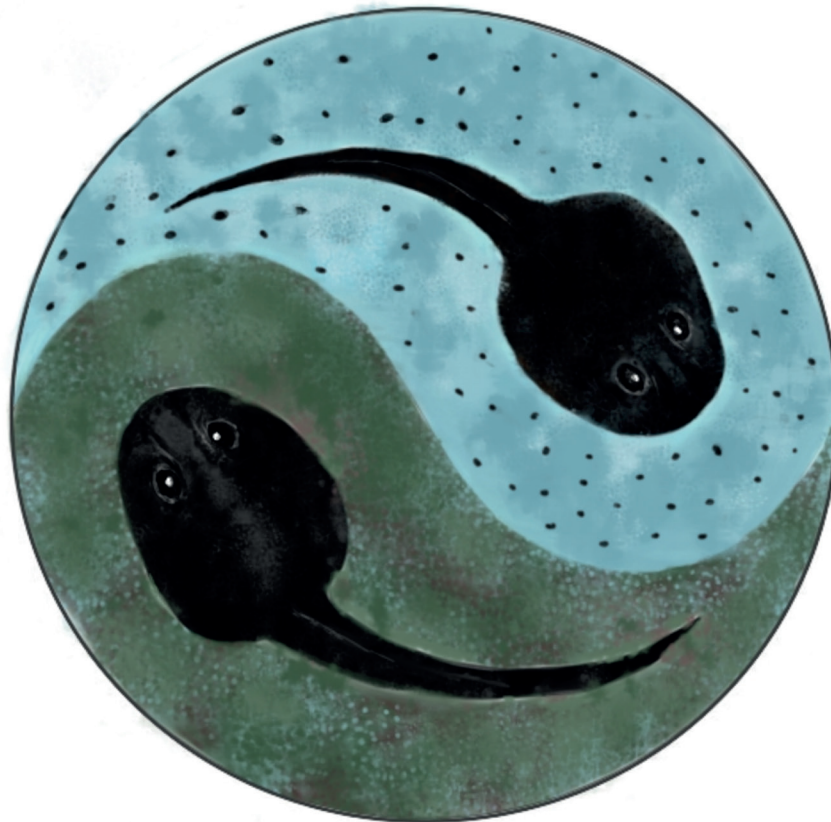Chloe Allison Fouilloux

Facing Enemies in an Ephemeral World

Tadpole Responses to Biological and Environmental Risk

UNIVERSITY OF JYVÄSKYLÄ

FACULTY OF MATHEMATICS
AND SCIENCE

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JYVÄSKYLÄN YLIOPISTO
UNIVERSITY OF JYVÄSKYLÄ

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Cover art design by Chloe A. Fouilloux, illustration by Zuzanna Zielińska.

ABSTRACT

Animals must successfully assess the world around them to survive. Quantifying how animals respond to their environment reveals traits (i.e., kin recognition, sensory plasticity) that have evolved to maximise an individual's fitness. In this thesis, I examine an amphibian system characterised by its intensive parental care and larval cannibalism. Males of the poison frog species *Dendrobates tinctorius* transport recently hatched tadpoles to pools of water formed by vegetation, called phytotelmata. Tadpoles are confined to phytotelmata chosen by fathers, and left to face the varied sources of risk associated with these microhabitats throughout development. The range in phytotelmata community and quality is vast and, thus, the possible rearing conditions experienced by tadpoles are variable. Faced with predators, poor visibility, and pathogens, tadpoles have had to evolve diverse strategies to assess risk. Here, I evaluate adult and larval decision-making through field observations and behavioural experiments. These studies are framed within the ecology of the Neotropics, and involve measures of broad multi-species comparisons as well as fine-scale characterisation of phytotelmata that range from the quantification of water chemistry and pool turbidity to the presence of pathogenic zoospores. The combination of laboratory and field methods allows for the study of behaviour within an ecologically relevant context. In addition to elucidating deposition strategies by adults, such an approach has allowed me to (1) demonstrate that *D. tinctorius* tadpoles are visually oriented and that the turbidity of rearing conditions influences their response to visual stimuli in novel conditions; and (2) that tadpoles are able to discriminate kin, and the interaction between relatedness and size are what governs aggression between these cannibalistic larvae. I close by considering an emerging infectious fungi both across life stages and within the environment to establish a framework of disease transmission for this species. As a whole, this work demonstrates how different sensory cues, physiology, and the surrounding environment shape an animal's response to risk in dynamic ecological contexts.

Keywords: Aggression; inclusive fitness; Neotropical ecology; parental care; poison frog; predator-prey interactions; risk.

Chloe Allison Fouilloux, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

TIIVISTELMÄ

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Vihollisten kohtaaminen epäennustettavassa ympäristössä: sammakonpoikasten vasteet biologisiin ja ympäristön aiheuttamiin riskeihin
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Diss.

Eläinten on selviytyäkseen pystyttävä onnistuneesti arvioimaan ympäristöään ja reagoimaan siihen. Eläinten reagointitavat ympäristöönsä paljastavat piirteitä (esim. sukulaisten tunnistaminen, aistiplastisuus), jotka ovat kehittyneet evoluution myötä maksimoimaan yksilön sopeutumista ympäristöönsä. Tutkin väitöskirjassani *Dendrobates tinctorius* nuolimyrkkysammakkoa, jolle on ominaista intensiivinen jälkeläishoito ja poikasten välinen kannibalismi. Sammakkokoiraat valitsevat vesionkalot, jonne ne kantavat ja jättävät poikasensa kasvamaan. Fytotelmojen laatu ja kasvuolosuhteet vaihtelee suuresti. Poikasille on kehittynyt erilaisia strategioita arvioida riskejä, kuten kannibaalit, pedot, sameus ja patogeenit. Tutkin aikuisten ja niiden jälkeläisten päätöksentekoa erilaisissa riskiympäristöissä sekä havainnoimalla että tekemällä käyttäytymiskokeita. Tämä empiirinen tutkimus asettuu Neotrooppisen ekologian viitekehykseen ja sisältää sekä monilajisia vertailuja että yksityiskohtaisia fytotelmojen ominaisuuksien karakterisointeja, kuten veden kemiallisen koostumuksen ja altaan sameuden määrityksiä. Yhdistelmä laboratorio- ja kenttämenetelmiä mahdollistaa eläinten käyttäytymisen tutkimuksen ekologisesti relevantissa yhteydessä. Tämä lähestymistapa on antanut mahdollisuuden osoittaa, että (1) *D. tinctorius* -lajin sammakonpoikaset käyttävät päätöksenteossa näköaistiaan, ja että veden sameus vaikuttaa negatiivisesti niiden kykyyn vastata visuaalisiin ärsykkeisiin, ja (2) että sammakonpoikaset pystyvät tunnistamaan sukulaisensa, mutta sukulaisuus ei yksin vaikuta kannibalismiin ja poikasten väliseen aggressioon. Lopuksi tarkastelen uuden tarttuvan taudin tuomia riskejä *D. tinctoriukselle*. Yleisesti tämä tutkimus osoittaa, kuinka eläimen ympäristö muokkaa sen vasteita erilaisia riskilähteitä kohtaan.

Avainsanat: Aggressio; jälkeläishoito; kokonaiskelpoisuus; myrkkysammakko; neotrooppinen ekologia, peto-saalissuhteet; riski.

Chloe Allison Fouilloux, Jyväskylän yliopisto, Bio- ja ympäristötieteiden laitos PL 35, 40014 Jyväskylän yliopisto

Author's address Chloe Allison Fouilloux
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
chloe.a.fouilloux@jyu.fi

Supervisors Associate professor Bibiana Rojas
Department of Interdisciplinary Life Sciences
Konrad Lorenz Institute of Ethology
Savoyenstraße 1
AT- 1160 University of Veterinary Medicine
Austria

Dr. Lutz Fromhage
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Dr. Janne Valkonen
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Reviewers Associate professor Iván Gómez-Mestre
Ecology, Evolution and Development Group
Estación Biológica de Doñana (CSIC)
41092 Sevilla
Spain

Assistant professor Ana Longo
University of Florida Gainesville
220 Bartram Hall Gainesville
32611, FL
USA

Opponent Prof. Rebecca Kilner FRS
Department of Zoology
University of Cambridge
Downing St
Cambridge CB2 3EJ
UK

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–V.

I   Fouilloux, C. A., Serrano Rojas, S. J., Carvajal-Castro, J. D., Valkonen, J. K., Gaucher, P., Fischer, M., Pašukonis, A., & Rojas, B. 2021. Pool choice in a vertical landscape: Tadpole-rearing site flexibility in phytotelm-breeding frogs. *Ecology and Evolution* 11: 9021– 9038.

II Fouilloux, C. A., Yovanovich, C. A. M., & Rojas, B. 2022. Tadpole responses to environments with limited visibility: what we (don't) know and perspectives for a sharper future. *Frontiers in Ecology and Evolution* 9:1–8.

III Fouilloux, C.A., Stynoski, J.L., Yovanovich, C.A.M., & Rojas, B. 2023. Visual environment of rearing sites affects larval response to perceived risk in poison frogs. *Journal of Experimental Biology* 226: 1–11.

IV Fouilloux, C. A., Fromhage, L., Valkonen, J. K., & Rojas, B. 2022. Size-dependent aggression towards kin in a cannibalistic species. *Behavioral Ecology* 22: 582-591.

V   Fouilloux, C. A., Almeida De Freitas, G., Dittrich, C., Hölzl, F., Pašukonis, A., Rosa, G.M., Serrano Rojas, S.J., Smith, S., Sonnleitner, R., Sundberg, L.R., Trafford J.D., Rojas, B. 2023. Pathogens hijacking parental care? A framework for chytrid transmission in Amazonian poison frogs. Manuscript.

Table of author contributions to the original publications.

	I	II	III	IV	V
Original Idea	BR, AP	CF	CF	BR	BR, CF
Study design	BR, AP	CF, CAM, BR	CF, CAM	JV, BR, CF	BR, JV, CF
Data collection	CF, SR, JC, JV, AP, BR	--	CF	CF	CF, RS, GR
Data analysis	CF	CF	CF	CF,LF	CF
Writing	CF, BR, AP	CF, CAM, BR	CF, JS, CAM, BR	CF, BR, LF	CF, BR

CF= Chloe Fouilloux, BR = Bibiana Rojas, LF = Lutz Fromhage, AP = Andrius Pašukonis, JV = Janne Valkonen, CAM = Carola Yovanovich, SR = Shirley Jennifer Serrano Rojas, JC = Juan Caraval-Castro, RS = Ria Sonnleitner, GR = Gonçalo Rosa, JS = Jennifer Stynoski

Song of Myself, 51
Walt Whitman

The past and present wilt—I have fill'd them, emptied them.
And proceed to fill my next fold of the future.

Listener up there! what have you to confide to me?
Look in my face while I snuff the sidle of evening,
(Talk honestly, no one else hears you, and I stay only a minute longer.)

Do I contradict myself?
Very well then I contradict myself,
(I am large, I contain multitudes.)

I concentrate toward them that are nigh, I wait on the door-slab.

Who has done his day's work? who will soonest be through with his supper?
Who wishes to walk with me?

Will you speak before I am gone? will you prove already too late?

1 INTRODUCTION

1.1 An inherently risky world

"Risk" is an ever-present force shaping animal decisions. The challenges individuals face throughout life are varied, and animals must weigh the potential costs versus the potential benefits of their decisions to ensure their survival and maximise their fitness (West and Gardner 2013). Animals must assess the quality of a habitat to make decisions about where to feed, breed, and invest long-term care in their offspring (I). In an ecological framework, sources of risk faced by an animal can be intertwined with the state of the environment (II). For example, the eutrophication of waterbodies impacts the visual landscape of animals and has cascading consequences on predator-prey dynamics and community composition (Chivers *et al.* 2012). In the case of competition between conspecifics, risk can be both indirect (in the form of decreased resources) and direct (as a result of physical altercations) (III). Individuals must decide when it is worth escalating antagonistic interactions: the benefits of such revolve around the acquisition of resources which are offset by the possibility of injury, death, and decreased inclusive fitness (Husak 2004; IV). Sometimes the most deadly sources of risk are microscopic. Pathogens are ubiquitous throughout the environment and, while some can be identified and avoided by animals, many cases of disease spread are dependent on mechanisms evolved by pathogens to access and infect an unsuspecting host (V).

Despite being faced with a myriad of ways to die, animals are generally good at doing the opposite. Flexible decision-making and adaptation are just a few of the tools animals have evolved to survive an inherently risky world, which I further explore in the context of this thesis.

1.2 Welcome to the jungle: A brief introduction to poison frogs

Amphibians are a useful model to consider how animals assess and respond to risk as their environment changes dramatically across the metamorphic boundary. In addition to the drastic change in physical form, amphibians have radiated across the entire globe, and are present on every continent (except Antarctica, Wells 2010). As a result of this dramatic radiation, amphibians have evolved diverse physical adaptations (e.g., aposematism, freeze-tolerance in overwintering frogs, vocal sacs for calling), reproductive modes (e.g., explosive breeding, lekking), and behaviours (e.g., parental care, navigation, territoriality). The Neotropics are a notable hotspot for animal and plant biodiversity, and the region contains more than half of the described amphibian species (Pillay *et al.* 2022).

Poison frogs evolved about 40 millions of years ago when they diverged from hyloid frogs (Carvajal-Castro *et al.* 2021), and transitioned to the use of terrestrial habitats from an ancestral stream-dwelling species (Summers and McKeon 2004). Inhabiting the Neotropical region, many of these species are known for their bright colouration and toxicity (Myers and Daly 1983, Summers and Clough 2001, Santos *et al.* 2003). This clade has shown to be experimentally tractable and proved to be a useful model to understand predator-prey dynamics (Maan and Cummings 2012, Paluh *et al.* 2014, Stuckert *et al.* 2014), navigation and homing behaviour (Pašukonis et al. 2022), territoriality (Crump 1972, Pröhl 2005), and communication (Bee 2003, Meuche *et al.* 2012, Ringler *et al.* 2017, Tumulty *et al.* 2022). Poison frogs distinguish themselves from the majority of anurans as they are part of the 10 percent of total species that display parental care (Schulte *et al.* 2020). In poison frogs, parental care can include the defence of clutches, protection of larvae, tadpole transport and tadpole feeding (Wells 1978, Brust 1993, Furness and Capellini 2019). The evolution of parental care is hypothesised, in part, to be an indirect effect of the predation of eggs and larvae in stable water sources by large predators (e.g., fishes; Heyer *et al.* 1975, Kats *et al.* 1988) which was the driving selective force to breed in small pools of water formed by vegetation (aka. phytotelmata) which exclude these large predators.

The escape from permanent water bodies was in part facilitated by the warm, humid climate of the Neotropics, wherein adults increasingly used terrestrial sites for reproduction. This resulted in complete terrestrial breeding in poison frogs, where small clutches of eggs are laid in the leaf litter and cared for intensively. To prevent desiccation, the moisture of a clutch is maintained by parents as the eggs mature. Upon hatching, as tadpoles that breathe using gills, individuals are transported dorsally by adults to small pools of water where they complete their development (Fig. 1). From an evolutionary perspective, the invasion of phytotelmata by poison frogs is believed to have been facilitated by their aposematic colouration (Carvajal-Castro *et al.* 2021). During this evolutionary time, brightly coloured poison frogs were rapidly diversifying many species traits (e.g., diet, communication; Toft 1995, Santos *et al.* 2003, Santos

et al. 2014) potentially including their reproductive specialisations within phytotelmata (Carvajal-Castro *et al.* 2021). As a result of the natural diversity of vegetation in the rainforest, the range of substrates that can serve as phytotelmata is equally variable. As such, many species have come to specialise on certain physical or chemical components of these microhabitats, which tends to naturally segregate species along the vertical gradient based on specific ecological niches (I).

FIGURE 1   **Dendrobates tinctorius father transporting a tadpole to a phytotelmata (treehole).** Tadpoles are contained within rearing-sites until they reach metamorphosis (Photo: Andrius Pašukonis).

There exist many forms of parental care by diverse poison frogs. It is hypothesised that biparental (e.g. *Ranitomeya amazonica*) and female-only (e.g. *Oophaga pumilio*) forms of care in this lineage have evolved from an ancestral state of male-only care (Summers and McKeon 2004). *Dendrobates tinctorius* is a Neotropical poison frog with year-round breeding that exhibits male-only parental care. This species is sexually dimorphic, where females tend to be larger and have thinner, more circular toe pads compared to males (Rojas and Endler 2013). *D. tinctorius* is a diurnal species from the Eastern Guiana shield (Rojas an Pašukonis 2019) that is coloured with variations of black, yellow, and blue as an adult. Their bright yellow aposematic signal is subject to impressive intra- (Rojas and Endler 2013) and inter- (Noonan and Gaucher 2006) population variation which allows for individual-level identification of adults.

Approximately two weeks after oviposition, fathers transport tadpoles singly from their oviposition sites to phytotelmata where they are left to develop until metamorphosis. Of the poison frogs, this species has both adult and larval physical and behavioural characteristics that are of particular evolutionary

interest. As adults, this species lack an obvious vocal repertoire (Rojas and Pašukonis 2019) which in other poison frog species is classically used to examine territoriality (Pröhl 2005, Tumulty *et al.* 2018) and sexual signalling (Erdtmann and Amézquita 2009). In addition to being a "voiceless" poison frog, male *D. tinctorius* are uniquely flexible in their microhabitat use. Observed from the leaf litter to the top of the forest canopy, the physical abilities of males on both the horizontal and vertical axes give them access to a wide range of phytotelmata; this flexibility creates many possibilities for the kinds of rearing sites available for them to choose for their offspring.

The larvae of *D. tinctorius* have also evolved extreme behavioural modifications which may contribute to their survival in these sometimes challenging developmental conditions. *D. tinctorius* tadpoles are aggressive predators that will readily consume heterospecific tadpoles and will also attack conspecifics (Rojas 2014). Extreme intraspecific aggression and cannibalism in young is taxonomically rare throughout the animal kingdom, as young individuals are not competing for mates, (typically) do not defend territories, and do not have the hormones/androgen levels expected of animals that are killing and consuming conspecifics (Fischer et al. 2020). Additionally, the fact that phytotelmata can be occupied in various densities of *D. tinctorius* tadpoles (Rojas 2014, 2015, Fouilloux *et al.* 2021) establishes a natural arena where tadpoles are faced with the cost and benefits of cannibalism as a function of the physical risk of being hurt and the potential benefit of a high-protein meal. These risks and benefits of cannibalism can also be contextualised within a broader evolutionary framework of inclusive fitness theory (Eickwort 1973, Pfennig 1997). In these cases, we can expect kinship between a cannibal and their potential meal could deter or eliminate a cannibal's aggressive behaviour in light of the benefit of a close relative surviving and passing on copies of their shared genes (Fouilloux *et al.* 2022).

In the broad understanding of poison frog biology, larvae have received much less attention than their parents. The phytotelmata-tadpole system allows us to study tadpole responses to environmental (turbidity, challenging chemistry) and biological (cannibalistic conspecifics, heterospecific predators) risk in a context of parental care and decision-making by adults. The occurrence of both adult and larval stages of *D. tinctorius* across the vertical gradient is a rare trait among amphibians, which opens up many questions with respect to parental care and community ecology.

1.3   The risks and challenges of an ephemeral world

Phytotelmata are small pools of water formed by vegetation (such as palm bracts, fallen trees, or leaf axils, Fig. 2). These points of accumulated rainwater are chosen by adults as nurseries for tadpoles to develop. From an evolutionary standpoint, phytotelmata have the obvious benefit of excluding large predators, but they are inherently risky as their small volume makes them prone to desiccation. Phytotelmata are extremely variable in volume, depth, and occurrence along the vertical gradient, but generally considered to be resource-limited. Different species have evolved various solutions to this problem by either consuming detritus and algae (e.g., *Allobates femoralis*), receiving food provisioning from parents (i.e. oophagy), or creating food opportunities themselves (i.e., predation, cannibalism).

Phytotelmata are seldom occupied by a single species. In fact, these natural mesocosms can house multi-trophic food webs that change as a function of phytotelm substrate and occurrence within the vertical gradient, from the ground to the canopy (Kitching 2001). In the phytotelmata at our study site in French Guiana, one can commonly find several species of amphibian larvae including toads (*Rhinella castaneotica*), other poison frogs (*Allobates femoralis, Ameerega hahneli*), as well as diverse invertebrates including mosquito and odonate larvae (such as dragonflies and damselflies) contained within the same pool. The trophic interactions between these animals can change depending on the resources available within a given phytotelm, but generally *D. tinctorius* are the predators of most other species (Rojas and Pašukonis 2019), except in the cases of odonate larvae, which can become tadpole predators if the size difference is great enough (Summers and McKeon 2004, Fouilloux *et al.* 2021). Tadpoles cannot escape or change nurseries if environmental conditions deteriorate (e.g. pools become damaged or full of debris) or if predators or competitors invade the nursery site. As a result of this, (1) the initial deposition by adults is a vital choice in contributing to the probability of tadpole survival and (2) tadpoles must manage the consequences of their parents' choices in order to survive.
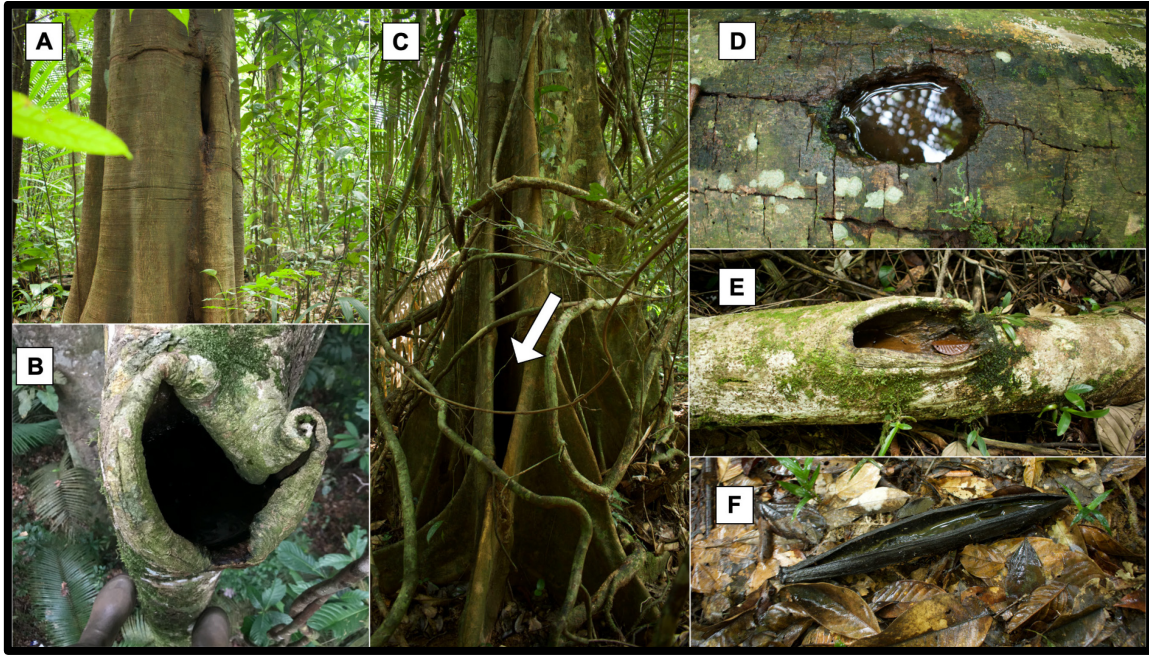
FIGURE 2   **The diversity of phytotelmata occupied by *D. tinctorius* tadpoles.** (A-C) Live treeholes that range from 0.5–20 m. in vertical height, (D-E) fallen trees, (F) dead palm bracts. Reprinted with permission from Ecology and Evolution.

In an ecological framework, phytotelmata are naturally occurring mesocosms. These small pools of water are relatively easy to access and extensively quantify with respect to both abiotic (water temperature, chemistry, turbidity) and biotic (species diversity, predator presence) factors. This natural set-up allows us to address questions about the species-specific patterns of pool-use, how chemical properties can predict community structure within pools, and the choices that larvae make as a consequence of being confined in an ephemeral, food-limited habitat.

1.4   How do tadpoles assess their environment?

1.4.1 Vision in tadpoles

Contained within phytotelmata, tadpoles must be able to recognise and respond to risk to survive. Amphibian larvae have been shown to frequently use chemical communication (in the form of water-borne cues) to recognise predators (Mathis and Vincent 2000, Weiss *et al.* 2021), discriminate kin (Blaustein and O'Hara 1982, Waldman 1985), and respond to food-provisioning mothers (multi-modal integration required for a full tadpole response: Stynoski and Noble 2012). Other amphibian larvae have also been hypothesised to use taste to identify kin (Pfennig *et al.* 1993), which may also play a role in kin recognition in dendrobatids (IV).

When we consider the bulk of sensory-related research in tadpoles from the past two decades, the majority of species that have been studied have been from temperate areas. For the most part, northern-latitude tadpoles inhabit permanent water bodies such as lakes and streams, which are subject to extreme eutrophication and agricultural runoff; these generally low-visibility turbid habitats, combined with early work that established that tadpole vision is generally myopic (Mathis *et al.* 1988, Hoff *et al.* 1999) and should be of low visual acuity on the basis of their small eye size (Caves *et al.* 2018), decreased general interest in studying vision in tadpoles. Nevertheless, tadpole vision could still play an important role in other ecological contexts.

With this in mind, in the volume of water contained within a phytotelmata there can be physical obstacles to navigate (pool debris) and, of course, members of the pool community which tadpoles must accurately assess. In these minute volumes, the visual impact of near-sightedness in tadpoles may not be biologically relevant and the clear water of pools (though this is not a given, nor is it constant) establishes a visual scene for tadpoles to explore. Given the increasing interest in phytotelm research in the past decade, studies have more consistently experimentally isolated and combined tadpole sensory modalities and have established the role of vision in conspecific communication and predator-prey interactions (*Oophaga pumilio:* Stynoski and Noble 2012, *Allobates femoralis:* Szabo *et al.* 2021, *Dendrobates tinctorius:* Kumpulainen 2022) in diverse poison frog tadpoles.

1.4.2 Vision in visually challenging conditions

The visual environment of phytotelmata is subject to enormous variation depending on physical (depth, pool type, canopy coverage) and biological (decomposed organic material) parameters of the pool. In the wild, the brightness of these natural pools can vary from being as clear as drinking water to a dark, turbid colour (II). In response to developing in turbid conditions there are generally two options that aquatic animals have evolved to maintain overall sensory perception acute enough to navigate their world: animals will opt for either (1) sensory compensation, i.e. investment into other sensory modalities such as olfaction (Hartman and Abrahams 2000), or (2) visual plasticity, where the eye, specifically the retina, is restructured to adjust spectral sensitivity to a more turbid (generally "redder", Corbo 2021) world. There has been evidence linking limited light and turbidity to the visual restructuring of fishes (Fuller *et al.* 2010, Ehlman *et al.* 2015), which in turn affected animal preferences and boldness.

When we consider the potential visual plasticity of tadpoles, we must limit our investigation to visual components that are able to respond to short-term changes in the visual environment. From a proximate perspective, one mechanism that has been shown to flexibly change the spectral tuning of the eyes is the vitamin A1/A2 chromophore exchange system in the retina (Reuter *et al.* 1971, Bridges 1972) which is manipulated by a single enzyme (Corbo 2021). Briefly, a higher proportion of vitamin A2 shifts sensitivity to the red parts of the

visual spectrum. The change of these ratios has been observed in amphibians both across the metamorphic boundary (see Donner and Yovanovich 2020 for a review) and within tadpoles as a function of the light levels of their environment (Bridges 1970). It should be noted that while a higher A2-chromophore ratio may confer an advantage to tadpoles in turbid environments, this pigment is thermally less stable, resulting in a noisier visual landscape (Donner and Yovanovich 2020). Overall, we hypothesise that this flexibility must confer a measurable advantage to tadpoles, where individuals with red-shifted spectra in turbid conditions are better able to recognize and optimally respond to the sources of risk they encounter.

1.4.3 Recognition in tadpoles

As tadpoles navigate their environment, they are often faced with diverse amphibians. For *D. tinctorius*, the importance of recognition works on two levels. First, as predators tadpoles need to be able to recognise heterospecific tadpoles in order to kill and consume them. The second level is a finer-scale discrimination between related versus unrelated conspecifics. As cannibals, there is an obvious benefit of consuming conspecifics as it both removes competitors from a resource-limited environment and may pay-off as an important nutritional benefit. However, killing closely related individuals decreases a cannibal's inclusive fitness; as such, there is a delicate balance between an individual's direct fitness potential and the inclusive fitness benefits of the survival and reproduction of their closely related kin.

Given this trade-off, we may expect kin discrimination to have evolved in *D. tinctorius*. Other species of amphibians are able to recognise kin (Pfennig *et al.* 1994, Pfennig and Frankino 1997), which has been found to influence cannibalism rates between individuals. In the social anuran species *Xenopus laevis*, Dulcis et al. (2017) show that tadpoles' dopamine expressing neurons changed significantly when exposed to either kin or non-kin odorants and in larval salamanders kin recognition disappears when an individual's nares are blocked (Pfennig *et al.* 1994). Together, these studies suggest that there may be an important olfactory component to kin discrimination.

1.5 A new frontier of risk: pathogens in the lowlands of the Neotropics

1.5.1 "Mama, where do zoospores come from?"

Some sources of risk are invisible to the naked eye. *D. tinctorius* tadpoles have evolved to face heterospecific predators, cannibals, and persist in highly variable environmental conditions; as such a flexible and resilient species, *D. tinctorius* seems primed to be resistant to both established and introduced sources of risk. *Batrachochytrium dendrobatidis* (hereafter, *Bd*) is an aquatic fungal pathogen that has been the source of decline of several hundred amphibian species and the cause of extinction of at least 90 (Lips *et al.* 2006, Scheele *et al.* 2019). In many ways, *Bd* is the ultimate source of risk. The pathogen cannot be avoided, as zoospores cannot be seen or heard, nor can it be escaped, making the water bodies where *D. tinctorius* tadpoles are confined to water bodies that may serve as an aquatic reservoir for the pathogen.

Infection consists of motile zoospores encysting onto the keratinised surfaces of amphibians, which include the skin of adults and the mouthparts of tadpoles (Kilpatrick et al. 2010). *Bd* is the causal agent of the emerging infectious disease, chytridiomycosis (colloquially called chytrid for short). Generally, the pathogenicity of chytrid in adults includes interrupted osmoregulatory function in the skin which can escalate to heart failure (Sewell et al. 2021). In pre-metamorphic stages, disease outcomes include deformities and degradation of tadpole mouthparts, which can inhibit feeding (Fisher *et al.* 2021). Although *Bd* is characterised as an aquatic pathogen, drastic species declines have been recorded in a large diversity of species that vary widely in their habitat-use (i.e. fully terrestrial, Burns *et al.* 2021; semi-arboreal, Miller *et al.* 2018; arboreal, Cádiz *et al.* 2019) and geography, highlighting the flexibility and virulence of this fungus. In modern history, *Bd* has been the single-greatest cause of biodiversity loss on a global scale (Fisher and Garner 2020)

Bd has a biphasic life cycle which is divided into a short (< 24 hour) aquatic motile phase, followed by a multi-week encysted period (Longcore *et al.* 1999). Free-swimming zoospores may disperse in the environment (laboratory reports state zoospore dispersal to be less than 2 cm, Kilpatrick *et al.* 2010); fungi of this stage are chemotactic and able to identify and encyst onto keratinized surfaces (Fisher *et al.* 2021). After finding a suitable surface, zoospores absorb their flagella and develop within the epidermal cells of amphibians. Following successful invasion of a host's epidermis, the remainder of *Bd*'s lifecycle unfolds within the skin cells of its host. Across a period of approximately two weeks, fungi develop by further anchoring their rhizomes into the hosts' skin and developing a sporangium in which cells begin to multiply and divide into future zoospores (Berger *et al.* 2005). Once fully mature, a discharge tube forms in the head of the sporangium and zoospores are then released into the environment.

Although the global impact of *Bd* has only been present since the turn of the 20th century, *Bd* is thought to have emerged from East Asia approximately 50 million years ago (Martel *et al.* 2014). While more than half a dozen lineages have been identified, a single lineage called *Bd*GPL (global panzootic lineage) is, as the name suggests, globally distributed and a highly virulent form of the pathogen that first emerged 10–40,000 years ago (Fisher and Garner 2020). This lineage of *Bd* predominates in the Neotropical regions and is hypothesised to have arrived via commercial pet-trade routes (O'Hanlon *et al.* 2018).

1.5.2 *Bd* in the Neotropics

One of the first isolates and extensive description of *Bd* originate from a preserved *D. tinctorius* [previously *D. azureus*] specimen (Longcore *et al.* 1999). Since its description, there have been a wealth of studies establishing the presence of the fungus throughout the Neotropical region (Berger *et al.* 1998, Lips 1998, Pounds *et al.* 2006) and its role in amphibian population declines or disappearances (La Marca *et al.* 2005, Kilburn *et al.* 2010, Lips 2016). When considering the infectiousness of the fungus, it is well-established that the virulence of *Bd* is driven by several environmental factors such as chemistry (ideal pH growth range from 6–7, Piotrowski *et al.* 2004) and temperature (thermal optima from 17–25°C, Woodhams et al. 2008), the latter of which covaries with altitude (Pounds *et al.* 2006, Miller *et al.* 2018). As such, it is unsurprising that the most extensive work on *Bd* has been done in mountainous regions (North American Sierra Nevada, Peruvian Andes, Spanish Pyrenees), where extensive population declines (and sometimes recoveries) have provided insight into *Bd* reservoirs and transmission routes (Rachowicz and Briggs 2007, Briggs *et al.* 2010, Catenazzi *et al.* 2013).

In light of this fundamental groundwork, *Bd*-infected animals still occur in lowland areas of the Neotropics where some impacted species are both fully terrestrial (keep in mind that *Bd* needs water to complete its life cycle) and subject to higher temperatures, especially during the dry season (Ruggeri *et al.* 2018, Whitfield *et al.* 2012). The occurrence of infected animals in apparently suboptimal infection conditions underlines the complexities of chytrid infection, which have earned this pathogen an infamous reputation of being challenging to work with.

While many surveys of *Bd* have been published in the lowland Neotropics (e.g., Kilburn *et al.* 2010, Courtois *et al.* 2015, Russell *et al.* 2019), determining the mechanisms of transmission both within and across life stages remains to be established for most amphibian species. One reason for the absence of a globally applicable transmission model is a result of the great diversity of amphibians in the region: we are faced with uncommon reproductive modes such as direct developers (i.e., species with no tadpole stage), species with intensive parental care, and species that have invaded unique habitats (i.e., forest canopy, phytotelmata), that may compromise particular transmission routes, and animal/environmental reservoirs that may not be relevant to other systems.

1.6   Aims of the thesis

No one goes through life unscathed. While animals must constantly manage and respond to various sources of risk throughout development, they have evolved an arsenal of traits to overcome varied biological and environmental threats. Built around a framework of parental care and offspring development, this work aims to measure animal decisions and their consequences. This thesis consists of five chapters, each of which explores a different facet of risk and animal decision-making.

In a system where fathers transport their tadpoles (sometimes over multiple days), what kinds of variables shape their rearing site selection? I begin with an overview of the system and an introduction to the phytotelm-breeding anuran species that co-occur with *D. tinctorius*. Having established the ecosystem where these animals occur, I explore the rearing environments that *D. tinctorius* fathers choose for their tadpoles. I approach this using a multi-year dataset to assess how physical, chemical, and biological variables predict deposition choices (I). Following deposition, tadpoles are left to acquire their own food and survive the various risks they may encounter in phytotelmata. The remainder of the chapters are focused on life inside these rearing sites and, ultimately, how tadpoles mediate the consequences of the decisions made by their fathers. I begin by proposing a series of hypotheses that consider how tadpoles are affected by the visibility of their rearing environment (II). I build upon these observations by specifically testing how tadpole behaviour and decision-making change as a function of (a) their physical environment and (b) their community. I address this by quantifying the turbidity of occupied phytotelmata and measuring how a tadpole's rearing environment affects their response to visual sources of risk when faced with either hetero- or conspecific stimuli (III). Once recognising that tadpoles are able to dynamically sample and respond to their environment, and seem especially reactive when faced with conspecifics, I further explore how tadpoles assess their cannibalistic counterparts. Specifically, I experimentally manipulate both size and relatedness between tadpoles and test how these differences mediate their interactions (IV). These empirical results are supplemented with a theoretical model, which I use to predict tadpole aggression in the system. Finally, I finish by considering how emerging infectious diseases present a new frontier of risk faced by poison frogs. In combining multiple seasons of field work and extensive field sampling, I propose possible mechanisms of disease transmission in an amphibian system characterised by its extensive parental care (V).

2 METHODS

2.1 Study Sites

Dendrobates tinctorius populations occur throughout the eastern Guiana shield (Courtois *et al.* 2015). Our field site is in Camp Pararé, Les Nouragues Field Station (4°02′N, 52°41′W) which is a primary lowland terra-firme forest in French Guiana. Due to the climbing ability of adults of this species, work with both adults and larvae was conducted from the leaf litter to the forest canopy (> 20 m.); higher microhabitats were accessed by tree climbing. In contrasting *D. tinctorius* behaviour with other poison frogs, I also worked with another population of poison frogs in Costa Rica. La Selva Biological station (10°26′N, 84°59′W) is a lowland Neotropical forest characterised by primary and secondary wet forests. Although *O. pumilio* adults are able to climb to impressive heights, only terrestrial samples were necessary within the context of their use. Field work in both locations was conducted throughout the wet season, which typically spans from January until April.

2.2 Environmental sampling

### 2.2.1 Chemical and physical quantification of phytotelmata

Phytotelmata were assessed in several ways. In order to quantify the desiccation risk of pools, we measured the physical dimensions of each phytotelm (maximum water depth, length, width) to estimate its water holding capacity. Chemically, we used a variety of tools to quantify pools: salinity, water conductivity, total dissolved solids were measured with electronic sensors (EZDO 7200) and pH was assessed using a digital reader (AMTAST Waterproof pH Meter), which was calibrated daily to provide more fine-tuned measurements of pools. Broad chemistry of pools was assessed by colour-matching water

quality measures such as KH, hardness, nitrates, and nitrates (JBL Easy Test) to universal standards.

2.2.2 Spectrophotometry and water colour (II, III)

Attempting to quantify a tadpole's visual environment is challenging. However, because the volume of water in phytotelmata is small, it is relatively easy to collect a representative sample that accurately characterises the pool. In Costa Rica, samples were analysed using both spectrophotometry and photographic methods. This was done in order to prove the validity of the photographic approach, which is a more field-friendly and ubiquitously available tool.

Spectrophotometry was performed using a Shimadzu (UV-1800) spectrophotometer. Water samples from the field were diluted with Milli-Q water before analysis (1:5), as pure samples led to absorption levels that were too high for accurate readings. Absorption measures were converted into transmittance values for ease of interpretation. There are many methods to quantify colour, some more fine-scaled than others. For example, a spectrophotometer measures wavelengths across an entire spectrum, generating hundreds of measures of how light is reflected across specific wavelengths while photos are composed of pixels that exist in a red/green/blue colour space (RGB). Each channel varies in brightness (range $0-255$), and the combination of the three values creates a digital colour of a pixel. In order to validate photographic quantification of pools, the RGB values were calculated from transmittance spectra based on human colour matching functions (Fig. 3A, spec2rgb in the R package called "pavo", Maia et al. 2019). Photographic assessment of pool turbidity involved transferring samples to glass vials which were then photographed on a white background using a Nikon D5300 (settings: ISO 500, f/13, shutter speed 1/125) next to a Macbeth XRite ColorChecker. Samples were then digitally evaluated using ImageJ software (Abramoff *et al.* 2004). When comparing the RGB values computed from spectrophotometer measures or from the linear normalised reflectance stacks generated by image analysis software, we find that the two measures are significantly correlated (Fig. 3B, see III for details).
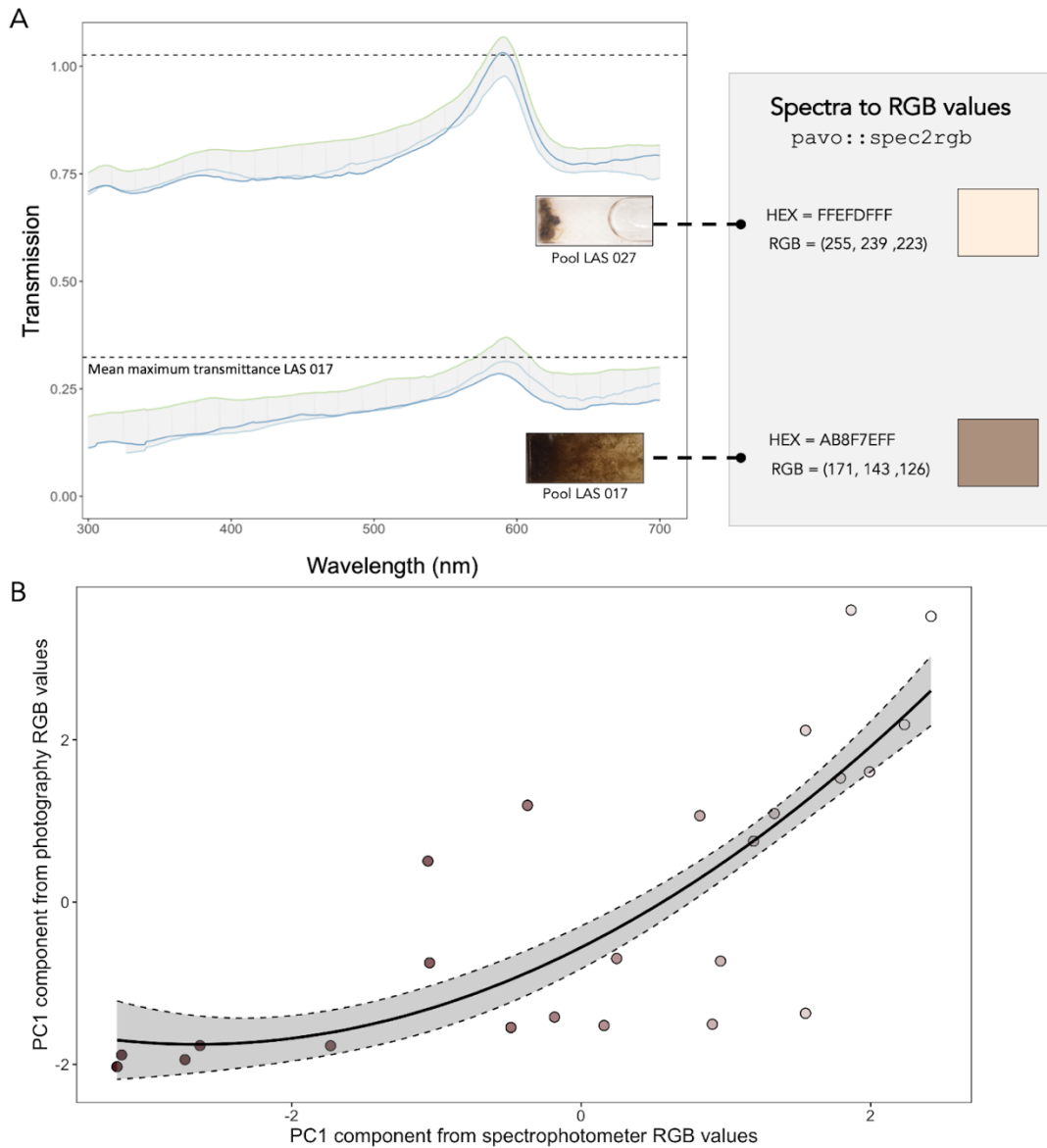
FIGURE 3   **Comparison between spectrophotometry and photography RGB values.** Panel (A) shows microhabitat transmittance spectra and their corresponding RGB values. Panel (B) compares PC1 values generated from RGB values from both spectrophotometer and photography data. A second order polynomial fit to data with shaded regions representing the 95 CI using a "gam" smoothing function. Reprinted with permission from the Journal of Experimental Biology.

