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Diversity and evolution of amphibian pupil shapes

KATE N. THOMAS^{1,+,*}, CAITLYN RICH^{2,+}, RACHEL C. QUOCK^{2,3,+},
JEFFREY W. STREICHER¹, DAVID J. GOWER¹, RYAN K. SCHOTT^{4,5},
MATTHEW K. FUJITA⁶, RON H. DOUGLAS⁷ and RAYNA C. BELL^{2,5,*}

¹Department of Life Sciences, The Natural History Museum, London SW7 5BD, UK

²Department of Herpetology, California Academy of Sciences, San Francisco, CA 94118, USA

³Department of Biology, San Francisco State University, San Francisco, CA 94132, USA

⁴Department of Biology & Centre for Vision Research, York University, Toronto M3J 1P3, Canada

⁵Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, 20560-0162, USA

⁶Department of Biology, Amphibian and Reptile Diversity Research Center, The University of Texas at Arlington, Arlington, TX 76019, USA

⁷Division of Optometry & Visual Science, School of Health Sciences, City, University of London, Northampton Square, London EC1V 0HB, UK

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Pupil constriction has important functional consequences for animal vision, yet the evolutionary mechanisms underlying diverse pupil sizes and shapes are poorly understood. We aimed to quantify the diversity and evolution of pupil shapes among amphibians and to test for potential correlations to ecology based on functional hypotheses. Using photographs, we surveyed pupil shape across adults of 1294 amphibian species, 74 families and three orders, and additionally for larval stages for all families of frogs and salamanders with a biphasic ontogeny. For amphibians with a biphasic life history, pupil shape changed in many species that occupy distinct habitats before and after metamorphosis. In addition, non-elongated (circular or diamond) constricted pupils were associated with species inhabiting aquatic or underground environments, and elongated pupils (with vertical or horizontal long axes) were more common in species with larger absolute eye sizes. We propose that amphibians provide a valuable group within which to explore the anatomical, physiological, optical and ecological mechanisms underlying the evolution of pupil shape.

ADDITIONAL KEYWORDS: activity period – Anura – aquatic – Caudata – fossorial – Gymnophiona – optics – scansorial – vision.

INTRODUCTION

The ability to detect light and form images is important for most animals. Almost all animal phyla have evolved light-sensitive organs, ranging from eye spots that simply detect the presence or absence of light to sophisticated eyes that detect fast movement, provide a wide field of view and allow images to be formed (Halder *et al.*, 1995). Vertebrates and cephalopods have camera-type eyes, in which the

aperture of the iris (the pupil) controls the amount of light reaching the retina. In most species, the iris muscles alter the size of the pupil in response to ambient light, enabling adjustment of the sensitivity and resolution of the eyes (Land & Nilsson, 2012). The configuration of the iris musculature determines the extent and speed of constriction of the pupil, as well as pupil shape. Although all dilated pupils are close to circular, constricted pupils can range from the circular and fixed pupils of most teleost fishes to the dynamic and complex pupil shapes of cephalopods (Mann, 1931; Douglas, 2018). Proposed functions of such differences in pupil shape include camouflaging the eye, allowing different ranges of constriction, minimizing chromatic

*Corresponding authors. E-mail: kate.thomas@nhm.ac.uk; rbell@calacademy.org

+These authors contributed equally.

aberration, enhancing object detection in various orientations and influencing the eye's depth of field (reviewed in Douglas, 2018). However, there is little direct evidence for any of these functions, and the evolutionary mechanisms underlying diverse pupil sizes and shapes, often among animals that occupy optically similar environments, are poorly understood.

Pupils in vertebrates include non-elongated shapes (e.g. circular) and elongated shapes with vertical or horizontal long axes (reviewed in Douglas, 2018). Some vertebrate groups exhibit little variation in constricted pupil shape: birds and turtles all have predominantly non-elongated, circular constricted pupils, and all crocodylian pupils constrict to a vertical slit. By contrast, within mammals, squamates (lizards and snakes) and amphibians (frogs, salamanders and caecilians), constricted pupils include different non-elongated, vertically elongated and horizontally elongated shapes (Douglas, 2018), and there is some evidence that different pupil shapes correspond to differences in visual ecology among species. For instance, circular pupils are typical of teleost fishes (Douglas, 2018) and aquatic amphibians (Cervino *et al.*, 2021), though the factors driving this are not well understood. Vertically elongated pupils in elapid snakes (cobras, mambas and marine snakes) are correlated with diel activity and foraging mode: the constricted pupils of nocturnal species that are ambush predators are vertical, whereas those of diurnal species that are active foragers are circular (Brischoux *et al.*, 2010). In mammals, pupil shape is also correlated with activity period, with horizontally elongated and non-elongated pupils occurring primarily in diurnal species, and vertically elongated pupils present in nocturnal and crepuscular species (Mann, 1931). Elongated pupil shapes (e.g. slits) are also hypothesized to enhance vision in particular orientations but with conflicting evidence. For instance, vertically elongated pupils have been proposed to increase depth of field in a horizontal plane (e.g. Brischoux *et al.*, 2010) or alternatively in a vertical plane (e.g. Hart *et al.*, 2006; Banks *et al.*, 2015). These hypotheses, however, have been explored in only a relatively small subset of the phylogenetic and ecological diversity of vertebrates. Here, we aim to quantify the diversity and evolution of pupil shapes among amphibians and test for potential correlations to ecology based on functional hypotheses.

