactylus fragilis* (B), terrestrial gelatinous clutches of *Agalychnis callidryas* that lack parental care (C) and terrestrial gelatinous clutches of *Hyalinobatrachium fleischmanni* that require parental care (D) to well-hydrated control and dry conditions.

2.2 Experimental induction of dehydration risk

We exposed egg clutches to ecologically relevant, species-specific control (well-hydrated) and dry conditions in the laboratory, based on their natural history. We collected foam nests of *E. pustulosus* from ephemeral pools in the field or by keeping amplectant pairs overnight in small containers with water-filled Petri dishes. We kept control nests on water in dishes and drained the water from dishes to simulate a dry pool for the dry treatment (Fig. 1A).

For *L. fragilis*, we monitored territories for mating activity and excavated their burrows to collect nests the morning after oviposition. We also measured soil water-content near nests using a soil-moisture smart sensor (S-SMC-M005, HOBO®). In the laboratory, we buried foam nests in soil collected from breeding sites (Fig. 1B). For controls, we matched soil water content to that measured near burrows (field conditions, mean ± SD: 0.386 ± 0.05 m^3/m^3, N = 30), spraying the soil with water daily to maintain hydration (control lab conditions: 0.35–0.40 m^3/m^3). For the dry treatment, we reduced soil water-content to simulate an extended period without rain (dry lab conditions: 0.25–0.30 m^3/m^3).

We collected *A. callidryas* egg clutches the morning after oviposition and transported them to the laboratory on the leaves where they were laid. We mounted the clutches (leaves) on plastic cards, set them over water in cups, and housed them in plastic containers with partly screened lids. To simulate ideal field conditions for the control treatment, we used an automated system to mist clutches hourly with rainwater, removing excess water twice daily to avoid submerging eggs. For the dry treatment, we manually misted clutches daily, providing just enough hydration to avoid mortality while exposing embryos to sublethal dehydration (Salica et al., 2017, Fig. 1C).

Because *H. fleischmanni* deposit egg clutches on the underside of leaves, embryos are protected from rain and depend on paternal care for hydration (Delia et al., 2020; Delia et al., 2013). Males deliver water via brooding eggs with their pelvic patch; this is required for embryo survival during early development and not effectively replaced by misting. Therefore, we allowed all clutches to receive parental care in the field for five days. We then collected clutches for the dry treatment and maintained them in the laboratory for 5 more days, providing minimal manual misting as above. We left control clutches in the field with their fathers for an additional five days of care, then collected them (Fig. 1D).

2.3 Plasticity in nitrogen excretion in response to dry conditions

2.3.1 Accumulation of N-wastes during early development

We quantified ammonia and urea concentration (mg/L) in developmental environments at multiple times to assess their accumulation over development and the effect of dry conditions. For all species, we collected samples of foam or egg jelly the morning after oviposition for baseline measurements of parental nitrogen-wastes. Subsequently, we collected samples of foam and perivitelline fluid (PVF) at species-specific ages (Fig. 2); these were based on key ecological and behavioral events, not morphological stages. Our last sampling age was just before the onset of dehydration-induced mortality in foam nests and drying-induced hatching in gelatinous clutches under laboratory conditions, matching sampling across treatments to this dry-treatment constraint.

We collected foam samples from *E. pustulosus* and *L. fragilis* nests with 3 ml plastic transfer pipettes. These samples contained accumulated nitrogen wastes from the excretion of multiple individuals in the nest. Samples collected near hatching (2.5 d and 4.5 d for *E. pustulosus* and *L. fragilis*, respectively) included embryonic wastes excreted into the PFV and released into the foam at hatching plus some early larval wastes. Subsequent samples at one (4.5 d for *E. pustulosus*) or two (8.5 d and 12.5 d for *L. fragilis*) ages during the extended time in the foam (Fig. 2A-B) included additional larval wastes. We collected foam samples from different parts of large foam nests of *E. pustulosus*; in the smaller nests of *L. fragilis* we collected most or the foam, especially at the latest age.

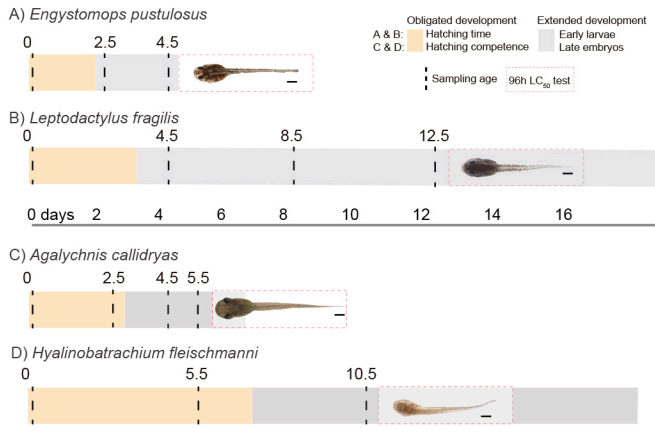


Fig. 2. Species-specific sampling ages (vertical dashed lines) for nitrogen excretion measurements in four frog species, each with a different reproductive mode. After the final N-waste sample, we conducted 96h-LC_{50} tests (dashed red box) for ammonia tolerance of

241 tadpoles (image scale = 1 mm) removed from the nest (A, B) or induced to hatch (C, D).
242 All four species have an obligate embryonic period (orange bars) and can extend
243 development on land (gray bars) as nest-dwelling larvae (A, B) or hatching-competent
244 embryos (C, D). Larvae must await (re)flooding to leave foam nests (A, B), while embryos
245 (C, D) fall to the water below upon hatching.
246
247 We collected PVF samples from *A. callidryas* and *H. fleischmanni* clutches with 0.3 ml
248 insulin syringes, pooling PVF extracted from several sibling eggs within a clutch. The first
249 samples (2.5 d and 5.5. d for *A. callidryas* and *H. fleischmanni*, respectively) included early
250 embryonic wastes, before hatching competence. Subsequent samples at one (10.5 d for *H.*
251 *fleischmanni*) or two (4.5 d and 5.5 d for *A. callidryas*) ages during the extended time in the
252 egg (Fig. 2C-D) also included late embryonic wastes.
253
254 We froze samples (-18°C) in micro-centrifuge tubes and measured ammonia and urea
255 concentration within two months. We quantified ammonia and urea concentrations using a
256 colorimetric approach (Fawcett & Scott, 1960) with a commercial enzymatic kit
257 (Boehringer Mannheim Cat. No. 10542946035). We thawed samples and centrifuged foam
258 for five minutes at 12000 rpm to obtain the liquid portion for analysis. PVF and jelly
259 samples were analyzed without centrifuging. We assessed ammonia and urea concentration
260 simultaneously, using 0.2 ml of stock of stock in two quartz test tubes (0.1 ml each) and
261 measuring changes in absorbance (at 340 nm) at room temperature with a UV-Visible
262 Spectrophotometer (Thermo-Scientific Evolution 60S). We ran controls without samples to
263 assess background absorbance (nm) of kit reagents for the ammonia and urea tests
264 (Supplementary Methods S1). Some samples produced zeros (under detection limit) or led
265 to high absorbances that did not allow us to quantify concentrations (NA; Supp. Methods
266 S2). We measured a subset of samples two or three times to assess measurement precision
267 (ammonia: mean CV = 7.3%, N = 21; urea: mean CV = 8.6%, N = 9).
268
269 **2.3.2 Amount of N-wastes accumulated at the last sampling age**
270 To test if dry conditions experienced during development led to a plastic change in the
271 amount of each nitrogen waste excreted, we estimated the amount (g = concentration ×
272 volume) of ammonia and urea in developmental environments at the latest sampling age for
273 *L. fragilis* and *H. fleischmanni*. This is necessary to determine if higher urea concentration