2.3 Wild and laboratory behavioural assays (III, IV)

In addition to the quantification of the physical space inhabited by tadpoles, I measured the behaviour of individuals. As *D. tinctorius* is diurnal, tadpoles were measured between 09:00 and 16:00, both in the field and in laboratory conditions. All of the behavioural assays done throughout this thesis are based on scan-

sampling, which is the categorisation of animal behaviour at defined intervals for an established amount of time (Martin and Bateson 2007). These data paint a general view of an animal's behaviour and assessment of their environment. Throughout this thesis I examined aggression (i.e., biting, chasing, IV), activity (i.e., resting, swimming, III, IV), and space-use (i.e., distance from stimulus, III). In addition to the frequency with which a behaviour is expressed, we can also consider the latency it takes for a behaviour to occur. The latter can provide valuable insight into the costs associated with risky decisions.

When considering the aggression between tadpoles, experiments in the laboratory allowed me to control variables necessary to demonstrate the role of kin discrimination in decision-making. For example, maintaining a consistent diet within the population to avoid confounding effects of hunger-driven aggression (Mayntz and Toft 2006). More importantly, establishing a controlled breeding system was fundamental to acquire known full, half, and non-siblings. This system enabled me to create pairings that varied both in size differences and relatedness between tadpoles (IV). Behavioural assays in the wild are more limited in the sense that we are unsure of the genetics or true age of an individual. However, biologically relevant proxies such as developmental stage (Gosner 1960) and weight are often more relevant quantifications of an individual's condition, allowing us to sidestep this limitation.

Fear is a difficult behaviour to quantify. The primary limitation of behavioural assays is that we are limited to ultimate-level observations and ignorant to the mechanistic, proximate-level understanding of "how" a behaviour is being expressed. For example, if an animal is shown a predator stimulus and is physically unresponsive, does this mean an animal is frozen in terror or simply indifferent to the experience (III)? Without a cognitive perspective of these responses, we are limited in our interpretation of animal behaviour. Nevertheless, we do observe the coarse outcome of interactions from which we can hypothesise their meaning. The advantage of working *in situ* is the availability of biologically relevant material, such as predators. Throughout this work I use varied tadpole predators, such as odonate larvae and *Cupiennius getazi/coccineus* spiders, that were collected and released within two days of testing. In the context of a tadpole's response to visual stimuli, "predators" were placed in clear containers in an experimental arena with all surfaces (except for the cover) wrapped in either black or white paper (Fig. 4). This manipulation changed the illumination conditions of the arena and thus the contrast of the stimulus.
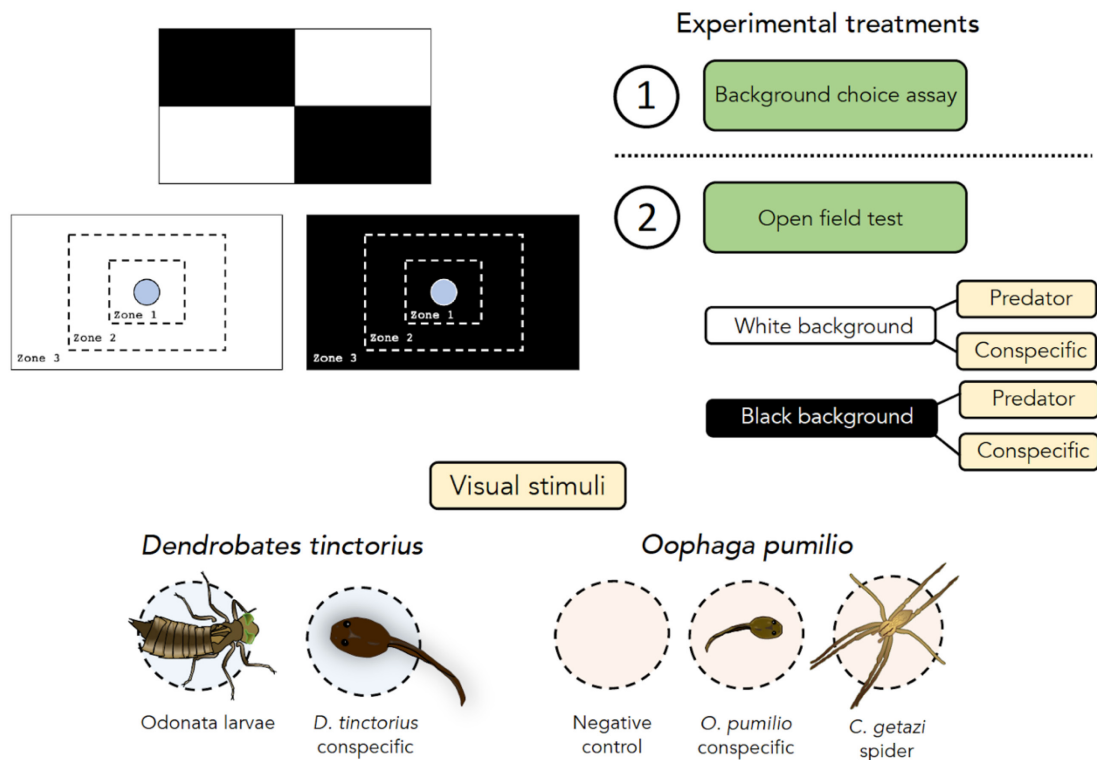
FIGURE 4   Experimental design for the experiments measuring the effect of the visual environment of phytotelmata on the response to diverse visual stimuli contrasted on different backgrounds (III). Reprinted with permission from the Journal of Experimental Biology.

2.4 Identifying *Bd* on animals and in the environment (V)

2.4.1 Sampling amphibians and phytotelm water

To understand the transmission dynamics of *Bd* between individuals and the environment, I sampled adult and larval *D. tinctorius* as well as phytotelmata water, as *Bd* is an aquatic fungus that requires moisture to complete its life cycle.

Amphibian sampling consisted of swabbing the keratinised regions of *D. tinctorius*, which includes the entire skin surface of adults and the mouthparts of tadpoles. Adults were swabbed (TS/19-J) on their lower mandible, front and back toe pads, and around the groin area following the Hyatt et al. (2007) protocol. Tadpoles' mouthparts were sampled using a fine-tipped swab (MWE, MW121 DRYSWAB with a rayon bud) to prevent injury to larvae. Swabs were air dried and stored in a 1.5 ml Eppendorf at room temperature while in the field. Samples were placed in -20°C for long-term storage once exported to the University of Jyväskylä, Finland. All adults and tadpoles were weighed, photographed, and released upon completed sampling. Both adults and tadpoles

were captured opportunistically throughout the vertical gradient. In order to assess the presence of *Bd* eDNA in the environment, I filtered phytotelm water weekly from pools (Millex-GS Syringe Filter Unit, 0.22 μm) over one month. Repeated pool sampling was required as the quantification of eDNA does not reveal if zoospores are dead or alive; by assessing the amount of *Bd* eDNA in pools over time, we can infer the condition of zoospores in pools, which is important to understanding if they are capable of infection. Immediately after sampling pools, ethanol was pulled through the filter to prevent any growth of biological materials. Water filters were stored at room temperature until analysis.

I sampled 15 unoccupied and 11 occupied pools by *D. tinctorius* tadpoles to assess if pool communities had any effect on zoospore dynamics in phytotelm water. Throughout the course of sampling, pools were monitored daily and no additional species were found therein.

2.4.2 Quantitative polymerase chain reactions (qPCR)

The presence of *Bd* on both swabs and filters was assessed using quantitative polymerase chain reactions (qPCR), a method for quantifying how much DNA of a specified target is present in a sample. Of course, a swab from *D. tinctorius* will not only potentially contain *Bd*, but may also contain a wide variety of bacteria or fungi that an adult encounters in the environment. Thus, the arguably most important part of qPCR is correctly defining primers and probes to amplify the correct species' DNA. Primers are engineered, predetermined sequences of DNA that, in our case, are complementary to the DNA of *Bd*. Fundamentally, DNA is a double-stranded helix that separates into two pieces when heated: this is the key component of qPCR (called denaturation). By separating and reconstructing DNA with free nucleotides through the repeated heating and cooling of samples, the qPCR process synthesises many copies of the original DNA and quantifies the DNA present in the original sample. Primers ensure that if a sample contains *Bd*, only the defined fungal DNA will be copied and amplified. It is important to note that there exist both forward and reverse primers that bind to the 5′ end of separated DNA strands (called annealing), which is because DNA polymerase (the enzyme that adds nucleotides and builds a complementary strand to a separated piece) can only synthesise DNA in one direction, moving from a 5′ to 3′ end. Probes are short, fluorescently labelled single-stranded DNA fragments that are complementary to the sequence of *Bd* DNA. When they bind to the target sequence, they fluoresce. This fluorescence allows for the detection of a positive sample, the intensity of which is proportional to the amount of target DNA. This signal is repeatedly measured as the sample goes through cycles of denaturation and annealing, allowing us to determine the quantity of *Bd* DNA in the original sample.

3 RESULTS AND DISCUSSION

3.1 Niche segregation along the vertical gradient (I)

What came first, parental pool choice or larval specialisations? We found that the occurrence of tadpole species in phytotelmata can be generally predicted by the physiological limitations or specialisations of their adult forms (Fig. 5). For example, *Allobates femoralis* is a small poison frog that is a poor climber (Roithmair 1992). Unsurprisingly, tadpoles from this species were only found in terrestrial phytotelmata. We did, however, find that tadpoles occur in terrestrial phytotelmata that are large enough to contain leaf litter and other debris, which may both serve as a shelter from tadpole predators and as a food source of decomposing plant matter. In species with biparental and female-only care, females will deposit unfertilised nutritive eggs in pools. Oophagous tadpoles are obligately dependent on their mother's eggs as a food source throughout development. The provisioning of food by mothers allows for these species to invade particularly small phytotelmata, such as the leaf axils of bromeliads, many of which have a pool volume of around 15 mL (imagine the volume of water contained in a Pasteur pipette). While these pools are especially vulnerable to desiccation, their volume excludes even invertebrate predators, such as odonate naiads (dragonfly larvae), that can occur in larger phytotelmata.
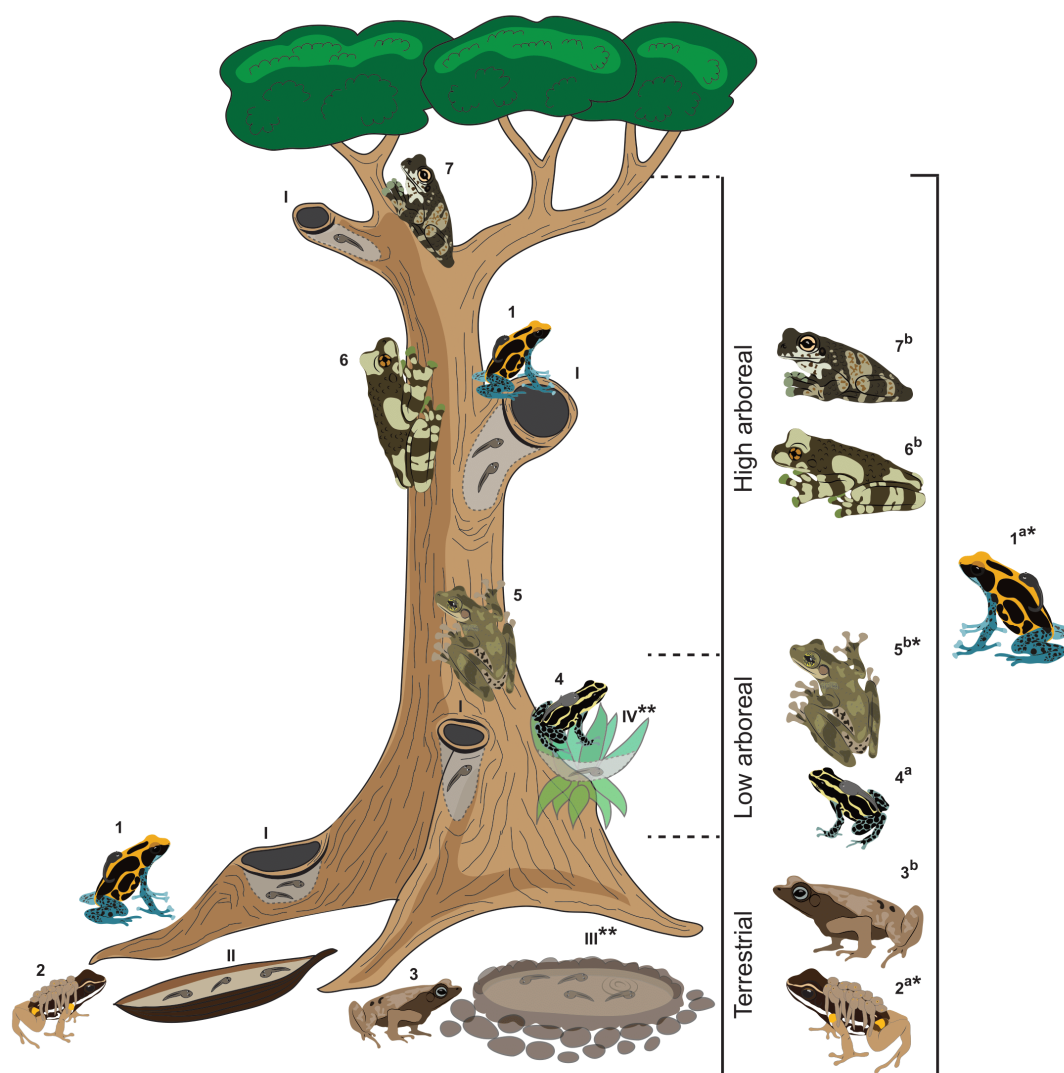
FIGURE 5 **Niche specialisation of phytotelm-dependent species across the vertical gradient.** The seven species commonly encountered throughout our sampling site include: 1. *D. tinctorius*, 2. *A. femoralis*, 3. *Rhinella castaneotica*, 4. *Ranitomeya amazonica*, 5. *O. oophagus*, 6. *Trachycephalus resinifictrix*, and 7. *T. hadropceps.* Letters indicate if species use terrestrial (a) or aquatic (b) oviposition sites. Roman numerals indicate common pool types. * indicates most commonly encountered species throughout our analysis and ** indicates pools that were not sampled throughout our study. Reprinted with permission from Ecology and Evolution.

D. tinctorius are the exception to these rather predictable species divisions. We found tadpoles in phytotelmata from the forest floor to more than 20 m high in the canopy (Gaucher 2002). Tadpole numbers ranged from one to more than 40 within a single pool, and pools spanned an impressive range of chemical (pH = 2.96 – 7, salinity = 0 – 1,000 ppm) and physical (pool volume = 15 ml to 270 l) parameters. The apparent lack of pool preference in *D. tinctorius* is counterintuitive considering that fathers have access to an unparalleled number of pools, and that tadpoles are aggressive cannibals. It appears that fathers are *opportunistic generalists* in the sense that males use a wide variety of phytotelmata,

but take advantage of unpredictable opportunities (i.e. new treefalls, very wet periods of time) to invade specific pool types. For example, males will invade pools in newly formed treefalls significantly faster and in higher densities when compared to pools under closed canopy (Rojas 2015).

While some pools may be excellent for the development of a single tadpole, the value of this pool would disappear if a father would use it for his entire clutch. The "survivability" of a single pool can vary based on variables such as seasonality or even pool age (i.e., substrate decomposition). As such, the generalist nature of males can lead to seemingly paradoxical deposition decisions: while the presence of a large conspecific may be risky in terms of the probability of cannibalism within a pool, more developed tadpoles may be an important indicator of pool stability (Rojas 2014). Overall, different pools tend to be characterised by different communities which can provide challenges and opportunities for *D. tinctorius* survival. High arboreal pools (i.e., >7 m) typically have more *D. tinctorius* tadpoles, but this risk of cannibalism may be offset by having a less stressful chemical environment (i.e., higher KH), a stable physical environment (i.e., they are extremely deep and, thus, less prone to desiccation) and a higher diversity of invertebrate and heterospecific eggs and tadpoles, which can serve as an alternative food source for *D. tinctorius* tadpoles.

The variable environments occupied by *D. tinctorius* tadpoles demonstrate that these tadpoles are resilient to diverse chemical and physical parameters. The diversity in rearing environments presents the opportunity to examine how rearing conditions affect the way larvae navigate their environment and respond to risk.

3.2 The effects of rearing conditions on behaviour (II + III)

There are many components of the aquatic habitats of phytotelmata that may drive the development and sensory fine-tuning of its inhabitants. The visual world of phytotelmata can be driven by pool type (i.e., tannins from tree bark can cause "coloured" water), water volume, canopy cover, and dissolved organic matter, all of which impact the visibility of an environment a tadpole must navigate to survive.

Because of the diverse communities that inhabit phytotelmata, we considered how the perception of risk may change as a function of the natural history of the species. How does the role of vision for *D. tinctorius*, a tadpole predator that must hunt its prey, compare to that of an oophagous species (*Oophaga pumilio*), that must recognise and respond to the visual cues of both positive stimuli (incoming mothers) and negative stimuli (predators). While similar to *D. tinctorius* in being a phytotelmata-dependent poison frog, *O. pumilio* is a species with maternal care which specialises in particularly small phytotelmata, such as the leaf axils of bromeliads. Most of this species' interactions are across the air-water interface as the minuscule volume of pools excludes the presence of predators and most other species. Thus, the risk that *O.*

pumilio tadpoles face tends to be from external sources such as incoming snake and spider predators.

Although the natural history of these two phytotelm-breeders is quite different, the rearing environment of both species has cascading effects on their behaviour in novel contexts (Fig. 6). For *D. tinctorius*, tadpoles that developed in turbid rearing environments did not respond to either the visual stimulus or background colour of the experimental testing condition. When faced with either a conspecific tadpole or a heterospecific odonate predator (that was large enough to be able to predate a tadpole) contrasted on either black or white backgrounds, tadpoles from turbid pools spent most of their time immobile. In comparison, tadpoles that developed in bright/crystalline microhabitats were significantly more active when they are paired with the visual stimulus of a conspecific compared to an odonate larva. The apparent discrimination of conspecifics is also reflected in tadpole space-use, where focal tadpoles spend significantly more time in the arena centre, close to the visual stimulus, when paired with a conspecific versus an odonate larva.

In the context of evolutionary ecology, what do these data mean? It is important to consider how developing in turbid environments affects an individual's perception of risk. For *D. tinctorius*, it appears that developing in turbid habitats affects an individual's overall activity, which has important implications for the foraging and, ultimately, the survival of a tadpole predator. If tadpoles are unable to identify or otherwise appropriately respond to visual stimuli, then they risk (1) being predated by species that are able to adapt to the dynamic photic environments of pools (i.e., non-vision dependent Odonata, Richards and Bull 1990), and (2) may fail to capture and subdue tadpoles that would have otherwise been profitable meals. This could have important effects on the rate of development, and thus, time to metamorphosis. When microhabitats can disappear within the span of a day, the cost of emerging a week or two later as a result of a nutrient-poor diet (CAF, *unpublished data*) can have deadly consequences, as tadpoles must continue to survive in their ephemeral microhabitats that may, for example, be decomposing or increasingly prone to desiccation as the wet season comes to an end.

Although we make no direct measure of vision in this study, we demonstrate that vision provides enough information to elicit a behavioural response in tadpoles. It is important to acknowledge that tadpoles from wild phytotelmata were tested in an arena with clear water (in order to facilitate behavioural observations). Thus, it is interesting to consider that the behaviour of tadpoles from turbid pools could be a result of the visual fine-tuning of the retina to turbid environments, which then becomes maladaptive in novel, clear conditions. Seemingly, *D. tinctorius* are able to recognise conspecifics based on visual information only, which is a trend that we first observed in the laboratory (Kumpulainen 2022) and now independently confirm with wild tadpoles in another experimental context.
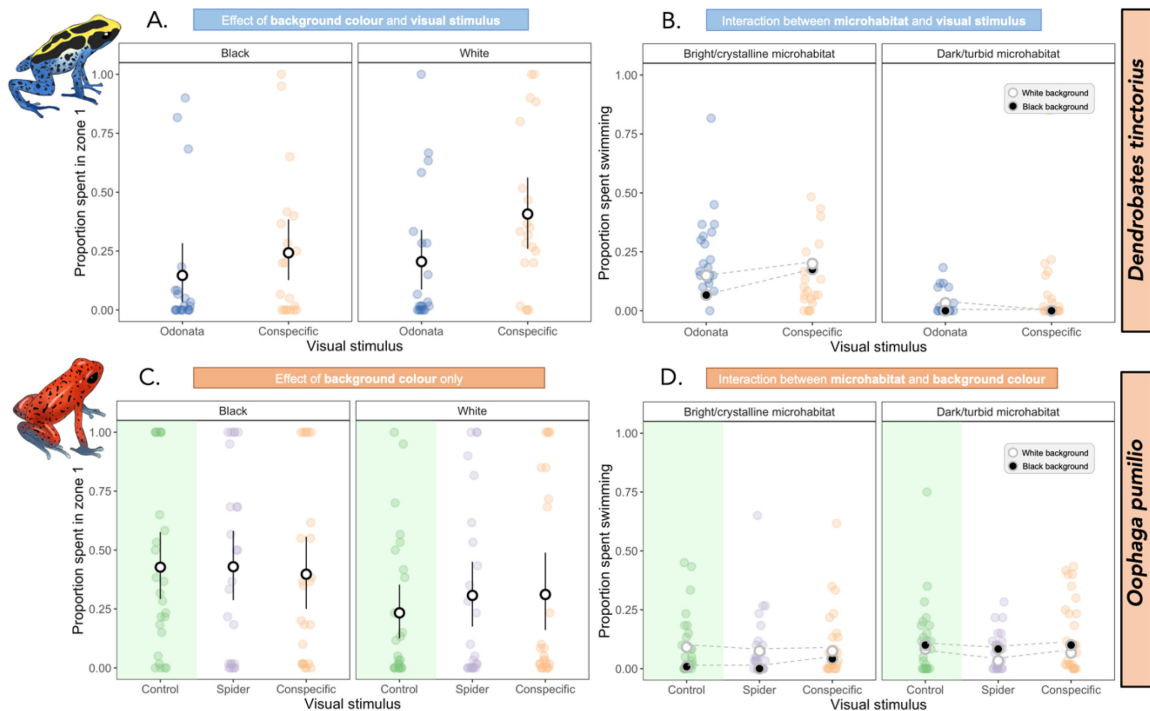
FIGURE 6   **Space use and activity in poison frog tadpoles.** Upper panels illustrate *D. tinctorius* space use and activity, and the bottom panels represent the same information for *O. pumilio* tadpoles. Characterisation of the phytotelmata photic environment as a factor is for visualisation purposes only. Dashed lines are to facilitate between-group comparisons. Reprinted with permission from the Journal of Experimental Biology.

In comparison, the oophagous species does not exhibit any difference in response based on the visual stimulus with which they were faced. However, *O. pumilio* tadpoles were significantly more active on backgrounds that more closely matched their rearing conditions. In other words, tadpoles that developed in dark/turbid microhabitats were more active on black experimental backgrounds while tadpoles from bright/crystalline microhabitats were more active on white experimental backgrounds. In comparing the two species from an evolutionary perspective, it is important to ground animal behaviour in their natural histories. Overall, our results do not reflect that one species is more visually-oriented than another but, instead, that each species' behavioural repertoire in response to risk reflects their biology. Specifically, as a predator and habitat generalist, *D. tinctorius* tadpoles must navigate diverse sources of risk. *D. tinctorius* tadpoles can cohabit pools with potential predators for extended periods of time, thus, it is valuable for individuals to accurately assess when to "attack" or "evade" their opponent. In contrast, in the vast majority of instances, *O. pumilio* tadpoles develop singly in miniscule pools of water. It has been shown that, when mothers come to pools to deposit unfertilised eggs, a combination of visual, chemical, and tactile cues are required to elicit a "vibration" response from tadpoles, which is needed to stimulate the deposition of nutritive eggs (Stynoski and Noble 2012). If *O. pumilio* tadpoles require such diverse information to respond to a positive stimulus, then a generalised fear response (i.e., escaping to the bottom of a

phytotelm pool and freezing at the bottom) to most incoming visual stimuli makes sense in the context of the species' biology.

From an ecological perspective, it is important to consider how communities are structured based on the modalities each species uses to navigate their environment. Phytotelmata are inherently dynamic habitats, so we would expect the communities that reside within them to be able to adapt to extreme and sudden changes. Yet, in both species their rearing environment played a role in their behaviour in a novel context (e.g., testing arena with clear water). These data indicate that tadpoles adapted to their rearing conditions and these proximate changes, while unquantified here, influenced their "success" in detecting risk in a new context. In order to draw a more complete understanding of the possible advantages of their presumed developmental adjustments to their visual environments, it would be useful to rear tadpoles in (1) constantly clear, (2) constantly turbid, or (3) dynamic clear/turbid conditions in a laboratory setting. Following a set development window, tadpoles could be paired with a visual stimulus in the photic environment that both matches and contrasts their rearing conditions. In addition to behavioural assays, the analysis of tadpole eyes would reveal the spectral tuning of an individual's retina, allowing us to link proximate-level changes (i.e., red-shifted spectra) to behavioural responses. While spectral tuning has been shown to be a biologically realistic mechanism in tadpoles (Bridges 1972, Corbo 2021) and previous studies have established that turbidity of rearing environment can induce spectral tuning in other aquatic animals (*fish:* Ehlman *et al.* 2015), the link between tadpole spectral tuning and their response to turbidity has yet to be established.

3.3   Inclusive fitness and kin discrimination (IV)

Understandably, many studies frame predator-prey interactions statically, but in reality the prey status of an animal can change depending on the phenotype or species with whom they are interacting. As cannibals, tadpoles must weigh the costs and benefits of killing and consuming conspecifics. In most cases, the latency to or frequency of cannibalism is driven by factors such as hunger/food availability (Mayntz and Toft 2006) or competition (i.e. conspecific density: Maret and Collins 1994). Once a cannibal makes a decision to attack a conspecific, a favourable outcome for the aggressor is not guaranteed. The outcome most often hinges on the physical capacities of an individual such as size, where larger individuals are more competitive (Mayntz and Toft 2006). From an evolutionary standpoint, the deposition strategy of placing smaller tadpoles in a pool full of large cannibals seems counterintuitive (I). Yet, despite *D. tinctorius* tadpoles being confined to the phytotelmata where they are deposited, tadpoles frequently coexist with other conspecifics.

While *D. tinctorius* tadpoles are aggressive, multiple individuals can successfully emerge from the same phytotelmata, and this species does not require a cannibalistic diet in order to reach metamorphosis. The benefits of

cannibalism in this species seem to be rather straightforward: eliminating other individuals reduces the competition within resource-limited conditions, and the potential benefit of a protein-rich meal could increase tadpole growth rate and reduce the latency to metamorphosis, both of which are important when existing in an ephemeral habitat. Further, cannibalism in *D. tinctorius* may also have carry-over effects in terms of the quality of aposematic signalling (i.e., signal size or signal brightness) in their terrestrial life stages (e.g., diet has been shown to affect the aposematic signal of ladybirds, Blount *et al.* 2012). The costs of cannibalism are a bit more convoluted. The risk of injury from aggressive encounters is evident, as injured tadpoles (missing/damaged tails, bruised bodies) are common in the wild, suggesting that either aggressive encounters do not always escalate to cannibalism or that cannibalistic attempts are not always successful. The other cost faced by cannibals is indirect: if they consume closely related individuals a cannibal decreases their *inclusive fitness*, otherwise understood as a loss of the number of shared genes with close relatives. Ultimately, removing a sibling who would have otherwise survived and reproduced reduces the overall evolutionary success of the cannibal, as fewer of its genes remain in a population.

In *D. tinctorius,* aggression is predicted by the interaction between tadpole size and relatedness, where larger non-siblings are significantly more aggressive than large siblings. These data show that some form of kin discrimination exists in the species, although the proximate mechanism underlying this recognition remains unknown. Using game theory models to infer what assumptions could explain the tadpoles' choices, we find that the models that most closely match our empirical data assume that aggression is size-dependent and overall costlier for small tadpoles. By testing different formulations, we are able to reject that direct fitness is either size-independent or proportional to size, and conclude that tadpoles trade off their own fitness for that of their relatives. As such, while discrimination appears to mediate aggressive encounters in this system, aggression levels in half-siblings was similar to non-siblings, indicating that recognition may not work on as fine of a scale for poison frog tadpoles compared to other species (e.g., larval tiger salamanders modify their behaviour even towards first cousins, Pfennig *et al.* 1994) or that recognition occurs but that this does not warrant a behavioural modification. There are some indications of taste or odour-based kin recognition in this species, as we observe different latencies to aggression in different relatedness treatments. Specifically, although siblings were the least aggressive overall, siblings with large size differences are among the fastest to exhibit aggression across treatments. As observed by Pfennig *et al.* (1994), it could be that there is some relatedness information that can be ascertained from the skin surface of tadpoles, although this remains to be experimentally verified.

Within the world of a phytotelmata, the fact that siblings are less aggressive towards each other helps explain the counterintuitive depositions made by fathers, as individuals may be more tolerant of closely related conspecifics in pools. However, the effects of kinship do not guarantee the safety of smaller tadpoles, as aggressive encounters occurred across all relatedness treatments and

sizes. Thus, there seems to be a dynamic balance between the costs and benefits of cannibalism for *D. tinctorius:* tadpoles do not behave as simple aggressive machines, but instead demonstrate an assessment of their surroundings to which they can gauge their response appropriately.

3.4 Tracking a deadly fungus through space (V)

When attempting to understand infection dynamics, biologists can approach the story from two different perspectives: that of the host or that of the pathogen. While some scientists may be interested in how an animal is infected others may instead consider the mechanisms a pathogen uses to infect a host; no matter the framing of the scenario, from a basic science perspective it is vital to underscore that there is no "correct" outcome to these interactions. There are simply players, acting in the grand theatre of evolution, and we as an audience have the great thrill of being able to sit and watch the performance. Of course, humans can have a (very active) hand in shaping panzootics, both for better and for worse. However, within the limits of this doctoral work let us consider only a single species, a single pathogen, and a snapshot of the environment in which they occur.

As previously established in this thesis, the *D. tinctorius* system consists of fathers that transport tadpoles (i) to pools of water (ii) that can be occupied by other tadpoles (iii). Naturally, these links establish nodes of possible pathogen spread within a single-species framework. The possible infection mechanisms include *horizontal transmission*, which is the spread of a pathogen via either cons- or heterospecifics (iii); *environmental transmission*, which is the acquisition from an physical reservoir (ii); and *pseudo-vertical transmission*, which is the transfer of infection from a parent to offspring via a care-related behaviour (i). In previous work investigating *Bd* it has been shown that both adults and larvae are able to become infected by zoospores in the water (Burns *et al.* 2021, Rumschlag *et al.* 2021). There has also been evidence of long-lived tadpoles serving as an animal reservoir for the pathogen, helping the fungus to persist throughout the cold winters in the Sierra Nevada Mountain range (Rachowicz and Vredenburg 2004, Rachowicz and Briggs 2007).

There are many reasons why there have been both taxonomic and geographical biases in understanding the impact of *Bd* on amphibian populations. Much of what we know about this disease and its possible mechanisms of spread are limited either to temperate species or to species that depend on permanent water bodies. This is unfortunate, as it has been established that about 30 percent of amphibian species with *Bd* undergoing population declines have "little to no aquatic association" (Scheele *et al.* 2019, Burns *et al.* 2021). Within the ecological context of many lowland Neotropical species, we must interpret previous findings with caution, as many poison frog species are fully terrestrial and do not occur near any permanent water bodies. This is especially puzzling when considering that *Bd* is an aquatic pathogen that

requires water to complete its life cycle (Longcore *et al.* 1999). While comparatively few studies on *Bd* in poison frogs have been published, the presence of *Bd* in *D. tinctorius* adults in French Guiana has been established (Courtois *et al.* 2015), and dendrobatids appear comparatively more frequently infected than other common genera in the region (e.g. Hylidae). Globally, the varying susceptibility of different species to the *Bd* has been well documented (Woodhams *et al.* 2007), and the natural history and ecology of *D. tinctorius* both limit and expand possible infection routes. On a positive note, dendrobatids are one of the most extensively-studied families in the region and the wealth of natural history information about these species (e.g., Ringler *et al.* 2015, Rojas and Pašukonis 2019) is pivotal for the exploration of relevant transmission routes.

Having sampled adults, tadpoles, and the water bodies in which they occur, we were able to rule out certain mechanisms of transmission and substantiate further investment into others (Fig. 7). Until now, the presence of this fungus on tadpoles or in tadpole rearing sites had not been established, and the potential mechanisms of transmission in an amphibian system with parental care have not been pursued past the point of speculation in published work (i.e., Holmes *et al.* 2014, Ruano-Fajardo *et al.* 2016). Because *Bd* is an aquatic pathogen, we originally hypothesised that phytotelmata could serve as environmental reservoirs for the fungus, as these pools are the only points of water in the area where *D. tinctorius* occurs. Repeated sampling of both occupied and unoccupied pools indicated that environmental *Bd* DNA in the water was present either (1) both inconsistently and in very low loads over a one-month period, or (2) completely absent from pools. In addition to low/non-existent *Bd* eDNA in pools, we were also able to look at the pH of pools, which revealed that the majority of infected tadpoles (10/13) were in phytotelmata with a pH (<6) that would either inhibit or completely kill *Bd* zoospores. There were also cases where infected tadpoles were detected in pools without eDNA, and pools with eDNA without any infected tadpoles: these data indicate that phytotelmata do not act as reservoirs for *Bd*. When we consider that a phytotelmata can dry out within a day, water temperatures can get quite warm when exposed to direct sun (>27 °C), and there could be *Bd*-ingesting micro-eukaryotes in pool water (shown by Blooi *et al.* 2017 in bromeliad axils), the finding that pools do not appear to be an environmental reservoir for the pathogen appears substantiated.

As *D. tinctorius* tadpoles can be aggressive cannibals, we hypothesised that encounters (involving biting, including mouth-mouth contact) may be a viable means of horizontal transmission between larval conspecifics. However, tadpoles from a wide range of sizes (which is a good proxy for development in this species) were positive for *Bd* (hereon, +*Bd*), the smallest being 0.05 g which is the standard weight of tadpoles at transport (Fouilloux *et al.* 2020). These data suggest that tadpoles can become immediately infected. Such findings are supported by the fact that zoospores contain a combination of proteases that allow fungi to degrade and invade epithelial cells almost immediately (Farrer *et al.* 2017, Fisher *et al.* 2021). When we consider the number of tadpoles in pools, we may expect pools with increased conspecific counts to have a higher frequency of infection (i.e., density-dependent infection). Yet, we do not find that

the number of tadpoles in pools predicts either tadpole infection status or infection intensity. In fact, many pools with more than 20 tadpoles had no signs of infection, and the highest infection load recorded was from a tadpole in a pool with only one other conspecific (that was uninfected). On this point, the proportion of infected tadpoles within a pool is highly variable. Thus, neither conspecific count nor tadpole age seem to be relevant in predicting tadpole infection in *D. tinctorius*, which does not substantiate the horizontal transmission hypothesis.

It is worth mentioning that when taking into account the presence of *Bd* on the vertical gradient, infected tadpoles can be found in arboreal pools over 9 m in height, and pools containing *Bd* eDNA in phytotelmata over 12 m above the forest floor. While these data do not contribute to our understanding of transmission *per se*, it is valuable to frame pathogen transmission in three dimensions, as amphibians exist both on the horizontal and vertical axis. Future work must consider how the flexible pool-use by *D. tinctorius* across the vertical axis may impact the diversity of *Bd* susceptible communities.
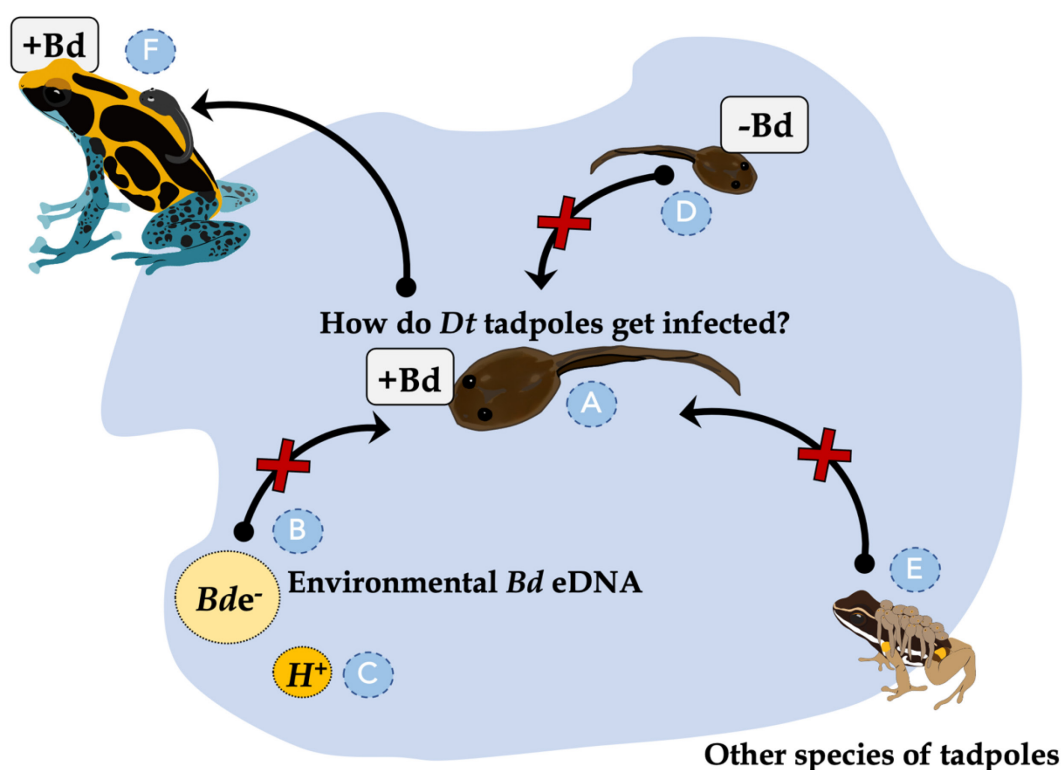

FIGURE 7   **Possible modes of *Bd* transmission in *D. tinctorius*.** (A) We found 13 tadpoles infected with *Bd*. (B) 8 of +*Bd* tadpoles were found in pools without *Bd* eDNA. (C) The majority of +*Bd* tadpoles were found in pools with a pH range that would impair/kill zoospores. (D) There is a variable proportion of infected tadpoles in each pool. (E) Phytotelmata were monitored continuously over 5 weeks, and only *D. tinctorius* tadpoles were found in occupied pools. (F) We found a +*Bd* tadpole in an artificial pool and males (the caring sex) have significantly higher infection loads than females.

As a system with intensive parental care and frequent interactions between adults in the form of mating or fighting, the behaviour of mature poison frogs is likely to be central in disease spread. Throughout our sampling, we found that both males and females were infected with *Bd*, although males had significantly higher zoospore loads. This is interesting to consider in the context of pseudo-vertical transmission of *Bd*. Before presenting data to substantiate the probability of pseudo-vertical transmission in the system, it is worth emphasising that establishing a framework of disease spread *in situ* is incredibly difficult, which is one of the main reasons why transmission data from terrestrial systems are so sparse (Burns *et al.* 2021). The data I present here are only a subset of a multi-year effort spanning multiple field seasons spent tracking frogs, climbing trees, and dealing with unexpected hurdles including (but not limited to) the loss of hundreds of samples due to contamination.

With the hurdles of collecting these data properly enumerated, I finish this chapter outlining possible transmission of *Bd* from tadpole-carrying adults to tadpoles. When fathers transport their tadpoles, there is contact between the oral disk of the tadpole and the dorsal surface/mucus of the adult. However, the mechanics underlying transport events are fuzzy: surprisingly, there is no published work examining how tadpoles physically (or chemically) attach (and remain alive) on the back of their parents for periods that can span from a couple of hours to multiple days. Despite this gap in knowledge, it is certain that the ventral surface of tadpoles comes into contact with the dorsal surface of adults, which introduces a significant opportunity for the transmission of zoospores from the epithelial cells of adults to the keratinised mouthparts of tadpoles.

In this work, two lines of evidence support the possibility of pseudo-vertical transmission in the system. Firstly, tadpoles that weigh as little as 0.05 g can be infected with *Bd*. As previously mentioned, the size of tadpoles upon transport is extremely uniform, making it easy to identify recently deposited tadpoles. Once deposited, tadpoles tend to grow very quickly. Having established that tadpoles do not appear to acquire zoospores from conspecifics or the water, there are few options from where the infection could have originated. Secondly, we have evidence of infected tadpoles in artificial pools. In this instance, plastic cups were filled with water from a nearby aquifer. Infected tadpoles were alone and collected within 24 hours of deposition. Although the direct evidence presented here is limited, when we consider the evidence against other transmission modes the validity of pseudo-vertical transmission in *D. tinctorius* seems biologically reasonable.

4 CONCLUSIONS

Throughout this work I explore the consequences of decisions made by parents who seemingly invest a great deal of energy to place offspring in environments that almost guarantee their struggle.
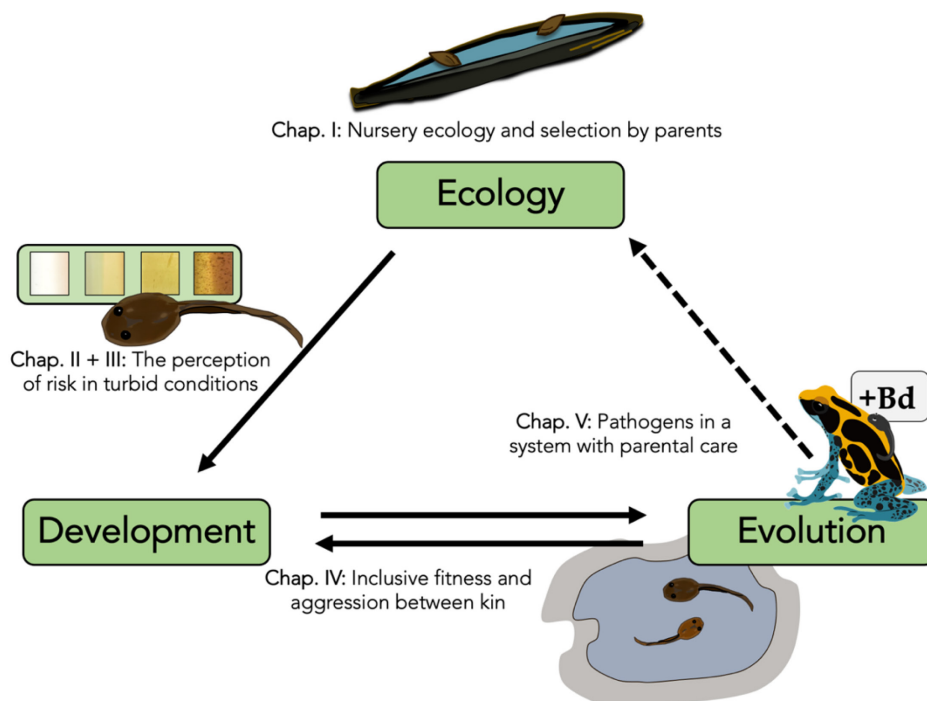

FIGURE 8   Thesis overview within an eco-evo-devo framework.