Amphibians are a speciose (*c.* 8300 extant species: AmphibiaWeb, 2022), diverse and ecologically rich radiation with repeated evolutionary transitions in activity period and habitat that influence the light environments in which they are active and have evolved. Although amphibian pupil shape has been studied in the context of species identification and systematics in some lineages (e.g. Drewes, 1984; Glaw & Vences, 1997; Nuin & do Val, 2005; Rödel *et al.*,

2009; Menzies & Riyanto, 2015), and more recently with respect to evolutionary lability (Cervino *et al.*, 2021), the functional consequences of different pupil shapes in amphibians are poorly understood. The limbless caecilian amphibians (order Gymnophiona, *c.* 200 extant species) are predominantly fossorial with greatly reduced visual systems, including eyes covered by skin and/or bone in many lineages (Walls, 1942; Wake, 1985; Wilkinson, 1997; Mohun *et al.*, 2010). In even the most extensively developed eyes of extant caecilians, the iris musculature is rudimentary (Mohun & Wilkinson, 2015) or absent (Himstedt, 1995), making changes in pupil size and shape unlikely (Douglas, 2018); consequently, in this study we focus on frogs (order Anura, *c.* 7300 extant species) and salamanders (order Caudata, *c.* 700 extant species). A recent study characterized variation in absolute and relative eye size across all anuran families, and determined that frogs generally have large eyes relative to other vertebrates and that variation in adult eye size is associated with differences in habitat, activity period and breeding ecology (Thomas *et al.*, 2020a). Variation in salamander eye size has not yet been quantified, but this lineage is also ecologically diverse with fully aquatic, arboreal and fossorial species that likely differ substantially in visual ecology. Frogs and salamanders are typically visual predators, and behavioural studies in both groups indicate that visual signals and coloration can play an important role in intraspecific communication (Jaeger & Forester, 1993; Haddad & Giaretta, 1999; Hödl & Amezcuita, 2001; Starnberger *et al.*, 2014; Yovanovich *et al.*, 2017). Likewise, both groups include species that are either primarily diurnal, primarily nocturnal or active under a range of light conditions (Anderson & Wiens, 2017). Consequently, both visual acuity and colour discrimination may be important for many amphibian species in bright and/or dim light conditions (e.g. Toledo *et al.*, 2007; Robertson & Greene, 2017). Furthermore, species that are active in both bright and dim light, and/or that have particularly large eyes, may rely on a large pupillary range to optimize visual performance relative to their surroundings, and may have slit pupils because these allow the largest range of contraction of the aperture (Walls, 1942).

Many amphibians have a biphasic ontogeny with an aquatic larval stage (termed tadpoles in frogs) and terrestrial adult life stages (e.g. McDiarmid & Altig, 1999), whereas others retain aquatic lifestyles as adults, have semi-terrestrial larvae or develop without a larval life stage (termed direct development). During amphibian metamorphosis, dramatic morphological and physiological changes occur, including alterations to the visual system (Hoskins, 1990). Changes in eye-body scaling (Shrimpton *et al.*, 2021) and lens shape (Sivak & Warburg, 1980, 1983) across ontogeny in frogs suggest that several structural aspects of the visual

system adapt to differing tadpole and adult visual requirements. Likewise, whole-eye differential gene expression of aquatic tadpoles vs. terrestrial juvenile frogs (Schott *et al.*, 2022) demonstrates changes in genes related to eye and retinal development, light detection, lens crystallins and phototransduction, indicating substantial decoupling between life stages at the level of gene expression. The biphasic ontogeny and shift between aquatic larval and terrestrial adult habitats in many amphibians is unique among tetrapods and thus presents the opportunity to investigate whether pupil shape is adaptively decoupled between life stages.

Here we survey and classify constricted pupil shape across adults of 1294 amphibian species, 74 families and three orders, and additionally for larval life stages for all families of frogs and salamanders with a biphasic ontogeny ($N = 56$). We first test the hypothesis that pupil shape changes across biphasic ontogeny in species that occupy distinct habitats before and after metamorphosis. Second, we identify evolutionary lineages with extensive pupil shape variation and quantify transition rates in pupil shape across the phylogeny. Finally, we test whether pupil shape exhibits correlated evolution with traits relevant to amphibian visual ecology. Specifically, we test whether (1) non-elongated pupils are correlated with aquatic or fossorial lifestyles; (2) non-elongated pupils are associated with diurnal activity; (3) vertically elongated pupils are correlated with navigating complex vertical (arboreal/scansorial) habitats; and (4) elongated pupils are more common in species with large absolute eye size.