under dry conditions simply reflects reduced water in developmental environments vs. higher urea (and lower ammonia) excretion in response to dehydration. For *L. fragilis*, we collected all foam and counted larvae in each nest. We centrifuged the foam and measured the volume of liquid. If there was not enough foam around nest-dwelling larvae to directly sample via a pipet, we collected the small nest with a clean spoon, taking care to not collect soil particles, dissolved it with a known volume of deionized water, and corrected by this dilution factor (Suppl. Methods S3). We were unable to separate and collect all foam and *E. pustulosus* larvae from their larger aquatic foam nests, limiting estimates of total and individual amount of N-wastes. For *H. fleischmanni*, we recorded the number of eggs per clutch from which we extracted the PFV and measured its pooled volume. Many *A. callidryas* embryos, especially in well-hydrated clutches, hatched during PVF collection, causing fluid loss and precluding estimates of total excretion. We calculated a predicted concentration of ammonia, that would have accumulated without urea excretion, for each sample (predicted ammonia = [ammonia]+2*[urea]) to test if this value predicted the excreted urea. Calculations were done using molar concentrations, then reconverted to mg/L for comparison with measured values. We also estimated the total N-waste accumulation in developmental environments (= mol ammonia + mol urea) to test if it was affected by dry conditions.

2.4 Ammonia toxicity in early larval stages at the latest sampling age

To assess if urea synthesis prevents ammonia toxicity, we conducted standard 96-h LC_{50} tests for ammonia in all the study species (Fig. 2). We selected our initial range of total ammonia nitrogen (TAN) concentrations based on the ammonia levels detected for each species at our latest sampling point in the dry treatment, where we observed no mortality that we could attribute to ammonia toxicity, but ammonia reached the highest levels (Table S1). We ran pilot toxicity trials to adjust concentrations across test solutions for each species in attempt to cover the full range of mortality (0 to 100%). For each trial, we randomly assigned 10 tadpoles per concentration into eight experimental TAN concentrations, except in five trials we had fewer concentrations or tadpoles per concentration. We also ran controls in aged (dechlorinated) tap water, for all but three trials, where we never detected mortality. For each TAN concentration, we filled small

plastic cups with 15 ml of an experimental solution made from ammonium chloride (NH_4Cl) in aged tap water. We measured the pH (Mettler Toledo) for one trial each in three species (excepting *E. pustulosus,* inferred from others; Table S2), to cover the full range of nominal TAN concentrations. We used these values for a descriptive assessment of the toxic effects of un-ionized ammonia (NH_3) to compare with other published studies (Suppl. Methods S4).

We tested individuals from our latest sampling age, after extended development on land, that we removed from foam nests or induced to hatch from egg clutches (hereafter tadpoles when referring to toxicity experiments). We placed siblings together in aged tap water for 2–6 h to eliminate nitrogen wastes accumulated in the body before the onset of each trial. All *E. pustulosus* and all but two *L. fragilis* trials used individuals from a single foam nest. For *H. fleischmanni* and all but one *A. callidryas* trial we pooled hatchlings from 2–3 developmentally matched clutches. To account for intraspecific variation we ran multiple trials, using tadpoles that came from both control and dry treatments. In total, we performed 12 trials for *E. pustulosus*, 14 for *L. fragilis*, 13 for *A. callidryas* and 5 for *H. fleischmanni*, some with different TAN concentration ranges (Table S2). Every 24 h, we recorded mortality, removed motionless subjects to a cup with aged tap water to confirm death, and replaced experimental solutions in test cups. Because embryos and foam-dwelling larvae subsist on yolk, test subjects were unfed. After 96 h, survivors were moved to aged tap water and fed *ad libitum* for at least one day before release at their collection site.

2.4 STATISTICAL ANALYSIS

We conducted statistical analyses in R v 3.6.1 (R Core Team, 2019). Because several samples fell below detection limits (Table S1; supporting information Methods S1) and were considered informative (Douma & Weedon, 2019), we rank-transformed N-waste concentrations and amounts in developmental environments to avoid a potential zero-inflation problem. We accounted for zeros in proportional data with a standard data transformation bounding all values between 0 and 1 (Douma & Weedon, 2019; Smithson & Verkuilen, 2006).

We tested for changes in ammonia and urea concentration with development (age), treatment (control and dry conditions), and their interaction, using a permutation approach to obtain P-values (5000 iterations). For *E. pustulosus*, we used linear mixed effect models (LMEM), including nest identity (ID) as a random effect to account for some repeated measurements (Bates et al., 2015; *lme4* package) and calculated permutated *p*-values for fixed factors (Luo et al., 2018; *predictmeans*). For *L. fragilis* and *A. callidryas,* we tested fixed effects with a permutated ANOVA (Frossard and Renaud 2019; *permuco*). For *H. fleischmanni*, we tested for an effect of age across sampling periods and treatment only at the last stage. We also performed permutated pairwise comparisons (fdr method), using the corresponding model structure in each case (*predictmeans*). Estimated parametric statistics and p-values are available in Supp. Material (Table S3-S6).

We tested for a difference in the amount of ammonia and urea excreted (total and per individual) across treatments using a permutated t-test (Hervé, 2020, *RVAideMemoire*). We also tested if the accumulated ammonia concentration (actual or predicted) in developmental environments at the last sampling age contributes or interacts with treatment to predict urea excretion amount, using an AIC approach (Ripley 2011; *MASS*); if so, we calculated permutated P-values of a LM (*permuco*). Finally, we tested if urea excretion as a proportion of total nitrogen wastes (ammonia + urea) increased in dry conditions using a generalized linear mixed model (GLMMs) with an underlying Beta error distribution (Brooks et al., 2017; *glmmTMB*) and ratio test (LRT) to obtain p-values and confirmation with a permutated t-test (Table S7).