From an evolutionary perspective, it seems counterintuitive that a parent is not attempting to minimise the risk faced by their offspring. Why put tadpoles in pools where they can be cannibalised? Why choose phytotelmata with a miniscule amount of water, when others with much larger volumes are available? Simply answered, the risks faced by animals are never singular and the benefits of an event never exist without an associated cost (I). *D. tinctorius* fathers adopt an opportunistic generalist pool deposition strategy: we find tadpoles in diverse

developmental environments whose physical (e.g., volume) and biological (e.g., occupation by other conspecifics) values pose differing advantages depending on the seasonal and environmental context. Because there exists a range of suitable deposition sites, the quality of which decreases with increased occupation, fathers may maximise their reproductive success by spreading their clutches across multiple sites.

In the face of such dynamic decision-making by fathers, *D. tinctorius* tadpoles have apparently evolved to be tolerant to challenging chemical conditions (ex. pH < 3, I), flexible with respect to the fine-tuning of their senses (III), dynamic in their decision-making (IV), and resilient to deadly pathogens (V)(Fig. 8). Tadpoles assess their environment, each other, and can process risk in a way that maximises their fitness. One way *D. tinctorius* may maximise their inclusive fitness is by being able to discriminate kin. This gives tadpoles the opportunity to opt-out or reduce aggressive behaviours towards conspecifics that can ultimately increase the number of shared genes in a population's genetic pool. Throughout this work, the concept of "altruism" in cannibals arose: by forfeiting a possible meal (thereby reducing possible direct fitness) and not cannibalising kin, are tadpoles being altruistic? In a pure sense of the word, the answer is no (West et al. 2007). Tadpoles face considerable risk of injury when attacking conspecifics, and tadpoles never fully abandon their aggressive behaviours towards each other, no matter their relatedness. Yet, there is some degree of kin selection at play within this system.

Tadpoles are also able to maximise their survival through their apparent sensory plasticity. While the photic environment can vary enormously among phytotelmata, tadpoles appear able to discriminate between visual stimuli. *D. tinctorius* can discern between odonate larvae and conspecifics, suggesting that tadpoles use vision to navigate the world and manage predator-prey interactions more than commonly assumed. From an experimental approach, tadpoles from a range of phytotelmata were tested in clear, controlled conditions and this experimental set-up had an impact on their behaviour. More specifically, tadpoles from clear phytotelmata discriminated between visual stimuli while turbid-pool tadpoles did not. The behaviour of clear-pool tadpoles demonstrates that (a) *D. tinctorius* uses vision to modulate their responses to their environment, and the behaviour of their turbid-pool counterparts suggests that (b) tadpoles' visual system adapts to the conditions they are raised in.

Phytotelmata where tadpoles develop are a mosaic of risks, none of the risks more poorly understood than the pathogenic fungi with whom they apparently co-exist. *Bd* is a deadly fungus that has decimated amphibian populations globally (Fisher *et al.* 2021), yet in this system appears present in inconsequential (and inconsistent) amounts. Whether the spread of *Bd* is limited by the resilience/resistance of the species (genetics/microbiome), the behaviour of the species, or the ecology of the environment (terrestrial lowlands) remains to be disentangled. In its totality, this work presents an overview of the natural history, behaviour, and ecology of a Neotropical amphibian. This thesis builds a foundation upon which future studies can further our knowledge of animal cognition, sensory development, and disease transmission in the wild.

SUMMARY (RÉSUMÉ FOR A GENERAL AUDIENCE)

The world that animals navigate is inherently risky. Animals must learn how to identify and avoid predators, make decisions about how to care for their offspring, and mediate interactions with other members of their species. All of these social decisions are tangled up in an animal's physical environment—the quality of which can impact the accuracy of their choices. For example, we can imagine how an animal's world must change when faced with dramatic environmental disturbances, such as the eutrophication of lakes: how is an individual's assessment of predators, mates, and competitors affected when an originally clear landscape becomes murky? Animals that go through metamorphosis are especially interesting to consider when we think about decisions, as the habitats occupied by larval stages are usually drastically different from those occupied by adults. Although this change in habitat usually entails profound changes in an animal's physical form, the larval stages of metamorphic animals typically do not receive as much attention as adult forms: compared to studies on butterflies, dragonflies and frogs or toads, not many have been interested in the secret lives of caterpillars, dragonfly nymphs, or tadpoles. We generally understand the juvenile state to be transitory—a means to achieving adulthood. However, caterpillars, nymphs, and tadpoles are adapted to the environment where they develop and, as such, their decisions, morphology, and senses are under similar selective forces as their adult counterparts.

Throughout this work, I explore decision-making in both the adult and larval forms of a tropical frog species called *Dendrobates tinctorius*, the dyeing poison frog. The biodiversity of the Neotropics has long fascinated biologists and for good reasons: the warm, humid climate has facilitated the rise of an amazing diversity of animals that have seemingly evolved to be brighter, louder, and simply more exuberant than animals we find in temperate forests. Amphibians are no exception to this beautiful radiation in form and function. For example, poison frogs have evolved both bright colours that serve to signal their unprofitability as prey and terrestrial breeding (most frogs lay their eggs in large bodies of water, such as ponds and lakes), which is hypothesised to go hand-in-hand with the unique rise of parental care behaviours that also characterises this group.

For *D. tinctorius*, parental care is displayed primarily by males and consists of fathers transporting recently hatched tadpoles from sites on the forest floor where the eggs are laid to small pools of water formed by vegetation (i.e., treeholes, fallen palm leaves) that serve as tadpole nurseries. While these small nurseries exclude large aquatic predators, such as fish, they entail their own risks in being both food-limited and prone to desiccation. Once a father chooses a nursery to deposit his offspring, tadpoles are confined to their microhabitat until they reach metamorphosis. This biological constraint means two things: (1) the nursery choice by fathers determines the developmental experience of their

young, and (2) tadpoles must navigate the diverse sources of risk in their nurseries to reach metamorphosis. This thesis explores adult decisions and follows how young mediate the challenges associated with the rearing environments chosen by their parents.

First, I explore what physical and chemical qualities of nursery sites are selected by parents. We observe many instances of apparently paradoxical decision-making by fathers that choose nurseries occupied by larger cannibals or that contain volumes of water that can evaporate within an afternoon. We find tadpoles in pools that vary enormously in volume (ranging from the amount of liquid contained within a shot glass to that of a bathtub), chemistry (ranging in acidity from a neutral pH to that as acidic as orange juice), height (ranging from the forest floor to the tops of forest canopies), and community (ranging from being alone to with more than 40 other tadpoles of diverse species). Are these deposition decisions mistakes or are they indicative of a somewhat oblivious strategy by fathers? While other amphibian species are consistent in their nursery-site selection, *D. tinctorius* appears exceptionally variable in their choices. Perhaps the remarkable flexibility of adults compared to other species allows fathers to play a game of chance with their own offspring. Ultimately, if *D. tinctorius* fathers can access a huge diversity of nurseries, why not deposit his tadpoles in a wide range of conditions? Diversifying his choices may maximise the number of offspring that survive and reach metamorphosis. As a result of these broad deposition patterns, tadpoles have had to evolve a range of strategies to survive the harsh environments where they are left to develop in.

One such challenge is the visual environment of nurseries. In the wild, we see that the water colour of pools can range from being as clear as drinking water to as turbid as an oxidised pinot noir. For a tadpole, the outcome of developing in such different conditions must have implications for the way they see the world. In order to understand this, I conducted experiments with tadpoles and assessed their behaviour as a function of their nursery turbidity. While I used various approaches to quantify the water contained within pools, the validation of all of my measures were rooted in spectrophotometry. Spectrophotometers quantify the wavelengths that are absorbed or transmitted through a sample across a defined spectrum. By knowing what colours are absorbed by a sample, we can very accurately define the colour of the water. From the wild, I collected tadpoles from a range of visual environments and then tested tadpoles in an arena where they could only *see* a predator (that was in a separate, transparent container). The experimental arenas were either white or black to see if the contrast of the visual stimulus changed tadpole activity and space use. In these experiments, I measured the behaviour of the cannibalistic *D. tinctorius* and separately repeated the same experiment with a different species of poison frog called *Oophaga pumilio.* The importance of this comparison lies in the natural history differences between these two species: *O. pumilio* is an *oophagous* tadpole, which means that they depend on their mothers to come and deposit unfertilised eggs in their nurseries throughout development. Thus, both species are hypothesised to use vision, but with different behavioural goals: *D. tinctorius* to hunt and *O. pumilio* to recognise incoming mothers.

I found that nursery conditions shape the way tadpoles perceive risk in new contexts. When raised in turbid conditions, the predatory *D. tinctorius* barely respond to any visual stimulus. In comparison, tadpoles raised in clear water are much more active when they see a tadpole of the same species versus a tadpole-predator (dragonfly nymph). *O. pumilio* tadpoles, on the other hand, are more active on experimental backgrounds that more closely match the nurseries where they come from. For example, tadpoles from clear nurseries are more active on white backgrounds than on black backgrounds (and vice-versa). This series of experiments reveals to us that (1) tadpole sensory systems may adapt to the conditions where they develop, revealing that how tadpoles see the world may change on a mechanistic level in response to their developmental environments, and (2) *D. tinctorius* tadpoles are able to recognise conspecifics using only vision, indicating that (contrary to previous suggestions) tadpole vision is useful enough to inform them about their environment.

Once I established the importance of vision in *D. tinctorius*, I wondered how this cannibalistic species makes decisions. Are cannibals always cannibals when given the opportunity? In this context, it is important to recognise that a meal is never free. If a tadpole attempts to feed on a conspecific, they risk serious injury as their target responds aggressively to the prospect of being eaten. The other cost they face is less obvious, but the logic is as follows: if cannibals consume a sibling they are directly pruning their family tree. This can be quite costly in evolutionary terms, as eliminating closely related individuals who may have otherwise survived and reproduced removes genes from the population pool that they would have shared. In this experiment I paired *D. tinctorius* tadpoles of various sizes with non-siblings, half-siblings, and full-siblings and measured the aggression between pairs. I found that tadpoles significantly reduce their aggression when paired with kin, suggesting that tadpoles can recognise siblings (the mechanisms underlying *how* they do this remains a mystery). With this said, being siblings never completely protects a tadpole from aggression: despite apparently being able to discriminate kin, if the size difference between tadpoles is large enough then the "protective effects" of kinship disappear and a small tadpole will be repeatedly attacked. What does this say about fathers? Are dads really placing their young offspring in nurseries as a meal for larger residents? A resident tadpole is a useful sign of nursery stability (i.e., a large tadpole indicates there has been enough water or resources in the pool) which may offset the risky decision of placing a young tadpole in an occupied nursery, especially if the residents are related.

Having explored the effects of parental choice, the environment, predators, and conspecifics I close here with a discussion of the future of amphibian biodiversity in the Neotropics, and the risks they face. Hundreds of amphibian populations have declined, a subset of which have gone completely extinct, as a result of the effects of a pathogenic fungus called *Batrachochytrium dendrobatidis (Bd)*. While the biodiversity losses entailed by this pathogen are historically unmatched, we lack a thorough understanding of where this pathogen occurs in some environments and how it is spread between animals. This is in part a result of the great global diversity of amphibians, and the range of ecosystems and

social structures that characterise them. Here, I explored the presence of *Bd* on adults, tadpoles, and in the water of tadpole nurseries, to find out how *Bd* is transmitted within the species. While a definite answer eluded me, I was able to show that the fungus occurs from the forest floor to the tops of canopies, infects adults and tadpoles of diverse stages, and apparently does not depend on tadpole nurseries as an environmental reservoir. One mechanism of transmission that remains compelling for further investigation is the infection of tadpoles via parental care of their fathers. In the wild, we find very small, recently transported tadpoles to be infected by the fungus. Having eliminated the possibility of infection by other tadpoles or from the environment, it could be that infected fathers transfer zoospores to their young while caring for them. This, however, remains to be experimentally proven.

This thesis explores the fascinating world of poison frogs. These animals are able to successfully navigate the complexity of the jungle, all while caring for their young. Both tadpole and adult forms of amphibians can make decisions which reveal that they assess their environment. With findings that have broader impacts in fields ranging from evolutionary theory to disease ecology and conservation, it is clear that better understanding life from an animal's perspective is a worthy endeavour.

Acknowledgements

Life is beautifully and frustratingly complex, and if there is only one thing you take away from this work, let it be this: **animals assess their surroundings and make decisions that, from my point of view, are incredibly perceptive**. If by the end you come out of this believing that frogs are simple hopping machines without much care for the world around them, then I have failed terribly at my job of telling you the most extraordinary story of parenthood, family, and growing up. I hope this work excites you to think about biology from a new perspective: one where science can flourish in a world filled with magic and fun.

The culmination of this work has lived through love and heartbreak, death and birth, and many events that have struck me as both miraculous and mundane in between. And I have a few people to thank for that. To begin with my mama. A mystic woman who always full-heartedly supported my adventures, no matter how dirty they got me or how far they took me. Thank you for always being so unabashedly proud and instilling the knowledge that life exists in many layers, and we must be brave enough to pull them back to see the truth. *Pour mon Papa, qui m'a élevé avec discipline et sagesse. Tu seras toujours mon guide. Pour Brigitte-Marie, ma tante adorée qui apporte toujours de la clarté et de la logique à mes décisions.* For my American family, Marcy and Dale, Kjersti and Nate, Kelly and the kiddos, Bryan and Karen, Naomi and Curtis, thank you all for loving me no matter the distance. Thank you for taking me out fishing, thank you for accepting me exactly how I am, and thank you always being excited to celebrate pretty much anything. To Riga for knowing me to my core and to Aislyn for loving me in every time zone.

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The majority of the world's population cannot place Jyväskylä on a map. And that's okay, because we must keep some of the world's treasures a secret. The thanks I owe to this city and the people in it could be the length of the entire thesis, but I will try to keep things brief (though this has never been a strong point of mine). Bibi, you took on a lot of roles throughout this journey—some more official than others. I am sorry for being so terrible with grammar. Though many have tried to teach me how to use a comma, you're the first one to nurture my inner-editor! Thank you for continually trying to teach me writing rules, despite my active efforts to apparently learn anything else first. Thank you for being such a source of warmth and joy and love. Thank you for building me up, and thank you for championing me through every stage and permutation of this path. You are a gift to science. To my *Doctor Pappa* and Sara and the babies, thank you for adopting me. I am so proud to be a part of your family. You are a model for balancing work, life, and love. I hope to be like you when I grow up someday. Sara, thank you for teaching me statistics and celebrating our shared nerdiness of loving magic, board games, and Zumba. Lutzian, thank you for always being there for me. You always know what to say, how to make me feel appreciated, and you even took me out fishing. More than once! Which in my head has built me up to be the best fisherwoman in all of Jyväskylä, though you still have to tie the knots for me. Thanks to both of you for all the hugs and always smelling nice (maybe it's a shared family soap?).

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ORIGINAL PAPERS

I

POOL CHOICE IN A VERTICAL LANDSCAPE: TADPOLE-REARING SITE FLEXIBILITY IN PHYTOTELM-BREEDING FROGS

by

Fouilloux C. A., Serrano Rojas S. J., Carvajal-Castro J. D., Valkonen J. K., Gaucher P., Fischer M., Pašukonis A. & Rojas B. 2021

ORIGINAL RESEARCH

Pool choice in a vertical landscape: Tadpole-rearing site flexibility in phytotelm-breeding frogs

Chloe A. Fouilloux[1] | Shirley Jennifer Serrano Rojas[2] |
Juan David Carvajal-Castro[3,4] | Janne K. Valkonen[1] | Philippe Gaucher[5] |
Marie-Therese Fischer[2] | Andrius Pašukonis[2,6] | Bibiana Rojas[1]

[1]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland

[2]Department of Biology, Stanford University, Stanford, CA, USA

[3]Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Bogotá, Colombia

[4]Department of Biological Sciences, St. John's University, Queens, NY, USA

[5]USR LEEISA—Laboratoire Ecologie, Evolution, Interactions des Systèmes Amazoniens, CNRS-Guyane, Cayenne, French Guiana

[6]Centre d'Ecologie Fonctionelle et Evolutive, CNRS, Montpellier Cedex 5, France

Correspondence
Andrius Pašukonis, Department of Biology, Stanford University, 371 Jane Stanford Way, Stanford, CA 94305, USA.
Email: apasukonis@stanford.edu

Bibiana Rojas, Department of Biology and Environmental Sciences, University of Jyväskylä, PO Box 35, Jyväskylä, FI 40001, Finland.
Email: bibiana.rojas@jyu.fi

Chloe Fouilloux, Department of Biology and Environmental Sciences, University of Jyväskylä, PO Box 35, Jyväskylä, FI 40001, Finland.
Email: chloe.a.fouilloux@jyu.fi

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Abstract

Many species of Neotropical frogs have evolved to deposit their tadpoles in small water bodies inside plant structures called phytotelmata. These pools are small enough to exclude large predators but have limited nutrients and high desiccation risk. Here, we explore phytotelm use by three common Neotropical species: *Osteocephalus oophagus*, an arboreal frog that periodically feeds eggs to its tadpoles; *Dendrobates tinctorius*, a tadpole-transporting poison frog with cannibalistic tadpoles; and *Allobates femoralis*, a terrestrial tadpole-transporting poison frog with omnivorous tadpoles. We found that *D. tinctorius* occupies pools across the chemical and vertical gradient, whereas *A. femoralis* and *O. oophagus* appear to have narrower deposition options that are restricted primarily by pool height, water capacity, alkalinity, and salinity. *Dendrobates tinctorius* tadpoles are particularly flexible and can survive in a wide range of chemical, physical, and biological conditions, whereas *O. oophagus* seems to prefer small, clear pools and *A. femoralis* occupies medium-sized pools with abundant leaf litter and low salinity. Together, these results show the possible niche partitioning of phytotelmata among frogs and provide insight into stressors and resilience of phytotelm breeders.

KEYWORDS

competition, niche partitioning, parental care, phytotelmata, poison frogs, tadpoles

1 | INTRODUCTION

The survival of young often hinges on the quality of the rearing environments created or chosen by their parents. Whether it is by building nests (birds: Brown & Brown, 1991; mice: Bult & Lynch, 1997, Zhao et al., 2016), digging burrows (rodents: Ebensperger et al., 2014; Svendsen, 1976), or depositing clutches/larvae (e.g., salamanders: Ruano-Fajardo et al., 2014, frogs: Pettitt et al., 2018), the ecology of rearing sites is fundamental in shaping offspring success. For animals with external fertilization, breeding site choice can be especially important, as optimal conditions for egg clutches may differ from the optimal environment for hatchlings and adults (fish: Ottesen & Bolla, 1998, Mikheev et al., 2001; salamanders: Nussbaum, 1987, Sih & Moore, 1993; frogs: Vági et al., 2019). Many of these animals assess and prefer biotic and abiotic properties of breeding sites that can enhance offspring survival (Brown & Shine, 2005; Marsh & Borrell, 2001; Mokany & Shine, 2003; Touchon & Worley, 2015). Thus, characterizing the nurseries where offspring occur and where they do not can provide information on the qualities parents assess when making these critical reproductive decisions.

The challenge of finding an optimal rearing site becomes especially apparent in terrestrial or arboreal breeding animals, whose larval forms are aquatic. For example, some treefrogs lay clutches overhanging water bodies. The placement of clutches is essential, as tadpoles from poorly placed clutches risk hatching and falling onto the ground (Warkentin, 2011; Wells, 2007). One remarkable amphibian strategy adapted to changing habitats between egg and larval stages involves parents that physically transport recently hatched tadpoles from terrestrial oviposition sites to small water-holding plant structures (phytotelmata), ponds, or streams (Schulte et al., 2020; Summers & Tumulty, 2014). Unlike other terrestrial breeding amphibians, the physical transport of young allows parents to select the ideal environment for their offspring to develop. Although it is difficult to extensively characterize streams and ponds due to their large size and interconnectedness with other water bodies, microhabitats like phytotelmata provide a unique opportunity to fully measure the biological, chemical, and physical aspects of a nursery, creating an opportunity to interpret deposition choices with a depth of ecological information that is rarely available for other rearing sites. Here, we investigate the chemical and physical properties of aquatic nurseries that predict the presence of Neotropical tadpoles in phytotelm-breeding frogs.

The use of phytotelmata as tadpole nurseries can seem counterintuitive, as their small volume makes them prone to desiccation and limited in food (Summers & McKeon, 2004; Summers & Tumulty, 2014). However, their small size provides protection from large predators and overall reduced interspecific competition (Kitching, 2001; Summers & Tumulty, 2014). Various species have evolved different strategies for their offspring to succeed in these pools (substrate specialization: von May et al., 2009; Pettitt et al., 2018; trophic egg feeding: Brown et al., 2010; Weygoldt, 1980; larval aggression/cannibalism: Gray et al., 2009; Poelman &

Dicke, 2007; Rojas, 2014; pool choice based on specific physical or chemical cues: Lin et al., 2008; Schulte et al., 2011). Despite the widespread use of phytotelmata (Lehtinen, 2021), and the non-random site selection shown by many frog parents, few studies go beyond quantifying basic pool dimensions and pool occupation to understand tadpole deposition decisions. Further, the bulk of phytotelm studies are focused only on bromeliads (Mageski et al., 2016; Pettitt et al., 2018; Ruano-Fajardo et al., 2014), while work exploring potential trade-offs associated between different phytotelmata (i.e., physical and chemical properties as well as food- and predator-related pressures), and how these change across a vertical gradient, has gone largely overlooked (but see Brown et al., 2008a).

To understand what variables drive phytotelm selection, we compared pool occupation by three Neotropical frogs (*Dendrobates tinctorius* (Dendrobatidae), *Allobates femoralis* (Aromobatidae), and *Osteocephalus oophagus* (Hylidae)) that were most frequently detected in phytotelmata throughout field surveys at our study site in French Guiana. Following broad species-wide comparisons, we focus on a more detailed analysis of pool choice in *D. tinctorius*, a phytotelm specialist with predatory and cannibalistic tadpoles which are deposited in a range of phytotelm types (e.g., palm bracts, tree holes, fallen trees; Figures 1 and 2) that occur from the forest floor to more than 20 m in vertical height (Gaucher, 2002; Rojas, 2014, 2015). The use of the high canopy pools is perplexing because *D. tinctorius* is commonly successful in terrestrial pools (Rojas, 2014). It is known that pool chemistry can change drastically depending on substrate ("dead" or "live"; see Figure 1), height, and community composition (Pettitt et al., 2018; Ruano-Fajardo et al., 2014). Thus, better understanding the ecology of high arboreal pools and characterizing phytotelmata across the vertical gradient could help explain both the apparent success of *D. tinctorius* in a wide range of pools and why parents sometimes carry their offspring to such heights. To our knowledge, this is the first study providing detailed biotic, physical, and chemical comparisons of phytotelm choice between Neotropical species.

2 | MATERIALS AND METHODS

The study was carried out in the primary lowland terra-firme forest near the Camp Pararé at the CNRS Nouragues Ecological Research Station in the Nature Reserve Les Nouragues, French Guiana (4°02′N, 52°41′W) over two field seasons: 1st February to 20th March 2019, and 30th January to 26th February 2020. The study area (approximately 0.2 km^2) was chosen specifically because of the high abundance of *D. tinctorius* (Rojas & Pašukonis, 2019). Pools were found with a combination of field methods. We opportunistically searched for pools targeting suitable microhabitats such as fallen trees, trees with buttresses, and palm trees. In addition, pools were discovered by using tracking to follow *D. tinctorius* during previous studies (Pašukonis et al., 2019). We also used experimentally induced tadpole transport in combination with tracking (Pašukonis
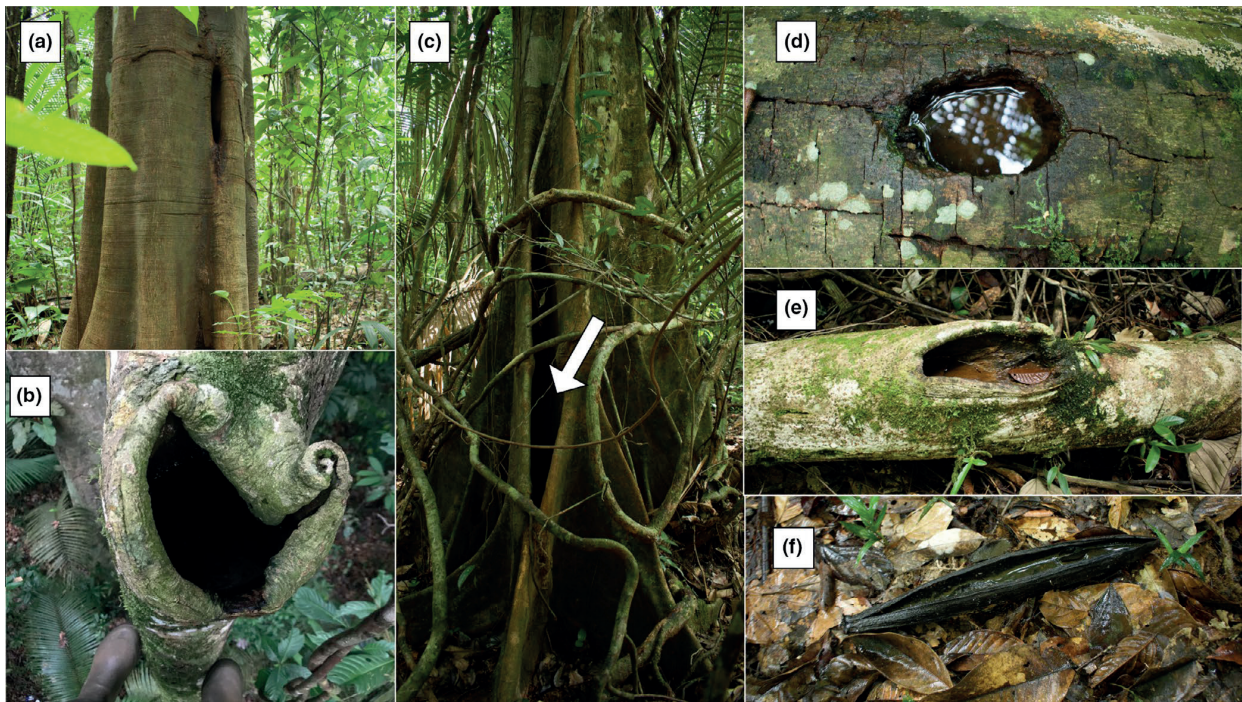
FIGURE 1 Visual overview of sampled pool diversity. Photographs show the diversity of pools across the vertical gradient. Phytotelmata used by frogs include "live" substrate pools such as tree holes (a), high arboreal pools (b), and buttresses (c). There were also commonly occupied "dead" substrate pools such as fallen trees (d, e) and palm bracts (f)

et al., 2017) to find additional pools used by *A. femoralis*. Trees with high arboreal pools were discovered by locating calls produced by the tree hole-breeding frogs *Trachycephalus resinifictrix* and *T. hadroceps* during night surveys.

2.1 | Study species

Throughout the course of this work, three species formed the core of our data. *D. tinctorius* and *A. femoralis* are both small poison frogs belonging to the superfamily Dendrobatoidea. *A. femoralis* is a terrestrial frog whose adult males aggressively defend territories during the rainy season (Narins et al., 2003; Roithmair, 1992), from which they carry recently hatched tadpoles to a variety of terrestrial pools including phytotelmata close to the ground (Ringler et al.,2009, 2013). Tadpoles of this species are omnivorous (McKeon & Summers, 2013), but not cannibalistic (Summers & McKeon, 2004). Similarly to *A. femoralis*, *D. tinctorius* males care for their offspring by carrying them to pools of water. Males of this species are adept climbers (depositing their tadpoles from the ground to more than 20 m in vertical height; Gaucher, 2002; Rojas, 2014, 2015), and their tadpoles are aggressive cannibals (Rojas, 2014; Rojas & Pašukonis, 2019).

Osteocephalus oophagus is a hylid treefrog with biparental care and obligately oophagous tadpoles (Jungfer et al., 2000; Jungfer & Weygoldt, 1999). As in our field site, adults have been found to call and breed in bromeliads, tree holes, and palm axils close to the forest floor (Jungfer & Weygoldt, 1999). Tadpoles of this species develop in the same pool in which the eggs are deposited.

2.2 | Sampled pools

We exclusively considered phytotelmata throughout this study. Pools could be classified into two categories: dead substrates, which included holes in dead branches, fallen trees, and fallen *Oenocarpus* palm bracts, and live substrates, which included live tree trunks, branches, roots, and buttresses. We did not sample bromeliads and nonphytotelm pools as these pools are not used by *D. tinctorius*. Based on the pools' height and accessibility to different frog species, we termed the pools as "ground access," "low arboreal," or "high arboreal" (Figures 1 and 2). Ground access pools did not require vertical climbing ability to reach and included dead fallen structures as well as pools in live roots or low buttresses. Low arboreal pools were inside vertical structures low on the trunk or on high buttresses. High arboreal pools were high on the trunk or in canopy branches and were accessed for sampling using rope-based canopy access methods. There was a clear vertical separation between ground access and low arboreal pools, which were all under 212 cm in height and between those and high arboreal pools, which were all above seven meters in height. In total, we sampled 84 unique pools across the 2019 and 2020 field seasons.

Several unique pools were sometimes found and sampled in the same tree. For all pools, we recorded the pool type, location (latitude/
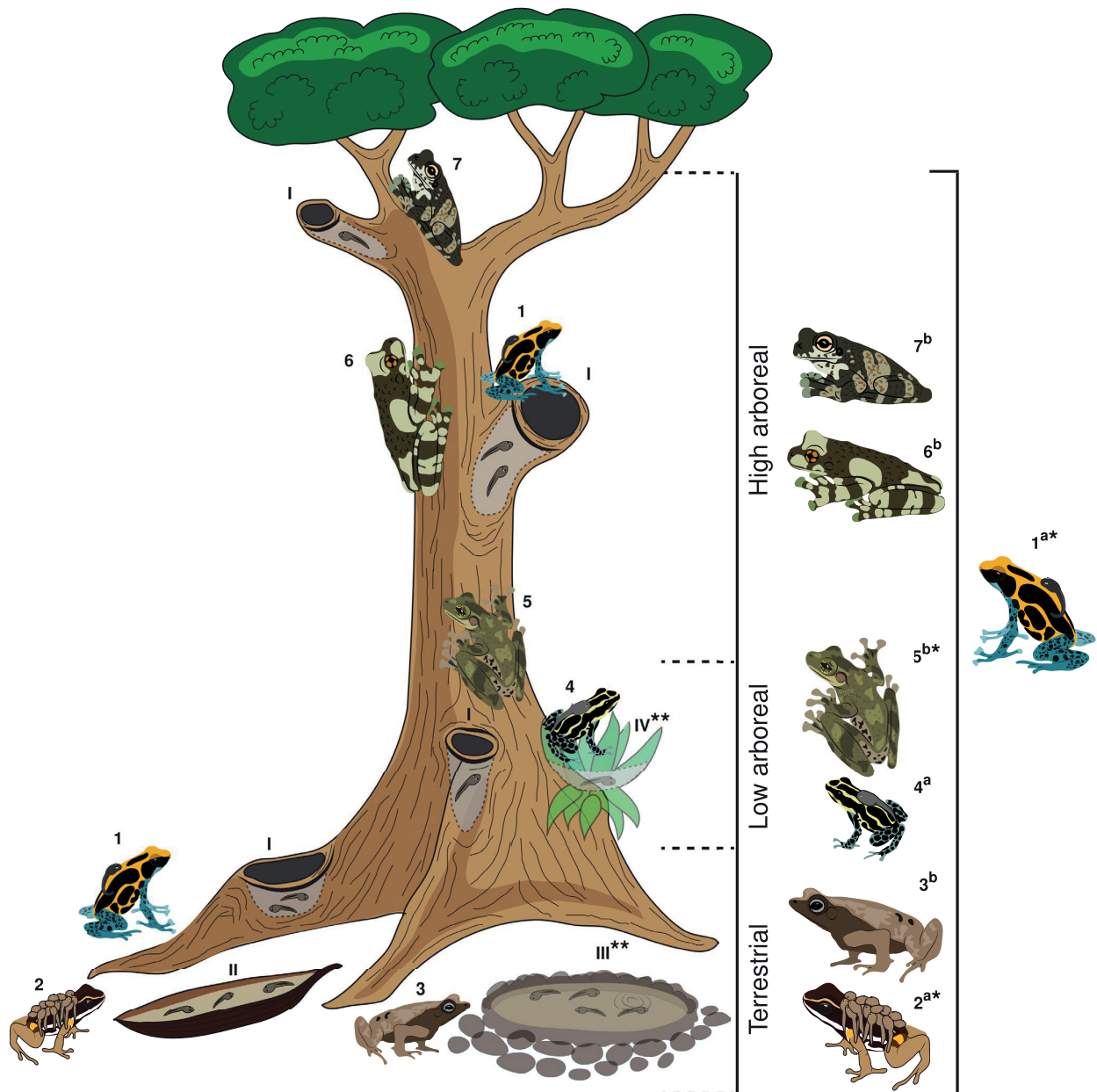
FIGURE 2 Vertical partitioning of the phytotelm-breeding anuran community in the Nouragues Nature Reserve, French Guiana. Numbers indicate seven species detected during this study: 1. _Dendrobates tinctorius_, 2. _Allobates femoralis_ 3. _Rhinella castaneotica_, 4. _Ranitomeya amazonica_, 5. _Osteocephalus oophagus_, 6. _Trachycephalus resinifictrix_, and 7. _T. hadroceps_. Letters indicate species with (a) terrestrial or (b) aquatic eggs. Latin numerals indicate commonly used pool types: I. tree holes at various heights, II. fallen palm bracts, III. ground puddles, and IV. bromeliads. *Most commonly encountered species included in the analysis and ** pool types not sampled in this study (see methods)

longitude), height from the ground to the pool edge, largest width and length parallel to the water surface, and the pool depth (maximum possible water depth of the phytotelmata) from the solid sediment bottom to the maximum water level line. Based on these measurements, we estimated the maximum water-holding capacity of each pool using the volume formula of a semi-ellipsoid as in Rojas (2014). Other sampling methods differed between the two field seasons.

2.3 | 2019 field season sampling

In 2019, we quantified physical measures (height, pool dimensions, leaf litter volume), biotic measures (amphibian and invertebrate counts and diversity), and chemical measurements (see Appendix 2 for description of all variables measured). For pools accessible from the ground and smaller arboreal pools, we attempted to sample all

tadpoles and Odonata larvae (predators of tadpoles; Caldwell, 1993; Fincke, 1999; Summers & McKeon, 2004) in each pool. Initially, we carefully observed the undisturbed pool and attempted to catch all tadpoles and Odonata larvae using a variety of tools. We then syphoned the entire volume of the water and sediment from the pool, emptied the leaf litter, and searched for tadpoles and Odonata larvae. The volumes of water, sediment, and leaf litter were measured. For deep arboreal pools, we repeatedly netted and observed the pool until no more tadpoles were caught during five minutes of continuous netting. We carefully scraped the inner walls of the pools and caught as many Odonata larvae as possible. The leaf litter volume could not be accurately measured for some deep arboreal pools, but they typically were protected from falling leaves and had little leaf litter in them.

We used visually apparent morphological traits to identify tadpoles, except for *Allobates femoralis*, *A. granti*, *and Ameerega hahneli*, which we could not reliably differentiate in the field. Because *Allobates femoralis* was more common in our study area than *A. granti* and *Am. hahneli* and we never observed *A. granti* and *Am. hahneli* directly at the pools, we classified all *A. femoralis*-like tadpoles as such. It is important to note that some *A. granti* and *Am. hahneli* tadpoles may have been misclassified as *A. femoralis*. However, this does not affect the interpretation of our results as all three species are cryptic terrestrial poison frogs similar in appearance, ecology, and behavior. We also opportunistically recorded all species of adult frogs heard or seen at each pool throughout the sampling period. This was used as an amphibian diversity index between 0 and 8 species observed at each pool. Tadpoles of only three out of seven recorded species, namely *D. tinctorius*, *O. oophagus*, and *A. femoralis*, were detected in pools with sufficient frequency for further analysis ($N = 34$ (2019), $N = 7$, and $N = 10$ pools, respectively).

Sampled invertebrates were counted, photographed, and classified only to a group level (usually order or class) apparent in the field. To estimate the predation pressure on tadpoles, we used the total count and average size of all Odonata larvae detected in the procedure described above. To estimate density and diversity of aquatic invertebrates, we carefully searched and counted invertebrates in a sample of up to 10 liters of water and up to one liter of sediment in proportion to the total estimated pool volume. For each liter of the water volume sampled, we sampled ~100 ml of sediment from the bottom of the pool. When the water volume was less than one liter or the amount of sediment was less than 100 ml, we sampled the entire pool and recorded the exact volumes. In the final analysis, we used the invertebrate density (count divided by the volume sampled) and the diversity index corresponding to our classification (between 0 and 12). The following 12 categories were used to quantify invertebrate diversity: Odonata Zygoptera larvae, Odonata Anisoptera larvae, surface Coleoptera adults, diving Coleoptera adults, Coleoptera Scirtidae larvae, Trichoptera larvae, Diptera Culicidae larvae, Diptera Chironomidae larvae, Diptera Tipulidae larvae, other Diptera larvae, small red Annelida, and other unidentified larvae. All water, sediment, tadpoles, and invertebrates were released back into the pool after sampling.

We measured water conductivity, salinity, total dissolved solids (TDS), dissolved oxygen, and temperature with electronic sensors (EZDO 7200 and pHenomenal OX4110H). Water chemistry (KH (also known as alkalinity), hardness, and NO_3) was recorded using aquarium water testing strips (JBL EasyTest). All measures were taken from the undisturbed surface water of the pool.

2.4 | 2020 field season sampling

The 2020 dataset focused solely on *D. tinctorius* tadpole counts and pH measurements of weekly resampled ground access phytotelmata ($N = 26$) over the time period of a month (February 2020). Rainfall data were provided by the Nouragues Ecological Research Station from an above-canopy weather station in the study area. High arboreal pools ($N = 8$, 2020) were only measured once. pH was recorded using a pH meter (AMTAST Waterproof pH Meter). The pH meter was calibrated once per day, prior to pool sampling, using both acidic (pH = 4) and neutral (pH = 7) calibration solutions. The pH of ground access pools was taken directly by submerging the pH probe into the pool, and the measurement was recorded once read-out stabilized. For arboreal pools, a sample of water was collected using a syringe, which was then sealed at both ends. Once on the ground, one end of the syringe was opened, and the pH was measured by submerging the pH probe into the syringe. Syringes were never reused. Between pool sampling, the pH probe was wiped with a clean cloth and rinsed with aquifer water.

2.5 | Statistical analyses

The presence of *D. tinctorius* in pools was analyzed using 2019 field data. As a result of the high collinearity between variables in the 2019 dataset (see Figure S1), we used a principal component regression to analyze phytotelm ecology data. We first checked data for a nonrandom structure following Björklund (2019); then, we established that the correlation matrices were significantly different from random ($\psi = 10.22$, $p = 0$; $\phi = 0.238$, $p < .001$) to ensure they were suitable for a PCA. Based on each PC difference from random matrices, we selected the first three principal components as predictors of probability for *D. tinctorius* tadpole presence as a binomial response in the principal component regression (PC1-3 explained about 53% of the variability of the data (where PC1 = 0.24 ± 0.48, PC2 = 0.17 ± 0.40, PC3 = 0.11 ± 0.33 (variance explained ± *SE*)). We evaluated the fit of negative binomial GLMs based on second-order AIC ranks (AICc) using the package AICcmodavg (Mazerolle, 2020) which are specialized for smaller sample sizes (Akaike, 1974; see Table S1). Models within two AIC scores of each other were further evaluated by assessing the significance of interactions between model terms.

To better understand which variables contributed significantly to each principal component, we calculated which variables had index loadings larger than random data. Following the methods outlined by

Björklund (2019) and Vieira (2012), we randomized the data and calculated new correlation matrices which we permuted 1,000 times. We then compared the index of loadings ($IL_{ij} = u_{ij}^2 \times \lambda_i^2$, see Vieira (2012) for details) between each observed PC and the randomly generated data to see which variables contributed significantly to each principal component.

The 2020 dataset consisted of weekly resampled pools throughout February 2020. Models took repeated measures of pool ID into account as a random effect. Both the presence of *D. tinctorius* tadpoles (count; negative binomial family) and pH (Gaussian family) from resampled pools were modeled using a mixed effects generalized linear model in the package "glmmTMB" (Magnusson et al., 2020). Predictor structure for both pH and *D. tinctorius* models was built based on biologically relevant variables (pool substrate, time, *D. tinctorius* tadpole count (for pH model), water capacity, surface area:depth ratio). Using these variables, models were first fit with relevant interactions (see Tables S2 and S3), which were then removed if they did not contribute significantly to the model using single term deletions (using base R function, `drop1`; Zuur et al., 2009). Residuals were diagnosed using the package "DHARMa" (Hartig, 2020). Final models were then checked for overdispersion and zero-inflation (using DHARMa); none of the final models required correction. All code was done in R (R Core Team, 2015).

3 | RESULTS

3.1 | Species-wide trends

We found 7 different species of frogs (either tadpoles or adults) from 4 families in the phytotelmata we surveyed for breeding (Figure 2): Dendrobatidae: *Dendrobates tinctorius* and *Ranitomeya amazonica*; Aromobatidae: *Allobates femoralis*; Hylidae: *Osteocephalus oophagus*, *Trachycephalus resinifictrix*, and *T. hardroceps*; and Bufonidae: *Rhinella castaneotica*. The tadpoles of only three species (*D. tinctorius*, *O. oophagus*, and *A. femoralis*, present in $N = 34$, $N = 7$, and $N = 10$ pools, respectively) were detected frequently enough for further analysis. The species-wide dataset is based on the sampling of 70 unique pools in 2019.