MATERIAL AND METHODS

SPECIES SAMPLING AND PUPIL CLASSIFICATION

To assess the diversity of pupil shapes across amphibians, we searched online photograph databases (primarily CalPhotos, <https://calphotos.berkeley.edu>) for images in which the eye and partially or fully constricted pupil was visible. Because dilated anuran pupils are always circular (Douglas, 2018; Supporting Information, Part B), we assumed that pupils that were elongated (contracting more along one axis) in photographs were at least partially constricted. For pupils that remain circular during constriction, it is more challenging to determine whether the pupil is constricted from a photograph in uncontrolled lighting, so we viewed as many photos as possible in these species to maximize our likelihood of seeing a constricted pupil. We also based our categorization on the photo with the smallest pupil area relative to exposed eye area, which tends to be smaller in a constricted circular pupil than a dilated one (Supporting

Information, Fig. S3, Fig. S4; Table S2). We aimed to sample at least one species per family or sub-family of all currently recognized amphibian orders with externally visible eyes (74 families; Frost, 2021). When suitable images for a target family or species were not available on CalPhotos, we searched for photographs on other user-upload sites (e.g. Flickr), field guides and primary literature, as well as verbal descriptions in the scientific literature (e.g. species descriptions). In a few instances, we relied on our personal photographs and field notes. Pupil shapes (circle, diamond, oval, slit, upside-down triangle, sideways triangle, upside-down tear) for each species were independently classified and reviewed by at least two observers. Shape assignments were made based on the number of vertices present in the aperture (e.g. three vertices = triangle) and the orientation of the aperture (e.g. one triangle side is dorsal and horizontal = upside-down triangle). See Supporting Information Part A for more details. In addition, some post-metamorphic amphibian irises include small projections (umbracula or opercula) from the dorsal edge into the pupil, and larval corneas can have dorsal accumulations of pigment (elygia) over the pupil (Kruger *et al.*, 2013). Although there are some proposed optical implications for these structures (reviewed in Douglas, 2018), we did not consider these additional features in our shape classifications because they are more difficult to identify from opportunistic photographs. Example photographs for all pupil shapes recognized in this study are depicted in Figure 1 and Supporting Information, Figure S2, and the list of references used in categorization are available in Supporting Information Part F.

Any discrepancies between observers were resolved with the input of additional observers and photographs when available, or were removed from the dataset. Larval frogs and salamanders, and adult caecilians, apparently lack or have a very weak pupillary response (Douglas, 2018; see Supporting Information, Part B; Fig. S5 for anecdotal observations of pupil response in anuran tadpoles), and thus our scoring in these instances was likely of permanently (or near-permanently) fixed pupil shapes. Likewise, we note that oval shapes in both horizontally and vertically elongated pupils may further constrict to a narrow slit under brighter light conditions. Because we relied on photographs to classify pupil shapes rather than on experimentally assessing pupillary response, our determination of 'oval' vs. 'slit' pupil shapes were limited by the available photos. However, our approach is similar to that of several recently published studies that quantified vertebrate pupil shape from photographs (Brischoux *et al.*, 2010; Banks *et al.*, 2015; Cervino *et al.*, 2021) and provides a more taxonomically and developmentally extensive survey of pupil

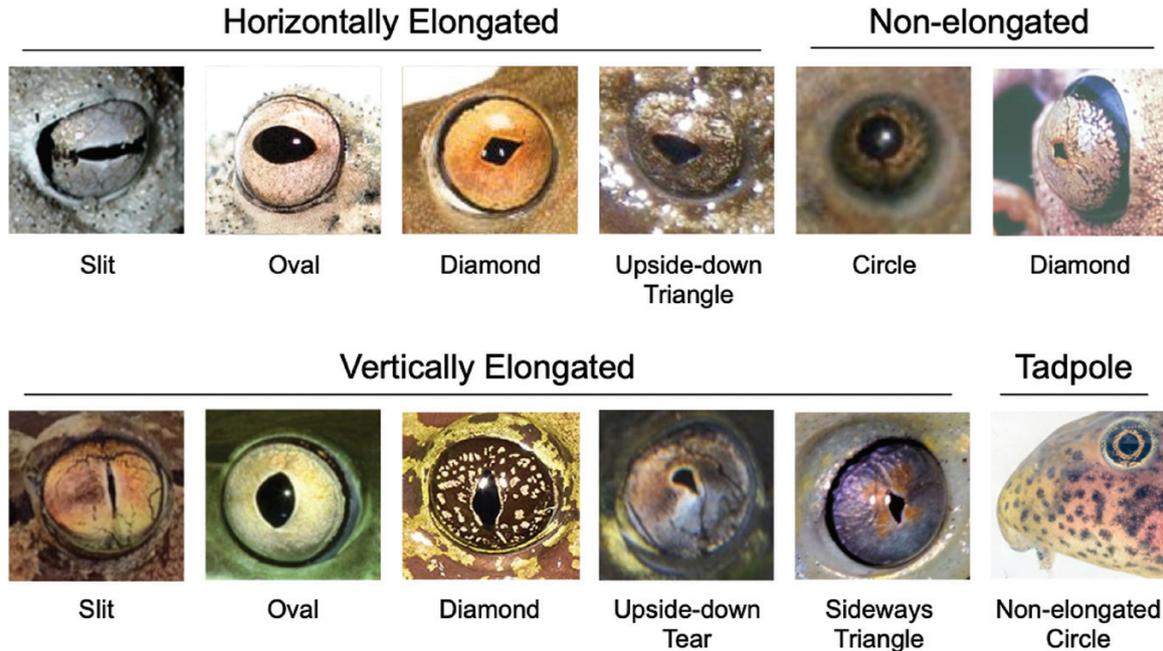


Figure 1. Examples of adult and larval anuran pupil shapes. Note that oval shapes may further constrict to a slit under brighter and/or longer duration light. For evolutionary analyses, this broader diversity of shapes was binned into a set of three shapes that may have functional consequences for vision (horizontally elongated, non-elongated and vertically elongated). Photography credits (left to right, top to bottom) *Breviceps macrops* (Arie van der Meijden), *Hyperolius thomensis* (Andrew Stanbridge), *Boana boans* (Twan Lenders) and *Geocrinia lutea* (Grant Webster); *Xenopus tropicalis* (Daniel Portik) and *Boana geographica* (Germano Woehl Jr); *Astylosternus batesi* (Greg Jongsma), *Tachycnemis seychellensis* (Gonçalo Rosa), *Heleophryne rosei* (Courtney Hundermark), *Calyptocephalella gayi* (Peter Janzen) and *Heterixalus betsileo* (Bernard Dupont); *Hylarana albolabris* (Christian Irian).

constriction diversity in amphibians than is currently feasible with experimental approaches.