We first estimated LC_{50} values for each species by fitting generalized linear models (GLM) using a binomial distribution with a probit-link function (*lme4*) with TAN concentrations as a fixed effect. We also included treatment (control or dry) and interaction effects in our models when they improved fit, based on AIC criteria (Table S8). Then, we used the best model to calculate LC_{50} values at 24 h, 48 h, 72 h, and 96-h LC_{50} and their 95% confidence intervals (Hlina, 2020, *ecotox*).

3. RESULTS

3.1 Ammonia and urea accumulation in terrestrial developmental environments

Foam nesting species

Ammonia was below the detection limit in newly laid *E. pustulosus* nests, with one exception, and all foam nests later accumulated ammonia (Fig. 3A, Table S1). Ammonia concentration changed with age ($p = 0.0002$), and treatment ($p = 0.01$), with no interaction effect ($p = 0.76$). Its concentration increased in foam nests from oviposition to 2.5 d (near hatching) and, again to 4.5 d (extended development) in both control and dry treatments (Fig. 3A, Table S3). Ammonia concentration was higher in dry foam nests at both ages (Fig. 3A, Table S3). We found urea in all newly laid foam nests. Urea concentration changed with age ($p = 0.0002$) and treatment ($p = 0.03$), with no interaction effect ($p = 0.59$). Its concentration decreased after oviposition with more nests falling below the urea detection limit over time, but with a marginally higher concentration in dry conditions (Fig. 3B, Table S3).

Ammonia was undetectable in newly laid *L. fragilis* nests and present in all older nests (Fig. 3C, Table S1). Ammonia concentration changed with age ($p = 0.0002$), treatment ($p = 0.02$), and their interaction ($p = 0.002$, Table S4). Its concentration increased in both treatments from oviposition to 4.5 d (near hatching) and again to 8.5 d (first extended development sample). However, it only continued to increase from 8.5 to 12.5 d (second extended development sample) in dry nests. Ammonia concentration was higher in dry conditions at the latest age (12.5 d, Fig. 3C, Table S4). We found urea in all recently laid foam nests (Fig. 3D, Table S1). Urea concentration changed with age ($p = 0.0002$), but not with treatment ($p = 0.07$) or their interaction ($p = 0.28$). First, its concentration decreased, falling below the detection limit by the first extended development sample (8.5 d). Then its concentration increased, especially in dry nests, by the second extended development sample (Fig. 3D, Table S4). Urea concentration was significantly higher in dry conditions only near hatching (4.5 d, Fig. 3D, Table S4).

Arboreal gelatinous clutches

Ammonia was undetectable in the jelly of newly laid *A. callidryas* clutches and the PVF at 2.5 d, except for one dry clutch (Fig. 3E, Table S1). Ammonia concentration in the PVF

changed with age, treatment, and their interaction (all $p = 0.0002$, Table S5). Its concentration increased more rapidly in dry clutches, from 2.5 d (early development) to 4.5 d (hatching competence), but we detected no change from 4.5–5.5 d. However, ammonia concentrations remained higher in dry clutches (Fig. 3E, Table S5). We found urea in the jelly of all newly laid clutches (Fig. 3F, Tables S1). Its concentration changed in the PVF with age ($p = 0.0002$), treatment ($p = 0.001$), and their interaction ($p = 0.02$). Urea concentration decreased over development and fell below detection limits by 4.5 d in control clutches, when it still remained detectable in the dry treatment. In both treatments, it was undetectable by 5.5 d (extended terrestrial development), with one exception in each condition (Fig. 3F, Tables S5). Urea concentration was higher in dry conditions at 2.5 and 4.5 d (Fig. 3F, Table S5).

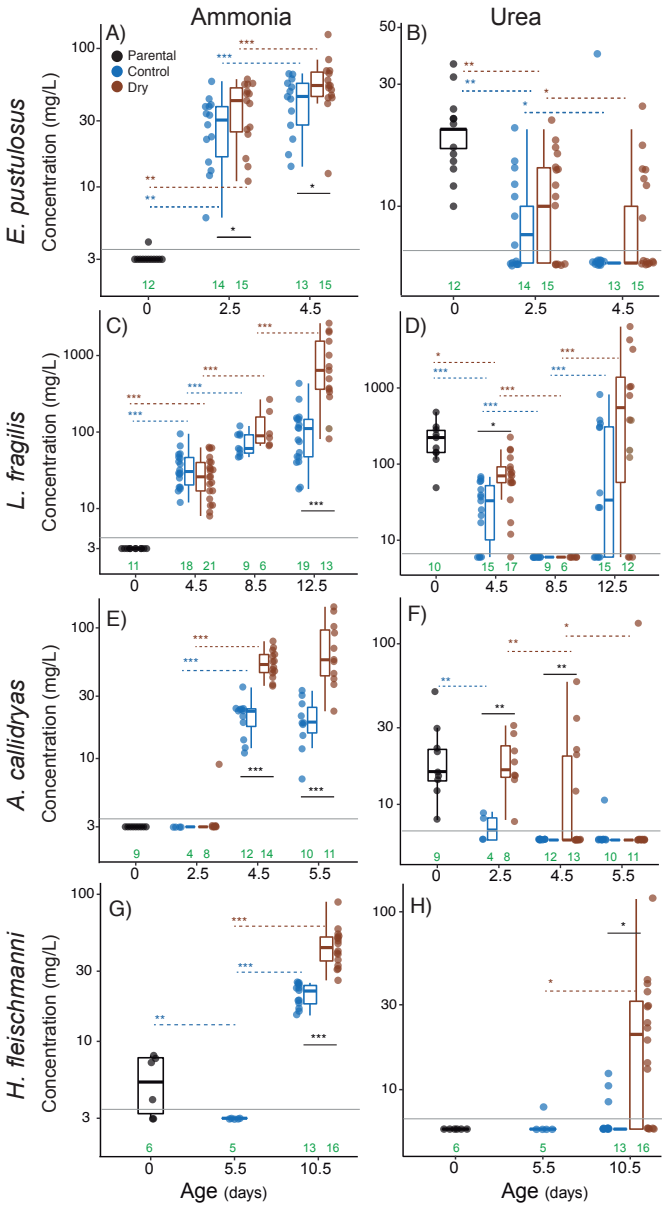


Fig. 3. Ammonia and urea concentration accumulated in developmental environments of four frog species: foam nests of A–B) *Engystomops pustulosus* and C–D) *Leptodactylus fragilis*; perivitelline fluid of E–F) *Agalychnis callidryas* and G–H) *Hyalinobatrachium fleischmanni*. Baseline measurements of N-wastes of parental origin were made shortly after oviposition (0 days; black points) from foam nests and egg jelly. Concentrations over development were measured from foam nests and PVF under species-specific control (blue) and dry (brown) conditions. Box plots show median, first and third quartiles, and extent of data to 1.5 X IQR; data points are also shown. P-values were obtained by permutation tests for pairwise comparisons (FDR correction), after fitting LMEM (A, B) and LM (C–H) for ammonia and urea concentration: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Table S3-S6). Significant differences between treatments are indicated with solid black lines and changes across consecutive ages with dotted blue (control) or brown (dry) lines. Solid grey lines represent detection limits of the enzymatic kit. Note log scale of Y-axes; we assigned

arbitrary sub-threshold values (Supplementary Methods 1) of 3 mg/L and 6 mg/L for zeros in this figure. Detailed descriptive analysis, including zeros, are in Supplementary material (Table S1).