Differences in pool accessibility are highlighted in Figure 3. Compared to *A. femoralis* and *O. oophagus*, one of the most striking aspects of *D. tinctorius* ecology is its flexibility with respect to site
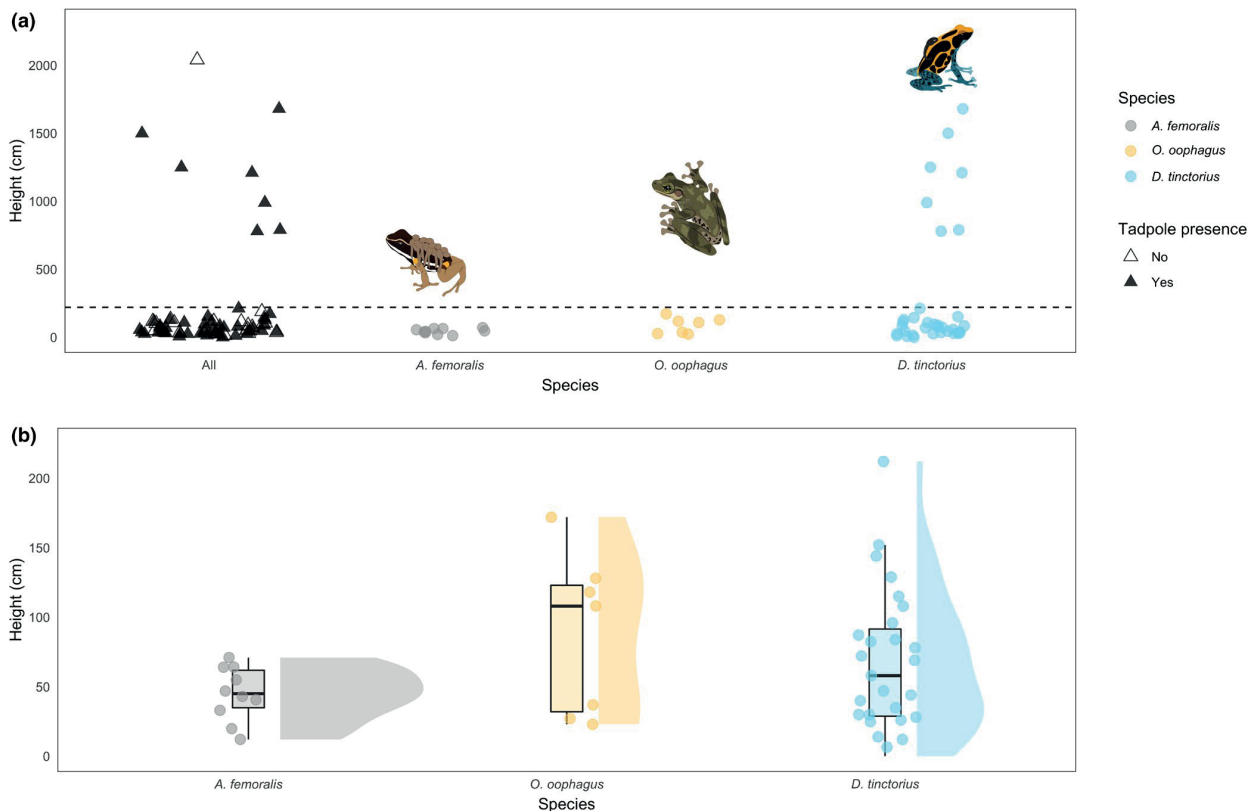
FIGURE 3 Tadpole presence across the vertical landscape. Panel (a) shows all sampled pools. For the "All" category, colored/empty triangles represent presence/absence data of at least one of the three species in the pool. Dashed line is drawn at 220 cm; pools above this limit are classified as high arboreal pools. Panel (b) highlights occupied pools below 220 cm. Dendrobates tinctorius ($N = 34$) tadpoles occur in pools across the vertical landscape. Distribution of *O. oophagus* ($N = 7$) and *A. femoralis* ($N = 10$) tadpoles shows possible vertical niche partitioning. Boxplot whiskers extend 1.5 * interquartile range. Violin plots represent density distribution for species occurrence. Data are from the 2019 field season

choice on a vertical axis. *Dendrobates tinctorius* tadpoles were found in pools from the forest floor to more than 15 meters in the canopy. For *O. oophagus*, a strictly arboreal frog in its adult stage, tadpoles were detected only in low arboreal pools where climbing is required for access, ranging from 20 cm to 1.7 m in height. In *A. femoralis*, tadpoles were only found in ground access pools where no vertical climbing is required and occurred at a maximum height of 71 cm.

Despite small sample sizes, we found clear trends: *O. oophagus* tadpoles are heavily biased toward small, clear pools and *A. femoralis* is present in medium and large pools with large amounts of leaf litter, whereas *D. tinctorius* occurs throughout the sampled range (Figure 4). With respect to density, we found that *A. femoralis* and *O. oophagus* tadpoles occur in higher numbers in pools (median = 7.5 and 10, respectively) compared to *D. tinctorius* tadpoles (median = 2), though a large range of tadpole densities was found for all species (*A. femoralis*: 1–51 tadpoles, *O. oophagus*: 1–50 tadpoles, *D. tinctorius*: 1–43 tadpoles).

As opposed to *A. femoralis* and *O. oophagus*, *D. tinctorius* can occupy chemically diverse pools, showing remarkable flexibility with respect to KH, salinity, and hardness that appears to limit the other species. *Allobates femoralis* and *O. oophagus* appear to exist in similar KH ranges (KH = 3–6), while *D. tinctorius* appears more tolerant of extreme values (KH = 3–20). *Allobates femoralis* tadpoles occurred in pools with a salinity range from 5 to 37 ppm, while *O. oophagus* tadpoles occupied pools with a range from 48 to 225 ppm (Figure 5, Panel C). *Dendrobates tinctorius* again appears to have no functional limitation, occupying pools with salinity from 11 ppm up to 955 ppm.

3.2 | Deposition site decisions: *Dendrobates tinctorius*

Because we detected *D. tinctorius* tadpoles much more frequently, we were able to conduct a more thorough analysis of the variables predicting tadpole presence in this species (see Appendix 2). We used principal components as predictors for *D. tinctorius* presence. Based on an AIC model comparison, we did not detect any significant interactions between components (Table S1). A negative binomial GLM only detected PC1 to play a significant role in predicting
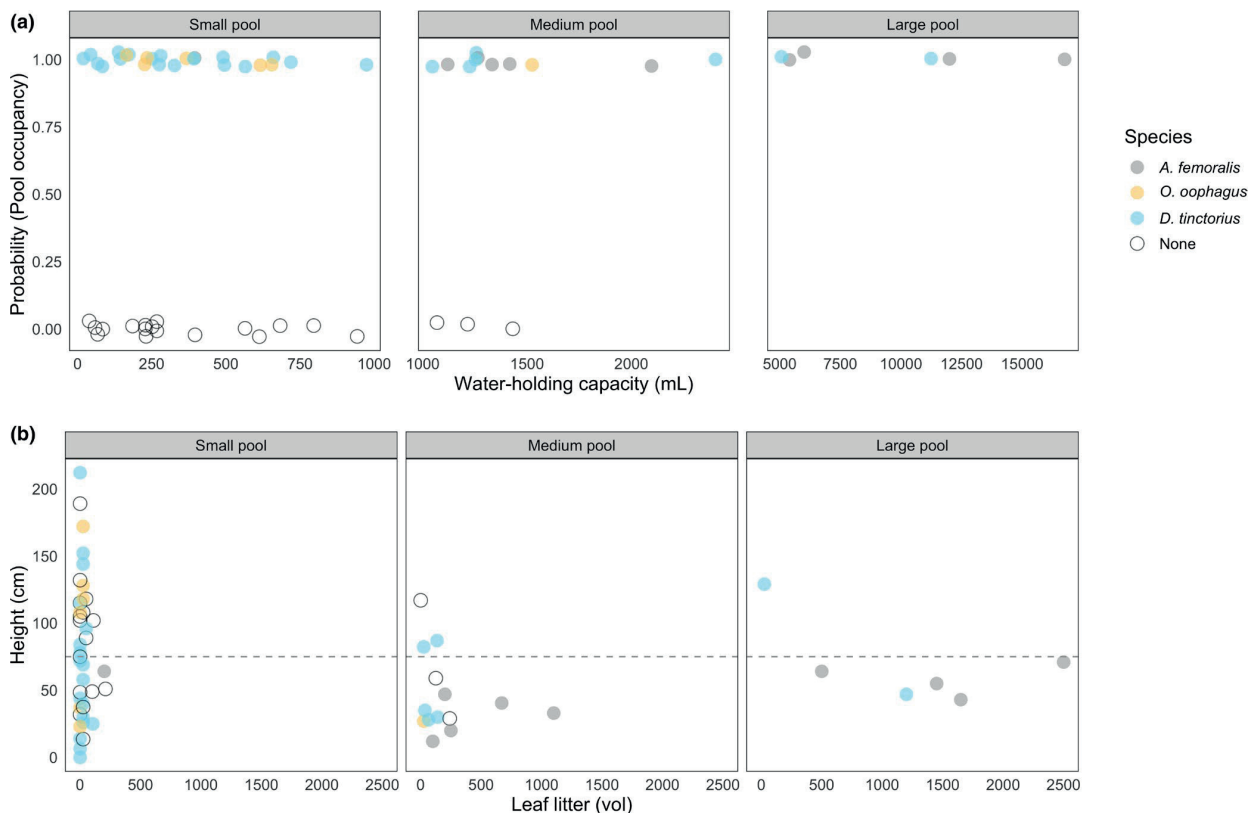
FIGURE 4 Pool occupancy based on water capacity and leaf litter volume of phytotelmata. All data are subsetted for low arboreal and ground access pools (<220 cm). Panel (a) is the probability of pool occupancy (binomial, 0/1) based on water capacity; data are faceted based on relative pool size (small = <1,000 ml, medium = <5,000 ml, and large = >5,000 ml). Points are plotted with a small amount of random noise on the y-axis to facilitate visualization of overlapping data. Panel (b) illustrates the correlation between leaf litter and height, faceted by the same pool categories as Panel (a). Points are colored by species presence. Dashed line indicates the vertical limit of *A. femoralis* (<75 cm). Out of the 62 ground access and low arboreal pools observed, *D. tinctorius* co-occurred once with *A. femoralis* and once with *O. oophagus*; *O. oophagus* and *A. femoralis* tadpoles were never found in the same pool
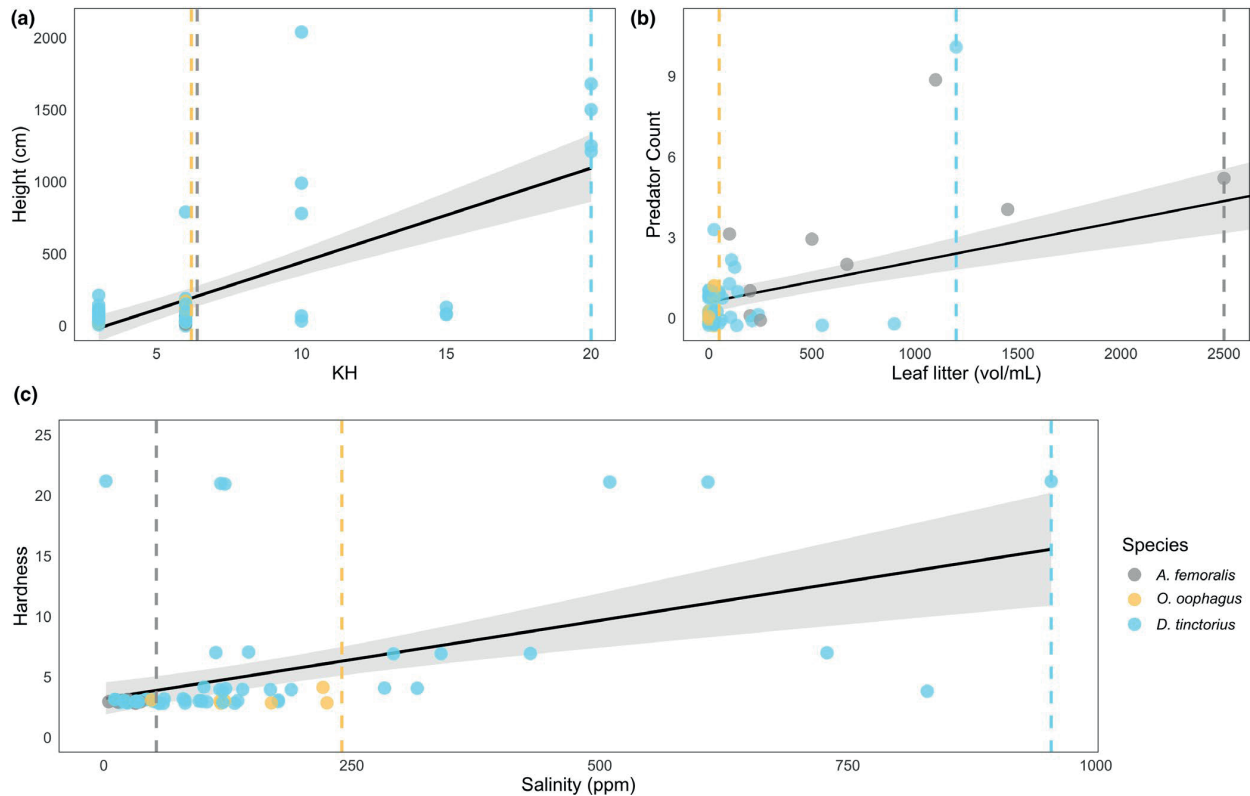
FIGURE 5 Chemical and physical predictors of tadpole presence in Neotropical tadpoles. We find that *D. tinctorius* tadpoles are tolerant to a wide range of KH, hardness, height, and salinity values, but appear to be limited with respect to high leaf litter volumes. Plots are based on variables with varied species limits (dashed lines). Colored points represent species presence. Black lines are fit with a GLM smoother, with 95% CI highlighted in light gray

TABLE 1 Principal component regression of tadpole presence in phytotelm pools. Using a negative binomial GLM, we found that only the first principal component is significant in predicting tadpole presence

Predictors	Tadpole presence (Y/N)		
	Estimates	**CI**	***p***
(Intercept)	−0.85	−1.28 to −0.49	**<.001**
PC1	0.25	0.07 to 0.42	**.003**
PC2	0.09	−0.19 to 0.40	.582
PC3	0.21	−0.07 to 0.48	.144

The bold text was to emphasize column names in the original tables.

tadpole presence (Table 1, CI: 0.08–0.42, $p = .003$), where an increase in component value increased the probability of detecting tadpoles.

Following Björklund (2019), we found that, when compared to randomly generated matrices, five out of the original 14 traits (see Appendix 2 for trait definitions) contributed significantly to the first principal component. The significant traits can be broadly categorized using three descriptors: (a) chemical (KH, $p < .001$; IL = 1.50, hardness, $p = .001$, IL = 1.30; salinity, $p < .001$, IL = 1.62); (b) physical (height, $p = .013$, IL = 1.06); and (c) biological (invertebrate diversity, $p < .001$,

IL = 1.20) (see Figure 6). Altogether, these results show that *D. tinctorius* tadpoles were found significantly more frequently in pools with higher levels of hardness, KH, and salinity; higher in the vertical gradient; and with more diverse invertebrate communities (Figure 6).

3.3 | *Dendrobates tinctorius* across temporal scales

Using both 2019 and 2020 datasets, we were able to follow phytotelmata across multiple timescales: 13 weekly resampled ground access and low arboreal pools, 13 annually resampled ground access and low arboreal pools, and 7 annually resampled high arboreal pools. Overall, we found that pools can persist over multiple sampling seasons. High arboreal pools appear to be the most stable with respect to both tadpole count and tadpole density compared to low arboreal and ground access pools sampled both years ($\bar{x}_{High}$ (2019) = 13.14 tadpoles, $\bar{x}_{High}$ (2020) = 10 tadpoles versus $\bar{x}_{Low}$ (2019) = 0.92 tadpoles, $\bar{x}_{Low}$ (2020) = 1.31 tadpoles). High arboreal pools also had the highest average pH and KH (pH$_{High}$ = 6.73, KH$_{High}$ = 15.14) compared to averages of other pool substrates (pH$_{(Low)Live}$ = 4.35, pH$_{(Low)Dead}$ = 5.68; KH$_{(Low)Live}$ = 5.69, KH$_{(Low)Dead}$ = 5.88). Due to difficult accessibility, high arboreal pools were sampled only once per year and thus were excluded from further analysis involving repeated sampling.

FIGURE 6 Binomial response of *Dendrobates tinctorius* presence to the first principal component. Dashed lines represent the 95% confidence interval. Significant variables within this component are detailed in Appendix 2
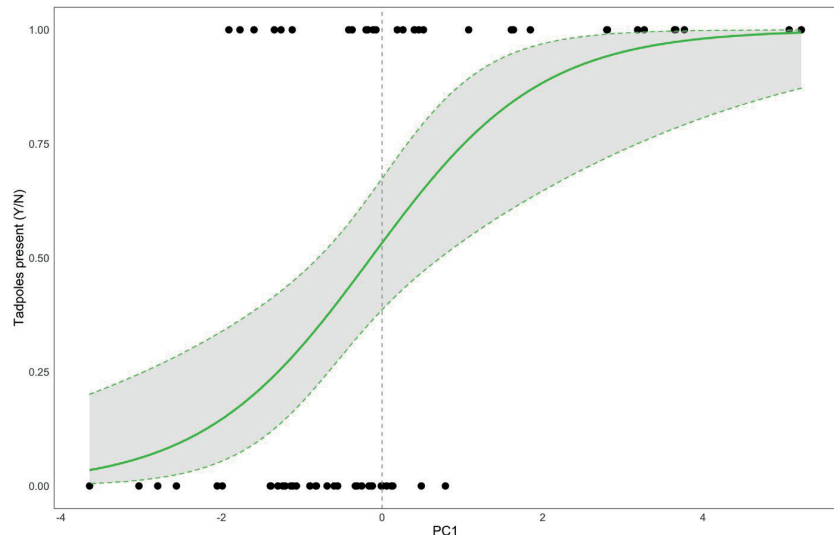
TABLE 2 Negative binomial generalized mixed model predicting pH over time based on pool substrate. Data include low arboreal and ground access pools. Pool type is a 2-level categorical variable ("Dead," "Live"); repeated sampling is taken into account by the random effect of pool_id, where pH is significantly higher in dead pool types versus live pools type and decreases over time

	pH			
Predictors	**Estimates**	**CI**	**z**	**p**
(Intercept)	4.66	4.13 to 5.20	17.09	**<.001**
Pool type [dead]	1.47	0.56 to 2.37	3.17	**.002**
Week	−0.11	−0.18 to −0.04	−2.93	**.003**
Random effects				
σ^2	0.20			
$\tau_{00\ pool\_id}$	1.13			

The bold text was to emphasize column names in the original tables.

When considering pools resampled weekly over the course of a month, two trends emerge: (a) pH is consistently higher in pools contained in "dead" substrates than in "live" substrates (odds ratio = 1.47, Table 2). For all substrate types, however, pH values decreased over the one-month sampling period (Figure 7a; odds ratio = −0.11, Table 2, which may be related to rainfall levels throughout the month); and (b) the number of *D. tinctorius* tadpoles can be predicted, in part, by the interaction between pool substrate and pH (Figure 7b). Dead pools have higher numbers of *D. tinctorius* tadpoles ($\overline{x}_{Live} = 0.72$, $\overline{x}_{Dead} = 4.03$, 2020 data). This pH/substrate relationship is clearly important, as tadpoles occur in higher numbers in high pH pools. Time (in weeks) was not detected as an important variable in determining *D. tinctorius* tadpole numbers throughout the month.

Several pools (N = 5, 2020) dried out completely during the month-long observation period, three of which were fallen palm bracts. Thus, although dead phytotelmata tend to have higher pH values and have a remarkable buffering capacity when filled, they also appear to dry out more easily during dry periods.

4 | DISCUSSION

Juvenile stages of development are particularly vulnerable for animals with external fertilization. Thus, the decision of where to breed

and raise young is vital to offspring survival. In this study, we investigated the tadpole-rearing site ecology of Neotropical phytotelm-breeding frogs. Out of seven detected species, five showed some form of parental care, three of which were sufficiently common to infer patterns of pool choice. Broadly, we found that the deposition choices of two of the three species are characterized by height and pool size, such that *O. oophagus* tadpoles occur in small, low arboreal pools below ~2 m and *A. femoralis* tadpoles occur in medium and large pools below ~1 m and with access only from the ground, which is not surprising considering that adults are poor climbers (Roithmair, 1992). *Dendrobates tinctorius* tadpoles, in contrast, occur in pools from the ground to the canopy and of sizes ranging from 19.6 ml to 270 L. When focusing on *D. tinctorius* pool choice, we found that despite being able to tolerate an impressive range of physical/chemical factors, tadpoles are more likely to be found in higher pools with greater levels of KH, salinity, and hardness, and higher invertebrate diversity.

4.1 | Interspecific comparison of rearing site choices

In line with previous research, we found that the preference for water capacity varies among species and that some of this variation is explained by parental behavior (Lin et al., 2008; McKeon
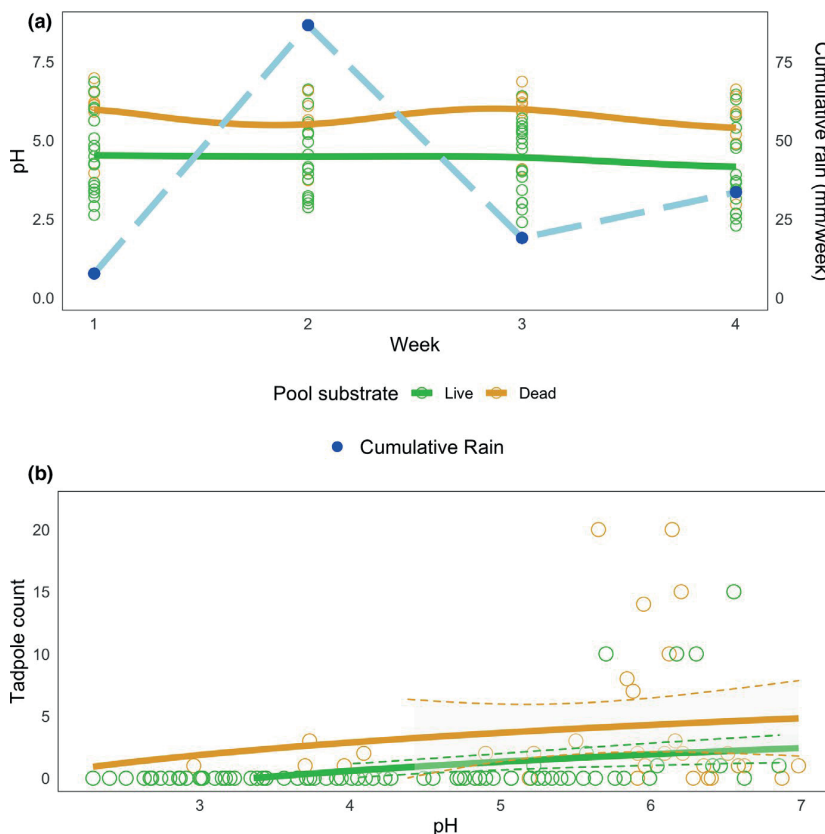
FIGURE 7 Phytotelm stability and tadpole presence across a month. Panels (a, b) are subsetted to exclude high arboreal pools and emphasize how pH is related to (a) pool substrate and (b) predicting *D. tinctorius* tadpole presence in resampled pools. Phytotelmata made of dead substrate have higher pH values than live substrate ($N_{Dead} = 8$, $N_{Live} = 18$; each resampled four times) (Table 2); more tadpoles are found in pools with a higher pH (Table 3). Dashed lines represent 95% CI. Blue points in (a) indicate the weekly accumulation of rainfall (February 2020). Tan lines are dead substrates, and light green lines are live substrates; lines in A are plotted with a "LOESS" fit and B with a "GLM" fit

& Summers, 2013; Summers & Tumulty, 2014). For example, *O. oophagus* provisions their tadpoles with trophic eggs (Jungfer & Weygoldt, 1999), which allows parents to choose very small arboreal phytotelmata with rather clear water and little food (Brown et al., 2008a, 2008b, 2010; Summers & Tumulty, 2014). Despite the desiccation risk associated with the notably small pools chosen by *O. oophagus*, their nurseries are predator-free, which is often suggested as the primary factor driving the invasion of phytotelmata (Crump, 1974; Gomez-Mestre et al., 2012; Magnusson & Hero, 1991; Summers & Tumulty, 2014).

In contrast, *Allobates femoralis* does not provision tadpoles and preferably transports them to medium-to-large ground access pools. These pools tend to have both high leaf litter concentrations and a high number of predators (Figure 5b; concurrent with McKeon & Summers, 2013). The effect of leaf litter on Neotropical tadpoles is unclear, but large amounts have been found to have both positive effects (increased growth rate in Malagasy tadpoles; Lehtinen, 2004) and negative effects (decreased growth rate and development in temperate-region tadpoles; Williams et al., 2008). Because *A. femoralis* are confined to ground access phytotelmata due to their inability to climb, choosing to deposit their tadpoles in pools containing high amounts of leaf litter may be their best option: despite the higher predation risk (which *A. femoralis* fathers try to minimize, Ringler et al., 2018), leaf litter provides a source of food and shelter/predator protection to tadpoles that do not exist in clear pools. Interestingly, the turbid leaf litter pools occupied by *A. femoralis* were functionally

available to *D. tinctorius*, who do not appear to use them. This could be the result of more accurate predator detection (potentially via chemical cues, Schulte et al., 2011) by *D. tinctorius* fathers, who then have the option to select other pools. This trend could also be explained by the predatory nature of *D. tinctorius* tadpoles, where clear pools may be better suited for visual foraging.

Contrary to our expectations, the water capacity of pools was not a key variable in predicting *D. tinctorius* tadpole presence, corroborating Rojas' (2014) findings. While a higher water-holding capacity is expected to decrease desiccation risk, pool volume and depth are not always reliable measures for water-holding persistence in phytotelmata (Rudolf & Rödel, 2005), making frogs adjust their preference based on other pool traits (see below). The presence of large conspecifics, for instance, may be used as a cue of pool stability and thus influence pool choice by males (Rojas, 2014).

An unexpected variable that segregated all three frog species was pool salinity, which tends to increase with height. Some high arboreal pools were particularly deep and a low turnover of stagnant water could explain high salinity levels, where ions (and similarly, salts) concentrate in pools over a long period of time (Sawidis et al., 2011). These pools were mostly occupied by *D. tinctorius* tadpoles, which appear to withstand salinity conditions of up to 1,000 ppm. *Allobates femoralis* tadpoles, in contrast, were only found in low-salt environments. Low salinity is likely a by-product of the high amount of leaf litter, which appears to buffer salt concentrations (Roache et al., 2006; see Appendix 3). As microbial

TABLE 3 Negative binomial generalized mixed model predicting *D. tinctorius* tadpoles in resampled pools in February 2020

	D. tinctorius tadpoles (count)			
Predictors	**Estimate**	**CI**	**z**	**p**
(Intercept)	0.06	0.01 to 0.35	−3.15	**.002**
Pool type [dead]	52.82	6.65 to 419.27	3.75	**<.001**
pH	4.92	1.61 to 15.00	2.80	**.005**
Week	0.85	0.68 to 1.07	−1.40	.160
Pool type [dead]: pH	0.22	0.05 to 0.93	−2.05	**.040**
Random effects				
σ^2	2.62			
$\tau_{00 \ pool\_id}$	1.87			

The bold text was to emphasize column names in the original tables.

activity is limited by high salinities, low-salt pools are ideal for the production of detritus (Roache et al., 2006), the main food source of *A. femoralis* tadpoles. *Osteocephalus oophagus* tadpoles were found in salinity ranges from 48 to 225 ppm (Figure 5c), and the relatively saline pools (>700 ppm) that occur within *O. oophagus* vertical ranges were only occupied by *D. tinctorius*. Adult *O. oophagus* deposit their egg clutches in the water as opposed to the other two species, which only use phytotelmata as tadpole-rearing sites. The saltier conditions of pools higher in the canopy may not be suitable for eggs (Albecker & McCoy, 2017; Christy & Dickman, 2002), which may limit the suitable conditions for oviposition in *O. oophagus*. Poison frogs, in contrast, can escape these limitations because their clutches are terrestrial. Although the small sample size does not allow any stronger interpretation, it appears that both chemical and physical variables influence ideal pool conditions in *A. femoralis* and *O. oophagus*.

4.2 | Pool choice and flexibility in *Dendrobates tinctorius*

Over the two sampling seasons, the bulk of our study focused on the factors that shaped *D. tinctorius* presence and persistence. Despite having sampled over 80 unique pools and found 350 *D. tinctorius* tadpoles (N = 208, 2019; N = 142, 2020), understanding the critical variables that drive *D. tinctorius* pool choice is difficult because of the wide range of physical and chemical properties in which these tadpoles occur. Further, the interactions between physical, chemical, and biological characteristics in phytotelmata are complex and collinear. Based on both our principal component regression and analysis, we found that the probability of detecting *D. tinctorius* tadpoles increases positively with specific physical (height), chemical (KH, salinity, and hardness), and biological (invertebrate diversity) properties. Interestingly, we found that salinity, hardness, and KH also tend to increase with increasing height (Appendix 4). Overall, these chemical components tend to vary in the same direction when moving up the vertical axis, suggesting a positive relationship between these chemical and physical traits. Invertebrate diversity of occupied pools, in contrast, tends to stay relatively constant across

heights and might serve as an important food source for predatory *D. tinctorius* tadpoles or vice versa.

In this study, we found that KH increases with height, and pools with high KH are more likely to have tadpoles in them. KH is a measure of a solution's buffering capacity or, in other words, a solution's resistance to pH changes (Yang et al., 2008). KH values in low arboreal and ground access pools usually ranged from 3 to 6 KH, while average KH in high canopy pools was 15. Interestingly, two of the five lower pools with a KH above 8 had *D. tinctorius* counts of over 10 tadpoles, demonstrating that, when these conditions are available terrestrially, *D. tinctorius* fathers take advantage of them. The apparent preference for high alkalinity environments is interesting, as work studying the formation of fungal granules has established that high alkalinity conditions inhibit fungal growth (Yang et al., 2008). The potential relationship of KH limiting the growth of fungi in phytotelm conditions is noteworthy as amphibian fungal pathogens such as *Batrachochytrium dendrobatidis (Bd)* spread aquatically (Rosenblum et al., 2010) and *Bd* presence (prevalence of ~5%) has been reported for *D. tinctorius* in our study area (Courtois et al., 2015). Thus, the consistent detection of *D. tinctorius* tadpoles in high KH pools could indicate that fathers are selecting environments less prone to fungal contamination. Although we are unsure of the proximate mechanisms driving the fathers' choice of particular chemical conditions in phytotelmata, we establish here that KH, hardness, and salinity play an important role in shaping *D. tinctorius* pool choice, and suspect that these chemical conditions may be linked to the long turnover time of high arboreal pools.

4.3 | The stability of ephemeral pools

In 2020, we were able to follow a subset of low arboreal and ground access pools over a month, recording the pH and *D. tinctorius* tadpole presence on a weekly basis. We found that pools made of dead substrate (fallen palm bracts, dead trees) had a higher pH than live substrates (tree holes). The gross average pH of dead phytotelmata across our sample was 5.68, which is almost exactly the value of unpolluted rainwater (pH = 5.65 when saturated with atmospheric CO_2; Koshy et al., 1997). In contrast to most live substrates (average

pH = 4.35), dead phytotelmata are usually in canopy gaps, where rain falls directly into the pools. When reported, the pH of most phytotelmata is acidic with ranges sometimes falling below a pH of 3 (Kitching, 2001, von May et al., 2009, Poelman et al., 2013, Ramos et al., 2017; but see Lehtinen, 2004, which shows that _Pandanus_ leaf axils were close to neutral pH). However, most studies on phytotelmata are biased toward living plants and trees and assess chemical/biological variables of pools at a single time point.

Throughout the month, all pool types decreased in pH (Table 2); a similar trend was also found in bamboo phytotelmata in Peru (von May et al., 2009), suggesting a time-dependent process causing pools to become increasingly acidic over time. Remarkably, some pools dried out multiple times during our sampling period and when refilled by rain were approximately at the same pH as before the drying event (ex. palm bract originally pH 6.98 (Week 1), dried out (Week 2), refilled pH 6.87 (Week 3); live tree hole pH 2.91 (Week 1), dried out (Week 2), pH 3.02 (Week 3)). This indicates that pool substrate may play an important role in establishing pool pH. Three out of the five pools that dried out were dead palm bracts, suggesting that this pool type, despite having favorable chemical conditions when filled, may also be at a higher risk for desiccation and decomposition.

Surprisingly, pools in dead substrates, such as palm bracts and fallen trees, contained more tadpoles than other pool types despite drying out more regularly across our month survey (Table 3). Such pools tend to occur in forest gaps, which makes them more prone to dessication because of their exposure to direct sunlight. However, pools in these lit areas may also have more microbial activity and less food limitation (Kitching, 2001; Rudolf & Rödel, 2005), making them attractive deposition sites for tadpoles. Suitable pools are a limiting resource for frogs and other animals (Donnelly, 1989a, 1989b; Fincke, 1992; Poelman & Dicke, 2007; Ringler et al., 2015), and new pools for _D. tinctorius_, such as those in tree-fall gaps, appear unpredictably and are rapidly occupied despite the high rates of competition and cannibalism (Rojas, 2015). Being the first occupant of a pool can be particularly beneficial when parents arrive early to recently established pools, as it allows their offspring to be predators rather than prey. Thus, the competition to be the first to deposit tadpoles might make pools in dead substrates that occasionally dry out additionally attractive.

Interestingly, the size range of tadpoles in dead substrates is much more variable than in low and high arboreal pools (CF, BR, AP personal observations), suggesting that the pools remain attractive even when already occupied by larger cannibals. This pattern corroborates the experimental evidence that _D. tinctorius_ preferably deposit newly hatched tadpoles in pools already occupied by conspecifics (Rojas, 2014, 2015); possibly, tadpole presence serves as an indicator of pool stability, which might be more valuable to a father's deposition choice than the risk of having his offspring cannibalized by conspecifics.

4.4 | High arboreal pools

While most of our work focused on low arboreal and ground access pools, this study provides one of the first thorough characterizations

of high arboreal phytotelmata in the Amazon. Gaucher (2002) unexpectedly found _D. tinctorius_ tadpoles in canopy pools up to 25 meters high while studying the treefrog _Trachycephalus hadroceps_. Other poison frogs, such as _D. auratus_, have been reported to use arboreal tree holes as well (Summers, 1990). We found large numbers of tadpoles in arboreal pools up to 20 m in height, which suggests some benefit of these pools given the presumed high energetic expense that fathers invest in transporting their tadpoles from terrestrial oviposition sites.

One key advantage of high arboreal phytotelmata may be a regular food source provided by _Trachycephalus_ treefrogs that specialize in these pools. During this study, all of the suitable high arboreal pools were found by locating nocturnal calls of _T. resinifictrix_ and _T. hadroceps_, indicating that these habitats were potentially used as breeding sites. Although the breeding frequency of these treefrog species is sporadic (Gaucher, 2002), successful breeding events result in clutches that consist of hundreds to thousands of eggs and tadpoles, which _D. tinctorius_ tadpoles readily consume (Gaucher, 2002, AP and BR, personal observations). As proposed by Gaucher (2002), it could be that _D. tinctorius_ fathers cue on _Trachycephalus_ calls for locating high arboreal pools, but this warrants further investigation.

Unexpectedly, we also found that dead substrate pools share some characteristics with high arboreal pools, particularly with respect to chemical qualities (a more basic pH), tadpole abundance, and being a limited or hard-to-access resource (Heying, 2004). As such, both pool types offer benefits that fathers may value: Despite having a shorter life, novel pools (such as fallen palm bracts and holes in fallen trees) are worth invading as deposition sites because they are easy to access and have a high probability of having food and a suitable chemical profile; high arboreal pools, on the other hand, may have sporadic food and are hard to access, but they are stable and less prone to chemical fluctuations. Together, these different pools are both worthy deposition sites, as they provide different stable environments which create a range of possible offspring success.

5 | CONCLUSIONS

When comparing the occurrence of tadpole species in pools, one of the first trends that emerges is the presence/absence on the basis of specific phytotelm characteristics. For example, _A. femoralis_ and _O. oophagus_ vertical ranges technically overlap, yet tadpoles never co-occur. In species that demonstrate a distinct morphological limitation or vertical preference, it may be that tadpoles occur in pools because that is what is available to their parents. These constraints play a role in the environment tadpoles are exposed to, and should affect their physiology and behavior. But what about when parents are completely unconstrained? _Dendrobates tinctorius_ occur across the vertical gradient and occupy pools that range from acidic (pH = 2.96) to neutral pH, with volumes from 19 ml to over 270 L and in pools that range from fresh to slightly saline (~1,000 ppm), which hints at a remarkable physiological flexibility that has been overlooked thus far. Therefore, physiological studies comparing

phytotelm-breeding tadpoles would be especially interesting to better understand parental decisions. It is also warranted to measure *D. tinctorius* growth in pools with different chemical compositions to see whether (despite surviving) these tadpoles are paying a cost for the deposition choices by their fathers.

In sum, natural history studies allow us to grasp species' flexibility; this is becoming increasingly relevant when we consider the effects of climate change in the Amazon. Forecasted changes in precipitation (Cochrane & Barber, 2009; Silvestrini et al., 2011) will have direct effects on animals dependent on plant-based water bodies and surely the chemical composition of the water bodies themselves. Further studies examining larval anuran responses to challenging environmental conditions (especially KH and pH) are necessary to better understand the potential resistance and adaptability of phytotelm-dependent frogs and how this may shape species resilience in the future.

5.1 | Significance statement

This two-year field project is a reflection of over a decade of natural history observation and experiments in the Neotropics. In this study, we expand our knowledge of the ecology of phytotelm-dependent frogs, this time focusing on a comparative overview between larval species and the precise ecological factors that shape the microhabitats in which eggs and larvae are deposited. The breadth of this work will surely engage a wide variety of readers who are interested in ecological dynamics in the tropics.

Here, we synthesize the occurrence and interactions between three different amphibian species, which contributes to understanding the dynamics of each species independently, but this work is also a perspective into the interactions between larvae and parents within the context of an ecological study. Species in this study are specifically plant-dependent as tadpoles, which provides a unique opportunity to thoroughly survey and measure the microhabitats in which they occur. Together, our findings show how biological, physical, and chemical components interact in predicting larval presence in species with parental care, which is a strategy present in 10% of anurans. We also provide the first detailed account of the ecology of high arboreal breeding pools. Accessing trees more than 20 meters in height is challenging, and conducting a thorough survey of these environments framed in comparison with microhabitats across the vertical gradient is a testament to the scope of this work. While our study is based on amphibians, it more broadly focuses on the ecology that shapes larval deposition sites and the wide array of species' flexibility we observe in the Neotropics.

Hopefully, a wide variety of researchers will be excited to learn more about the diversity of microhabitats in the Amazon and a cross-species comparison of the amphibians that depend on them.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Chloe A. Fouilloux: Data curation (equal); Formal analysis (lead); Investigation (equal); Writing-original draft (lead). **Shirley Jennifer Serrano-Rojas:** Data curation (equal); Formal analysis (supporting); Investigation (equal); Writing-review & editing (supporting). **Juan David Carvajal-Castro:** Investigation (equal); Writing-review & editing (supporting). **Janne K. Valkonen:** Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Philippe Gaucher:** Investigation (supporting). **Marie-Therese Fischer:** Investigation (supporting); Writing-review & editing (supporting). **Andrius Pašukonis:** Conceptualization (equal); Funding acquisition (equal); Methodology (equal); Supervision (equal); Writing-review & editing (equal). **Bibiana Rojas:** Conceptualization (equal); Funding acquisition (equal); Investigation (supporting); Methodology (equal); Supervision (equal); Writing-review & editing (equal).

ETHICAL APPROVAL

The study was approved by the scientific committee of the Nouragues Ecological Research Station and covered under a partnership agreement between BR, AP, and the Nouragues Nature Reserve (No. 01-2019). We strictly adhered to the current French and European Union law and followed the Association for the Study of Animal Behaviour's (ASAB) Guidelines for the use of live animals in teaching and research (ASAB, 2017).

DATA AVAILABILITY STATEMENT

All data are publicly available in Dryad (https://doi.org/10.5061/dryad.7wm37pvst). All raw data, analysis, and R code are also available on GitHub (https://github.com/chloefouilloux/frogpool).

ORCID

Chloe A. Fouilloux https://orcid.org/0000-0003-1265-5824

Shirley Jennifer Serrano Rojas https://orcid.org/0000-0001-6811-8265

Juan David Carvajal-Castro https://orcid.org/0000-0002-3182-9474

Janne K. Valkonen https://orcid.org/0000-0002-2177-6612

Marie-Therese Fischer http://orcid.org/0000-0002-6693-8662

Andrius Pašukonis http://orcid.org/0000-0002-5742-8222

Bibiana Rojas https://orcid.org/0000-0002-6715-7294

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

APPENDIX 1

SPECULATIONS AND ALTERNATIVE VIEWPOINTS

Dendrobates tinctorius males typically father egg clutches of 2–5 tadpoles per clutch and breed year-round (Rojas & Pašukonis, 2019). As a result of the presumed high energetic expense from carrying each tadpole from each clutch singly, we hypothesize that tadpoles transported later may be subject to bet-hedging by fathers.