Once we had surveyed representatives of each family or subfamily, we expanded our sampling to encompass species that were the focus of recent studies on anuran visual biology (e.g. Thomas *et al.*, 2020a, 2022a; Shrimpton *et al.*, 2021) to maximize overlap with existing datasets. Preliminary assessments of this diversity suggested that pupil shape was diverse and/or evolutionarily labile in particular lineages, and thus we elected to sample these groups in more depth. This included families in the Afrobatrachia radiation (Arthroleptidae, Brevicipitidae, Hemisotidae, Hyperoliidae), and the families Hylidae, Microhylidae and Myobatrachidae. For families with extensive ecological diversity as adults (i.e. aquatic, semiaquatic, ground-dwelling, arboreal, fossorial) we aimed to sample species representative of this diversity. Our final dataset included pupil shape observations for 1241 species of Anura (58 families), 43 species of Caudata (nine families) and ten species of Gymnophiona (seven families). Images and references used to assess pupil shape for each species are given in the supplementary data on the Natural History Museum (London, UK) Data Portal (Thomas *et al.*, 2022b).

The primary aim of our study was to investigate whether pupil shape exhibits correlated evolution with traits relevant to amphibian visual ecology. Thus, for evolutionary analyses, we binned the broader diversity of shapes into a set of three that may have functional consequences for vision (non-elongated, horizontally elongated and vertically elongated; Fig. 1; Supporting Information, Figs S1, S2). We also compared our classifications to two recently published datasets that categorized pupil shape in adult frogs and salamanders (Yovanovich *et al.*, 2020; Cervino *et al.*, 2021; Supporting Information, Part C).

PHYLOGENY

We used the phylogenetic hypothesis of Jetz & Pyron (2018) for visualizing trait distributions and modelling trait evolution across species. This phylogeny used a molecular backbone as well as taxonomic information to infer proposed relationships among 7238 amphibian species. We matched the phylogeny to our dataset and performed all subsequent analyses using R v.4.1.0 (R Core Team, 2021) in RStudio v.1.4.1717 (RStudio Team, 2021). We used the R package AmphiNom v.1.0.1 (Liedtke, 2019) to match tip labels in the phylogeny to

species names in our dataset by converting both to the taxonomy of [Frost \(2021\)](#) and manually checking and matching any species with multiple synonyms. For the 46 species in our dataset not represented in the phylogeny, we used the published literature to find the closest sister taxa that were represented in the tree ([Supporting Information, Part D; Table S3](#)) and then added the missing species to the node representing the most recent common ancestor of these taxa using the `getSisters`, `findMRCA` and `bind.tip` functions in `phytools` v.0.7.70 ([Revell, 2012](#)). Finally, we pruned the phylogeny to the 1294 species in our dataset using `drop.tip` in `ape` v.5.4.1 ([Paradis *et al.*, 2004; Paradis & Schliep, 2019](#)) and randomly resolved polytomies with the `multi2di` function in `ape`. The resulting tree and associated species data can be viewed in [Supporting Information Part E](#).

ADULT HABITAT AND ACTIVITY PERIOD CLASSIFICATION

Adult ecology was categorized into binary states for activity pattern and different aspects of habitat and lifestyle using peer-reviewed literature, online natural-history resources, field guides and field observations, as: (1) primarily diurnal or non-diurnal; (2) aquatic or non-aquatic; (3) fossorial or non-fossorial; and (4) scansorial or non-scansorial. Categorizations were simplified versions of those used by [Thomas *et al.* \(2020a\)](#). Species were classed as primarily diurnal if adults were primarily active in daylight above ground; arrhythmic, cathemeral, crepuscular and nocturnal species were all classified as non-diurnal. Species in which adults were primarily active underwater were categorized as aquatic. Species were classified as fossorial if adults were active underground, typically in soil (as opposed to only aestivating or sheltering underground). Finally, species in which adults climbed up off the ground onto vegetation were classified as scansorial. Pupil shapes, habitat classifications and associated references are listed in the supplementary data ([Thomas *et al.*, 2022b](#)) and [Supporting Information Parts E and F](#).