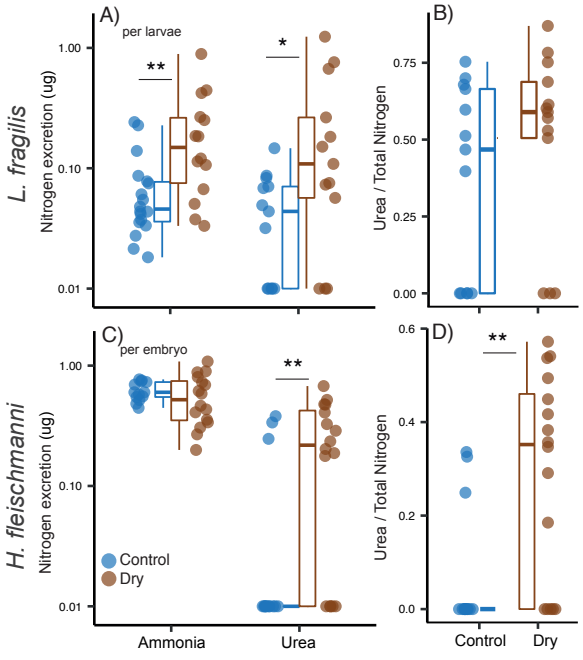
Ammonia was detected in the jelly of some, but not all, recently laid *H. fleischmanni* clutches (Fig. 3G, Table S1). Ammonia concentration in the PVF differed among age and treatment categories ($p = 0.0002$). It was undetectable after 5.5 d of paternal care in the field (1.5 days before hatching competence). Ammonia concentration then increased as development continued in both treatments, but concentrations were greater in the dry treatment without fathers (Fig. 3G, Table S6). Urea was undetectable in the jelly of newly laid clutches (Fig. 3H, Table S1). Urea concentration in the PVF differed among age and treatment categories ($p = 0.002$). At 5.5 d (before hatching competence) urea was detectable in the PVF of only one clutch. However, by 10.5 d (3.5 days into extended terrestrial development) urea was detected in more clutches and significantly higher in the dry treatment (Fig. 3H, Table S6).

3.2 Urea excretion in response to dry conditions and predicted ammonia accumulation

We found evidence of embryonic/larval urea excretion in *L. fragilis* and *H. fleischmanni,* that we could separate from parental urea (Fig. 3). We also found that dry foam nests and jelly clutches contained less water than controls (*L. fragilis* nests: 0.02 ± 0.01 ml, N = 8 vs. 0.11 ± 0.06 ml, N = 13; $t_{16.4}$=3.91, $p = 0.001$; *H. fleischmanni* clutches: 0.29 ± 0.1 ml, N = 14 vs. 0.61 ± 0.2 ml, N = 6; $t_{6.18} = 3.32$, $p = 0.0152$). Water volume was negatively correlated with ammonia and urea concentration in foam nests of *L. fragilis* (ammonia: S = 2638, $p = 0.0003$; urea: S = 1757, $p = 0.0167$) and with ammonia but not urea concentration in the PVF of *H. fleischmanni* (ammonia: S = 2175.5, p = 0.0026; urea: S = 1633.7, $p =$ 0.3329). Therefore, we calculated the amount of ammonia and urea (total and per-individual) excreted by embryos/larva of these two species to test for plastic changes (Fig. 4).

In *L. fragilis*, total and individual ammonia excretion differed between treatments (total, $p = 0.006$; individual, $p = 0.008$; Table S7, Fig. 4A), while urea excretion was marginally higher in the dry treatment (total, $p = 0.059$; individual, $p = 0.049$, Table S7, Fig. 4A).

455 However, the proportion of total nitrogen wastes excreted as urea did not differ between
456 control and dry foam nests (Table S7, Fig. 4B). In analyses including actual ammonia
457 concentration, there was a significant interaction as well as a main effect of treatment on
458 total urea excretion (interaction $p = 0.045$; treatment $p = 0.009$; ammonia $p = 0.085$), but no
459 ammonia or interaction effect on individual excretion (Table S8). However, higher
460 predicted ammonia concentration (total, $p = 0.004$; individual, $p = 0.015$; Table S8, Fig.
461 5A) and dry conditions (total, $p = 0.035$; individual, $p = 0.049$) both increased urea
462 excretion, with a significant interaction (total, $p = 0.014$; individual, $p = 0.037$).
463 Furthermore, we found that dry foam nests accumulated a higher total N-waste amount than
464 controls (dry:1.401 ± 1.067 µmol, N = 13 vs control: 0.477 ± 0.286 µmol, N = 13; t = -3.01
465 $p = 0.004$).
466



467

468 **Fig. 4.** Amount of ammonia and urea excreted per individual for (A) early larvae of
469 *Leptodactylus fragilis* and (C) late embryos of *Hyalinobatrachium fleischmanni*, and the
470 proportion of total nitrogen waste (ammonia + urea) excreted as urea in both species (B, D)
471 during development in control (blue) and dry (brown) conditions at the latest sampling age
472 (12.5 d and 10.5 d, respectively). P-values were obtained from permutation tests *$p < 0.05$,
473 **$p < 0.01$, ***$p < 0.001$ (Table S7).
474

475 In *H. fleischmanni*, neither total nor individual ammonia excretion differed between
476 treatments (Table S7, Fig. 4C), but urea excretion was higher in the dry treatment (total $p =$

0.008; individual, $p = 0.022$). Moreover, the proportion of total nitrogen wastes excreted as urea was higher in dry clutches ($p = 0.006$, Table S7, Fig. 4D). Higher predicted ammonia concentration and dry conditions both increased urea excretion, with a significant interaction (total: ammonia $p = 0.003$, treatment $p = 0.005$, interaction $p = 0.011$; individual: ammonia $p = 0.002$, treatment $p = 0.007$, interaction, $p = 0.009$; Table S8, Fig. 5B). Actual ammonia concentration did not explain urea excretion in this species, neither as a main effect nor an interaction with treatment (Table S8). Furthermore, total N-wastes did not differ between control and dry conditions (dry: 0.846 ± 0.314 µmol, N=13 vs control: 0.778 ± 0.235 µmol, N=16, t = -1.21 $p = 0.228$).