Combined with the important chemical aspects of pools shown from 2019 data, it seems that fathers can cue on either chemical (KH, salinity, hardness) or biological components (the presence of conspecifics) as information about pool stability. We speculate that the importance of chemical and physical cues changes with respect to pool age. For example, in new uninvaded pools, chemical cues of the pool may be more important (left side of figure), while in older, established pools that are more densely occupied, density serves as a main cue for transporting fathers (right side of figure). Finally, the value of these cues may vary with the amount of offspring fathers are caring for. Hypothetically, fathers who must transport more offspring are less discerning of where they transport latter tadpoles because they can afford to make less "ideal" deposition decisions because of their large reproductive output.
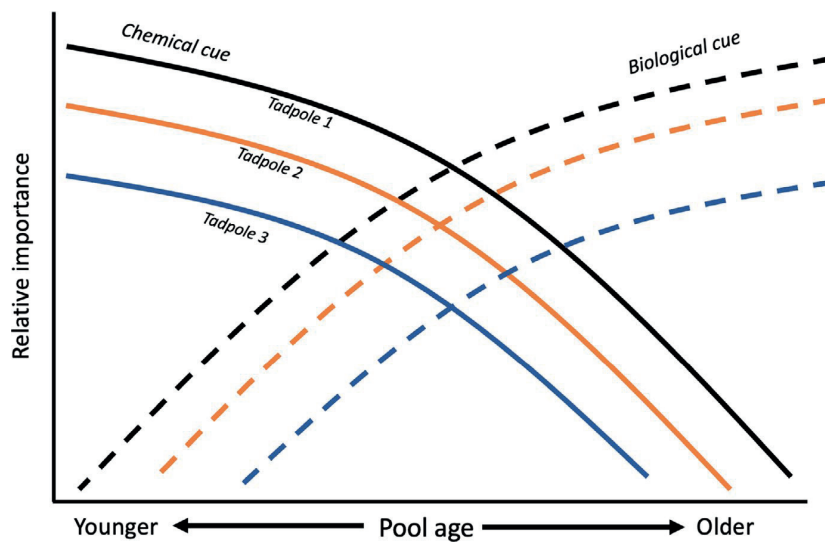
FIGURE A1 Hypothetical succession of relative cue importance in phytotelmata across time
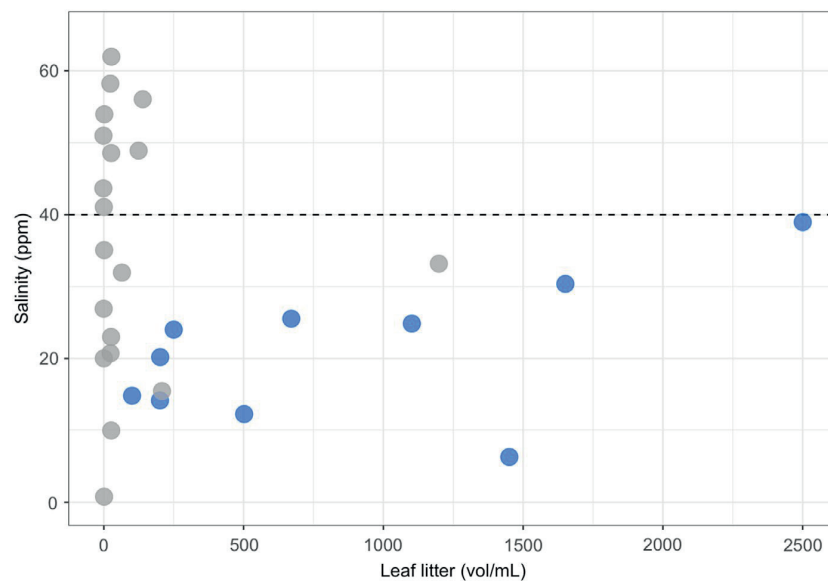
APPENDIX 2

DEFINITION OF VARIABLES (TRAITS) CONSIDERED IN THE PRINCIPAL COMPONENT ANALYSIS

Variable	Category	Description
Salinity	Chemical	Quantification of salt in solution. Range = 0–10,000 ppm.
KH (alkalinity)	Chemical	Quantification of pool bicarbonate/carbonate in solution. Range = 0–25 dKH
NO_3^-	Chemical	Quantification of nitrate in solution. Range = 0–160 ppm.
Hardness	Chemical	Quantification of ions in solution (e.g., calcium). Range = 0–425 ppm.
Height	Physical	Vertical height from the ground to the pool entrance. Measured in cm.
Water capacity	Physical	Water-holding capacity of the pool. Estimated from pool width, length, and depth using a semi-ellipsoid formula.
Surface area to depth ratio	Physical	Surface area to depth ratio. Surface area calculated from semi-ellipsoid formula.
Leaf litter volume	Physical	The measure of leaf litter volume in each pool.
Amphibian diversity	Biological	The sum of all species observed using each pool including adults, calling, dead tadpoles, and opportunistic observations after the sampling.
Invertebrate density	Biological	Sum of all invertebrate densities (counts divided by sampling volume)
Invertebrate diversity	Biological	Number of distinct invertebrate categories observed in each pool (between 0 and 12)
Predator count	Biological	Number of Odonata larvae in each pool.
Average predator size	Biological	The average size of Odonata larvae in each pool. Size is calculated by dividing (pred_size_sum)/(pred_count)
Total other	Biological	Sum of *O. oophagus* and *A. femoralis* tadpoles co-occurring in the pool.

Colors were just to highlight the change in categories (chemical, physical, ect.).

APPENDIX 3

Relationship between leaf litter volume and salinity. Dashed line is at 40 ppm which is the limit where we detected *A. femoralis* tadpoles. Below this level, it appears that leaf litter and salinity have a slightly positive relationship, though interpretation is limited by sample size (blue points, $N_{Femoralis}$ = 10). Data are subsetted for ground access pools, and salinity upper bound was limited to 70 ppm to emphasize potential leaf litter effect.

APPENDIX 4

Relationship between height and salinity, invertebrate diversity, and hardness. There is a positive relationship between salinity and hardness with height. We see that invertebrate diversity does not meaningfully change with height. Dashed line represents 95% CI. GLM line fitted with a $y \sim x$ formula.
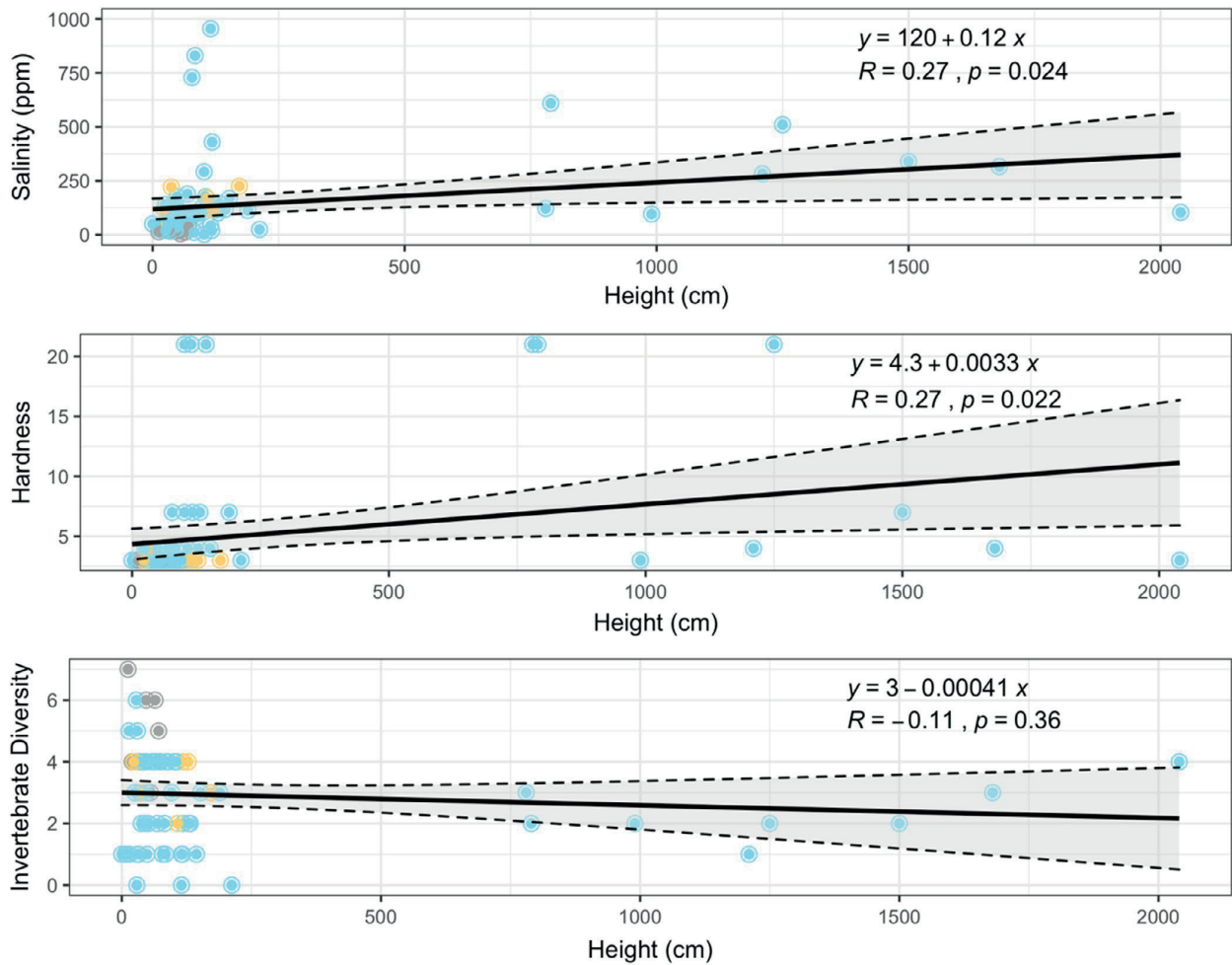
1 **Supplementary Materials**

2

3 **Supp. Table 1.** PCA model analysis rank using AIC. All models were coded with a negative

4 binomial family in a GLM framework. Two models fell within 2 AIC of each other; the

5 interaction in the second model was not significant, so we chose the simplest model of the

6 two (Rank 1).

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Rank	Model family	Interaction	Structure	AIC	ΔAIC	*w*
1	**GLM (NB)**	**+**	**PC1 + PC2 + PC3**	**113.49**	**0.00**	**0.47**
2	GLM (NB)	*	PC1 * PC2 + PC3	115.09	1.60	0.21
3	GLM (NB)	*	PC1 * PC3 + PC2	115.64	2.15	0.16
4	GLM (NB)	*	PC2 * PC3 + PC1	115.83	2.34	0.15
5	GLM (NB)	*	PC1 * PC2 * PC3	121.83	8.34	0.01

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Supp. Table 2. drop1 model selection for the predictors of *pH* measure after repeated pool observations (2020 data). Bolded components of each row indicate dropped part of each model iteration. Pool type was a binomial categorical variable (dead/alive), Week indicates week of sampling (numerical variable: 1-4), water capacity was a continuous variables (depth*length*width) of each pool, and Dt_Tadpole_Num was a continuous whole number of *Dendrobates tinctorius* tadpole counts. Interaction term indicated the interaction between covariates. Model family was coded as "Gaussian" for all pH models. Models are ranked in decreasing AIC order. Random effect of pool ID was included in all models.

| Rank	Structure	AIC
8	**pool_type * Week * Dt_Tadpole_Num * water_capacity * sa_depth**	**NA**
7	**pool_type * Week * Dt_Tadpole_Num + water_capacity * sa_depth**	**232.47**
6	**pool_type * Week * Dt_Tadpole_Num + water_capacity + sa_depth**	230.47
5	**pool_type * Week + Dt_Tadpole_Num**	225.20
4	pool_type + **Week * Dt_Tadpole_Num**	225.16
3	pool_type + Week + **Dt_Tadpole_Num**	223.27
2	**pool_type * Dt_Tadpole_Num** + Week	222.09
1	pool_type + Week	221.61

Supp. Table 3. **drop1 model selection for the predictors of *Dendrobates tinctorius* tadpole numbers after repeated pool observations (2020 data)**. Bolded components of each row indicate dropped part of each model iteration. Pool type was a binomial categorical variable (dead/alive), Week indicates week of sampling (numerical variable: 1-4), and pH was a continuous variable, water capacity is pool volume based on semi-ellipsoid equation, and sa_depth is the surface area to depth ratio of each pool. Interaction term indicated the interaction between covariates. Models are ranked in decreasing AIC value. Random effect of pool ID was included in all models. Models were fit with a quadratic (nbinom2) negative binomial family.

Rank	Structure	AIC
8	**pool_type * Week * pH* water_capacity * sa_depth**	**NA**
7	**pool_type * Week * pH + water_capacity * sa_depth**	**NA**
6	**pool_type * Week * pH + water_capacity + sa_depth**	NA
5	**pool_type * Week * pH + water_capacity**	NA
4	**pool_type * Week * pH + sa_depth**	233.08
3	pool_type + **Week * pH**	232.12
2	**pool_type * Week** + pH	231.34
1	pool_type * pH + Week	228.2

48
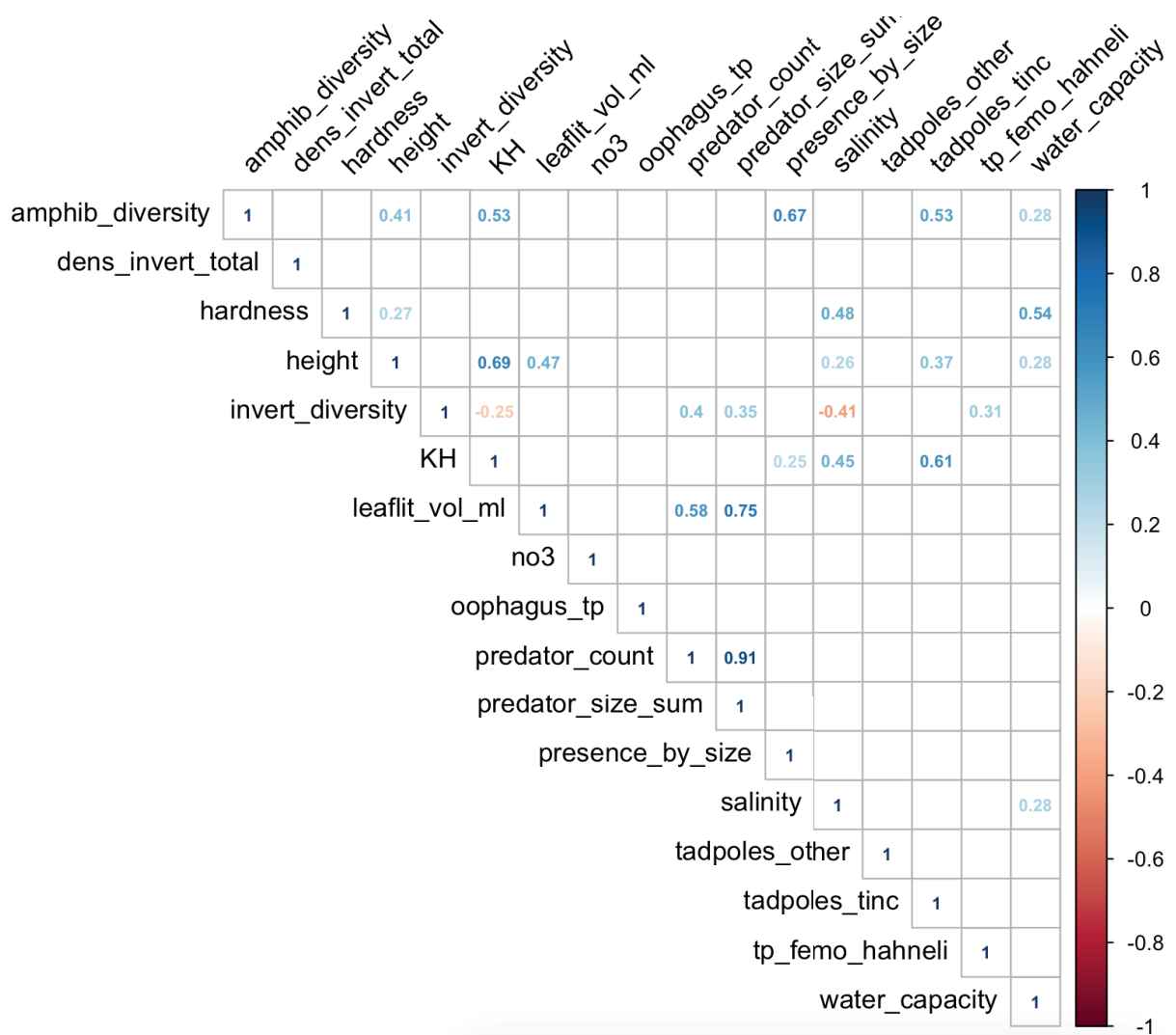
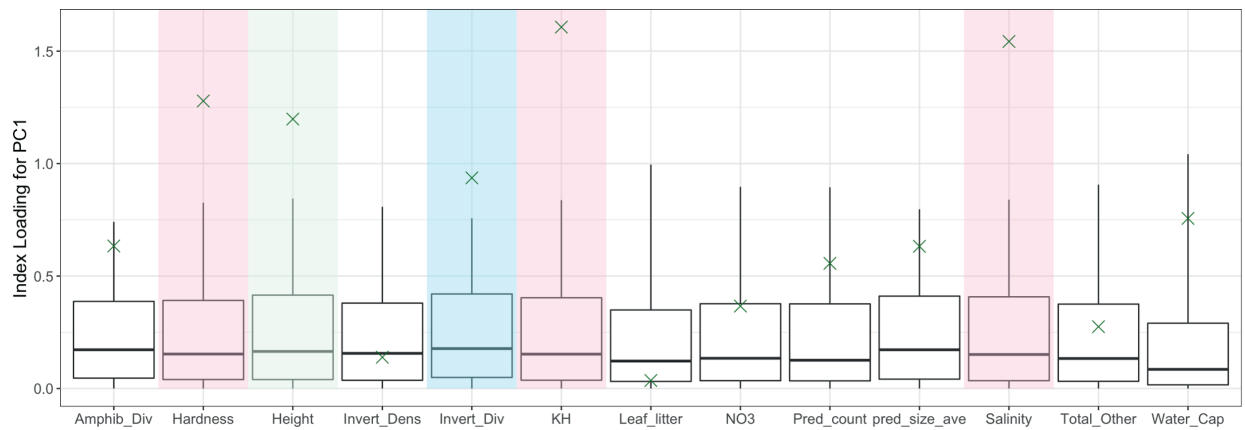Supplementary Fig 1. High correlation between numeric variables in frogpool data set (2019).

Only significant correlations ($p > 0.05$) from a Pearsons's correlation test are visualized. Cooler

colors represent positive correlations and warmer colors represent negative correlations.

Variables are ordered alphabetically.

53

Supp. Figure 2. Results from the PCA correlation procedure. Boxplots are generated from random data, where whiskers range 95% confidence intervals; green "X"s are observed PCA index loading values. Variables where the observed PCA index loading are significantly different from the random confidence interval are highlighted. Pink highlight represents chemical variables, green highlight represents physical variables, and blue highlight represent biological variables.

II

TADPOLE RESPONSES TO ENVIRONMENTS WITH LIMITED VISIBILITY: WHAT WE (DON'T) KNOW AND PERSPECTIVES FOR A SHARPER FUTURE

by

Fouilloux C. A., Yovanovich C. A. M. & Rojas B. 2022

Tadpole Responses to Environments With Limited Visibility: What We (Don't) Know and Perspectives for a Sharper Future

Chloe A. Fouilloux[1], Carola A. M. Yovanovich[2,3]* and Bibiana Rojas[1,4]

[1] Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland, [2] Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil, [3] School of Life Sciences, University of Sussex, Brighton, United Kingdom, [4] Department of Interdisciplinary Life Sciences, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine Vienna, Vienna, Austria

Amphibian larvae typically inhabit relatively shallow freshwater environments, and within these boundaries there is considerable diversity in the structure of the habitats exploited by different species. This diversity in habitat structure is usually taken into account in relation to aspects such as locomotion and feeding, and plays a fundamental role in the classification of tadpoles into ecomorphological guilds. However, its impact in shaping the sensory worlds of different species is rarely addressed, including the optical qualities of each of these types of water bodies and the challenges and limitations that they impose on the repertoire of visual abilities available for a typical vertebrate eye. In this Perspective article, we identify gaps in knowledge on (1) the role of turbidity and light-limited environments in shaping the larval visual system; and (2) the possible behavioral and phenotypic responses of larvae to such environments. We also identify relevant unaddressed study systems paying special attention to phytotelmata, whose small size allows for extensive quantification and manipulation providing a rich and relatively unexplored research model. Furthermore, we generate hypotheses ranging from proximate shifts (i.e., red-shifted spectral sensitivity peaks driven by deviations in chromophore ratios) to ultimate changes in tadpole behavior and phenotype, such as reduced foraging efficiency and the loss of antipredator signaling. Overall, amphibians provide an exciting opportunity to understand adaptations to visually limited environments, and this framework will provide novel experimental considerations and interpretations to kickstart future research based on understanding the evolution and diversity of strategies used to cope with limited visibility.

Keywords: larval vision, turbidity, chromophore shift, phytotelmata, phenotypic plasticity

INTRODUCTION

An incredible diversity of animals inhabits aquatic environments for either part or the entirety of their lives. As a medium, water poses unique challenges and opportunities compared to air, which has resulted in distinct physiological adaptations of even temporarily aquatic animals. For example, short-range communication using chemical signals is facilitated in water

(Wisenden, 2000), whereas communication using sound in shallow water environments is compromised (Ladich and Winkler, 2017). The visual world underwater is shaped by the way in which light gets refracted and attenuated, and by the inherent optical properties of water (e.g., high absorbance of blue light). In this default scenario, physical variables such as pool type, volume, and geometry, and biological variables, such as the amount and nature of organic matter, interact in shaping the luminic environment (Jerlov, 1976; Lythgoe, 1979). Here, we will use "limited visibility" and "turbidity" in the sense implied throughout Lythgoe's (1979) "The Ecology of Vision," as follows. Limited visibility refers to a number of situations in which increased scattering and/or selective absorption alter the quality and quantity of light available for a visual system, including, but not limited to, depth-dependent filtering of spectral bands at the boundaries of the visible spectrum, darkening/coloring due to absorption by dissolved pigments, etc. Turbidity, in this context, is a particular case of limited visibility; its most defining feature is the presence of a "veil" of scattered light originating from suspended particles in the visual scene, and its direct consequence is the decrease of brightness contrast of objects against the background. Thus, even though suspended matter can also absorb light, the main challenge it poses to visual performance is decreased visibility via an increase in "noise" in the visual scene -rather than a decrease in light availability as is the case of other visibility-reduced environments, such as the "deep" or "colored" waters mentioned just above.

Despite the intricate visual systems that aquatic animals have evolved throughout time, individuals can be challenged by the rapid and unexpected change in the structure of their visual environments. Sudden increases in dissolved organic matter through algal blooms, agricultural/anthropogenic run-off, or from resuspended particulate matter can drastically change the visibility of aquatic habitats (Granqvist and Mattila, 2004; Engström-Öst and Candolin, 2006; Chivers et al., 2012). In these situations, the question is no longer about the refinement of an animal's visual system for a specific habitat, but about the flexibility of that system to maintain image quality and visual performance in the face of change. Research on the plasticity of visual systems so far has mostly dealt with its biochemical and genetic bases (see Corbo, 2021 and Carleton and Yourick, 2020, respectively, for reviews on examples of these topics), whereas focused research on its consequences for ecologically relevant, visually guided behaviors is still missing in the scientific literature. In an era where the stressors of agricultural practices, land use, and climate change are more present than ever before, understanding the responses to turbidity from both proximate and ultimate perspectives will help to better understand the responses of aquatic animals in these increasingly stressed environments (Rowe and Dean, 1998; Sundin et al., 2010).

Tadpoles can rely on vision, at least to some degree, for social behavior (Katz et al., 1981; Caldwell, 1989; Sontag et al., 2006) and complex space use (Beiswenger, 1977). Despite this, they are nearsighted (Mathis et al., 1988), and have typically been assessed as having poor vision overall (Hoff et al., 1999). This mindset has shaped both the experimental design and interpretation of results of studies testing their reliance on different sensory modalities

(Rot-Nikcevic et al., 2006; Saidapur et al., 2009). However, other recent work has begun to question this, providing support for a more prominent role of visual cues in predator detection (Hettyey et al., 2012), identification of conspecifics (Gouchie et al., 2008; Kumpulainen, 2021) and habitat assessment (Rot-Nikcevic et al., 2005; Hettyey et al., 2012). For many amphibian species, tadpoles are aquatic and adults are (semi-)terrestrial. While "immature" in the grand scheme of ontogeny, larvae are adapted to their aquatic habitats with the same degree of refinement with which adults are adapted to their environments post-metamorphosis (McDiarmid and Altig, 1999). Such a clearly defined biphasic life cycle, unparalleled among vertebrates, presents a unique opportunity to study the plasticity of visual systems: even within species the visual system can be differentially tuned to match the surrounding environment before and after metamorphosis, as has been shown for relative eye size (Shrimpton et al., 2021) and expression levels of genes that determine spectral sensitivity (Schott et al., 2021).

Overall, amphibian vision is relatively well documented in adults from the point of view of phototransduction mechanisms, spectral tuning, retinal topography and signal processing, and optical performance, but less work has been done on larval stages (see Donner and Yovanovich, 2020 for a review). To the extent to which tadpoles' visual systems have been studied, they expectedly share the organization and functionality of vertebrates in general, and amphibians in particular, and showcase some differences with their adult selves in aspects that impact performance in aquatic vs. aerial media. For example, tadpoles' lenses tend to be spherical, while those of terrestrial adults become flattened (Mathis et al., 1988), and their visual pigments tend to be shifted to long wavelengths compared to adults (Crescitelli, 1958), with implications discussed in the upcoming sections. Amphibian larvae constitute a unique model to understand the evolution of vertebrates' visual systems in the transition from aquatic to aerial environments, and the effect of ontogeny on the maintenance or change of ocular adaptation. Thus, their visual ecology and the behavioral adaptations resulting from environmental changes provide an exciting and relatively unexplored framework of questions to address.

DIVERSITY OF VISUAL ENVIRONMENTS AND ITS IMPLICATIONS FOR TADPOLE ECOMORPHOLOGY

To date, researchers have focused primarily on fishes to understand how predator-prey interactions, mate choice, and foraging adaptations are affected in environments with limited visibility (e.g., turbid). The bulk of these studies show that when challenged with turbidity animals demonstrate a relaxed selection in mate choice (Järvenpää and Lindström, 2004; Sundin et al., 2010), poorer foraging efficiency (Rowe and Dean, 1998; Horppila et al., 2004), and reduced perception of predation risk (Kimbell and Morrell, 2015). Overall, these trends are not without exception, as turbidity can both provide cover to prey while also impairing reaction time/evasive maneuvering, thus the (dis)advantage of turbidity is more generally determined by the

modalities used by the community. The majority of turbidity studies have been conducted in North America and Northern Europe, leaving most of the equatorial regions and its diverse (semi-) aquatic fauna understudied. Thus, much of the diversity with respect to species, geography, and life histories, is available for novel comparisons and new insights into animal adaptations in visually challenging environments.

One such environment that has yet to be addressed in studies on limited visibility are phytotelmata. These ephemeral pools of water are formed in plant structures, and distributed throughout the vertical gradient. Phytotelmata, which are notably different from ponds and lakes, commonly occur in tropical regions and are frequently invaded by insects and amphibian tadpoles (Kam et al., 2001; Lehtinen, 2004; Summers and Tumulty, 2013; Biju et al., 2016; Cossio et al., 2021; Fouilloux et al., 2021) and constitute a model system that is relatively easy to access and extensively quantify, and lends itself very well to experimental manipulations (e.g., Serrano-Rojas and Pašukonis, 2021). Occupied pools range widely in size and shape (Fouilloux et al., 2021), and in turbidity and/or water color as well, according to qualitative field observations (unpublished data recorded by CF, **Figure 1**). Additionally, phytotelmata themselves contribute to defining the light environment in pools. For example, light reflected from fresh green bromeliad leaves will be different from that reflected from decomposing bark or decaying palm bracts. To our knowledge, thus far there have been no quantitative measurements of light or turbidity levels in phytotelmata, which only emphasizes the knowledge gap within this system.

One could expect that a consistent decrease in light availability (based on denser canopy cover) and visibility would have a measurable impact on larval development. For instance, tadpoles of the Amazonian phytotelm-breeder *Dendrobates tinctorius* that develop in pools with dark-tinted water appear to be collectively smaller than those deposited in clear waters despite an abundance of prey availability in both cases (e.g., mosquito larvae, BR *unpublished obs.*). We hypothesize that the decreased body condition of these *D. tinctorius* larvae may indicate that, in environments with limited visibility, phytotelm-reared tadpoles may experience decreased hunting efficiency, and shift their feeding strategies from omnivorous/predacious to mostly herbivorous. The constraints of turbid environments may not only affect predatory behavior by carnivorous tadpoles, but could also impact other species with specialized feeding strategies like oophagy, which relies on the ritualized parent-offspring feeding behavior initiated after tadpoles visually recognize an adult and increase activity to indicate hunger (Stynoski and Noble, 2012). Ultimately, we hypothesize that turbid or otherwise visually limiting microhabitats could have profound effects on both body condition and survival in a variety of phytotelm-based species (**Figure 2**).

Turbidity could also have notable effects on phytotelmata communities. In estuary systems, for example, it appears that biodiversity decreases in turbid conditions, where the few species that use chemoreception (crabs) outcompete those that rely more heavily on visual cues (fish; Reustle and Smee, 2020). Diverse tadpoles (Kitching, 2001; Summers and McKeon, 2004), insects (Fincke, 1992; Caldwell, 1993), and crustaceans (Pettitt et al., 2018) (co-)exist in phytotelmata, yet the effect of limited visibility on community-wide changes has not been tested in these systems. We propose that phytotelmata provide natural mesocosms to investigate and compare the responses of different community structures (and the diverse visual systems of the animals that these communities contain) to habitat disturbance. Out of the handful of studies that have considered amphibian responses to visually limited conditions, most have followed trends found in fishes (i.e., reduced anti-predator behavior in salamanders, Zabierek and Gabor, 2016; decreased growth rates in frogs, Wood and Richardson, 2009). In amphibians, these experiments could be taken one step further by considering potential carry-over effects of how rearing environments shape behavior through metamorphosis. Overall, it has been shown that environmental stress has significant carry-over effects from larval to adult stages in amphibians (size: Scott, 1994; reproductive organs: Harper and Semlitsch, 2007). Thus, tadpoles raised in the stress of turbid conditions may not only be physically smaller as a result of reduced foraging performance but also have reduced fitness in adulthood compared to their counterparts raised in clear water conditions.

WAVES AND VITAMINS: STRATEGIES TO RESPOND TO VISUALLY CHALLENGING ENVIRONMENTS

In turbid habitats, the best option to improve visual function is to filter out the added background light arising from scattering that does not contribute to image formation (Lythgoe, 1979). While scattering from large particles tends to be wavelength-independent, scattering coming from small particles (Rayleigh scattering) that also contributes to image quality degradation is enriched in short wavelengths (i.e., ultraviolet, violet, blue) (Douglas and Marshall, 1999). Thus, a decreased sensitivity in the "blue" part of the spectrum, be it at the level of the eye as a whole or the individual retinal photoreceptor cells, would help to some degree to deal with the detrimental effects of turbidity. Conversely, an increased sensitivity in the long-wavelength (i.e., "red") part of the spectrum would arguably improve vision in freshwater aquatic environments which tend to be redder than marine ones (Lythgoe, 1979). Thus, we would expect considerable overlap between the strategies that visual systems can use to cope with turbid environments and with red-colored environments (such as the ones we show in **Figure 1**), even if they differ substantially in overall light availability.

Several species of frogs have some kind of pigment in their crystalline lenses, many of them resembling those of fishes in their absorbance profile, which selectively remove short wavelength light before it reaches the retina (Yovanovich et al., 2020). However, because these data come from adult individuals, it remains to be investigated if these filtering properties are already present in the tadpoles of relevant species, and whether larval stage could provide a compelling biological explanation for those cases in which the adult filtering pattern does not follow any immediately obvious logic. Interestingly, other recent work has found differences in expression levels of genes related to lens

FIGURE 1 | Water samples (collected by CF) from phytotelmata occupied by *Dendrobates tinctorius* tadpoles in French Guiana. Based on water samples, it appears that tadpoles are deposited in pools with a wide range of visibility. Colored labels refer the water sample (top of figure) to the pool from which it was collected. We hypothesize that in darker waters tadpoles' spectral sensitivity is red-shifted (lower Vitamin A1:A2 ratio). Photos: Chloe A. Fouilloux.

composition in tadpoles versus juveniles of the frog *Lithobates sphenocephalus* (Schott et al., 2021). If these gene products were ones to affect light transmittance in a wavelength-dependent manner (e.g., Röll, 2001), the finding would argue against the speculation outlined just above, and highlight the need to explore the issue in more species to find -or rule- out potential patterns.

At the level of the retina, the availability of photoreceptors with peak sensitivities at short wavelengths is another potential point to control scattering-related visual noise. As it turns out, the highly blue-sensitive rods unique to amphibians, which allow color discrimination in extremely dim light (Yovanovich et al., 2017), only appear toward the end of the larval phase in *Xenopus laevis* (Chang and Harris, 1998; Parker et al., 2010). It is tempting to speculate that such a delay in the acquisition of blue sensitivity "protects" the tadpoles' visual system from the degradation of image quality caused by short wavelength scattering, but here again we must take this finding as an encouragement to study photoreceptors' ontogeny synchronization in a variety of tadpoles from different ecomorphological backgrounds, rather than settling on it as the default pattern.

The components discussed so far cannot be expected to adjust to short-term changes in the visual environment, and so the spectral absorbance profile of individual photoreceptor cells remains the best candidate to allow tadpoles' visual systems to adapt to changes in turbidity in physiological time-scales. This profile is a property of the visual pigment (opsin apoprotein + Vitamin A-derived chromophore) housed in the photoreceptors membranes, and depends solely on the amino acid sequence of the opsin and the type of Vitamin A (1 or 2) used (Govardovskii et al., 2000). The opsin sequence is, naturally, genetically encoded, and thus cannot be changed during the lifetime of the animal in response to changes in the environment. Overall photoreceptor spectral sensitivity can, in fact, be worked around by regulating the gene expression levels in cases when several copies with slightly different sensitivity peaks are available such as the fast-speciating cichlid fishes radiations from the African great lakes (Carleton and Yourick, 2020). Furthermore, opsin expression levels within individuals can fluctuate as a consequence of manipulations in the light environment
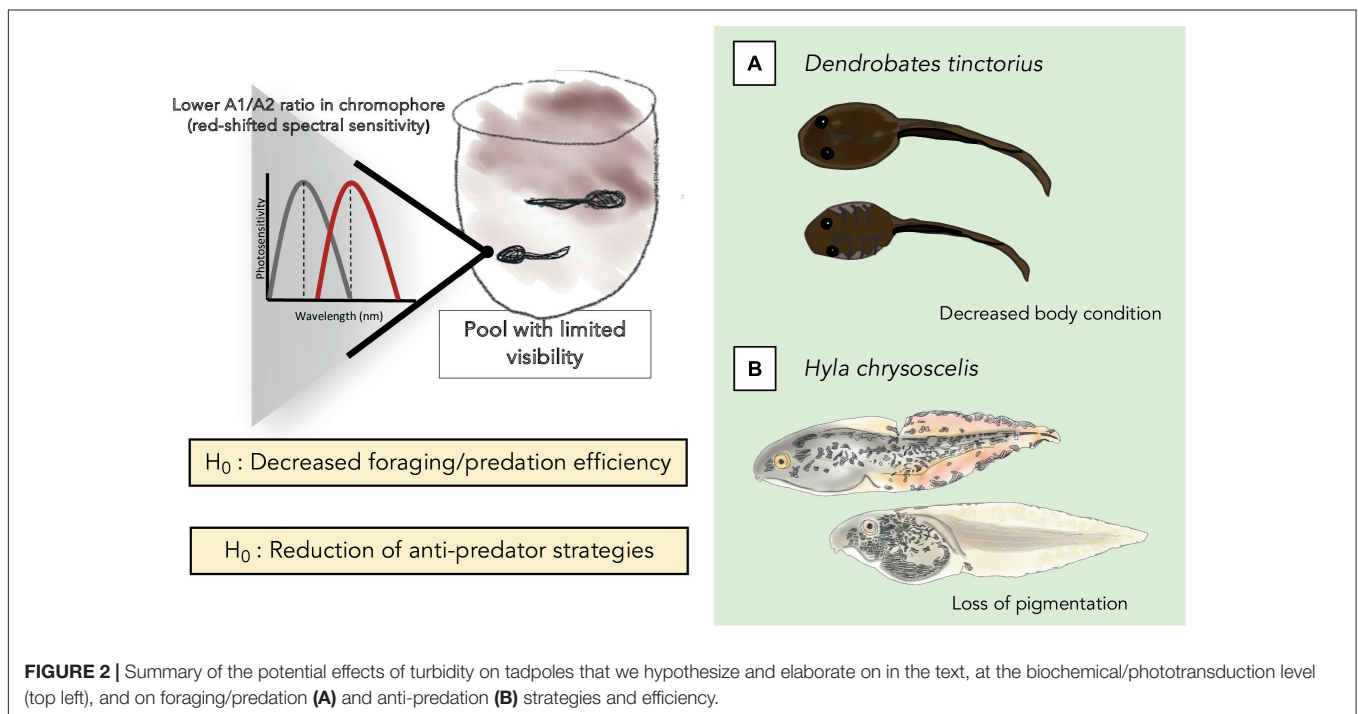
FIGURE 2 | Summary of the potential effects of turbidity on tadpoles that we hypothesize and elaborate on in the text, at the biochemical/phototransduction level (top left), and on foraging/predation **(A)** and anti-predation **(B)** strategies and efficiency.

in killifish (Fuller et al., 2010; Fuller and Claricoates, 2011). However, such a diversified opsin gene pool is not typically available in amphibians, but all the opposite: the sensitivity peak for rhodopsin sits on a particularly narrow range in the spectrum compared to vertebrate standards (Crescitelli, 1958; Donner and Yovanovich, 2020). In contrast, switching from Vitamin A1 to Vitamin A2 (which confer higher sensitivity toward the blue and red parts of the spectrum, respectively) is quite straightforward and achieved by a single enzyme conserved across vertebrates, the expression levels of which are quite reactive to changes in the photic environment, even though the underlying mechanism remains unknown (Corbo, 2021). Documented cases among fishes include change of chromophore ratios in response to water temperature (Ueno et al., 2005), seasonality (Temple et al., 2006), and turbidity (Bridges, 1972; Beatty, 1984).

In the case of amphibians, the general trend is to go from a Vitamin A2-rich tadpole to Vitamin A1-rich adults, except in species who remain aquatic throughout life such as *Xenopus laevis* (see Donner and Yovanovich, 2020 for a review), supporting the view that Vitamin A2-rich retinas are beneficial in typically "red-shifted" freshwater environments (Corbo, 2021). The evidence that the A2/A1 transition occurs both at different ontogenetic stages and at different rates depending on the species (Hödl, 1975), and that the vitamin ratio can reversibly change during the tadpole phase in response to manipulations of light levels (Bridges, 1970), suggests that the Vitamin A system is the most readily available modulator of visual function in response to unpredictable changes in visually challenging environments.

The ultimate effect of shifting amphibian chromophore ratios on behavior remains unknown, but is a fascinating consideration for evolutionary ecologists to approach. For example, we have found an impressive range of tint and turbidity levels in the pools of water in which *Dendrobates tinctorius* tadpoles are deposited (**Figure 1**). Based on previous work on visual pigments, we would hypothesize that tadpoles deposited in more turbid pools of water would have a red-shifted chromophore system. *Dendrobates tinctorius* tadpoles are aggressive predators and appear to partially rely on vision to attack each other (Fouilloux et al., 2020), and thus presumably rely on vision to hunt other prey as well. Ultimately, with a noisier visual scape, red-shifted tadpoles may not be as effective at hunting, cannibalizing, or even foraging in general, but may perform better in visually challenging environments over having not shifted at all; overall, this flexibility could have remarkable impacts on tadpole fitness and survival (**Figure 2**).

ENVIRONMENTALLY INDUCED PHENOTYPIC ADAPTATIONS AND THEIR CASCADING EFFECTS

One of the most covered topics in turbidity literature is predator-prey dynamics. The majority of work considers prey activity (reduced antipredator behavior: Meager et al., 2006; Kimbell and Morrell, 2015), and predator accuracy (Bonner and Wilde, 2002; Gadomski and Parsley, 2005) under various turbidity conditions. Although interactions between predator and prey most often hinge on whether the animal is more dependent on chemical or visual cues, or a combination of the two (Swanbrow Becker and Gabor, 2012), visually guided behaviors often break-down in turbid environments (Chivers et al., 2012; Swanbrow Becker and Gabor, 2012). This results in different advantages

for predators or prey as a function of the modalities used for detection/evasion. For example, some tadpoles have adapted anti-predator phenotypes such that, when under high predation risk, individuals will express redder-colored fins, which have been hypothesized to serve either as camouflage or to prevent lethal attacks, compared to those that are not (*Hyla chrysoscelis*, McCollum and Van Buskirk, 1996; *Dendropsophus ebraccatus*, Touchon and Warkentin, 2008). The underlying rationale is that the redder fins may direct predators to strike non-lethal parts of the tadpole's body, or that the spotty coloration could act as some kind of camouflage by "breaking up" the contour of the tadpole's body. The effects of turbidity on the expression of anti-predator signaling in tadpoles remain unknown.

As turbidity interferes with color vision (Wilkins et al., 2016), its fluctuations can impact the interpretation of color as (including, but not limited to) a proxy for distance (Bartel et al., 2021). Until now, the change of color-based signals in response to visually challenging environments has been demonstrated only in a handful of studies, and limited to the context of adult signaling. In gudgeon fish, a two-week transplant experiment showed the overall expression of melanin decreased in turbid conditions, leading to lighter colored fishes (i.e., economy of pigments hypothesis; Côte et al., 2019). We hypothesize that increased turbidity levels will induce a "relaxed" phenotype where tadpoles do not express camouflage/disruptive coloration despite the potential presence of predators. The direction of which actor would benefit from this phenotype is dependent on the detection mechanisms used by predators and prey.

The change of color expression as a function of visually challenging environments could also be interesting in the context of aposematic signaling. In fire salamanders, it has been shown that the background albedo of larval environments influences juvenile phenotype (Sanchez et al., 2019), indicating that the light quality of larval environments can have impacts that persist through metamorphosis. The carry-over effects of larval rearing environment on adult coloration have yet to be explored in frogs; based on salamander responses, it is plausible that anuran tadpoles who have lived in turbid/red-shifted environments could carry over to the color expression in their (semi-) terrestrial adult forms, perhaps in having less saturated coloration as a result of developmental stress and a low-quality diet during their larval stages. This could have remarkable implications for aposematic amphibians who rely on color as an anti-predator (or sexual) signal (Brown, 2013; Segami Marzal et al., 2017).

In response to the impressive variation in color displayed among poison frogs and the interest in aposematic signaling

throughout the clade, establishing a novel driver of color variation through turbidity-induced carry-over effects has the potential to be valuable across research disciplines. Exploring how colors change in the face of increased turbidity would be a great opportunity to understand the plasticity of color-based signals in antipredator contexts, and how these are shaped by optically challenging scenarios. Overall, amphibian larvae provide an exciting opportunity for exploring adaptations to visually limited environments from both proximate and ultimate perspectives, and framing these responses comparatively across clades, habitats, and geography will surely challenge evolutionary ecologists for years to come.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CF gave shape to the initial draft and designed both figures. All authors were directly involved in all stages from conceptualization to final editions.

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III

VISUAL ENVIRONMENT OF REARING SITES AFFECTS LARVAL RESPONSE TO PERCEIVED RISK

by

Fouilloux C.A., Stynoski J.L., Yovanovich C.A.M., & Rojas B. 2023

The visual environment of rearing sites affects the larval response to perceived risk in poison frogs

Chloe A. Fouilloux[1,*], Jennifer L. Stynoski[2], Carola A. M. Yovanovich[3] and Bibiana Rojas[1,4,*]

ABSTRACT

Turbidity challenges the visual performance of aquatic animals. Here, we use the natural diversity of ephemeral rearing sites occupied by tadpoles of two poison frog species to explore the relationship between environments with limited visibility and individual response to perceived risk. To compare how species with diverse natural histories respond to risk after developing in a range of photic environments, we sampled wild tadpoles of (1) *Dendrobates tinctorius*, a rearing-site generalist with facultatively cannibalistic tadpoles and (2) *Oophaga pumilio*, a small-pool specialist dependent on maternal food-provisioning. Using experimental arenas, we measured tadpole activity and space use first on a black and white background, and then on either black or white backgrounds where tadpoles were exposed to potentially predatory visual stimuli. The effects of rearing environment on *D. tinctorius* tadpoles were clear: tadpoles from darker pools were less active than tadpoles from brighter pools and did not respond to the visual stimuli, whereas tadpoles from brighter pools swam more when paired with conspecifics versus predatory insect larvae, suggesting that tadpoles can visually discriminate between predators. For *O. pumilio*, tadpoles were more active on experimental backgrounds that more closely matched the luminosity of their rearing sites, but their responses to the two visual stimuli did not differ. Larval specialisation associated with species-specific microhabitats may underlie the observed responses to visual stimuli. Our findings demonstrate that light availability in wild larval rearing conditions influences risk perception in novel contexts, and provides insight into how visually guided animals may respond to sudden environmental disturbances.