PUPIL SHAPE ACROSS BIPHASIC ONTOGENY

To assess variation in pupil shape among larval frogs and salamanders, we searched through field guides, primary literature and online photograph databases (e.g. CalPhotos, Flickr), and categorized pupils as described above. We classified larval pupil shape for at least one species in every family that has species with a larval life stage, including representative species with different larval habitats (i.e. semiterrestrial, phytotelm-, pond- or stream-dwelling). To identify which lineages exhibit changes in pupil shape between

larval and adult life stages, we classified pupil shape in adults for all species for which we determined larval pupil shape ($N = 92$). As with larval habitat diversity, we also aimed to maximize adult habitat diversity in this paired sampling (i.e. aquatic, semiaquatic, ground-dwelling, scansorial, fossorial). Both larval and adult habitat classifications were determined based on field guides, primary literature and expert knowledge. To visualize variation in an evolutionary context, we mapped tadpole and adult pupil shapes and habitats on the modified [Jetz & Pyron \(2018\)](#) phylogeny using `ape` ([Paradis *et al.*, 2004; Paradis & Schliep, 2019](#)).

EVOLUTIONARY TRANSITIONS IN PUPIL SHAPE ACROSS THE AMPHIBIAN PHYLOGENY

To gain insights into the evolutionary history and lability of adult pupil shape across the amphibian phylogeny, we implemented stochastic character mapping ([Bollback, 2006](#)) for the three categories of pupil shapes that may have functional consequences for vision (non-elongated, vertically elongated, horizontally elongated). We used the `fitDiscrete` function in `phytools` to fit equal-rates, symmetrical-rates and all-rates-different models of character evolution ([Revell, 2012](#)). To select the 'best' model, we compared Akaike information criterion (AIC) scores and weights and then used `make.simmap` with the best-fit transition model (all-rates-different) to simulate character evolution across 100 trees. We plotted the phylogeny with branches coloured based on the highest likelihood state of the node it originated from, and summarized mean pairwise transitions between each set of states across the 100 simulations.

EFFECTS OF SPECIES ECOLOGY ON PUPIL SHAPE

We implemented multivariate phylogenetic logistic regression in the R package `phylolm` v.2.6.2 ([Paradis & Claude, 2002; Ives & Garland, 2010; Tung Ho & Ane, 2014](#)) to examine the correlation structure among binary discrete states for pupil shape and categorical discrete states for ecology. We used the `logistic_MPLE` method, which maximizes the penalized likelihood of the logistic regression, and ran 1000 bootstrap replicates to estimate coefficients. First, we tested whether vertically elongated pupils are associated with scansorial lifestyles in a model of binary pupil shape (0 = horizontally elongated or non-elongated, 1 = vertically elongated) vs. scansoriality (0 = non-scansorial, 1 = scansorial). Then, we tested whether non-elongated pupils are associated with aquatic habitats, fossorial lifestyles or diurnal activity patterns. Because we predicted that three different covariates may be associated with non-elongated pupils, we fit ted three models with different predictors

(habitat, activity period and habitat + activity period) and determined the best-fit model by ranking AIC scores for each model, with a $\Delta\text{AIC} \geq 2$ between models considered a significant difference in model fits. These models fit binary pupil shape (0 = horizontally or vertically elongated, 1 = non-elongated) vs. habitat (aquatic, fossorial, or neither) and/or activity period (0 = non-diurnal, 1 = diurnal).

To test the prediction that species with large eyes would benefit from having a large pupillary range facilitated by elongated pupils, we tested whether eye size differed in species with non-elongated and elongated (horizontal or vertical) pupils using a phylogenetic least squares (PGLS) regression in caper v.1.0.1 (Orme *et al.*, 2018). Eye size (maximum externally exposed eye diameter) data for 207 anuran species representing 54 families were obtained from Thomas *et al.* (2020b); details on the collection of these data can be found in Thomas *et al.* (2020a). We used phytools (Revell, 2012) and ggplot2 v.3.3.3 (Wickham, 2016) to visualize the data.

RESULTS

PUPIL SHAPE DIVERSITY ACROSS AMPHIBIANS

The variety of pupil shapes within the three living orders of Amphibia corresponded with the diversity of their habitats. We examined pupil shape in ten species of Gymnophiona that occupy aquatic or fossorial habitats, all of which had non-elongated, circular pupils (Fig. 2; Supporting Information, Fig. S6). Pupil shape was more diverse across the 43 species of aquatic, fossorial or scansorial Caudata we classified, with non-elongated (circle) and horizontally elongated (oval, slit, upside-down triangle) pupil shapes (Fig. 2; Supporting Information, Figs S2, S6). The greatest diversity of pupil shape was observed in the 1241 species of Anura we examined, including non-elongated (circle, diamond), horizontally elongated (oval, diamond, slit, upside-down triangle) and vertically elongated (oval, diamond, sideways triangle, slit, upside-down teardrop) shapes (Figs 1, 2; Supporting Information, Fig. S6; Table S1). Anurans include the greatest diversity of species and ecologies (i.e. aquatic, semiaquatic, ground-dwelling, scansorial, fossorial), and we sampled species representative of this diversity within each family where possible. Pupil shape was notably diverse in the Afrobatrachia radiation (Arthroleptidae, Brevicipitidae, Hemisotidae, Hyperoliidae) and the families Hylidae, Microhylidae and Myobatrachidae, with all the different non-elongated, horizontally elongated and vertically elongated shapes represented in each of these lineages (Fig. 2; Supporting Information, Fig. S6). By contrast, other speciose and ecologically diverse lineages, such