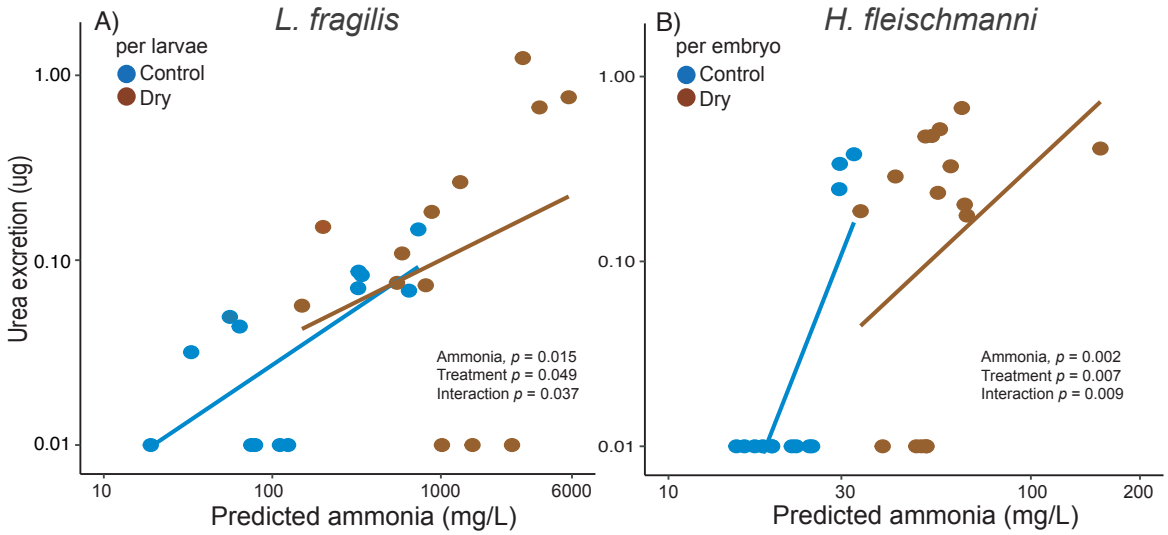


Fig. 5. Amount of urea excreted per individual *Leptodactylus fragilis* larva (A) and *Hyalinobatrachium fleischmanni* embryo (B) in relation to the ammonia concentration predicted to have accumulated in their developmental environments without the urea cycle, under control (blue) and dry (brown) conditions, by the latest sampling age (12.5 d and 10.5 d, respectively). P-values were obtained from permutation tests from a LM in both species (Table S8).

3.3 Ammonia tolerance (LC_{50}) in early life stages during terrestrial development

Early larvae of *L. fragilis* had the highest LC_{50} level at 24 and 96 hours (2260 and 2070 mg/L, respectively, Table 1, Table S9), while *H. fleischmanni* had the lowest LC_{50} value at 24 hours and 96 hours (709 and 428 mg/L, respectively; Table 1, Table S9). In general, LC_{50} decreased over time from 24 to 96-h in all study species (Table 1).

Table 1. Ammonia LC_{50} values (mg/L) for early life stages of anurans with terrestrial development (mean and 95% CI; this study; Table S10) and highest values for anuran embryos and tadpoles and multiple life stages of fishes with aquatic development (from literature review of 15 anuran and 19 fish species, Table S11).

	Species	24 h LC_{50}	96 h LC_{50}	Life stage	Reference
Anurans	*E. pustulosus*	1650 (1460–2100)	859 (693–986)	Hatchling	This study
	L. fragilis	2260 (2100–2560)	2070 (1960-2230)	Hatchling	This study
	H. fleischmanni	709 (566–2450)	428 (305–693)	Hatchlings	This study
	A. callidryas	1121 (1060–1630)	648 (567–753)	Hatchlings	This study
	Pseudacris regilla	—	26.48—77.67	Embryos	Schuytema & Nebeker, 1999
	Anaxyrus americanus	—	17.56—495.5	Tadpoles	Hecnar, 1995; Xu and Oldham, 1997
Fishes	*Opsanus beta*	—	1083	Embryos	Barimo and Walsh, 2005
	Opsanus beta	—	93	Larvae	Barimo and Walsh, 2005
	Oncorhynchus mykiss	—	161	Juveniles	Thurston et al., 1981; Walsh et al., 1993
	Alcolapia grahami	—	13.23		
	Monopterus albus	—	3481	Adults	Ip et al., 2004; Walsh et al., 1993
	Alcolapia graham	12.77	—		
	Notropis topeka	—	21.4		

The mean ammonia concentration accumulated in developmental environments (foam nests or PVF, Table S1) at the latest sampling age did not overlap with LC_{50} values and their CI (Table 1), even under dry conditions, nor did it with any concentration that caused mortality in any of the four study species (Fig. 6). However, some *L. fragilis* nests and one *H. fleischamnni* clutch in the dry treatment had ammonia concentrations that could cause mortality (Fig. 6B, D), and levels in some dry *A. callidryas* clutches reached the onset of mortality (Fig. 6C). The predicted ammonia concentration, without urea excretion, under dry conditions did overlap with the lethal range in the two species where urea excretion was confirmed. In *L. fragilis* the mean predicted ammonia level was slightly below the LC_{50}, with the 95% CI extending to near 100% mortality (Fig. 6B). In *H. fleischmanni* the 95% CI for predicted ammonia overlapped with the onset of mortality at 96-h (Fig. 6D).

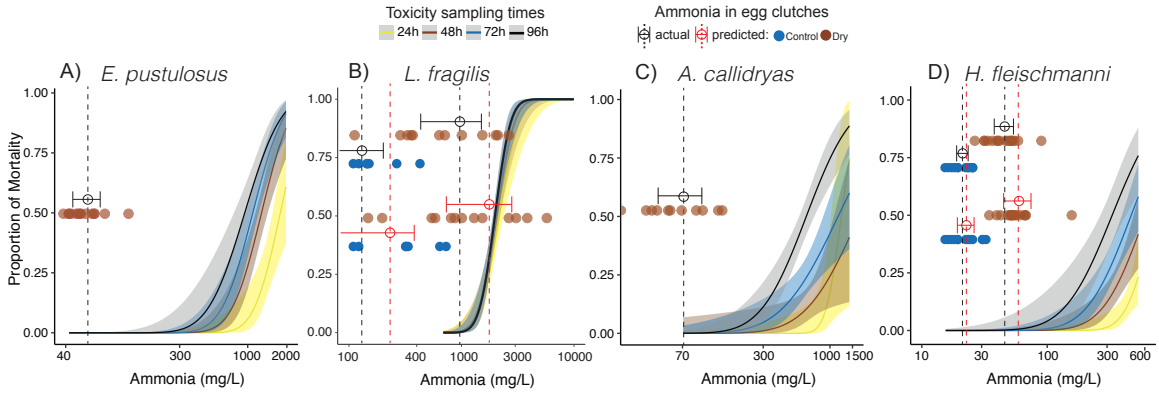


Fig. 6. Mortality as a function of ammonia (TAN) concentration for early larvae (A, B) and hatchlings (C, D) in four frog species, each with a different reproductive mode. Mortality curves, from adjusted binomial functions (probit link), and their 95% confidence intervals are color coded for four exposure durations. Means (with vertical dashed lines) and 95% CI represent actual ammonia concentrations (black) and ammonia concentrations predicted to have accumulated without the urea cycle (red), at the latest sampling age. Data points represent actual and predicted values for individual control (blue) and dry (brown) foam nests and egg clutches. Ammonia levels for control *E. pustulosus* nests and *A. callidryas* clutches were below the plotted range.