KEY WORDS: Larval vision, Phytotelmata, Poison frog, Predator–prey interactions, Sensory ecology, Limited visibility

INTRODUCTION

Sensory recognition is crucial for the success of predators and the survival of prey. While predators and prey are in an evolutionary arms race to refine their detection of each other, there are factors outside their control that can limit the accuracy of their perception, such as chemical contamination (Weis and Candelmo, 2012) and light

pollution (Minnaar et al., 2015). Nevertheless, developmental plasticity associated with the growth of organs and tissues creates the opportunity for bodily systems to optimise their function in a given environment. For example, the optic tecta of the brain in both fishes and amphibians enlarges when they develop in habitats with higher conspecific densities (Gonda et al., 2009; Trokovic et al., 2011), whereas nutritionally poor diets in mammals (Lee and Houston, 1993) and birds (Savory and Gentle, 1976) induce an increase in gut size, optimising digestion. Sensory systems are particularly susceptible targets of selection, as they are energetically expensive structures (Niven and Laughlin, 2008) whose performance is fundamental to individual survival and reproductive success (Streinzer et al., 2013). Bentho-pelagic elasmobranchs, for example, have denser and larger olfactory surfaces than benthic species, which supposedly compensates for the scarcity of visual cues in the open ocean (Schluessel et al., 2008). Male net-casting spiders (*Deinopis spinosa*) have enlarged principal eyes (and a more developed integration centre to process their inputs) compared with females and juveniles; the change in eye size in males is hypothesised to enhance predator detection as a result of switching from a sedentary to a wandering lifestyle (Stafstrom et al., 2017). These examples illustrate how sensory organs can compensate to adjust sensory function when faced with pressures from contrasting ecological niches (Schluessel et al., 2008).

Turbidity is a ubiquitous yet often underappreciated environmental pressure that shapes the dynamics of predator–prey interactions in aquatic environments (Abrahams and Kattenfeld, 1997; Horppila et al., 2004; Van de Meutter et al., 2005). In experimental tests on the impact of turbidity, both background colour and the turbidity of rearing conditions have been found to influence the perception of risk by prey, affecting both their activity (invertebrates: Van de Meutter et al., 2005; fish: Leris et al., 2022) and space-use (invertebrates: Horppila et al., 2004), while generally reducing capture rates by predators (reviewed by Ortega et al., 2020). Laboratory studies have shown that the photic environment during development affects the fine-tuning of visual systems in tadpoles (Bridges, 1970) and fishes (Kröger et al., 1999; Fuller et al., 2010). From a proximate perspective, one mechanism that has been shown to flexibly change the spectral tuning of vertebrate eyes is the vitamin A1/A2 chromophore exchange system in the retina (Bridges, 1972; Reuter et al., 1971). In this system, a lower A1/A2 ratio generates a red-shifted spectral sensitivity that can be advantageous in reddish environments (Corbo, 2021) such as freshwater (Lythgoe, 1979) and turbid waters (Jerlov, 1976). Ultimately, although environmental conditions affect immediate (activational) individual responses, they also play a long-term (organisational) role in shaping the development of sensory systems throughout an individual's lifetime (Snell-Rood, 2013). Previous work on both killifish (Fuller et al., 2010) and guppies (Ehlman et al., 2015) has linked the developmental impact of limited light and turbidity with the spectral tuning of the visual system, which in turn has cascading effects on the behaviour of fishes in novel contexts. These studies establish an

[1]University of Jyväskylä, Department of Biology and Environmental Science, 40014 Jyväskylä, Finland. [2]Clodomiro Picado Institute, University of Costa Rica, Coronado, 2060 San José, Costa Rica. [3]School of Life Sciences, University of Sussex, Brighton BN1 9QG, UK. [4]Department of Interdisciplinary Life Sciences, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine Vienna, 1160 Vienna, Austria.

*Authors for correspondence (chloe.a.fouilloux@jyu.fi; bibiana.rojas@vetmeduni.ac.at)

C.A.F., 0000-0003-1265-5824; J.L.S., 0000-0002-9132-8567; C.A.M.Y., 0000-0002-0589-3499; B.R., 0000-0002-6715-7294

important connection between proximate-level restructuring of the visual system and ultimate-level changes in behaviour that can be used to predict outcomes of predator–prey interactions.

Phytotelm-breeding amphibians provide an exciting vertebrate model to further explore the relationship between environments with limited visibility and visually guided behaviours. This is in part because tadpoles are confined to isolated pools of water in the vegetation where they remain until metamorphosis. Phytotelmata are water-filled vegetal structures such as treeholes, palm bracts and seed capsules, which are inhabited by diverse communities (Kitching, 2000), and can appear as any combination of bright/ dark and crystalline/turbid (Fouilloux et al., 2022a). Tadpole deposition sites are a unique system because the conditions in lentic phytotelm environments can change suddenly and then persist for long periods of time (Leris et al., 2022; Fouilloux et al., 2021). In turn, the 'darkness' can be a result of light absorption by pigments and substances in the water itself, or the inner walls of the container, or both. Despite this remarkable variation, tadpole vision has never been explored in the context of the photic environment of rearing conditions. This is at least partly because earlier studies suggest that tadpole vision is generally 'poor' (e.g. myopic: Hoff et al., 1999; Mathis et al., 1988; low visual acuity expected on the basis of small eye size: Caves et al., 2018; Butler et al., 2022 preprint) and it had been well established that tadpoles rely heavily on chemical cues to navigate and assess their environment (Mathis and Vincent, 2000; Weiss et al., 2021). More recently, however, experiments that isolate and combine sensory modalities have uncovered an important role of tadpole vision in conspecific communication and predator–prey interactions (Hettyey et al., 2012; Stynoski and Noble, 2012; Kumpulainen, 2022) and even some extent of illumination-dependent differential expression in vision-related genes has been reported (Schott et al., 2022). Previous work demonstrating plasticity in tadpole vision substantiates the hypothesis that environments with selective light absorption may induce spectral shifts in their visual system (Donner and Yovanovich, 2020; Schott et al., 2022) rather than eliciting other adaptive responses, such as sensory compensation (Ehlman et al., 2015). Ultimately, although turbidity is frequently used to characterise permanent aquatic habitats, it has been an overlooked feature of ephemeral ones; as such, the impact of turbidity on the sensory development and visual recognition in species evolved to use ephemeral habitats remains unknown.

In this study, we investigated how wild-caught larvae of two phytotelm-breeding poison frog species (*Dendrobates tinctorius* and *Oophaga pumilio*), reared in pools with different optical characteristics, respond to predator and conspecific visual stimuli in novel contexts. *Dendrobates tinctorius* tadpoles are aggressive predators that will consume each other facultatively (Rojas, 2014; Rojas and Pašukonis, 2019; Fouilloux et al., 2022b). They are deposited in a wide range of microhabitat types and frequently cohabit pools with other tadpoles and insect larvae that can be either predators (e.g. dragonflies: Odonata) or prey (Rojas and Pašukonis, 2019). In contrast, *O. pumilio* tadpoles depend on their mothers to deposit unfertilised, nutritive eggs in their pools (Stynoski, 2009), which serve as their primary food source throughout development (Brust, 1993). In addition to being oophagous, *O. pumilio* tadpoles are small-pool specialists, primarily using bromeliad and other plant axils as pools, which they occupy singly; if multiple depositions occur, smaller tadpoles are cannibalised within a couple of days (Brust, 1993; Stynoski, 2009).

Here, we aimed to measure how tadpole response to visual cues changes as a function of the photic environment of their rearing
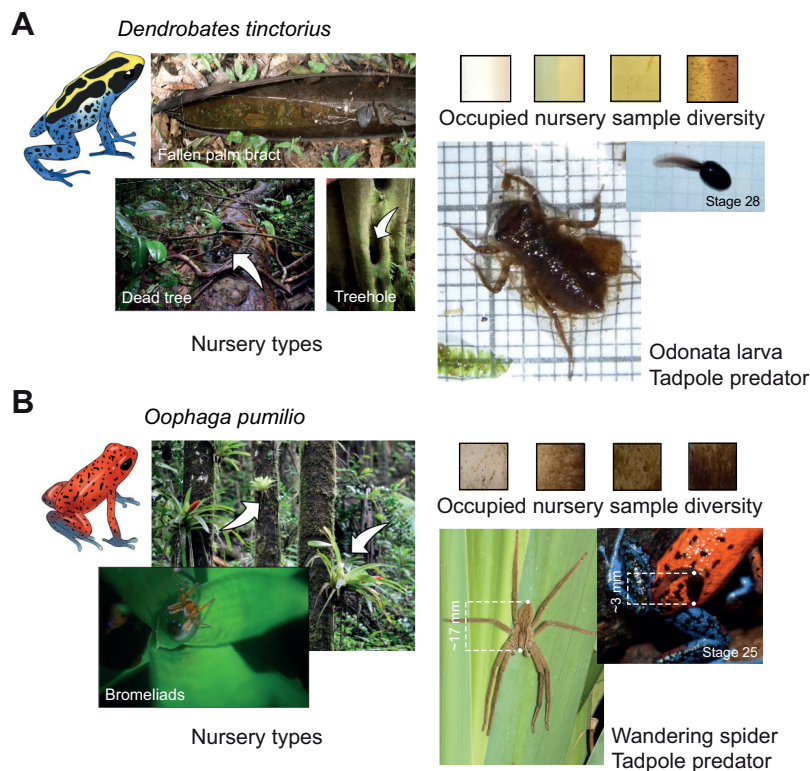
conditions. We measured individual space use and activity in artificial arenas where visual stimuli were contrasted on either a white or a black background. As such, we could quantify how the visual environment within phytotelmata influences tadpole response to risk in novel conditions with contrasting light environments. Given the variability in light availability and visibility inside phytotelmata, we hypothesise that the visual environment of rearing sites influences the development of sensory systems, and thus, the behaviour of larvae in novel conditions. Predatory tadpoles such as *D. tinctorius* may rely on vision (Kumpulainen, 2022) and thus low visibility during development could impact the phenotype of *D. tinctorius* visual systems (e.g. by red-shifting spectral sensitivity; Fouilloux et al., 2022a), which may mitigate the loss of acuity and improve predator performance in turbid conditions. However, a red-shifted spectrum in tadpoles is not a failsafe way of overcoming the challenges of a visually limited environment. We thus predict that *D. tinctorius* reared in habitats with poor visibility would be generally less active (reduced prey response: Kimbell and Morrell, 2015) and less discerning between visual stimuli (poor learning in turbid conditions: Chivers et al., 2013) than tadpoles reared in clear habitats. In comparison, given the restricted surface area of pools and high predation rates (67%; Maple, 2002) in *O. pumilio* rearing sites, tadpole responses to perceived risk are better contextualised in terms of 'seeking refuge and freezing' instead of the 'escaping or attacking' response that more accurately describes the behavioural repertoire of *D. tinctorius*. Thus, although *O. pumilio* tadpoles have been shown to respond differently to positive (food-bearing mother) and negative (predator) visual stimuli (Stynoski and Noble, 2012), we expect a more generalised fear response where we predict that experimental contexts that are perceived as safer (i.e. more similar to rearing conditions) will elicit stronger behavioural responses (i.e. more activity) from *O. pumilio* tadpoles.

MATERIALS AND METHODS
Study species
Dendrobates tinctorius (Cuvier 1797) and *Oophaga pumilio* (Schmidt 1857) are Neotropical poison frog species with intensive parental care. In *D. tinctorius*, fathers care for the terrestrial clutches and transport tadpoles from oviposition sites to phytotelmata. *Dendrobates tinctorius* pool use is especially flexible compared with other phytotelm-breeding species, as these tadpoles live in pools from a wide range of substrates that can be vastly different sizes (Fig. 1, volume: 19 ml to 270 l; height: 0 to >20 m; Fouilloux et al., 2021). Despite being facultative cannibals, *D. tinctorius* tadpoles are frequently placed in pools occupied by larger conspecifics (Rojas, 2014). In these pools, tadpoles also encounter heterospecific tadpoles (generally *Allobates femoralis*) and insects (predatory Odonata larvae; Rojas, 2014; Rojas and Pašukonis, 2019; Fouilloux et al., 2021). For this study, *D. tinctorius* tadpoles were taken from dead palm bracts, treeholes and fallen trees found around Camp Pararé, Les Nouragues Field Station, French Guiana from March to April 2022. Tadpoles were captured using small aquarium nets and spoons. Focal tadpoles ranged from stages 25 to 41 (Gosner, 1960) and weighed 0.05–0.88 g.

Unlike in *D. tinctorius*, *O. pumilio* mothers are the primary caregivers, transporting tadpoles from terrestrial clutches to phytotelmata (Brust, 1993). *Oophaga pumilio* are much more specialised in their pool use than *D. tinctorius*, generally opting for small bromeliads and other axil-forming plants (volume: 10–100 ml: J.L.S., unpublished data; Maple, 2002). In addition to using much smaller pools, *O. pumilio* mothers feed deposited tadpoles with nutritive eggs, which serve as an obligate food source until

A

Dendrobates tinctorius

Occupied nursery sample diversity

Nursery types

Odonata larva
Tadpole predator

B

Oophaga pumilio

Occupied nursery sample diversity

Nursery types

Wandering spider
Tadpole predator

Fig. 1. Characterisation of the visual environment of two phytotelm-bound tadpole species. Schematic compares pool diversity in terms of both structure and turbidity, tadpole morphology and frequently encountered predators in (A) *Dendrobates tinctorius*, a rearing-site generalist with facultatively cannibalistic tadpoles and (B) *Oophaga pumilio*, a small-pool specialist dependent on maternal food-provisioning. All photos by C.F. except for Odonata larva (B.R.), forest epiphytes (US Forest Service - Southern Region, CC BY-SA 2.0) and spider (Stuart J. Longhorn, CC BY-SA 2.0). Line drawings kindly provided by Lia Schlippe Justicia.

metamorphosis (Brust, 1993; Stynoski, 2009). Tadpoles generally do not co-occur with other amphibian species. The predation rate of *O. pumilio* tadpoles is high, primarily as a result of snakes, aquatic beetle larvae (Family: Elateridae), and spiders (Family: Ctenidae, Maple, 2002; Stynoski et al., 2014). Trials were conducted in La Selva Biological Station, Costa Rica from June to July 2022. *Oophaga pumilio* tadpoles were captured from the field using pipettes and were tested the day of capture. Focal tadpole stages ranged from 25 to 41 (Gosner, 1960) and their mass from 0.01 to 0.12 g.

Experiments in French Guiana were approved by the DGTM (arrête No. R03.2022-01-07-00002), the Scientific Committee of Les Nouragues Ecological Research Station, and the Nouragues Nature Reserve (partnership agreement no. 01-2019 with BR). Experiments in Costa Rica were approved by the Resolución 292 of the Comisión Institucional de Biodiversidad de la Universidad de Costa Rica and SINAC-ACC-VS-16-2021 from MINAE, and ethics clearance CICUA-004-2021 from the Universidad de Costa Rica.

Basic set-up

Dendrobates tinctorius trials (*n*=20) were conducted outdoors under a white tarp to both prevent rainfall from affecting measures and to attempt to standardise ambient light above the arena (dimensions: 28.5 cm×21.0 cm×9.0 cm, 3 mm thickness Rotho LOFT filled with 2.9 l rainwater). *Oophaga pumilio* trials (*n*=25) were conducted in a laboratory with fixed fluorescent overhead lighting where temperature was held at 26°C (arena dimensions: 25 cm×20 cm×14.5 cm, 5 mm thickness plexiglass, filled with 800 ml rainwater). After capture, tadpoles were housed singly and were given >1 h to acclimate before testing. All trials were conducted from 12–18 h, when both of these diurnal species are naturally active.

Background choice assay

All tadpoles (*D. tinctorius*, *n*=20; *O. pumilio*, *n*=25) were placed in the centre of an arena with a 2×2 black/white chequered background

(Fig. 2). The activity and background choice of tadpoles were quantified by scan samples every 15 s for 15 min.

Open field tests

Following the background choice assay, the same individuals were used for multiple open field tests. These consisted of an arena with either an entirely black or white background used as extreme cases of 'crystalline dark' and 'crystalline bright' visual environments, respectively (Fig. 1), containing different visual stimuli in the arena centre, i.e. either a conspecific tadpole or a known predator placed in an isolated container filled with rainwater. We recorded activity (swimming or not swimming) and space use (zone occupancy) of the focal tadpole at 15 s intervals for 15 min in each treatment. Zones were delineated by distances from the edge of the stimulus container in the arena centre, where zone 1 was up to 2 cm away from the centre, zone 2 was 2–5 cm away from the centre, and zone 3 was beyond 5 cm.

We used a complete factorial design where all tadpoles were tested with all visual stimuli under both background conditions (Fig. 2). Between individual stimulus trials within each background treatment, tadpoles were given 5 min to rest in a plastic container filled with rainwater. When changing between background colours, tadpoles were allotted at least 15 min of rest. After each 15 min treatment, the water in the arena was changed to avoid the possibility of chemical-borne cues affecting tadpole behaviour.

To capture the variation of tadpole interactions in natural conditions, and also as a result of the limitation of available tadpoles, the size of the focal tadpole was at times smaller or larger than the tadpole that served as the visual stimulus. Mass differences between the focal and stimulus tadpoles ranged from −0.35 g (where the focal tadpole was smaller than the stimulus) to 0.75 g (where the focal tadpole was larger) for *D. tinctorius*, and from −0.10 g to 0.054 g for *O. pumilio*. Statistically, using the absolute mass of the focal tadpole or the mass difference between the focal and stimulus tadpoles predicted identical tadpole

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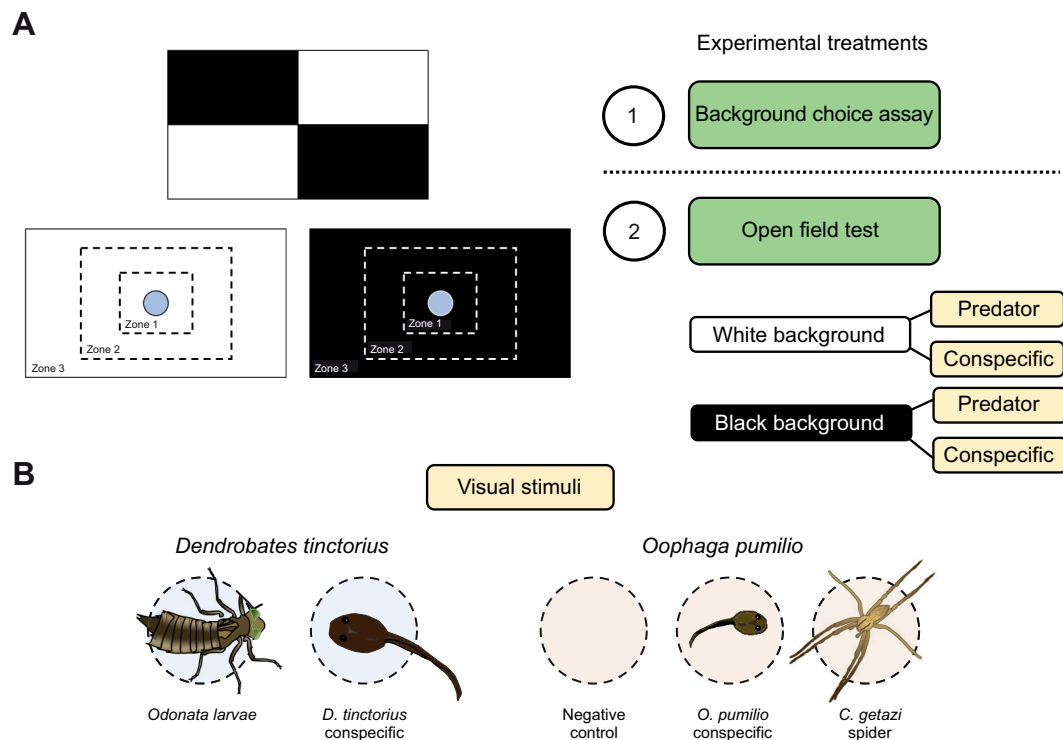
Fig. 2. Schematic detailing experimental overview. A background choice assay was followed by open field tests with either white or black background treatments (A) where tadpoles were exposed to either two or three visual stimuli (B) in successive trials. Background order and the visual stimuli within each background block were randomised.

behaviour. All else being equal, we used focal tadpole mass instead of mass difference in all analyses for ease of interpretation (Table S1). For *D. tinctorius* experiments, in 18 out of 20 trials Odonata larvae were the same size (n=1) or larger (n=17) than the focal tadpole.

Once all trials were finished, the experimental tadpole was weighed and staged. After each day of testing, all *D. tinctorius* tadpoles and odonate larvae were returned to their original pools. *Oophaga pumilio* tadpoles were kept in the lab for an ongoing study. *Cupiennius getazi* or *C. coccineus* spiders were released at their capture point. All trials with each individual were completed on the same day. No tadpoles were used as a visual stimulus before serving as a focal tadpole. For *D. tinctorius*, ecological data about phytotelmata, including conspecific count and the presence of heterospecific predators were available as a result of parallel monitoring surveys.

Standardised water sample photography, image analysis and validation
Water samples from *D. tinctorius* rearing sites (n=9 pools) were agitated and transferred to 1.5 ml glass vials and then photographed indoors under laboratory fluorescent lighting on a white background next to a Macbeth XRite ColorChecker. Water samples from *O. pumilio* rearing sites (n=25 pools) were transferred to a spectrophotometer cuvette and photographed in a lightbox (Puluz Ring Led Portable Photo Studio) on a white background next to a Macbeth XRite ColorChecker. In all cases, raw photographs were taken using a Nikon D5300 digital camera (settings: ISO 500, f/13, shutter speed 1/125) from a 24 cm vertical distance (see supplements for details of sample collection and storage, S1). Even though there is no specific information about the visual systems of the tadpoles used in our study, previous work on adults of *O. pumilio* has shown that they have a trichromatic cone system made of blue, green and red channels and they lack a dedicated UV channel (Siddiqi et al., 2004).

Thus, the RGB channels of 'standard' photographic cameras match the expected dimensionality of their colour space.

All available water in *O. pumilio* phytotelmata was taken for spectrophotometry/photography measurements. Spectrophotometric readings of *O. pumilio* phytotelm water were analysed at the Clodomiro Picado Institute of the University of Costa Rica [see Supplementary Materials and Methods for details on spectrophotometry and full transmittance spectra of *O. pumilio* pools (Fig. S1)]. Water spectra were taken in addition to standardised water sample photographs to validate the colour analysis of photos that were evaluated using ImageJ software (Abramoff et al., 2004). Such spectrophotometric quantification was not available for the *D. tinctorius* phytotelm water samples. Both methods aim to characterise light availability in the rearing sites to allow, albeit loosely, sorting of samples by visibility *sensu* Lythgoe, 1979. For both *D. tinctorius* and *O. pumilio*, images were calibrated using the greyscale (standard reflectance set at 7% and 97%) and converted into .mspecs using the MicaToolbox plugin in ImageJ (Troscianko and Stevens, 2015). Images were measured with objective camera vision, normalised by reflectance, and analysed as a linear normalised reflectance stack. Phytotelm samples were selected as regions of interest and their percentage of reflectance (relative to the standards) in the RBG spectra was computed, which then generated the reflectance of each colour channel relative to the standards.

Statistics
Background preference was assessed using a Wilcoxon signed rank test with a continuity correction. The null-hypothesis (μ) was set to 0.5 and the alternative hypothesis was specified as "greater" to compute if tadpoles spent significantly more than half of their time on black backgrounds. Reflectance values from RGB photos (for both *D. tinctorius* and *O. pumilio*) were assessed using a principal

components analysis (see Supplementary Materials and Methods and Fig. S2 for methods to compare photography and spectrophotometer quantification of water appearance). For both *D. tinctorius* and *O. pumilio* models the first component (PC1) explained more than 95% of the variance and thus were used as the predictor to represent differences in phytotelm photic environment (Fig. 3; Fig. S3). *Oophaga pumilio* models were coded with either mean maximum transmittance from spectrophotometer readings or PC1 from photos (see Supplementary Materials and Methods). All statistical models were parameterised as count events; activity and space-use responses were predicted by experimental background, visual stimulus, the principal component (PC1) of microhabitat RGB % reflectances from standardised photography, and tadpole mass. *Dendrobates tinctorius* additionally included the conspecific count and the presence/absence (1/0) of natural predators in the original phytotelmata. All statistical models included tadpole identity (Tad_ID) as a random effect. *Dendrobates tinctorius* models also included phytotelm (Pool_ID) as a random effect as multiple tadpoles were sampled from the same phytotelmata. The best fitting interactive parameterisation was assessed via a backward stepwise algorithm on a full three-way interactive model (interactions between experimental background, PC1 and visual stimulus were considered), followed by analysing the significance of two-way interactions by comparing model formulations via ANOVAs and assessing model ranks through AICc model selection. All models for both *D. tinctorius* and *O. pumilio* were coded using a negative binomial family. Final models passed quality checks for residual patterns, over/underdispersion, and zero-inflation using the simulation-based package 'DHARMa' (https://CRAN.R-project.org/package=DHARMa). All models and figures were coded in R (v.4.1.1; https://www.r-project.org/). For ease of comparison with *D. tinctorius,* the PC1 data from photos are reported in the results.

RESULTS
Characterisation of phytotelmata and validation of photographs for the quantification of pool visual environment
Tadpoles of both *D. tinctorius* and *O. pumilio* occupy a range of phytotelmata that vary widely in the appearance of their water

contents (Figs 1 and 3). They all have a red tint (see position of the transmittance peak in the red part of the spectrum, ~600 nm in Fig. 3 and Fig. S1), such that the perceived differences lie on the bright–dark axis (see overall transmittance values in Fig. 3 and Fig. S1). For *D. tinctorius*, out of the nine pools sampled, three contained odonate predators and the total number of tadpoles ranged from one to ten. All 25 *O. pumilio* pools were singly occupied and none contained potential predators.

We validated the use of the reflectance values from photographs for classification of microhabitats as an alternative to spectrophotometer readings from paired measurements of samples from *O. pumilio* pools (see details in Supplementary Materials and Methods).

Background choice assay
Dendrobates tinctorius and *O. pumilio* tadpoles spent significantly more time on black backgrounds than predicted by random chance (one-sample Wilcoxon signed-rank test: *D. tinctorius*, $P=0.044$; *O. pumilio*, $P<0.001$). There was no apparent trend of an effect of tadpole mass or phytotelm photic environment on background choice for either species (Figs S4 and S5).

Space use
Space use in *D. tinctorius* was predicted by both experimental background colour and visual stimuli, yet not in an interactive manner. Overall, tadpoles spent more time in the arena centre when on a white background (GLMM, $z=2.36$ $P=0.018$, Table 1) and when exposed to a conspecific versus an odonate larva (GLMM, $z=3.07$, $P=0.002$, Fig. 4A). Neither phytotelm photic environment, nor focal tadpole mass or predator presence in pools affected *D. tinctorius* space use (Table 1). Tadpoles from pools with more conspecifics spent significantly more time away from the arena centre (GLMM, $z=-2.59$, $P=0.010$). For *O. pumilio*, only experimental background colour predicted space use: tadpoles spent significantly more time in the arena centre while on a white versus a black background (GLMM, $z=-3.22$, $P=0.001$, Fig. 4B). Visual stimuli, phytotelm photic environment and focal tadpole mass did not significantly influence *O. pumilio* space use (Table 2).
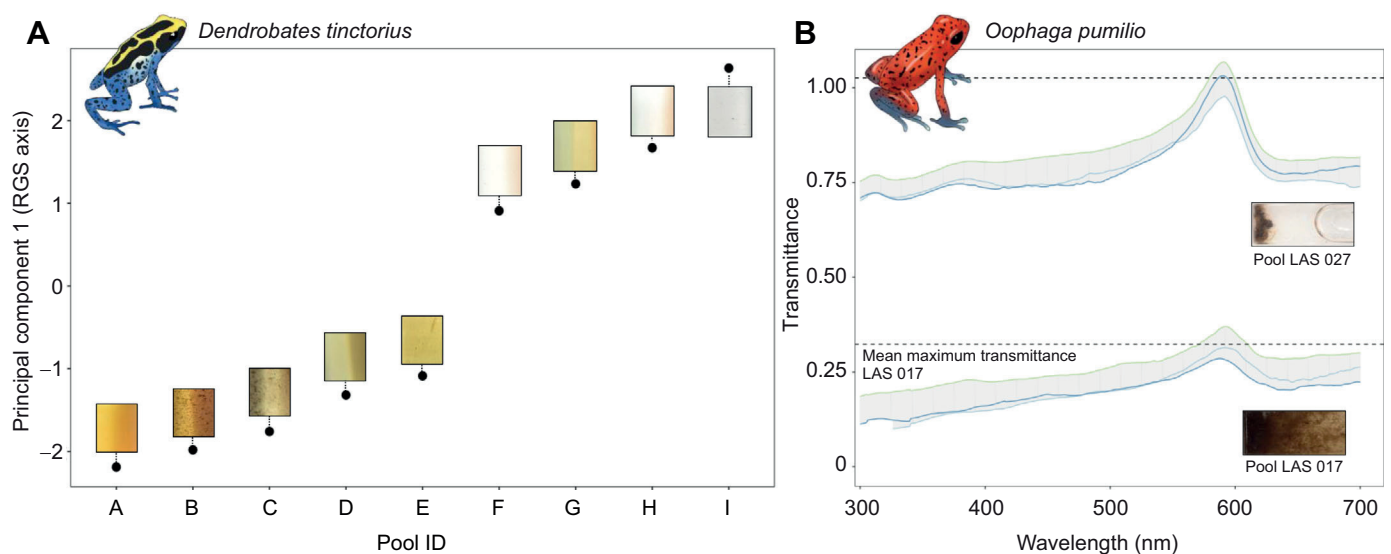
Fig. 3. Diversity of tadpole pools with respect to photic environment. (A) The range of colour/brightness (PC1 with corresponding photographs, unedited) in pools occupied by *D. tinctorius*. (B) The variation in full absorbance spectra of *O. pumilio* microhabitats. Selected transmittance spectra show one of the darkest and one of the brightest pools (dashed lines: mean transmittance at the peak).

Table 1. Model output for space use dynamics (zone 1 counts) in *Dendrobates tinctorius*

Predictors	log-mean	s.e.	z-value	P
(Intercept)	3.31	0.40	8.32	**<0.001**
Condition [White]	0.60	0.25	2.36	**0.018**
PC1	0.10	0.11	0.94	0.347
Predator [Conspecific]	−0.77	0.25	−3.07	**0.002**
Wild conspecific count	−0.21	0.08	−2.59	**0.010**
Wild predator presence (Y/N)	0.79	0.54	1.46	0.143
Mass	0.01	0.84	0.01	0.994
Random effects				
σ^2	1.07			
$\tau_{00\ Pool\_ID}$	0.00			
$\tau_{00\ TadID}$	0.18			

In all tables, bold values are statistically significant. The best fitting model was based on an additive parameterisation.

Table 2. Model output for space use dynamics (zone 1 counts) in *Oophaga pumilio*

Predictors	log-mean	s.e.	z-value	P
(Intercept)	3.00	0.34	8.77	**<0.001**
Condition [White]	−0.54	0.17	−3.22	**0.001**
Predator [Spider]	−0.03	0.20	−0.17	0.862
Predator [Conspecific]	−0.10	0.20	−0.48	0.629
PC1	0.06	0.08	0.68	0.498
Mass	2.21	3.91	0.56	0.572
Random effects				
σ^2	1.09			
$\tau_{00\ Tad\_ID}$	0.36			
ICC	0.25			

The best fitting model was based on an additive parameterisation.

Activity

Dendrobates tinctorius tadpoles from darker pools swam less than tadpoles from lighter pools independently of the visual stimulus in the arena centre (GLMM, z=3.71, *P*<0.001; Fig. 5A, Table 3). We found a significant interaction between the visual stimulus and phytotelmata photic environment, where tadpoles from lighter pools (higher PC1 values) swam significantly more around conspecifics (GLMM, z=−2.19, *P*=0.029; Fig. 5A) than around odonate larvae. We found no effect of experimental background colour, focal tadpole mass, number of conspecifics in microhabitats or the presence of predators in microhabitats on *D. tinctorius* swimming behaviour (Table 3). *Oophaga pumilio* tadpoles from lighter pools
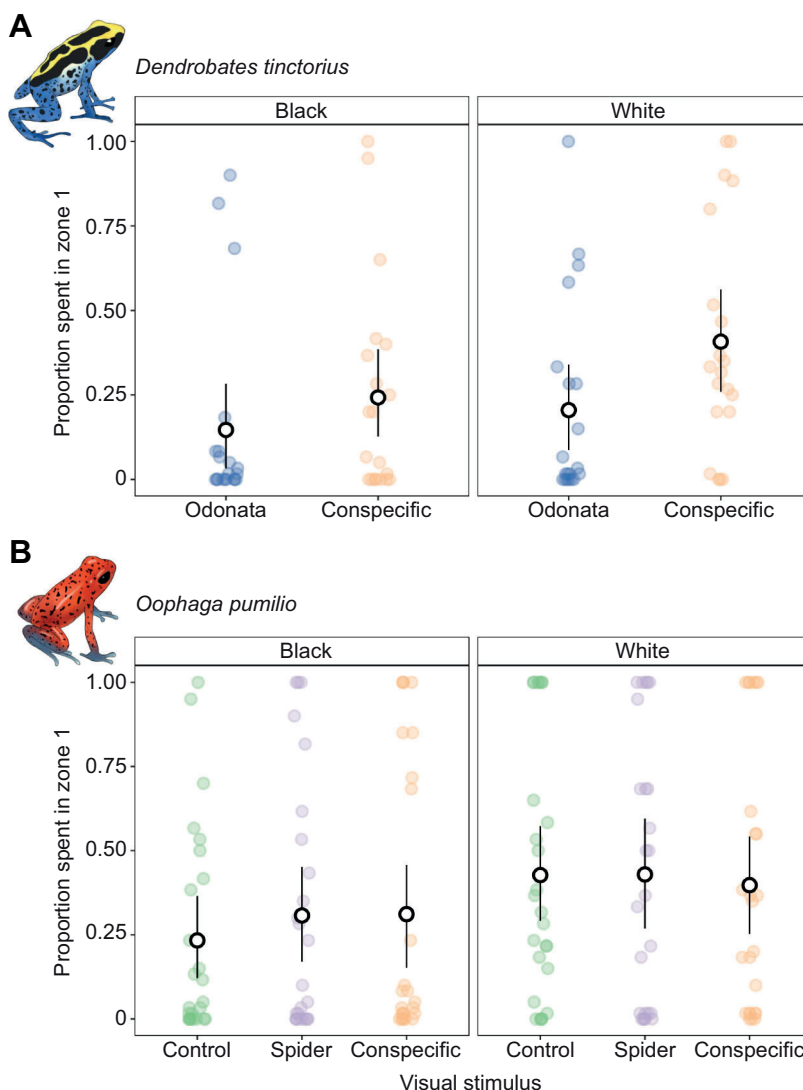
Fig. 4. Space use in poison frog larvae. The proportion of time spent in the arena centre (zone 1) is predicted by background colour (GLMM, z=2.35, *P*=0.018) and the visual stimuli (GLMM, z=−3.07, *P*=0.002) in *D. tinctorius* (*n*=20) (A) and only predicted by background colour (GLMM, z=−3.11, *P*=0.001) for *O. pumilio* (*n*=25) (B). Points represent treatment means and bars illustrate 95% CI. Tadpoles were tested once in each treatment combination (background+species-specific stimulus).

Table 3. Model output for activity (swim counts) in *D. tinctorius*

Predictors	log-mean	s.e.	z-value	P
(Intercept)	1.61	0.48	3.33	**0.001**
Condition [White]	0.34	0.21	1.59	0.111
Predator [Conspecific]	0.05	0.24	0.21	0.837
PC1	0.22	0.14	1.51	0.130
Wild conspecific count	0.01	0.11	0.09	0.926
Wild predator presence (Y/N)	0.05	0.70	0.08	0.938
Mass	−1.15	1.04	−1.10	0.269
Predator [Conspecific]×PC1	0.31	0.14	2.19	**0.029**
Random effects				
σ^2	0.89			
$\tau_{00\ Pool\_ID}$	0.00			
$\tau_{00\ TadID}$	0.37			

Best fitting model included an interaction between the visual stimuli and PC1 value representing microhabitat samples.

Table 4. Model output for activity (swim counts) in *O. pumilio*

Predictors	log-mean	s.e.	z-value	P
(Intercept)	2.29	0.31	7.41	**<0.001**
Condition [White]	0.43	0.16	2.65	**0.008**
PC1	−0.09	0.09	−0.94	0.346
Predator [Spider]	−0.17	0.19	−0.87	0.385
Predator [Cons.]	0.02	0.19	0.12	0.901
Mass	−12.97	3.62	−3.58	**<0.001**
Condition [White]: PC1	0.18	0.09	2.02	**0.044**
Random effects				
σ^2	0.92			
$\tau_{00\ Tad\_ID}$	0.26			
ICC	0.22			

Best fitting model included an interaction between background colour and PC1 value representing microhabitat samples.

were significantly more active on white backgrounds than on black backgrounds (GLMM, z=2.018, P=0.044; Table 4, Fig. 5B). These trends were consistent irrespective of the visual stimulus (Table 4). We also found that larger *O. pumilio* tadpoles were less active in general (GLMM, z=−3.622, P<0.001; Fig. S7B). A similar, yet non-significant, trend was found for *D. tinctorius* tadpoles (Fig. S7A).

## DISCUSSION

Developmental conditions shape the way in which many organisms perceive risk. Throughout our sampling of phytotelmata we found that both *D. tinctorius* and *O. pumilio* tadpoles occur in a wide range of microhabitats that vary in their brightness and turbidity (Figs 1, 3) and that these differences have measurable effects on tadpole behaviour when paired with relevant visual stimuli.
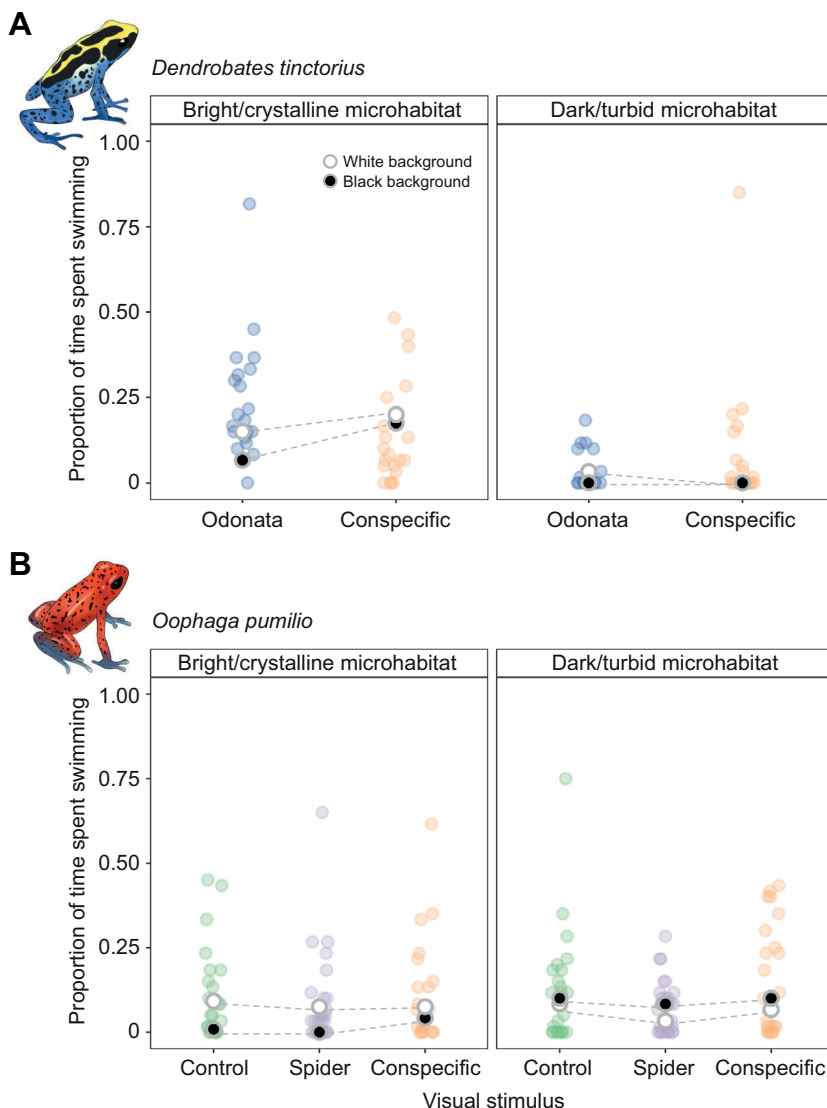
Fig. 5. Activity in poison frog larvae. Microhabitat influences activity in both species, where *D. tinctorius* is also influenced by visual stimuli (GLMM, z=2.19, P=0.029) (n=20) (A) and *O. pumilio* tadpoles by background colour (GLMM, z=2.02, P=0.044) (n=25) (B). Points represent group means for each background treatment. Microhabitat photic environment is coded as a factor for visualisation purposes only, where a PC1 above 0 was categorised as 'bright/crystalline' and below was categorised as 'dark/turbid'. Dashed lines help to visualise slopes.

The effect of photic environment on activity across species

Both species of tadpoles use vision to assess their surroundings. Visual environment has different consequences on the behaviour of *D. tinctorius*, a predatory tadpole, versus *O. pumilio*, a species dependent on maternal provisioning throughout larval development. When assessing an individual's preference for brightness, our choice experiments established a tadpole's preference for black versus white backgrounds. The majority of tadpoles of both species spent more than half of their time on black backgrounds, where their dark brown bodies are largely concealed. Similar results of animals choosing concealing environments have been shown in killifish (Kjernsmo and Merilaita, 2012) and other amphibian larvae, all of which avoided non-concealing backgrounds (Eterovick et al., 2018) and had higher survival rates on concealing backgrounds when faced with predators (Espanha et al., 2016). Having established this preference, we followed up by introducing an additional risk component (predatory visual stimulus) on either entirely white or black backgrounds.