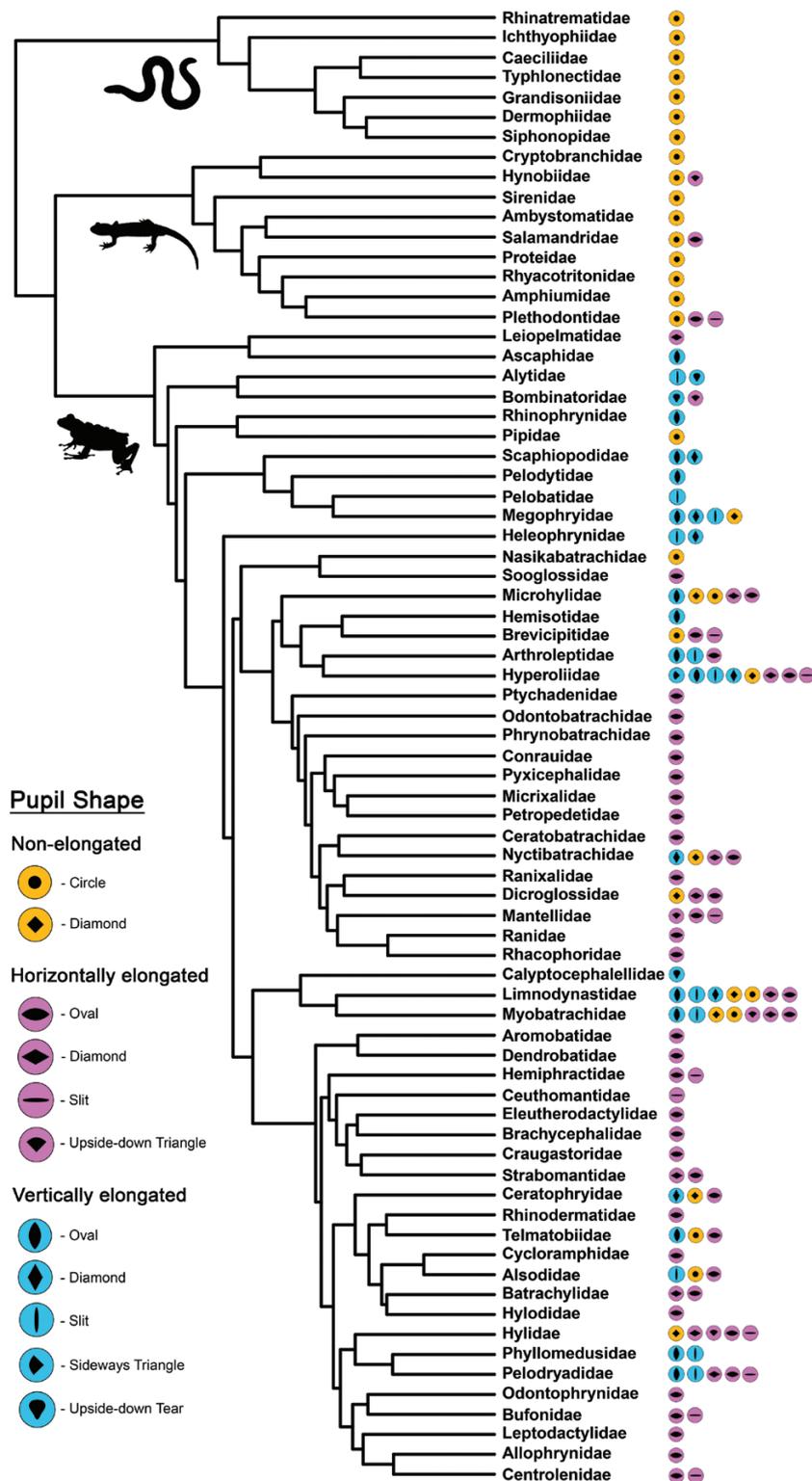
as Bufonidae, all exhibited horizontal oval pupil shapes (Fig. 2; Supporting Information, Fig. S6). Adults of the fully aquatic clawed frogs (Pipidae), giant salamanders (Cryptobranchidae), sirens (Sirenidae), amphiumas (Amphiumidae) and torrent salamanders (Rhyacotritonidae) all had circular pupils (Fig. 2; Supporting Information, Fig. S6) as did the fossorial pignose frogs in the family Nasikabatrachidae (Fig. 2; Supporting Information, Fig. S6).

PUPIL SHAPE ACROSS BIPHASIC ONTOGENY

Larval pupil shape was circular in all 92 species of frog and salamander that we surveyed regardless of their habitat (Figs 1, 3). For instance, the larvae of the reed frog *Hyperolius thomensis* develop in small pools of murky water that collect in tree cavities in dense, primary forest (Drewes & Stoelting, 2004; Gilbert & Bell, 2018) and the tadpoles have non-elongated, circular pupils like those of the larvae of the congeners *Hyperolius endjami*, which develop in ponds and streams in more open canopy habitats (Amiet, 2012; Supporting Information, Fig. S6). Likewise, semi-terrestrial tadpoles that develop in the splash zones of waterfalls (e.g. rock river frog *Thoropa miliaris*), in terrestrial nests (e.g. nurse frog *Allobates magnussoni*) or in dorsal pouches (e.g. marsupial frog *Gastrotheca piperata*) all have circular pupils. The only exception was the fossorial tadpoles of the dancing frog *Micrixalus herrei*, which hide within the gravel of streambeds, and appear to have skin-covered eyes as larvae but well-developed, uncovered eyes with horizontal pupils as adults (Senevirathne *et al.*, 2016). In the ten species (three Anura and seven Caudata) in our dataset that inhabit aquatic habitats as both larvae and adults, pupil shape remained circular in adults (Fig. 3; Supporting Information, Fig. S6). In the species that transition from an aquatic larval stage to a fossorial, scansorial or ground-dwelling adult life stage, we observed non-elongated, horizontally elongated and vertically elongated pupil shapes in adults (Fig. 3; Supporting Information, Fig. S6). Collectively, these observations indicate that pupil shape changes across biphasic ontogeny in many frog species that occupy distinct habitats before and after metamorphosis.