DISCUSSION

Our study revealed that exposure to dry conditions during terrestrial development affected urea excretion, mediated by a risk of ammonia toxicity during extended development on land. First, we determined that ammonia from embryonic and early larval excretion accumulated in terrestrial developmental environments and reached a higher concentration under dry conditions in all four species (Fig. 3). We found that urea excretion occurred in the two species with the longest developmental periods on land, nest-dwelling early larvae of *L. fragilis* and late embryos of *H. fleischmanni*. We also found a plastic increase in urea excretion under dry conditions, in foam nests in dry soils (Fig. 4A) and egg clutches without parental care (Fig. 4B). We present evidence for an adaptive role of urea excretion during development. This is supported by the increase in amount of urea excreted as predicted ammonia concentration rises (i.e., the ammonia concentration that would occur without the urea cycle, Fig. 5) and by the overlap between these concentrations and their ammonia LC_{50} values (Table 1; Fig. 6B, D). Our toxicity experiments also showed that actual levels of ammonia in developmental environments do not overlap with the ammonia LC_{50} values (Table 1, Fig. 6), which may explain the lack of urea excretion in *E. pustulosus* and *A. callidryas*. Thus, plasticity in urea excretion appears adaptive; it prevents

accumulation of toxic ammonia levels during extended development on land, especially under dry conditions where such levels are more likely to occur. Variability of urea excretion among and within species suggests that it is not simply terrestrial development but more specifically the risk of ammonia toxicity on land (Fig. 6) that regulates urea synthesis. Our results also suggest that plasticity in urea excretion may facilitate extended terrestrial development in some contexts, by preventing toxic levels of ammonia under the common risk of dehydration.

Ammonia accumulation in developmental environments

Our data supported our initial hypothesis that terrestrial development can generate a waste-disposal problem, particularly when prolonged, since ammonia accumulated in developmental environments (Fig. 3). This accumulation over development is presumably the product of protein breakdown from yolk reserves used in embryonic and early larval differentiation, as reported in fishes (Dworkin & Dworkin-Rastl, 1991; Finn et al., 1995) and *Xenopus* (Jorgensen et al., 2009). We detected ammonia levels up to 145, 126, and 89 mg/L in *A. callidryas*, *E. pustulosus*, and *H. fleishmanni*, respectively (Fig. 3), and above 2600 mg/L in *L. fragilis*, with higher concentrations under dry conditions. These values of ammonia exceed both assessed tolerances (i.e., LC_{50}) reported for aquatic tadpoles (Table 1, Table S10) and levels considered safe to avoid toxicity in freshwater environments (acute, 17 mg/L TAN; chronic, 1.9 mg/L TAN; EPA, 2013). Our results reveal that, even in a rainforest context, terrestrial development exposes early life stages of anurans to ammonia levels that can produce toxic effects in many species. The drier conditions that occur during short periods without rainfall during the reproductive (rainy) season, which are increasingly frequent with climate change, may exacerbate this (Lowe et al., 2021; Touchon & Warkentin, 2009).

Early onset of urea excretion during terrestrial development – context matters

The higher risk of ammonia toxicity is considered a key factor that selects for urea excretion when vertebrates move onto land (Wright, 1995; Wright & Turko, 2016), and transitions to terrestrial development are widespread in frogs, particularly in wet tropical environments (Gomez-Mestre et al., 2012). Amphibians that move to land at

577 metamorphosis are able to shift from ammonia to urea excretion at that stage (Cohen, 1970;
578 Munro, 1953). The urea we found in perivitelline fluid and foam nests in our four study
579 species (Fig. 3) suggests its excretion by early life stages but, alone, is not definitive
580 evidence. Although urea was undetectable in newly laid *H. fleischmanni* clutches, it was
581 present in *A. callidryas* clutches and foam nests of both *Leptodactylus* species long before
582 the onset of embryonic ammonia excretion. This presumably parental urea gradually
583 disappeared from developmental environments as embryos developed; however,
584 measurements at a single later stage would not distinguish urea from embryonic vs. parental
585 sources. This could be the case for at least some studies that reported urea in PVF and foam
586 nests during terrestrial development (Alcocer et al., 1992; del Pino et al., 1994; Shoemaker
587 & McClanahan, 1973). Other studies reported an early capacity for urea excretion from
588 indirect measurements, after initially terrestrial individuals were moved to water (Grafe et
589 al., 2005; Martin & Cooper, 1972; Shoemaker & McClanahan, 1982), or upon detection of
590 arginase activity in embryonic tissues (Alcocer et al., 1992; Shoemaker & McClanahan,
591 1982). Nonetheless, arginase can be involved in other biochemical pathways during early
592 development, so activity of rate-limiting enzymes such as CPS1 is necessary to confirm
593 urea cycle activity (Srivastava & Ratha, 2010). Our finding that urea is present in terrestrial
594 nests and clutches of some species without, or before, embryonic or larval excretion
595 suggests that, beyond simple detection, measures of changes in urea concentration over
596 development will be necessary to clarify the distribution and prevalence of the capacity for
597 urea excretion at early life stages, as well as its potential environmental regulation.
598
599 Our results revealed urea synthesis by embryos of *H. fleischmanni*, where urea was absent
600 in early development and higher amounts accumulated in clutches removed from parental
601 care. We also demonstrated urea synthesis by early larvae of *L. fragilis* during extended
602 development in foam nests (8–12 d), after parental urea disappeared. At these stages larvae
603 can produce their own foam if needed (authors, unpublished); our measurements of urea in
604 new larval foam, produced in a urea-free environment, provide further evidence of larval
605 urea excretion (authors, unpublished). Embryos of *H. fleischmanni* can remain *in ovo* up to
606 19 d with reliable paternal care (Delia et al., 2019), while early larvae of *L. fragilis* can
607 slow development after 8 d and survive for an extended period on land (two or three weeks;