For both species, the visibility (including, but not limited to turbidity) of rearing conditions significantly influenced tadpole activity in a novel context. In *D. tinctorius*, tadpoles from darker pools moved less irrespective of the experimental background colour or visual stimuli (Fig. 5A) with which they were paired. This behavioural pattern has generally been established across the animal kingdom, where animals that were reared in more turbid conditions are less active and social in novel contexts (Fuller et al., 2010, but see work on damselfly responses reported by Van de Meutter et al., 2005). The implications of darker pools on activity are worth considering, as visually limited environments tend to negatively impact predators (Ortega et al., 2020). Predatory species such as *D. tinctorius* can consume both con- and heterospecifics (Rojas and Pašukonis, 2019); lower tadpole activity could have cascading effects on cannibalism rates, diet quality and thus time to metamorphosis (C.F., unpublished data; Kupferberg, 1997), which may have negative implications for the survival of tadpoles, as pool stability is never guaranteed in ephemeral habitats (Fouilloux et al., 2021). From a community perspective, a shift in composition of the predator diet may affect the richness of the microhabitat, where species able to use other modalities to navigate their environment may outcompete vision-dependent species when faced with turbid conditions.

Oophaga pumilio tadpoles were significantly more active on background conditions that more closely matched their rearing conditions (Fig. 5B). A similar trend was also found in Trinidadian guppies, where fish raised in clear water aquaria were more active in clear conditions compared with turbid conditions (Ehlman et al., 2015). Ultimately, the visual landscape of phytotelmata can drastically change depending on various biotic and abiotic factors, and it is not far-fetched to imagine a crystalline pool suddenly becoming turbid (or vice versa), as pools regularly dry out and fill up again with clear rainwater, and debris regularly falls from above (Romero et al., 2020). Based on our results, we would hypothesise that an *O. pumilio* tadpole from a brighter pool would consequently move less in dark conditions. This change in activity as a function of a changing environment has important implications for *O. pumilio* tadpoles, and may impact both the begging ritual between tadpoles and their mothers and the ability of tadpoles to detect and evade terrestrial predators entering their pools (Stynoski and Noble, 2012; Stynoski et al., 2018). As most of *O. pumilio* tadpole interactions occur at the air–water interface, manipulating object size, distance and colour from the edge of a turbid versus a crystalline arena could disentangle the strength of tadpole responses to positive/negative

stimuli and the acuity of tadpole vision in visually limited environments.

While we cannot conclude that the behavioural changes in either species are a result of visual restructuring, previous work has found lighting conditions of rearing environments (tea-stained versus clear) to affect both foraging behaviour and opsin expression in the eyes of killifish (Fuller et al., 2010). Ultimately, there exists a wealth of mechanistic oriented vision research, primarily in fish, showing that environmental light and colour affect the expression of visual pigments during development (Fuller et al., 2005; Shand et al., 2008). Thus, although we do not assess the visual system from a proximate perspective, multiple studies have shown that tadpoles rely on vision to assess risk (Hettyey et al., 2012; Szabo et al., 2021; this study), substantiating the role of vision as an important modality in the larval umwelt. While the link between turbidity as a particular case of 'red-shifted' environment (Jerlov, 1976; Corbo, 2021) and visual restructuring remains to be experimentally tested in tadpoles, recent interest in this field (Donner and Yovanovich, 2020; Corbo, 2021) is starting to yield exciting insights. It is well established that the challenge in turbid waters is not light availability (and thus it cannot be overcome straightforwardly with increased sensitivity) but rather distance-dependent contrast degradation due to non-image forming scattered light (Lythgoe, 1979). In this scenario, myopic eyes such as those of tadpoles might not be so disadvantageous after all (Mitra et al., 2022), as the image quality of the visual scene is proportionately better when viewing objects close by. There are neither theoretical nor empirical estimates of tadpole visual acuity. However, the recent confirmation that they tend to have spherical lenses even in species in which the adult lenses are flattened (Mitra et al., 2022) opens up the possibility for tadpoles to present adaptations to maximise (within the constraints imposed by eye size) their spatial resolution and thus have a better chance to deal with turbid visual environments.

Ecology shapes species' responses to perceived risk

For each species, only one variable influenced consistently both activity and space use: the visual stimulus with which tadpoles were paired for *D. tinctorius* and background colour for *O. pumilio*. The implication of these variables can be contextualised by comparing the biology of a habitat generalist versus a habitat specialist.

The outcome of most of *D. tinctorius* tadpole biological interactions is context-dependent, as tadpoles from this species can not only readily cannibalise conspecifics (Rojas, 2014) but can also co-exist with them (Fouilloux et al., 2021); they can survive when odonate larvae are present and can also easily become their prey when the naiads are large enough (Rojas and Pašukonis, 2019). Within the continuum of perceived risk for *D. tinctorius*, we found that in undesirable contexts (i.e. white background: an experimental environment tadpoles avoid when given the choice) a tadpole's behavioural response changes depending on the visual stimuli with which they are faced. When faced with conspecifics, a tadpole's behavioural response is to 'engage' with a potential threat. *Dendrobates tinctorius* tadpoles from clear pools swam more and spent more time in the arena centre where the stimulus was placed, suggesting that tadpoles recognise and seek to interact with conspecifics, even when provided with only visual cues (similarly to findings in a laboratory setting; Kumpulainen, 2022). In comparison, when paired with an odonate larva that was larger or the same size as focal tadpoles 90% of the time, tadpoles from clear pools spent more time frozen at the edges of the arena. Previous work using open field tests associates elevated stress levels and risk aversion with arena centre avoidance (Champagne et al., 2010), suggesting that tadpoles might occupy the arena edges in response to the potential risk posed by the

naiad. While *D. tinctorius* tadpoles from dark coloured pools seem to have adopted a 'sit and freeze' strategy no matter what the condition, tadpoles from lighter pools are able to recognise and change strategies depending on the visual stimulus with which they are faced. As a result of the flexibility of *D. tinctorius* deposition strategies, tadpoles are more likely to encounter diverse heterospecifics (tadpoles, dragonflies, mosquitoes) on varied backgrounds (dead bark, young plants, treeholes) and thus, need to be able to distinguish different types of visual stimuli.

These findings do not suggest that *D. tinctorius* tadpoles are more visually oriented than *O. pumilio* but instead highlight how the natural history of different species shapes their response to perceived risk. For example, the effect of previous predator exposure generating stronger responses in ensuing encounters has been shown before in tadpoles (via chemical cues; Fraker, 2009). In *D. tinctorius*, focal tadpoles from pools containing more conspecifics spent less time in the arena centre, suggesting that the strength of stimuli avoidance may be informed by previous antagonistic experiences. In comparison, *O. pumilio* are obligately oophagous tadpoles which, in most instances, develop singly and in particularly small water volumes. In the context of their natural history, a tadpole's response to risk is to seek refuge. In the wild, this can be done by diving down to the detritus at the bottom of a leaf axil, for example. Previous work has established that *O. pumilio* tadpoles see and respond distinctly to diverse visual stimuli (Stynoski and Noble, 2012; Stynoski et al., 2018); in the context of this study, the potential effect of a visual stimulus may have been overridden by the design of the experiment itself, as tadpoles were in an arena larger than any of their natural deposition sites with nowhere to hide (800 ml in this study versus ~15–150 ml in previous experiments; Stynoski et al., 2018; Khazan et al., 2019). While this design was intended to create a comparable context to *D. tinctorius*, its role in contributing to a tadpole's perceived risk should be acknowledged. Overall, *O. pumilio* tadpoles spent less time in the arena centre when on a white background compared with black backgrounds (even in the control instance when there is no stimulus; Fig. 4B). For an *O. pumilio* tadpole, it could be that when on white backgrounds the potential 'refuge' provided by edge effects of the arena draws tadpoles away from the arena centre. In a novel context, 'freezing' appeared to be the general strategy adopted by tadpoles; without a refuge to hide in, there was no reason for individuals to modulate their activity in response to the distinct risks of each stimulus.

Phytotelmata as models for environmental disturbance

Phytotelmata provide natural mesocosms to test and manipulate diverse habitat quality indicators on the development of animals. The natural diversity in phytotelm turbidity can be used as a small-scale model to predict how other aquatic animals may respond to sudden changes in habitat quality (Busse et al., 2018; Romero et al., 2020). Ecologically, predator or prey advantage in turbid conditions depends on the short-term adaptability of either player's detection/evasion systems (Abrahams and Kattenfeld, 1997); ultimately, here, two visually guided species respond in ways that could negatively impact their survival in the form of decreased foraging or hindered access to parental care. Although the plasticity of the visual system may mitigate these effects to some degree, based on our data we would expect animals dependent on other modalities to better succeed in visually limited environments. To our knowledge, this is the first study to quantify phytotelm photic environment: we validate that digital photography (with a colour standard) generates the same ranking of pool brightness/turbidity as full spectral

readings obtained with a spectrophotometer, demonstrating the accuracy of photography as a quantification tool for microhabitat visual environment and opening the door for future studies to pursue other turbidity-related questions independently of access to expensive laboratory devices.

Conclusions

We compare the perception of risk in a flexible predatory species with that of a microhabitat specialist that has an adapted oophagous diet, testing the responses of both species to visual stimuli after they spent their early larval stages in varied photic environments. The perception of risk in animals is context dependent, and the light quality of rearing conditions affects the response to risk in novel contexts. As animals face increasingly disturbed habitats, these results highlight how animals with varied natural histories depend on vision and how they might respond to sudden environmental disturbances.

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Summary: The visual environment of rearing sites used by poison frogs affects tadpole responses to risky visual stimuli in novel contexts.

Visual environment of rearing sites affects larval response to perceived risk in poison frogs.

Chloe A. Fouilloux, Jennifer L. Stynoski, Carola A. M. Yovanovich, and Bibiana Rojas

SUPPLEMENTARY METHODS

1. Testing conditions

D. tinctorius trials (n = 20) were conducted outdoors under a white tarp to both prevent rainfall from affecting measures and to attempt to standardise ambient light above the arena (dimensions: 28.5 cm x 21.0 cm x 9.0 cm, 3mm thickness Rotho LOFT filled with 2.9 L of rainwater). *O. pumilio* trials (n = 25) were conducted in a laboratory with overhead light where temperature was held at 26°C (arena dimensions: 25 cm x 20 cm x 14.5 cm, 5 mm thickness plexiglass, filled with 800 mL of rainwater). After capture, tadpoles were housed singly and were given > 1 hour to acclimate before testing. All trials were conducted from 12-18h, when both of these diurnal species are naturally active.

2. Collection of water samples from pools and storage of *O. pumilio* water samples

Water from occupied phytotelmata was collected and photographed on the same day that tadpoles were tested. *D. tinctorius* samples (n = 9 pools) were collected in syringes that were completely submerged in a disturbed pool. Samples, which ranged from 1.5 mL to a maximum of 10 mL, were then transported to the field station. For *O. pumilio,* after tadpoles were captured, nursery water was collected by submerging a pipette with the tip cut off into the bottom of the leaf axil and immediately transferred to a 1.5 mL glass vial (n = 25 pools). *O. pumilio* phytotelm water was photographed both the day of collection and the day of spectrophotometry measurements to confirm that samples had not changed or degraded between sampling and processing dates. After sampling and initial photography, water samples were refrigerated at 4°C until processing with a spectrophotometer at the Clodomiro Picado Institute of the University of Costa Rica.

3. Spectrophotometry measurements of water from *O. pumilio* phytotelmata

For spectrophotometry measurements, *O. pumilio* phytotelm water samples were agitated and then diluted 1:5 with Milli-Q water before analysis, as pure samples led to absorbance levels above 1, at which point the samples are too concentrated and do not provide accurate absorbance outputs. We measured the full absorbance spectra of three technical replicates of each microhabitat sample, with the exception of three samples for which only two replicates could be measured due to insufficient volume. We used a Shimadzu (UV-1800) spectrophotometer with both ultraviolet range deuterium (D2, Type: L6380) and a 20W Tungsten halogen (W1, Type: NA55917) lamps with a silicon photodiode detector. The spectral absorbance of microhabitat samples was measured in quartz cuvettes (0.01m pathlength) against a Milli-Q water blank at 0.5 nm intervals. For ease of interpretation, absorbances were converted to transmittance values (Abs = log(1/T)). The maximum of the 300-700 nm full transmittance spectra were averaged between the three (or two) replicates for each pool (Fig 3A). This single value, the mean maximum transmittance, was then used as a representative value for phytotelm turbidity, where lower average transmittance represented darker/more turbid pools.

4. Photography and spectrophotometry measures and validation

To verify the similarities in using photography and spectrophotometry to quantify optical characteristics of the water samples, spectrophotometer and photography values were compared by converting spectrophotometer spectra to RGB values based on human colour matching functions using the `spec2rgb` function from the R package "pavo" (Maia et al. 2019). Then, the relationship between the first principal components generated for spectrophotometer and photography RGB values (Supp. Fig 8) were compared by calculating Spearman's correlation coefficient *rho*. When comparing the correlation between the PC1 of the RGB values generated from spectrophotometer data and the RGB values of photographs of the same *O. pumilio* microhabitats, we found the two measures to be significantly and positively correlated (Spearman's *rho* = 0.84, p < 0.001, Supp. Fig 8). These data support the relationship between photography and spectrophotometry, and substantiate the accuracy of the reflectance values taken for *D. tinctorius* pools (Supp. Fig 8).

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. *D. tinctorius* activity or space use is not predicted by mass difference between tadpoles or focal tadpole mass. Model parameterizations yielded identical results (significant variables highlighted), were all within 2 AICc of each other, and passed all suitability checks using the DHARMa package (residuals, dispersion, inflation). All models included random effect structure described in main text.

| Statistically significant predictors highlighted				
	Response	Final model structure	AICc	DHARMa Check
Absolute mass (Mass)	Zone 1	Background + Predator + PC1+ #conspecifics + Predator (Y/N) + **Mass**	509.06	Pass
Mass Difference (Focal-Center)	Zone 1	Background + Predator + PC1+ #conspecifics + Predator (Y/N) + **Mass difference**	509.76	Pass
Absolute mass (Mass)	Swim	Background + Predator * PC1 + #conspecifics + Predator (Y/N) + **Mass**	440.36	Pass
Mass Difference (Focal-Center)	Swim	Background + Predator * PC1 + #conspecifics + Predator (Y/N) + **Mass Difference**	440.36	Pass

Supplementary Table 2. Model parameterizations with either mass difference between tadpoles or focal tadpole mass for *O. pumilio* yielded identical results (significant variables highlighted). Neither mass difference nor absolute mass was significant in predicting tadpole space use, but did play a role in overall activity. All models passed suitability checks using the DHARMa package (residuals, dispersion, inflation). Models included random effect structure described in main text.

| Statistically significant predictors highlighted				
	Response	Final model structure	AICc	DHARMa Check
Absolute mass (Mass)	Zone 1	Background + Predator + Mean Absorbance + Mass	1140.03	Pass
Mass Difference (Focal-Center)	Zone 1	Background + Predator + Mean Absorbance + Mass difference	1139.65	Pass
Absolute mass (Mass)	Swim	Background * Mean Absorbance + Predator + Mass	823.14	Pass
Mass Difference (Focal-Center)	Swim	Background * Mean Absorbance + Predator + Mass difference	818.35	Pass

Supplementary Figure 1. Effect of size difference between focal and Odonata stimulus in *D. tinctorius* tadpoles.
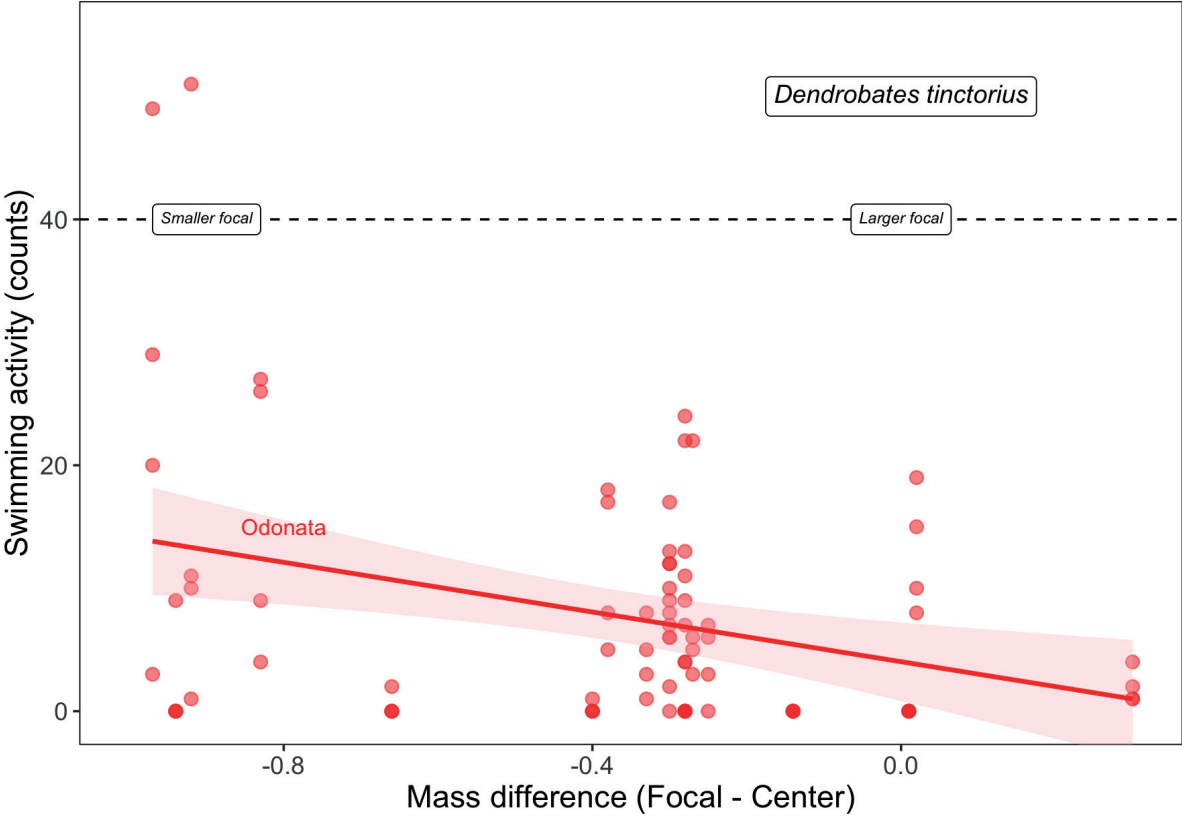
Supplementary Figure 2. The full transmittance spectra from spectrophotometer readings of each of the sampled microhabitats from La Selva, Costa Rica. Each panel represents an individual pool. Facets are ordered by increasing mean maximum transmittance.
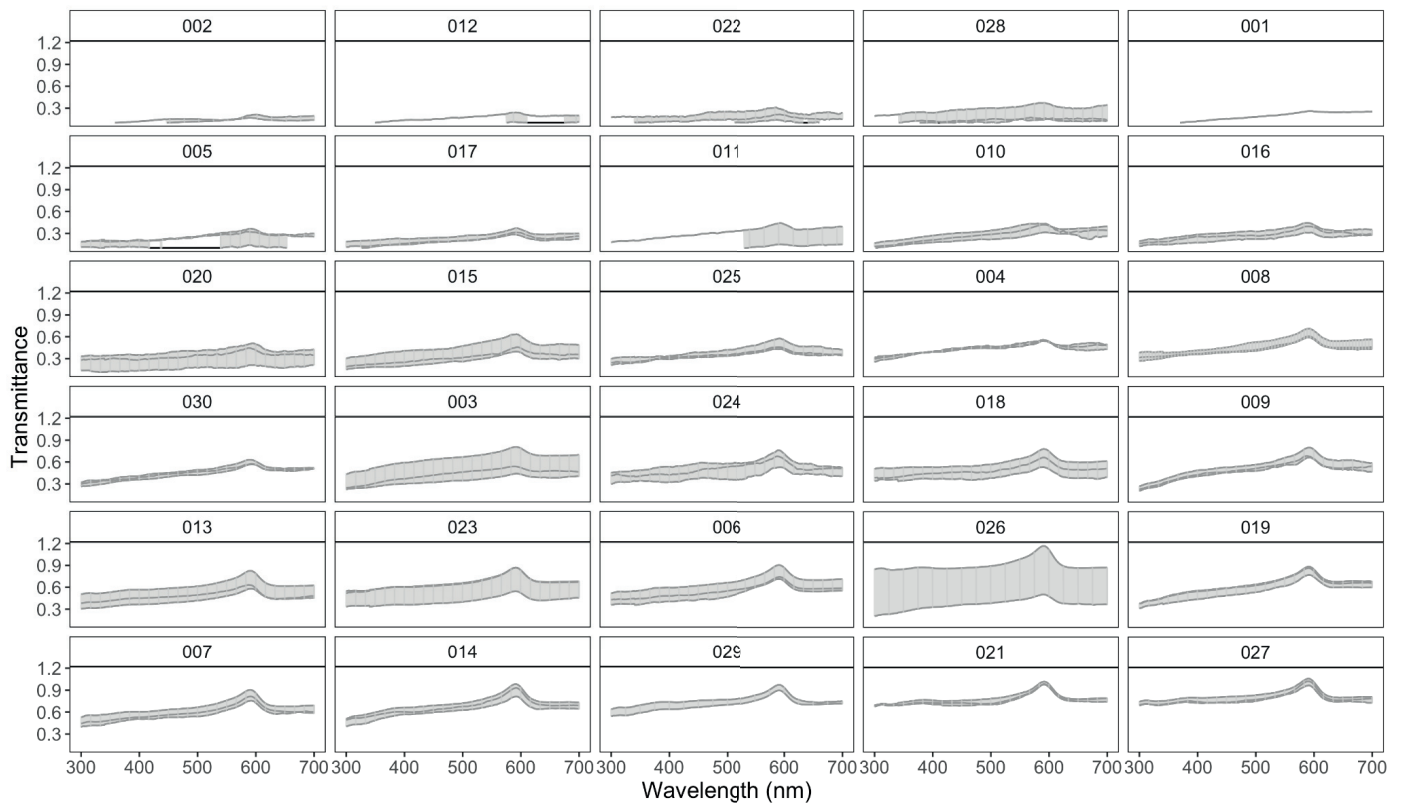
Supp. Figure 3. Comparison between spectrophotometry and photography RGB values. Panel (A) shows microhabitat transmittance spectra and their corresponding RGB values generated by the `spec2rgb` function from the "pavo" package. Panel (B) compares PC1 values generated from RGB values from both spectrophotometer and photography data. Second order polynomial fit to data with shaded regions representing the 95 CI using a "gam" smoothing function.
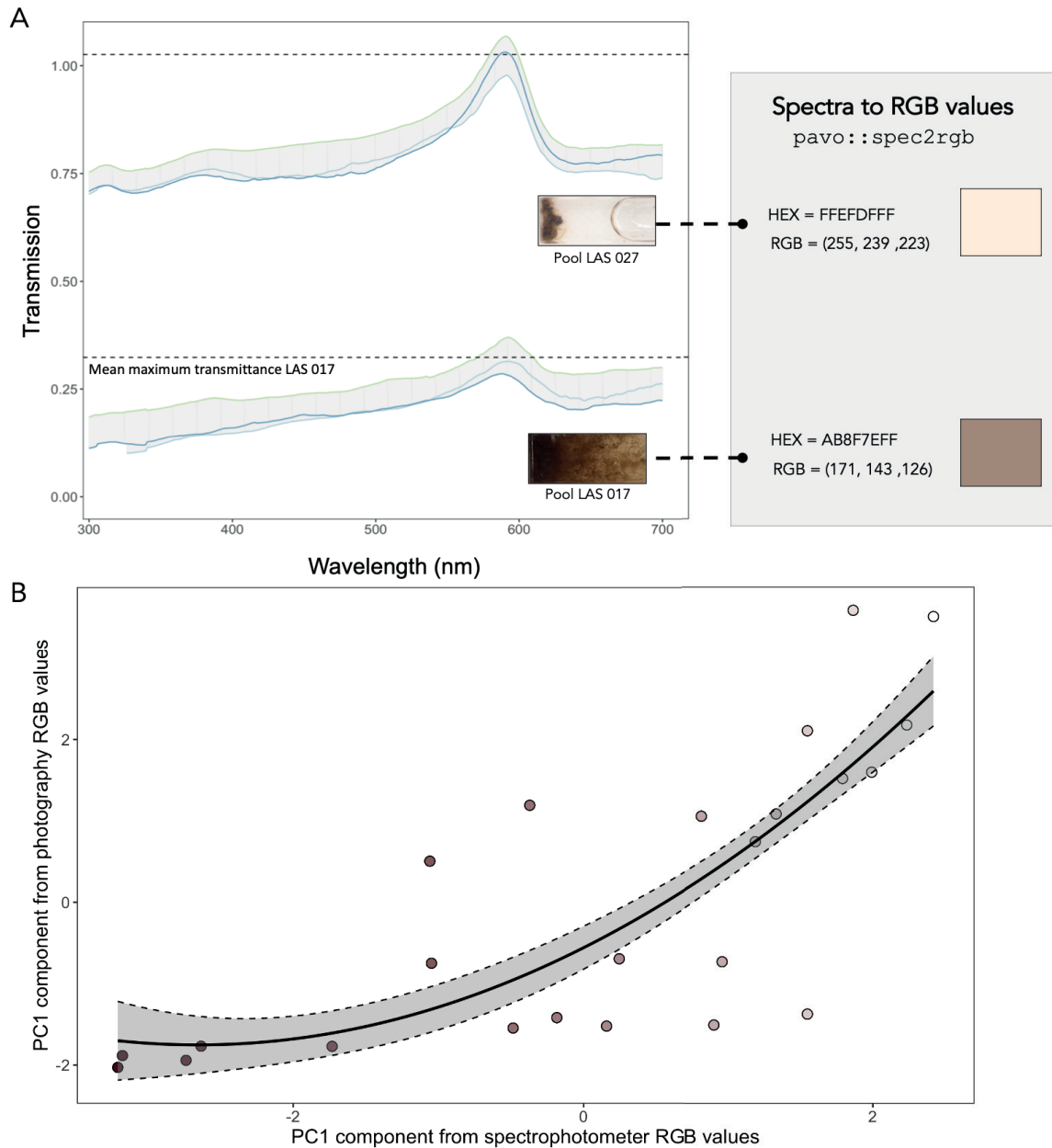
Supplementary Table 3. Model parameterizations with either spectrophotometry or photography data for *O. pumilio* yielded identical results (significant variables highlighted). All models passed suitability checks using the DHARMa package (residuals, dispersion, inflation). Models included random effect structure described in main text.

Statistically significant predictors highlighted				
	Response	Final model structure	AICc	DHARMa Check
Mean Max. Trans. Zone 1		Background + Predator + Mean Absorbance + Mass	1140.03	Pass
PC1	Zone 1	Background + Predator + %R-channel reflectance + Mass	1142.08	Pass
Mean Max. Trans. Swim		Background * Mean Absorbance + Predator + Mass	823.14	Pass
PC1	Swim	Background * %R-channel reflectance + Predator + Mass	821.14	Pass

Supplementary Figure 4. Principal component 1 (PC1) explaining the RGB values from photos of each pool occupied by *Oophaga pumilio* tadpoles. We see that negative PC1 values capture more turbid pools while positive values show more clear pools. The same trend is found in *Dendrobates tinctorius* pools (main text Fig 3).
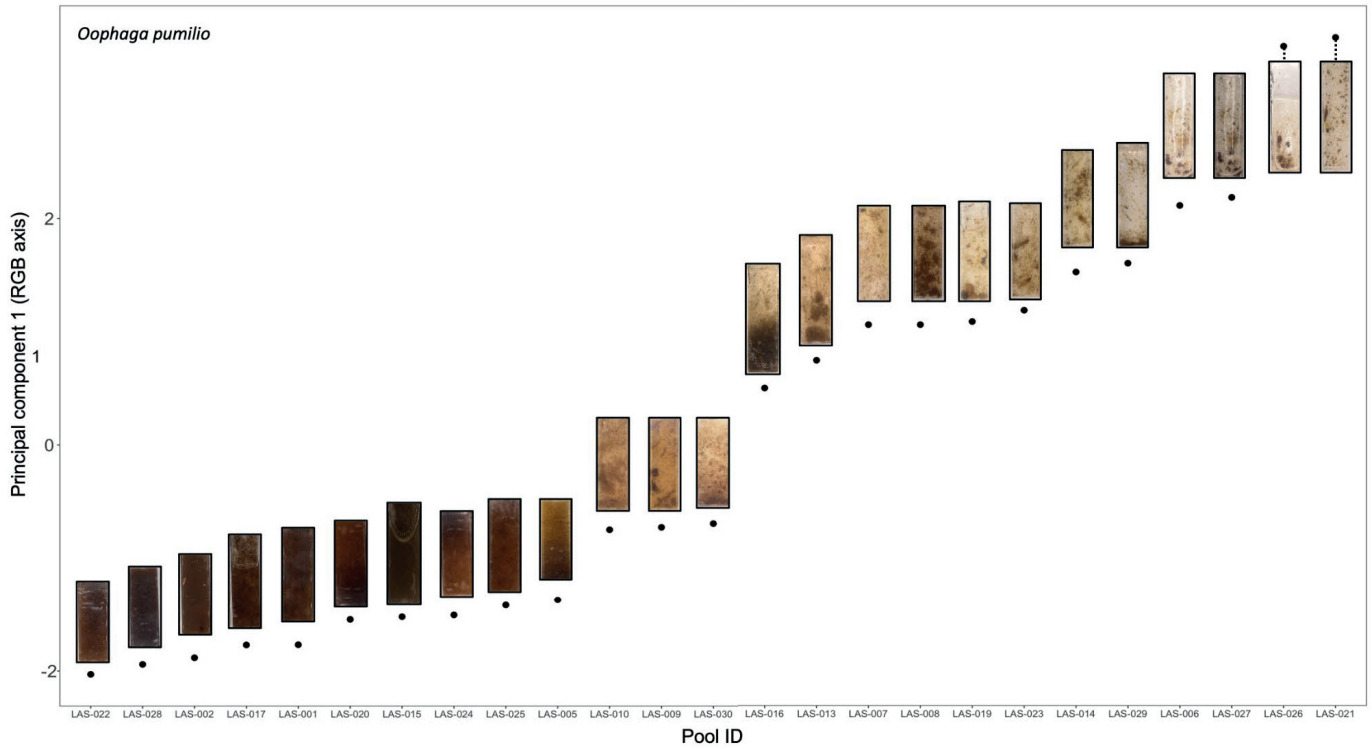
Supplementary Figure 5. Proportion of time spent on black background for *D. tinctorius* tadpoles. Points coloured by mean RGB PC1 from photos.
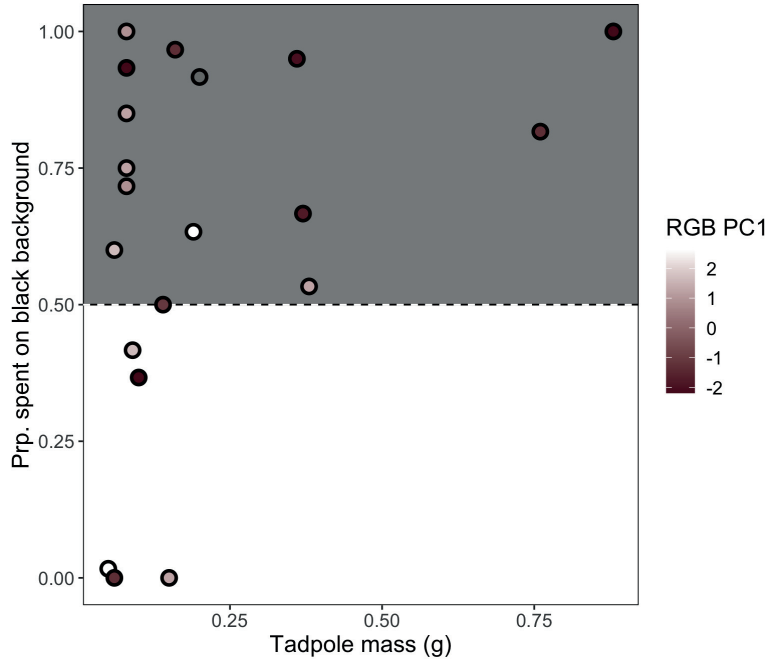
Supplementary Figure 6. Proportion of time spent on black background for *O. pumilio* tadpoles. Points coloured by mean RGB PC1 from photos.
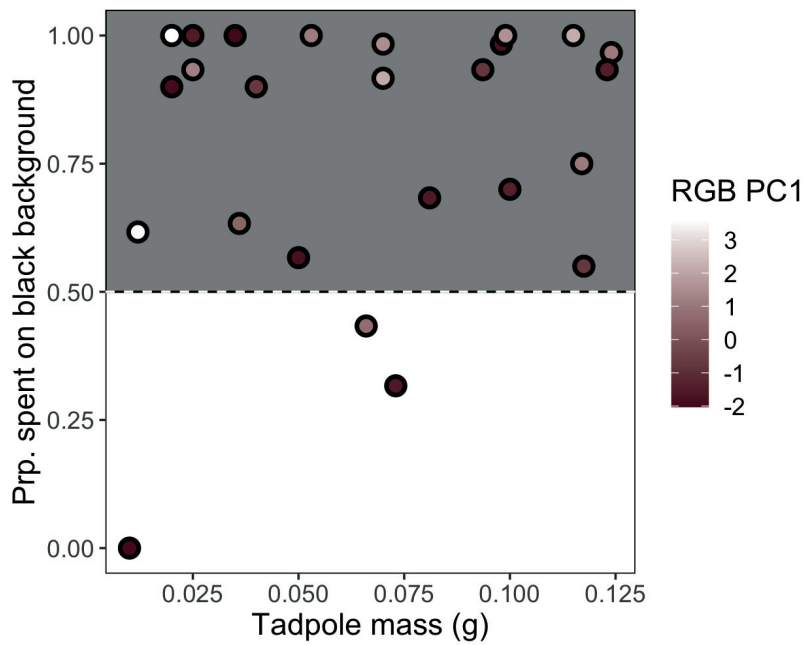
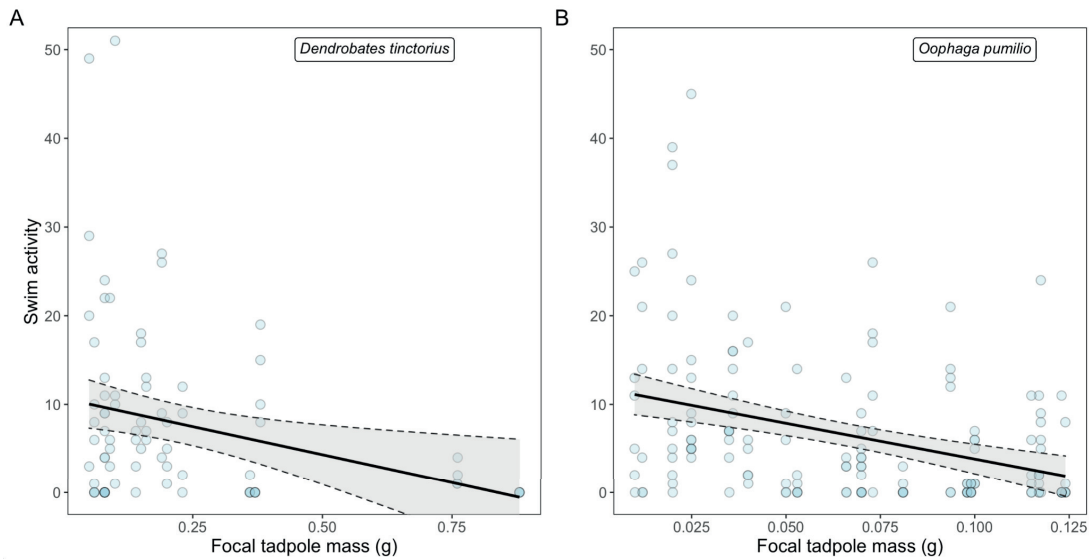**Supplementary Figure 7.** Activity and focal tadpole mass correlation. In both species, we find a negative relationship between tadpole swimming and tadpole size (where size correlates to developmental stage in both species), where larger tadpoles tend to move less. Note the difference in scale on the x-axis between species.

Supplementary Figure 8. Relationship between nursery conspecific counts and space use in *D. tinctorius*. Tadpoles from larval nurseries with more conspecifics spend less time in the arena centre. Interestingly, nursery conspecific count is highly correlated with the presence of predators (Pearson coefficient $r = 0.83$)
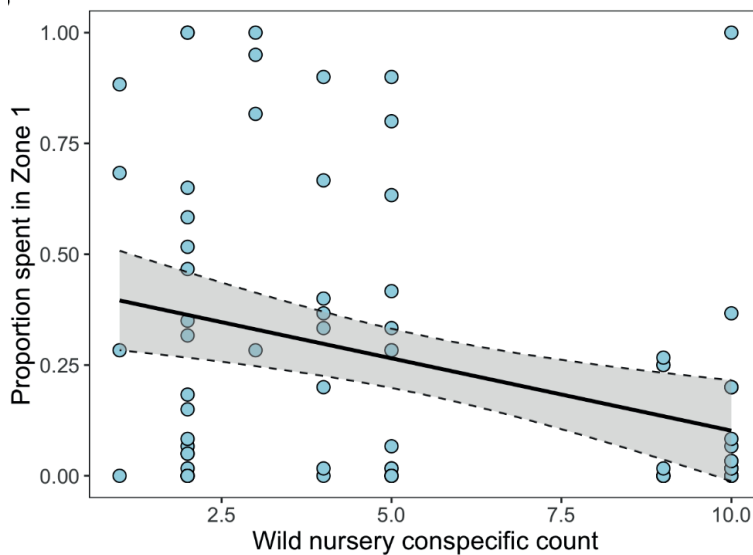
IV

SIZE-DEPENDENT AGGRESSION TOWARDS KIN IN A CANNIBALISTIC SPECIES

by

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Original Article

Size-dependent aggression towards kin in a cannibalistic species

Chloe A. Fouilloux,[a,©] Lutz Fromhage,[a] Janne K. Valkonen,[a] and Bibiana Rojas[a,b,©]

[a]Department of Biology and Environmental Science, University of Jyvaskyla, P.O. Box 35, 40014 Jyväskylä, Finland and [b]Department of Interdisciplinary Life Sciences, Konrad Lorenz Institute of Ethology, University of Veterinary Medicine Vienna, Savoyenstraße 1, 1160 Vienna, Austria

In juveniles extreme intraspecies aggression can seem counter-intuitive, as it might endanger their developmental goal of surviving until reproductive stage. Ultimately, aggression can be vital for survival, although the factors (e.g., genetic or environmental) leading to the expression and intensity of this behavior vary across taxa. Attacking (and sometimes killing) related individuals may reduce inclusive fitness; as a solution to this problem, some species exhibit kin discrimination and preferentially attack unrelated individuals. Here, we used both experimental and modeling approaches to consider how physical traits (e.g., size in relation to opponent) and genetic relatedness mediate aggression in dyads of cannibalistic *Dendrobates tinctorius* tadpoles. We paired full-sibling, half-sibling, and non-sibling tadpoles of different sizes together in an arena and recorded their aggression and activity. We found that the interaction between relative size and relatedness predicts aggressive behavior: large individuals in non-sibling dyads are significantly more aggressive than large individuals in sibling dyads. Unexpectedly, although siblings tended to attack less overall, in size-mismatched pairs they attacked faster than in non-sibling treatments. Using a theoretical model to complement these empirical findings, we propose that larval aggression reflects a balance between relatedness and size where individuals trade-off their own fitness with that of their relatives.

Lay Summary
Before you eat someone, you have to attack them first. Here, we investigated the factors that shape aggression in the cannibalistic tadpoles of the dyeing poison frog. We find that aggression depends on both size and relatedness: when set in pairs, large tadpoles are half as aggressive towards their smaller siblings than to nonsibs. It looks like belonging to the same family provides some protection against aggression, though no one is ever truly safe.

Key words: cannibalism, kin discrimination, larval aggression, poison frog, tadpole.

INTRODUCTION

Aggression is often a necessary precursor to cannibalism, as individuals must subdue their counterpart before consuming them (Sakakura and Tsukamoto 1997; Caldwell and de Araujo 1998; Lund et al. 2016). In juveniles, which typically do not hold territories nor compete for mates, the function of escalated aggression is primarily to monopolize nutritional resources (either realized or potential) as most of their energy is invested into growth. In systems with sibling aggression, fighting represents an important potential advantage in early development for securing resources (Drummond et al. 2003; Naidenko and Antonevich 2009); in cannibalistic species, the factors that shape opponent assessment are vital, as there is the threat of interactions escalating to death. Thus,

cannibalism is often conditional on the assessment of either the environment (food availability: Mayntz and Toft 2006; Dugas et al. 2016a, conspecific density: Maret and Collins 1994, Frankino and Pfennig 2001; or a combination of the two: Wildy et al. 2001) or the opponent (size and relatedness: Dugas et al. 2016b, condition: Ibáñez and Keyl 2010).

Empirically, many studies have found that winners of cannibalistic interactions are larger than losers (Claessen et al. 2004; Ibáñez and Keyl 2010; Barkae et al. 2014; Rojas 2014), although exceptions exist when larger individuals are weakened (Richardson et al. 2010) or when individuals compensate for their size with increased aggressiveness (Issa et al. 1999). Kinship between individuals can also explain aggression. This has been shown to be an important factor in several cannibalistic species that demonstrate kin discrimination and avoid eating kin (Pfennig et al. 1994; Pfennig and Frankino 1997; van den Beuken et al. 2019), although there are

Address correspondence to C.A. Fouilloux. E-mail: chloe.a.fouilloux@jyu.fi; B. Rojas. E-mail: bibiana.rojas@vetmeduni.ac.at.

also examples of cannibals consuming their kin without avoidance (Boots 2000; Gray et al. 2009). Although differences in opponent size and relatedness have individually been identified as variables that shape cannibalistic decisions, the interaction between these two variables has yielded diverse results across taxa where, for example, studies have reported a strong interactive effect in earwigs (Dobler and Kölliker 2011), the absence of size effect in spiders (Bilde and Lubin 2001; Roberts et al. 2003), and both a stage and phenotype dependent adversity where spadefoot toads are less likely to cannibalize other cannibals (Pfennig 1999) as well as more developed siblings (Dugas et al. 2016b). Ultimately, more work is needed to tease apart the factors influencing decision-making in juvenile cannibals in a broader range of taxa. Notably, in low-fecundity systems where each case of cannibalism may represent a substantial loss to the parents, understanding the adaptive significance of cannibalism seems all the more pressing.

Dendrobates tinctorius is a Neotropical poison frog with parental care whose larvae are facultative cannibals (Rojas 2014). Tadpoles are often deposited by their fathers in ephemeral pools of water, where they are confined until metamorphosis (Rojas and Pašukonis 2019). While tadpoles are most often transported singly, the ephemeral pools in which they are deposited can have multiple tadpoles of various developmental stages (Rojas and Pašukonis 2019) and degrees of relatedness (Rojas B, and Ringler E, unpublished data). In these environments, cannibalism is common (Rojas 2014, 2015), but not necessary for the successful development and metamorphosis of an individual tadpole. In closely related poison frogs, cannibalism is usually an outcome of sequentially intensified attacks (Summers and Symula 2001; Gray et al. 2009), although exceptions where tadpole aggression does not include cannibalism exist (i.e., obligate egg-feeders with parental care, Dugas et al. 2016a). Here, we aim to better understand what drives cannibals to express aggression towards conspecifics and disentangle the apparent variation that exists in this behavior.