EVOLUTIONARY TRANSITIONS IN PUPIL SHAPE ACROSS THE AMPHIBIAN PHYLOGENY

The 'all-rates-different' model of character evolution was by far the best fit to our data for pupil shape (Table 1), and there were high transition rates between pupil shapes across the phylogeny (average of 87.68 changes between states across 100 total simulations) demonstrating the high evolutionary lability of this



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Figure 2. A phylogeny of all amphibian families that have externally visible eyes (some caecilian eyes are under bone), showing the pupil shapes we found within that family. Note that oval pupil shapes may further constrict to a slit under brighter and/or longer duration light, but this was not always possible to assess from the available photographs. The phylogeny is modified from [Jetz & Pyron \(2018\)](#) and the complete dataset is in the [Supporting Information \(Fig. S6\)](#).

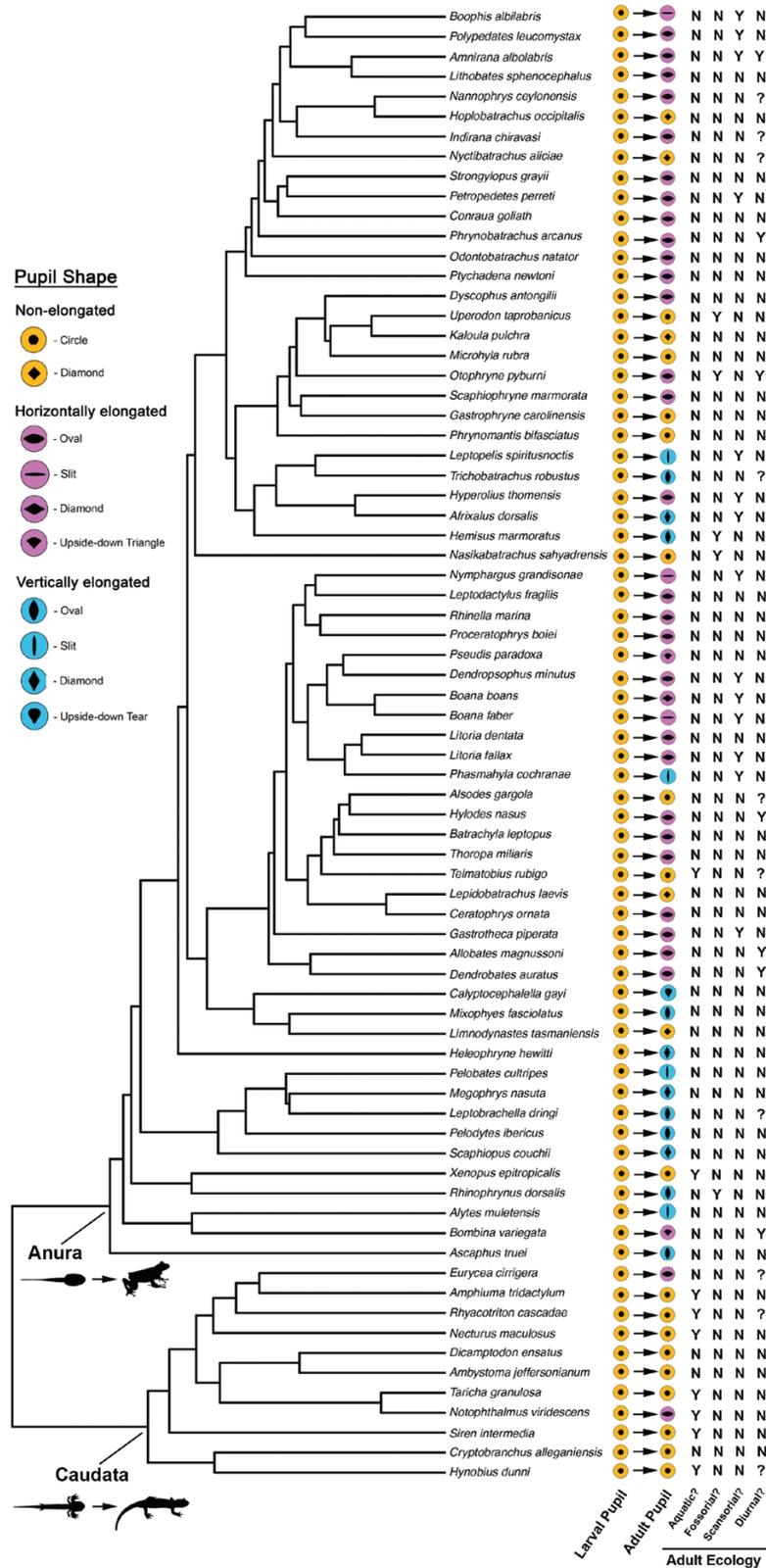


Figure 3. A phylogeny of larval and adult species pairs in our dataset (including representative species for all anuran and caudatan amphibian families that have a larval life stage with developed eyes) with observed pupil shapes. The phylogeny is modified from [Jetz & Pyron \(2018\)](#) and the complete dataset is in the [Supporting Information \(Fig. S6\)](#). For adult ecology, Y = yes, N = no, and ? = unknown.