608 authors, unpublished). In both cases, the ability to excrete urea may reduce the risk of
609 ammonia accumulation to toxic levels during this extended terrestrial period. We found no
610 evidence for urea synthesis in *A. callidryas* or *E. pustulosus*, which have shorter total and
611 facultative periods of terrestrial development (Fig. 2). Thus, urea excretion may occur and
612 improve survival in certain terrestrial development contexts and be disfavored in others
613 where its costs exceed any benefits (Shambaugh, 1977).
614
615 Embryos of *A. callidryas* hatch early in response to drying (Salica et al., 2017). The
616 specific cue mediating this response is unknown, but our results suggest that ammonia is a
617 candidate worth testing. Early hatching has been suggested to occur in fish embryos in
618 response to high ammonia accumulation in the PVF (Wright & Fyhn, 2001). Experiments
619 to date do not support this (Steele et al., 2001), but combinations of cues such as ammonia
620 and hypoxia should also be considered (Dhiyebi et al., 2013; Ortiz-Santaliestra et al., 2010).
621 We were only able to obtain samples of *A. callidryas* PVF under moderate levels of egg
622 dehydration, but embryos can experience – and survive – more extreme egg drying
623 (authors, personal observation). Moreover, ammonia levels in drying *A. callidryas* clutches
624 approached lethal levels more closely than they did for *E. pustulosus* foam nests. Thus, we
625 cannot reject the possibility that urea synthesis may occur under more extreme dehydration.
626 These embryos could also employ alternative physiological mechanisms to limit toxic
627 ammonia accumulation in the PVF. For instance, some fish embryos under high
628 environmental ammonia sequester ammonia in the yolk (Braun et al., 2009; Steele et al.,
629 2001) and others remove it by synthesizing glutamine (Essex-Fraser et al., 2005; He et al.,
630 2010; Sanderson et al., 2010; Wright et al., 2007). Some adults may even avoid ammonia
631 toxicity by using partial amino acid catabolism to produce alanine for an energy souce
632 during periods of terrestrial exposure (Ip & Chew, 2010). Nonetheless, the more limited
633 capacity of *A. callidryas* to extend development *in ovo*, compared to *H. fleischmanni* and
634 other glassfrogs (Delia et al., 2019), may limit the benefits these embryos could gain by
635 urea excretion and other ammonia detoxification strategies.
636
637 **Urea excretion plasticity in response to dry conditions and risk of ammonia toxicity**

Our study demostrated a plastic increase in the amount of urea excreted by both *L. fragilis* and *H. fleischmanni* under dry conditions (Fig. 4). Embryos of *H. fleischmanni* can remain *in ovo* up to 19 d with care (Delia et al., 2013) or hatch as early as 7 d after parental desertion (Delia et al., 2014). Our results show that parental egg brooding, which provides hydration, prevents the accumulation of high ammonia concentrations within eggs. They also suggest that embryos' ability to shift from ammonia to urea excretion may help them to cope with the dehydration that can occur without brooding, facilitating a several-day plastic delay in hatching even without care (Delia et al., 2013; Delia et al., 2020). Embryos of *L. fragilis* may reach water and survive as early as 3 d if flooded or hatch at 3.5 d and remain in the foam for weeks. While late embryos of *H. fleischmanni* can hatch to escape a deteriorating clutch environment, falling into the stream below, larvae of *L. fragilis* cannot leave their burrow until it floods; instead, they arrest development and produce new foam to prolong their survival on land (authors, personal observations; Downie, 1984). The inability of *L. fragilis* larvae to control when they leave the nest, thus higher potential N-waste accumulation (Fig. 3C, G), may explain their greater ureotelism even in control conditions (almost 50% N-waste excreted as urea; Fig. 4A, B). In contrast, *H. fleischmanni* show a strong increase in urea production under dry conditions (Fig. 4C, D). Urea was detectable in most of the control *L. fragilis* nests (61%, vs. 23% of *H. fleischmanni* controls; Fig. 4). A subset of these nests, like the dry treatment nests, had almost completely lost their parental foam by the last sampling age (authors, personal observation) and had urea excretion and predicted ammonia levels resembling the dry treatment (Fig. 5A, Table S10, Fig. S1); even so, differences between control and dry clutches in urea excretion were still evident (Fig. 4).

Our results support a role for ammonia accumulation, and its potential toxicity, in mediating a plastic increase in urea excretion under dry conditions. In both *L. fragilis and H. fleischmanni,* the ammonia concentration that would have occurred without the urea cycle predicted urea excretion (Fig. 5; Table S8). Because N-wastes concentrate as water is lost, if the wastes remain as ammonia drying increases risk of toxicity. Experimentally increasing environmental ammonia increases urea synthesis in ureotelic fish species, via upregulation of the activity of urea cycle enzymes (Barimo et al., 2004; Barimo & Walsh,

669 2005; Chew et al., 2005; Ip et al., 2005). In aquatic bullfrog tadpoles, urea excretion
670 increases with environmental ammonia, apparently without upregulating urea cycle
671 enzymes (Wright & Wright, 1996). However, precocious activation of urea cycle enzymes
672 has been reported in embryos of some fishes (Chadwick & Wright, 1999; Kharbuli et al.,
673 2006; Wright et al., 1995), where it is hypothesized to have evolved to prevent high
674 perivitelline ammonia levels where the chorion and surrounding water chemistry limit
675 ammonia diffusion (Dhiyebi et al., 2013; Rahaman-Noronha et al., 1996). In both *H.*
676 *fleishmanni* and *L. fragilis*, exposure of early larvae to sublethal ammonia levels in water
677 increases urea accumulation in tissues and activity of some urea cycle enzymes, compared
678 to siblings in water (authors, unpublished). This is consistent with an ammonia-induced
679 plastic increase in urea excretion as risk of ammonia toxicity increases in terrestrial
680 developmental environments.
681
682 We also detected a higher total amount of N-wastes (and thus potential ammonia level) in
683 dry nests of *L. fragilis*, but not in dry clutches of *H. fleischmanni*. Development rates are
684 the same in control and dry nests of *L. fragilis* (authors, unpublished). However,
685 adaptations that improve survival on land, enabling extended terrestrial development, may
686 involve specific metabolic demands that vary with hydration. We hypothesize that N-waste
687 production may increase as larval foam production increases in the dry treatment. Larval
688 foam-making is a key behavior facilitating extended survival on land (Downie, 1984;
689 Kokubum & Giaretta, 2005) and may require considerable energy for bubble blowing, as
690 well as glycoprotein for mucus production.
691
692 Moreover, interspecific variation in ammonia tolerance, combined with risk of ammonia
693 accumulation to toxic levels in terrestrial developmental environments, may explain
694 variation in urea synthesis. For *A. callidryas* and *E. pustulosus,* we found no evidence for
695 urea excretion, and ammonia levels in their PVF and foam nests did not overlap the lethal
696 range (Fig. 6A, C). We cannot, however, rule out urea synthesis under more extreme
697 dehydration. These embryos and larvae had a substantial margin of safety under control
698 conditions and for *E. pustulosus* even in dry conditions, but some *A. callidryas* clutches
699 were close to the onset of mortality in our dry treatment. In contrast, for both *L. fragilis* and