For *D. tinctorius*, the costs of cannibalism are direct, as attacking kin can reduce inclusive fitness and the potential for injury (even with a small counterpart) is high. The long-term potential benefits, on the other hand, are yet to be established in detail. Fundamental work in systems with sibling aggression posits that aggression towards kin evolves when the benefits are greater than the (in)direct fitness costs associated with fights (Parker et al. 1989). For example, consuming a conspecific could shorten the cannibal's time to metamorphosis and increase size thereafter (as observed in frogs: Crump 1990; spiders: Mayntz and Toft 2006; salamanders: Wildy et al. 1998). This could, in turn, translate into escaping precarious conditions and improving fitness (Wissinger et al. 2004). Here we build game theory models post hoc to theoretically investigate the evolutionary forces that shape aggressive encounters in *D. tinctorius*. As we will show in the theoretical part of this study, small changes in the assumptions about the size-dependent costs of aggressiveness can lead to qualitatively different predictions of behavior. We therefore derive a range of alternative predictions to serve as alternative hypotheses for the empirical component of our study. In this experiment we considered size and relatedness to better understand the basis of aggression in a cannibalistic species. We conducted behavioral assays between dyads of *D. tinctorius* tadpoles, and measured aggression and activity in response to changes in relative size differences and relatedness between pairs.

In the context of cannibalism, recording general activity levels in addition to aggression itself can help to elucidate the underlying behavioral mechanisms (Kralj-Fišer et al. 2012; Adriaenssens and Johnsson 2013; Vallon et al. 2016); for example, an increase in activity could be a result of either attacking or evasion by tadpoles, whereas a decrease could be either stealth or a freezing response. It is important to note that although aggression has often been used as a proxy for cannibalism throughout this family (Caldwell and De Araujo 1998; Summers and Symula 2001), we cannot fully disentangle whether conspecific aggression is truly an attempt at predation or an act of resource defense, where more aggressive tadpoles would acquire a foraging benefit. Nevertheless, because both tadpoles are confined to the same pool of water throughout development, either predation or resource-holding behaviors converge on the same outcome of additional feeding opportunities. Together, these experiments and models contribute to our understanding of how intraspecies aggression is shaped by the relatedness and size differences of competitors that may cannibalize each other.

METHODS

Study species

Dendrobates tinctorius has elaborate parental care. Males attend small terrestrial clutches and transport newly hatched tadpoles, one or two at a time, to pools of water where they are left until metamorphosis. Males carrying more than one tadpole at once can be seen either depositing both tadpoles in the same pool or distributing tadpoles between pools (Rojas and Pašukonis 2019). The tadpoles are omnivorous and frequently demonstrate cannibalism (Rojas 2014, 2015); despite this, it is not unusual to see several tadpoles, at various stages of development, coexisting within the same pool in the wild (Rojas and Pašukonis 2019; Fouilloux et al. 2021).

We used tadpoles from a breeding laboratory population of *D. tinctorius* kept at the University of Jyväskylä, Finland. We used a paternal half-sibling design as it could be expected that paternal half-siblings are more likely to co-occur as a result of fathers reusing pools after multiple transport events. Tadpole dyads were assigned in response to 1) individuals needing to be visually distinguishable from each other (i.e., size), and 2) the laboratory mating schedule/network, which was prioritized so as to not stress the animals from overbreeding. Most breeding pairs laid clutches (3–7 eggs) weekly, which allowed us to use tadpoles of diverse sizes throughout the experiment. Adult pairs were each housed in a 115L terrarium that contained layered expanded clay, leaf-litter, moss substrate and were equipped with a shelter, logs, and live plants. Terraria were maintained at 26 °C (±2 °C) and were automatically misted with reverse osmosis water four times a day (maintaining a humidity around 95%) and lit with a 12:12 photoperiod. Frogs were fed live *Drosophila* fruit flies coated in vitamin supplements five times per week. Tadpoles were raised singly in $10 \times 6.5 \times 5$ cm containers that were filled with spring water, and fed ad libitum a diet of fish food (JBL NovoVert flakes) three times a week. Adult and tadpole health and water levels were checked daily.

Behavioral trials

Pairs of tadpoles of different degrees of relatedness (full-sibling, half-sibling, non-sibling) were placed together in an arena. Tadpoles in early larval development were used, that is, before the toe differentiation in hind legs development to control for possible life-history effects (stage 31, Gosner 1960). Experimental tadpole weight ranged from 0.04 g to 0.38 g, and mass differences between pairs ranged from 0.03 g to 0.30 g. Blinding in the experiment was not possible, as the set-up and experiment were conducted by the same person,

but the order of trials was assigned randomly. The arena was an 18.5 cm by 12 cm clear plastic container filled with 400 mL of spring water. Initially, each tadpole was placed on either side of an opaque partition dividing the arena; this partition kept tadpoles separated but allowed water to flow throughout the container. After an acclimation period of one hour, tadpole activity (resting, swimming) of the separated individuals was recorded every 15 s for 10 min.

After the acclimation and separated observation, the barrier was removed and tadpole interactions were recorded for 60 min. Behaviors (resting, swimming, biting, and chasing; see Supp. Table 1 for descriptions) were recorded for both tadpoles every 15 s. Tadpoles were visually distinguishable due to size differences, as heavier tadpoles were larger. Individuals were photographed and weighed before the beginning of each trial to establish initial tadpole condition, and were only used once (n_{Trial} = 15 for each relatedness level, n = 90 tadpoles for the entire experiment).

Trials were ended prematurely if tadpoles demonstrated aggression levels that would cause severe damage or death (where bites lasted for more than 2 s, recorded as "potential lethal attack"). Although aggression was common, potential lethal attacks were rare, occurring in only 3/45 trials. There were no tadpole deaths as a result of the behavioral trials, and all tadpoles were kept and reared in the laboratory after the experiment. Assay methods followed the Association for the Study of Animal Behaviour's guidelines for the treatment of animals in behavioral research and teaching (ASAB 2018), and were done with the approval of the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland (ESAVI/9114/04.10.07/2014).

Statistical analysis

All models and statistics were performed in the program R (v. 3.6.1, R Development Core Team 2019) with additional packages "glmmTMB" (Brooks et al. 2017), "coxme" (Therneau 2020), "dplyr" (Wickham et al. 2018), "tidyr" (Wickham et al. 2019). Activity and aggression analyses (see below) took into account pair identity (Pair_ID) and family (breeding pair) level random effects. We included pair ID as a random effect because we needed to consider that individuals were not independent within pairs; similarly for family, the behavior of siblings was likely partly correlated, and we wanted to account for that possibility. Differences in duration of trials during experiments (n = 3/45 trials ended early due to potential lethal attacks) were taken into account by offsetting models with a trial duration. The structure of the aggression and activity models was based on the interaction between relative size (two-level categorical, where tadpoles were assigned a relative size (large/small) within a dyad) and relatedness (three-level categorical, sibling/half-sibling/non-sibling). Based on the size effect having predicted cannibalism in previous experiments in the wild (Rojas 2014), which reported the effect of size on the latency to cannibalism, we hypothesized that relative size differences must play a part in shaping aggressive decisions between kin. Residual diagnostics (zero-inflation, residual patterns, and over/underdispersion) were checked using the "DHARMa" (Hartig 2020) package, all of the final models passed diagnostic checks.

Activity levels

Tadpole activity was categorized as "resting" and "swimming" (see Supp. Table 1 for details). Tadpole activity was observed during post-acclimation (10 min) and experimental (max. 60 min) periods. These measures provided an assessment of how tadpoles behaved before and after visual/physical contact, and help contextualize the role of activity versus aggression. Activity was coded as counts and was modeled in a generalized linear mixed model framework (GLMM). Because these data were overdispersed, they were modeled using a negative binomial parameterization, which adjusts the variance independently of the mean.

Overall aggression

Aggression between tadpoles was observed as chasing or biting, which were recorded as counts. These two behaviors were combined to represent "total aggression". These data provide a direct measure of aggression between dyads, which have generally been considered as a precursor to cannibalism in this family. These data were fit with a Poisson family with a log link.

Latency to first bite

Latency data were built by selecting the "first biter" within a pair, which involved subsetting the original data set. We modeled latency to first bite using a mixed effect Cox proportional hazards model. Survival object was parameterized with respect to latency to first bite event and absolute biting (0/1, where 0 represents no biting occurred during the trial) in response to the interaction of relatedness and mass difference between tadpole dyads. The first bite within a dyad is of interest because taste may play a role in kin recognition (as with salamanders; Pfennig et al. 1994); thus, this behavior could serve as an initial assessment, but is risky as it exposes tadpoles to potential attacks. Mass difference was calculated as the difference between tadpole pairs: this value was always positive because large tadpoles were always heavier. Using subsetted data, each pair identity was independent, so only "Family" was used as a random variable.

Game theory model

We modeled pairwise interactions between tadpoles arbitrarily labeled as 1 and 2. We assumed that only one tadpole per pair survives ("wins"), and that the probability of winning depends on each individual's competitive strength. Competitive strength θ_i of tadpole i was calculated based on its relative **size**, s_i and its **aggressiveness**, a_i as $\theta_i = s_i \cdot a_i$. This multiplicative formulation reflects the biological idea that a given increment in aggressiveness should have a greater effect on a large than a small tadpole's competitive strength. Individual 1's probability of winning is given by its **relative competitive strength**, as $\omega_1 = \frac{\theta_1}{(\theta_1 + \theta_2)}$. The reproductive success ("**direct fitness**", v_i) of the winning tadpole was modeled under three assumptions: (*1a*) v_i is size-independent, as $v_i = 1 - a_i^2$; (*1b*) v_i is proportional to size (for a given level of aggressiveness), as $v_i = s_i - a_i^2$; and (*1c*) v_i is size-dependent due to aggressiveness being costlier for smaller tadpoles, as $v_i = 1 - \left(\frac{a_i}{s_i}\right)^2$ (see Figure 4 for visualization). In all three formulations costs increased at an accelerating rate, such that low levels of aggression had low costs whereas high levels of aggression could be extremely costly; this was done to account for the increasing danger and energy expense associated with more violent behaviors.

Finally, the inclusive fitness of the surviving tadpole was calculated as $v_1 - r\, v_2$, where r is the relatedness between the pair. This formulation reflects the idea that winning involves the killing of a relative that would have had reproductive success v_2 had it survived. The inclusive fitness of the losing tadpole is zero, because the losing tadpole neither reproduces nor affects the other tadpole's reproduction.

We calculated the expected (i.e., probability-weighted mean) inclusive fitness of tadpole 1 as $F_1 = \omega_1(v_1 - r\, v_2)$. For given values of s_1, s_2, and a_2 we numerically determined individual 1's optimal aggression level as the value of a_1 that maximizes its expected inclusive fitness. By computing individual 1's "best response" aggression level for any given a_2 which its opponent might exhibit, we then identified pairwise optimal aggression levels that are best responses to each other.

RESULTS

Activity levels

We observed tadpole activity during both post-acclimation and experimental phases. While tadpoles were separated by an opaque barrier during the post-acclimation phase (but water still freely moved throughout the arena) we did not detect any differences in activity between any of the experimental treatments. During the experiment, however, we found that large tadpoles across all relatedness treatments were significantly more active than small tadpoles (negative binomial GLMM, CI: 1.79–3.58, $z = 5.23$, $P < 0.001$; see Figure 1, Table 1).

When comparing models, we found that random effects of pair ID had higher between-subject variance ($\tau_{00} = 0.19$) than tadpole family ($\tau_{00} = <0.001$) during post-acclimation activity (Table 1, Panel A), indicating that when separated, there was less variation in behavior on a family level. Yet, while interacting during the experiment this difference disappears (Table 1, Panel B). In both cases, between-subject variance is low, indicating that across families and pairs of tadpoles, activity levels are similar.

Overall aggression

The total aggression expressed by individuals could be predicted by the interaction between relative size and relatedness between dyads. We found that the interaction term of the model was significant overall (ANOVA, $P = 0.004$, $\chi^2 = 10.905$, df = 2). Large tadpoles from sibling dyads were significantly less aggressive than the large tadpoles from non-sibling dyads, exhibiting almost half the amount

of aggressive behaviors as large non-siblings (Figure 2, Poisson GLMM, $z = -3.170$, $P = 0.002$, Table 2). Half-siblings were not significantly different from either treatment. After our expectations of creating unique pair interactions, the random effect of pair identity had a high between group-variation ($\tau_{00Pair\_ID} = 1.04$, Table 2), but families differed little from each other ($\tau_{00Family} = 0.13$, Table 2).

Latency to first bite

The initial aggression between tadpoles depended on the interaction between mass difference and relatedness between dyads. We used biting as a measurement of first aggression because it consistently represented the first aggressive contact in pairs. Based on a mixed effect Cox proportional hazards model, we assessed the risk of first attack when considering relatedness and mass difference between pairs. We detected a significant interaction between relatedness and mass difference, where closely related pairs displayed more immediate aggression when dyad mass differences were large. In other words, siblings bit their counterpart faster when mass differences between pairs were greater (Cox mixed effects, $z = 2.209$, $P = 0.022$, see Table 3). For example, at a large mass difference (>0.15 g between tadpoles) siblings were more than 40% more likely to bite than non-siblings within the first 5 min of a trial. Interestingly, non-siblings demonstrated a seemingly inverted behavioral trend, where dyads with large mass differences had delayed aggression. Half-siblings did not behave significantly differently from either treatment. In trials where biting was exhibited, large tadpoles were most often the first aggressor ($n = 8/13$ for siblings; $n = 10/13$ for half-siblings; $n = 11/12$ for non-siblings).

Game theory model

Based on our three formulations (1a–c) we varied the impact of size to model aggression levels of tadpoles with different degrees of relatedness. The version where aggression was both size-dependent and costlier for the smaller tadpoles (Figure 4, third row) appeared
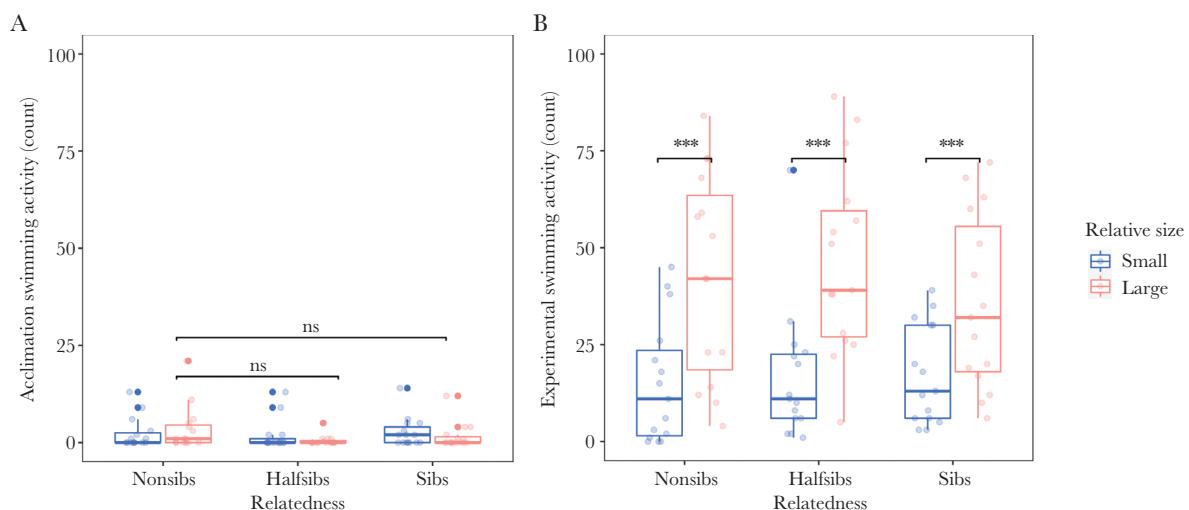
Figure 1

Tadpole activity levels before and during experimental trials. Panel (**A**) shows the post-acclimation activity of tadpoles. We found no difference in swimming between large and small tadpoles or relatedness treatments during this phase. Panel (**B**) shows experimental activity throughout behavioral trials. Large tadpoles were significantly more active than small tadpoles during assays. $N_{Trial} = 15$ for each relatedness level. Large tadpoles are in pink and small tadpoles in blue. Boxplot medians are depicted by thicker lines, whiskers span ± 1.5 * interquartile range.

Table 1

Summary of negative binomial GLMM with linear parameterization of tadpole activity. (A) activity of tadpoles was not affected while tadpoles were physically separated. (B) large tadpoles were overall more active once the barrier was removed. Models for (A) and (B) were predicted by interactive effects of relative size and relatedness. Tadpole dyads (Pair_ID) and family were accounted for as random effects, CI represents 95% confidence interval. Differences in trial time during the experiment ($n = 3/45$) were accounted for by using duration as offset in the model. σ^2 represents residual variance and τ_{00} represents random intercept variance

(A)	Post-acclimation activity			
Predictors	Estimate	CI	z	P
(Intercept)	0.72	−0.14–1.58	1.64	0.101
Half-siblings	−0.63	−1.87–0.62	−0.98	0.326
Siblings	0.24	−0.78–1.27	0.47	0.639
Size (large)	0.39	−0.57–1.34	0.79	0.428
Half-siblings: size (large)	−0.63	−2.33–1.07	−0.73	0.466
Siblings: size (large)	−0.90	−2.30–0.51	−1.25	0.211
Random effects				
σ^2	1.68			
$\tau_{00\ \text{Pair\_ID}}$	0.19			
$\tau_{00\ \text{Family}}$	< 0.001			
(B)	Experimental activity			
Predictors	Estimate	CI	z	P
(Intercept)	0.00	0.00–0.01	−22.21	**<0.001**
Half-siblings	1.25	0.66–2.36	0.69	0.489
Siblings	1.52	0.82–2.80	1.34	0.181
Size (large)	3.46	1.96–6.09	4.30	**<0.001**
Half-siblings: size (large)	0.81	0.38–1.73	−0.54	0.588
siblings: size (large)	0.51	0.24–1.08	−1.76	0.079
Random effects				
σ^2	0.43			
$\tau_{00\ \text{Pair\_ID}}$	<0.001			
$\tau_{00\ \text{Family}}$	0.01			

Bold values represent significant values.
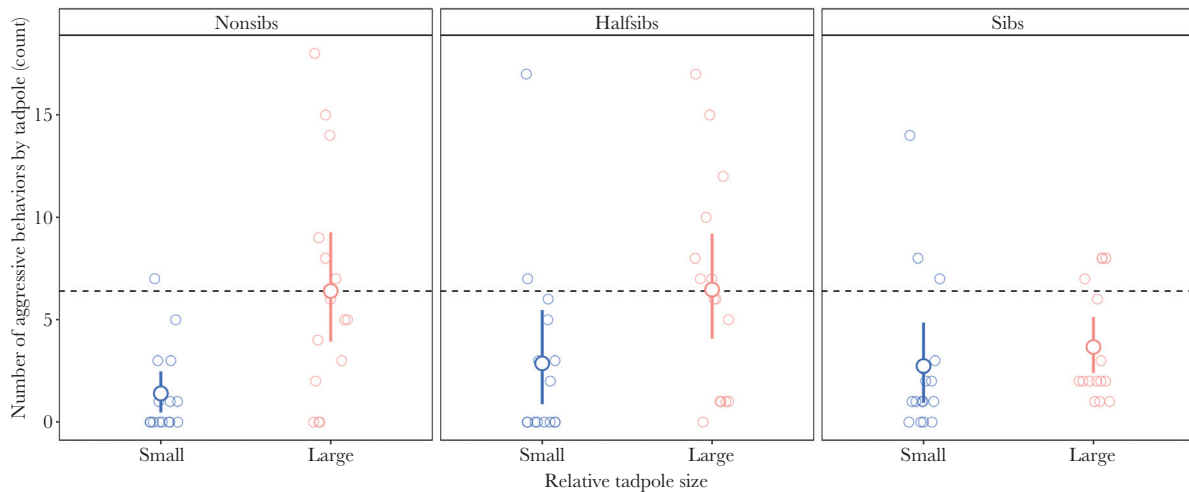
Figure 2

Differences in aggression across relatedness treatments with respect to relative size between dyads. Point ranges indicate mean for each category with bootstrapped 95% confidence intervals. Pink points represent large tadpoles and blue dots represent small tadpoles. Dashed line indicates mean aggression for large tadpoles from non-sibling dyads. $N_{\text{Trial}} = 15$ for each relatedness level. There was significantly less aggression by large tadpoles from siblings dyads when compared with large tadpoles from non-siblings dyads.

most consistent with our empirical data (Figure 2), in that larger tadpoles were consistently predicted to be more aggressive than their smaller counterparts, and overall aggression by large tadpoles decreased with relatedness.

DISCUSSION

Dendrobates tinctorius tadpoles are subject to their parents' deposition decisions, where males—counter-intuitively—will frequently deposit smaller conspecifics with larger cannibals (Rojas 2014). In

Table 2

Summary of Poisson GLMM of tadpole aggression. Total aggression (total count of biting and chasing) was predicted by the interaction between relative size (two-level categorical variable) and relatedness. Tadpole dyads (Pair_ID) and family were accounted for as random effects, CI represents 95% confidence interval. Differences in trial time during the experiment ($n = 3/45$) were accounted for by using duration as offset in the model. σ^2 represents residual variance and τ_{00} represents random intercept variance

Predictors	Total aggression			
	Estimate	CI	z	P
(Intercept)	−8.03	−8.79−−7.28	−20.87	**<0.001**
Half-siblings	0.42	−0.60–1.45	0.81	0.416
Siblings	0.54	−0.43–1.50	1.09	0.275
Size (large)	1.42	0.85–1.98	4.92	**<0.001**
Half-siblings: size (large)	−0.40	−1.21–0.40	−0.98	0.327
Siblings: size (large)	−1.12	−1.82−−0.43	−3.17	**0.002**
Random effects				
σ^2	0.34			
$\tau_{00 \ \text{Pair\_ID}}$	1.04			
$\tau_{00 \ \text{Family}}$	0.13			

Bold values represent significant values.

Table 3

Mixed effects Cox proportional hazards model. Time to first aggressive behavior was predicted by the interaction of the mass difference between tadpoles and their relatedness; family is taken into account as a random effect. There is a significant interaction between relatedness and mass, where siblings of similar masses have a shorter latency to aggression than non-siblings. Mass_Diff is the difference in weight between large and small tadpoles

Predictors	Latency to first bite			
	Estimate	CI	z	P
Half-siblings	−1.27	−2.83–0.30	−1.59	0.113
Siblings	−1.44	−3.12–0.24	−1.68	0.093
Mass_Diff	0.89	−8.65–10.44	0.18	0.854
Half-siblings: Mass_Diff	9.62	−2.14–21.38	1.60	0.109
Siblings: Mass_Diff	16.32	1.80–30.83	2.20	**0.028**

Bold values represent significant values.

this system, the study of how tadpoles interact and manage their aggression is crucial to understanding their father's unexpected deposition behavior which differs from poison frogs that avoid pools occupied by predatory tadpoles (Schulte et al. 2011). Here, we observed aggression between *D. tinctorius* tadpoles in resource-abundant, low-density conditions. Empirically, we found that aggression is common (Rojas 2014; Fischer et al. 2020), and depends on the interaction between relative size and relatedness between tadpoles. From a theoretical perspective, we found that aggression in this system is probably costlier for smaller tadpoles, as making this assumption yielded predictions that qualitatively matched the empirical observations (formulation 1c, bottom row of panels in Figure 4). Combining empirical and theoretical methods, we found that relatedness and physical attributes interact in shaping overall aggression, latency to aggression, and even activity levels in a context-dependent way.

Interacting predictors of aggression

In animals where aggression can escalate to cannibalism, the majority of studies focus on the causes that underlie the killing and consumption of conspecifics. This previous work has been primarily done in insects (but see Dugas et al. 2016b for a similar study done with spadefoot toads) and has yielded a variety of results (interaction between relatedness and size: Dobler and Kölliker 2011, relatedness effect only:

Bilde and Lubin 2001; Roberts et al. 2003), providing no consistent pattern to extrapolate to cannibalistic vertebrates. In *D. tinctorius*, where there is high offspring investment (i.e., male parental care and low fecundity), we found that large tadpoles (where size is relative between pairs) from sibling dyads were the least aggressive, expressing almost half the amount of aggression compared with large tadpoles from non-sibling dyads (Figure 2). The importance of size differences in predicting aggression was expected: Rojas (2014) established that cannibalism between *D. tinctorius* tadpoles occurs faster with increasingly size-mismatched pairs. In fact, across the animal kingdom, the aggressor in a pair/group is most often the larger individual, which typically faces a smaller risk of injury (Mock et al. 1987; Mayntz and Toft 2006; Ibáñez and Keyl 2010). However, our findings highlight that in this system aggression is not solely mediated by size differences, but that some form of kin discrimination is also at play. To understand the influence of potential kin recognition in modulating aggression, we designed our experiment to include a range of relatedness coefficients between pairs ($r = 0.5$ full-sibling; $r = 0.25$ half-sibling; $r = 0$ non-sibling). Surprisingly, these half-siblings did not differ significantly from either of the other relatedness treatments. Overall, large tadpoles from the half-sibling treatment exhibited similar mean levels of aggression as large tadpoles from the non-sibling treatment, suggesting that if kin recognition does occur it may not function on as fine a scale as for other cannibals (i.e., aversion to cannibalizing cousins in salamanders, Pfennig et al. 1994).

When we compare activity and aggression results, these data reveal a potential mechanism by which tadpoles assess one another. Initially, during the acclimation period we found no differences in activity across treatments (Figure 1A). At this stage, individuals were separated by an opaque physical barrier, therefore removing visual information, but shared the same water, allowing for the transmission of chemical cues. Once the experiment began, the barrier was removed and the tadpoles were allowed to physically interact: here, all large tadpoles within dyads were significantly more active than their smaller counterpart (Figure 1B). Intriguingly, though all large tadpoles across relatedness treatments had similar levels of activity, only non-siblings frequently shifted action into attack. Non-siblings exhibited twice the amount of aggression towards their smaller counterpart than siblings, despite swimming the same amount (see Supp. Fig 2). While we are unsure what cues are being used to discriminate kin in this species, it appears that the visual assessment of conspecifics could play a role in aggressive decisions (the role of vision in activity has also been shown by Kumpulainen et al. in preparation). Overall, recognition amongst larvae is relatively common in amphibians (Waldman 1984; Blaustein and Waldman 1992) and in combination with our latency data (that suggests that initial aggression is shaped by the interaction between relatedness and size differences between pairs), we hypothesize that *D. tinctorius* tadpoles may be using both olfactory or taste cues to discriminate kin (as shown in salamanders, Pfennig et al. 1994 and *Xenopus* sp., Dulcis et al. 2017). Kin discrimination then appears to be used in a context-dependent manner depending on size differences between pairs, which through visual assessment can serve to initiate or escalate aggression. When we consider this from an evolutionary perspective, the context-dependent nature of aggression suggests that the value of kin discrimination is

lower in this species; it may be that aggression provides an overall benefit in securing resources or that, in some cases, escalated aggression (and eventual cannibalism) benefits individual survival enough to outweigh the fitness costs of consuming kin.

The escalation of aggression

The escalation of aggression between individuals is often overlooked or dismissed in systems where cannibalism occurs. These behavioral data can be valuable in understanding opponent assessment and decision-making in cannibals, as there may be unexpected costs paid in terms of energy expenditure and opponent retaliation that are shaping aggressive encounters. Intuitively, one might expect that there would be less aggression between size-mismatched pairs as large size differences may provide a cue to the smaller individual that it is unlikely to win (seen in salamanders: Brunkow and Collins 1998); however, in cases of extreme intraspecific aggression (i.e., the possibility of escalating to cannibalism), aggressive interference models predict aggressive encounters to occur more frequently with increasing size differences (Persson 1985; Polis 1988).

Here, aggressive attacks between pairs were recorded across all relatedness treatments and sizes. Although less common, small tadpoles were sometimes quicker to exhibit aggression than their larger counterparts (Figure 3) and, in some instances, were even more aggressive than large tadpoles (this was observed only in sibling and half-sibling treatments). Latency to attack changed as a function of mass differences between pairs and the magnitude of this change was dependent on relatedness. When pairs were closer in weight, non-siblings attacked faster; in contrast, when mismatched in weight, non-siblings delayed aggression (Figure 3).
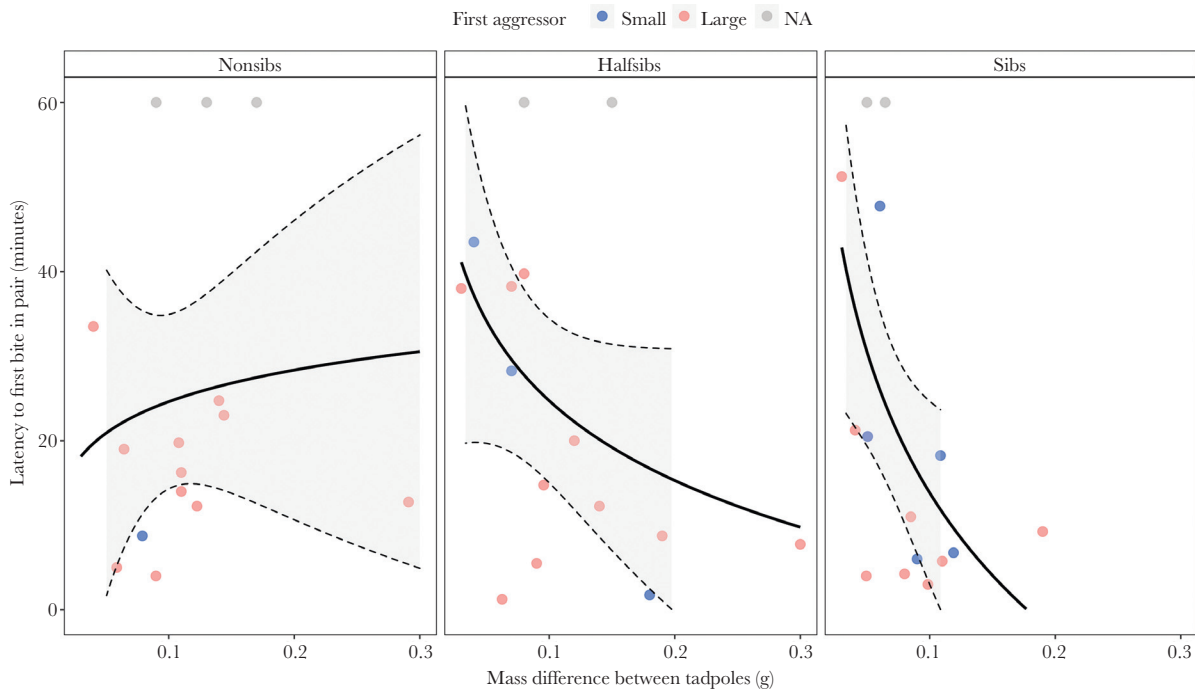
Figure 3

Latency to first bite between tadpole dyads. Points are colored by the first biter's relative size within dyads. Lines are fit with a GLM smoother with a y ~ log(x) formula and shaded regions represent 95% confidence intervals. There is an inversion in behavior as weight difference between dyads increases, where sibling pairs with large weight differences attacked significantly faster than non-siblings. Dyads where there were no aggressive behaviors were accounted for by assigning them the maximum time limit (60 min). N_{Trial} = 15 for each relatedness level.
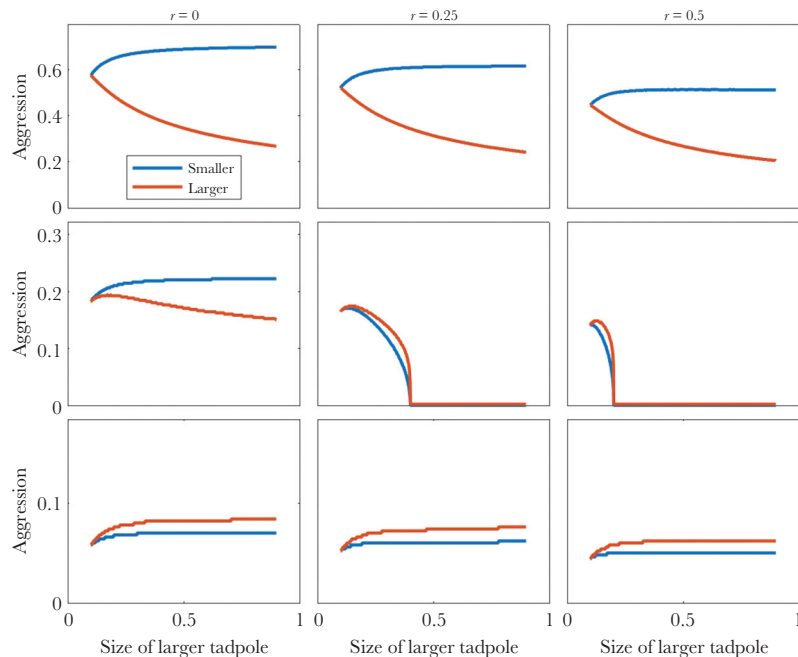
Figure 4

Optimal aggressiveness of dyads of tadpoles as a function of relative size difference for three different levels of relatedness (represented in panel columns) and three sets of assumptions (represented in panel rows). **First row:** direct fitness was assumed to be size-independent. **Second row**: direct fitness was assumed to be proportional to size. **Third row**: aggressiveness was assumed to be costlier for smaller tadpoles. The smaller tadpole's size was held fixed at $s_i = 0.1$; plotted lines show aggression levels in response to the increasing difference in size between dyads. The larger tadpole's size is shown on the x-axis.

This trend was inverted for siblings, which were tolerant of a similarly sized counterpart, but were quickly aggressive in pairings with large differences in weight. We speculate that fast "attacking" may serve different functions in different contexts. For example, when performed between mass-mismatched siblings, quick aggression may serve not to initiate cannibalism but to ascertain by taste the first impression of relatedness. While these data may be useful in unraveling potential assessment mechanisms, they should be interpreted with caution as considerable variation occurred throughout non-sibling treatments.

When comparing our empirical data with our inclusive fitness models, we are able to reject several theoretical possibilities for the *D. tinctorius* system. For example, if (adult) reproductive success and aggressiveness costs were independent of tadpole size, smaller tadpoles should compensate for their size disadvantage by being more aggressive (top row of panels in Figure 4). Empirically, we continuously see that small tadpoles are the least aggressive across treatments, suggesting that aggression for small tadpoles is costlier and/or less beneficial. Additionally, if tadpole size strongly predicted adult reproductive success, then above certain size difference smaller tadpoles should let their larger relative win without fighting (middle row of panels in Figure 4). This altruistic behavior of "sacrificing" oneself to a larger relative seemed plausible a priori in light of the observation that, in this system, fathers deposit younger individuals in occupied pools which could function to feed older siblings (Rojas 2014). However, this possibility can be rejected based on our empirical observations, where neither the small nor large tadpoles in pairs fully abandoned their aggressive behaviors towards one another (although we do observe a reduction in aggression in related tadpoles with larger size differences; see Supp. Fig 1 for aggression plotted across mass differences between pairs). When we frame these results in the context of deposition decisions by parents, we could hypothesize that some form of bet-hedging by fathers is occurring when choosing larval nurseries; ultimately, the benefit of a high-quality nursery may be worth the risk of cannibalism when that risk is minimized by being placed with siblings.

CONCLUSIONS

In this study, we explored aggression under resource-abundant, low-density conditions, which differs from the experimental set-up through which extreme intraspecific aggression is usually reported, such as in response to starvation (Mayntz and Toft 2006; Ibáñez and Keyl 2010; Dobler and Kölliker 2011), pathogens (Pfennig et al. 1991; Wang and Daane 2014), and high population densities (Moksnes 2004). We show here that intraspecific aggression (which may escalate to cannibalism) by *D. tinctorius* is not random, and that the interaction between relative size and relatedness shapes a cannibal's decision to attack.

We found that large tadpoles from sibling dyads were significantly less aggressive than large tadpoles from non-sibling dyads towards their smaller counterpart, presenting evidence for context-dependent kin discrimination in *D. tinctorius*. These findings are complicated by latency to aggression, which showed unexpected trends based on dyad relatedness, but may be related to the modalities involved in kin recognition. These results set the stage for studies to consider aggression in cannibals in more complex ways, and to better understand the value and purpose of kin discrimination in cannibals.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Behavior	Description
Resting	Non-movement under normal, healthy conditions
Swimming	Movement of the tadpole's tail in over two consecutive oscillations without 'intent' (see below) with respect to the other tadpole. Sometimes tadpoles contract their muscles or displace by floating up or down the water column; these behaviors did not qualify as swimming in this experiment.
Biting	A tadpole attempting or successfully grasping/clutching any body part of another. This behavior was allowed if it was isolated and its duration lasted less than 2 seconds. If the bite was followed by a "hold" by a tadpole that could result in permanent injury or death of the counterpart, the trial was terminated and categorized as ended by a means of "potential lethal attack".
Chasing	A tadpole swimming after its counterpart with intent; "intent" was perceived by the observer as rapid changes in swimming speed and direction of swimming being towards the other tadpole

Supplementary Table 1. Categorization of behaviour observed during aggression trials between tadpoles.

Supplementary Figure 1. *Differences in aggression across relatedness treatments with respect to weight difference between dyads. Pink dots represent large tadpoles and blue dots represent small tadpoles (sizes relative to dyads); N_Trial = 15 for each relatedness level. There was a higher level of aggression by large tadpoles overall, but significantly less aggression by large siblings when compared to non-siblings. Lines fit with GLM smoother (y ~ log(x)) for relative sizes, shaded regions represent 95% confidence intervals around model estimate.*
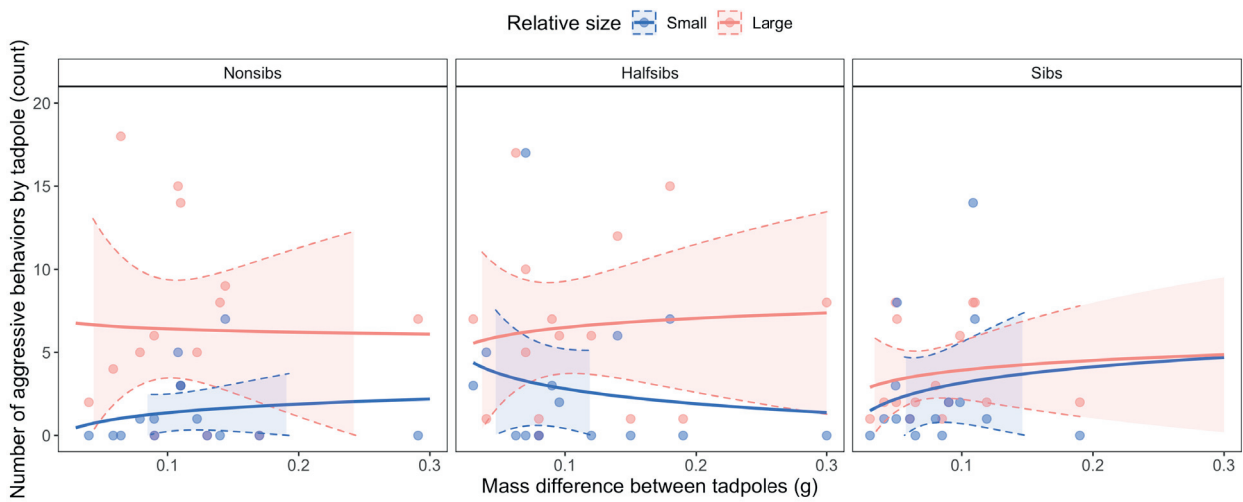
Supplementary Figure 2. The relationship between experimental activity and tadpole aggression based on relative size. We find for large tadpoles within dyads that activity is strongly related to aggression, especially for non-siblings and half-siblings (pink and red points). Large tadpoles from sibling dyads swim just as much as other treatments, but attack only half as much (Fig 2, 3 main MS). These data provide support for the hypothesis that tadpoles assess their counterpart (swimming, Fig 2. of main text) which informs their overall aggression. Lines fit with a GLM smoother, shaded regions represent 95% confidence intervals.
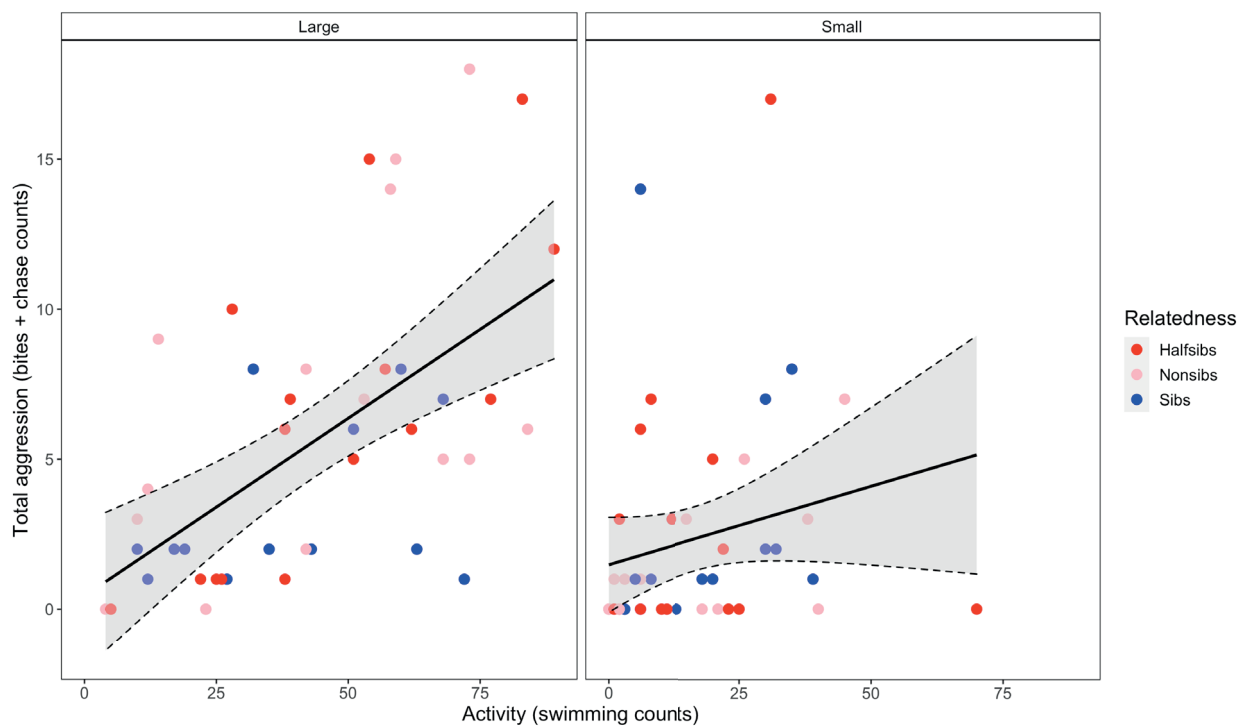
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PATHOGENS HIJACKING PARENTAL CARE? A FRAMEWORK FOR CHYTRID TRANSMISSION IN AMAZONIAN POISON FROGS

by

Fouilloux C. A., Almeida De Freitas G., Dittrich C., Hölzl F., Pašukonis A., Rosa G.M., Serrano Rojas S.J., Smith S., Sonnleitner R., Sundberg L.R., Trafford J.D. & Rojas B. 2023

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