H. fleischmanni the potential ammonia concentration without urea synthesis overlapped the lethal range (Fig. 6 B, D), reaching as high as 100% predicted mortality in *L. fragilis*. Although *L. fragilis* larvae can tolerate high ammonia levels, without urea excretion during extended development on land they could face lethal toxicity; thus, we consider their high prevalence of urea excretion and its increase in dry conditions to be adaptive. Embryos of *H. fleischmanni*, which require parental care, show much lower ammonia tolerance. Without care, predicted ammonia levels can reach the onset of mortality in their toxicity curve (Fig. 6D) and, in many clutches, embryos excrete urea. If parental care is intermittent, urea excretion may enable embryos to survive a period of neglect and then benefit from further care, rather than simply hatching to escape a deteriorating egg environment; this plastic increase in urea excretion also appears to be adaptive. Overall, these results confirmed the hypothesis that a plastic increase in urea excretion is associated with the risk of ammonia toxicity under water constraints on land, likely improving survival and facilitating extended development of embryos and early larvae on land.

High ammonia tolerance also prevents ammonia toxicity with development on land

Our results suggest that terrestrial frog embryos/early larvae have evolved substantially higher ammonia tolerance than early life stages of aquatic-breeding frogs and fishes (Table 1, Table S11). Like ammonia levels in developmental environments, ammonia tolerance varied among our study species (Table 1, Table S9), but even the most sensitive, *H. fleishmanni*, showed greater tolerance than reported for other anuran larvae. The only comparable ammonia tolerances we found at early stages are for ureotelic embryos of some fishes, in particular the toad fish *Opsanus beta* (Barimo & Walsh, 2005; Rice and Strokes, 1975). Some adult fishes that live with high environmental ammonia or low water availability also show high ammonia tolerance (Chew et al., 2004; Ip et al., 2005; Saha & Ratha, 1994). Urea excretion was suggested to prevent toxicity during terrestrial development in two frogs, *Leptodactylus bufonius* and *Gastrotheca riobambae* (del Pino et al., 1994; Shoemaker & McClanahan, 1973). However, toxicity tests found low ammonia tolerance in these tadpoles, with lethal ammonia levels orders of magnitude lower than the levels reported in their developmental environments (Table S11). This mismatch and potential methodological limitations of the studies (e.g., chronic ammonia exposure and

high pH) suggest a need to re-examine these species (Brinkman et al., 2009; Thurston et al., 1981) and limit comparisons with other research. A pattern similar to our results occurs in *O. beta* and *Oncorhynchus mykiss*, where ammonia levels in their developmental environments (nests and PVF) do not reach toxic levels, most likely due to their early urea excretion (Barimo & Walsh, 2005; Dhiyebi et al., 2013; Rice and Strokes, 1975).

Evolutionary implications of early onset of urea excretion in vertebrates

The transition from aquatic to terrestrial life at metamorphosis in anurans (Wilbur & Collins, 1973), and concurrent shift from ammonotelism to ureotelism (Munro, 1953), has been a strong focus of ecological, physiological and evolutionary research (Laudet, 2011; Lowe et al., 2021; Wassersug, 1975). However, frogs have evolved a wide array of life history traits and parental strategies that allow them to reproduce and develop out of water (Gomez-Mestre et al., 2012; Haddad & Prado, 2005). Our results suggest that reproductive colonization of land by frogs was enabled not only by parental adaptations, such as water provisioning and thermal buffering of eggs (Delia et al., 2020; Méndez-Narváez et al., 2015; Pyburn, 1970), but also by embryonic and larval physiological adaptations. These include physiological responses to terrestrial conditions, such as an early onset of urea excretion, its upregulation under dry conditions, and elevated ammonia tolerance, all of which can help to prevent ammonia toxicity under water constraints. Such physiological traits and plastic responses seem most likely to evolve in terrestrial embryos and larvae that must, or are able to, spend more extended periods on land; indeed, such mechanisms may be a key component of this ability. The benefits of urea excretion for early life stages should be balanced against the metabolic cost of urea synthesis (Shambaugh, 1977; Wright & Fyhn, 2001) in analyses of overall cost–benefit trade-offs across ecological and developmental transitions, including from terrestrial to aquatic environments in early development (Delia et al., 2019; Touchon & Warkentin, 2010; Warkentin, 1995) as well as from aquatic to terrestrial development at metamorphosis (Bouchard et al., 2016; Gomez-Mestre et al., 2010; Touchon et al., 2013; Vonesh & Bolker, 2005).

Enzymatic mechanisms and genetic regulation of urea excretion have been studied in some teleost fishes under high risk of ammonia toxicity during terrestrial emersion (Chew et al.,

2003; 2004; Loong et al., 2005; Loong et al., 2012) and aquatic embryonic development (Barimo et al., 2004; LeMoine & Walsh, 2013; 2015; Steele et al., 2001; Wright et al., 1995). However, few studies have explored such mechanisms in tetrapod lineages in the context of ammonia toxicity (Ip et al., 2012; Janssens, 1972; Wright & Wright, 1996) and the transition to terrestrial life (Brown et al., 1959; Weng et al., 2004). Understanding physiological mechanisms of plasticity may be important to understand evolutionary change (Ledón-Rettig & Ragsdale 2021; Suzuki and Nijhout 2006;). For labile traits that may change during individual lives (Rundle & Spicer, 2016), changes in physiological tolerance across environments in response to diverse abiotic factors (Braun et al., 2009; Hopkins et al., 2016; Mendez-Sanchez & Burggren, 2017; Peña-Villalobos et al., 2016), might also contribute to colonization of and survival in new environments (Kulkarni et al., 2017; Lande, 2015; Velotta & Cheviron, 2018). We suggest that repeated independent evolution of terrestrial development in frogs offers an excellent opportunity to study developmental mechanisms of physiological plasticity and their role in the reproductive colonization of land, considering ammonia toxicity as a common environmental threat and cue during early development.

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793

794 **Conflict of interest**

795 Authors acknowledge that there are not conflict of interest

796

797 **Author Contributions**

798 Designed the study: J.M.N. and K.M.W.; Collected the data: J.M.N.; Analyzed data:

799 J.M.N.; Wrote the manuscript: J.M.N.; Revised and edit the manuscript: J.M.N. and

800 K.M.W.

801

802 **Data Availability Statement**

803 Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.866t1g1r2

804 (Méndez-Narváez & Warkentin 2021) *in Process*

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