Table 1. Comparison of three Mk models of discrete character evolution for pupil shape (non-elongated, vertically elongated, horizontally elongated) across sampled amphibian species ($N = 1294$). Models include an equal-rates model with one transition rate parameter, a symmetric-rates model with three transition rate parameters and an all-rates-different model with six transition rate parameters

Model	Log-lik	AICc	Δ AICc	AIC weight
Equal-rates	-355.6	713.3	50.3	0
Symmetric-rates	-351.2	708.3	45.4	0
All-rates-different	-325.5	663.0	0	1

trait. The majority of transitions occurred from non-elongated to horizontally elongated pupils, whereas transitions from horizontally elongated to vertically elongated pupils were the least common (Fig. 4). Many of the evolutionary transitions were concentrated within the Afrobatracha radiation (Arthroleptidae, Brevicipitidae, Hemisotidae, Hyperoliidae) and the families Microhylidae and Myobatrachidae.

CORRELATED EVOLUTION OF SPECIES ECOLOGY AND PUPIL SHAPE

Tests for correlation between pupil shape and ecology indicated that some aspects of habitat and activity period are associated with pupil shape in

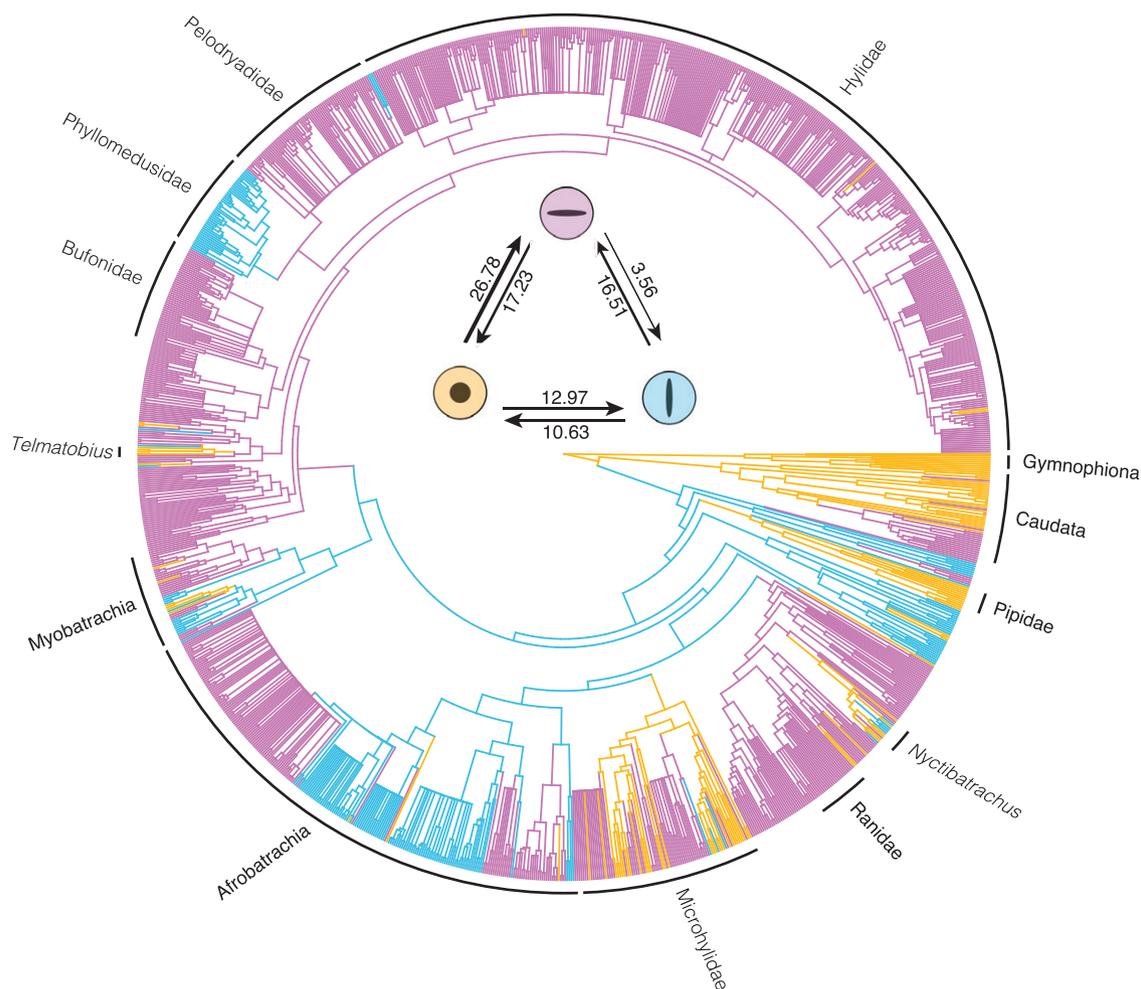


Figure 4. Distribution of non-elongated and elongated pupil shapes in adult life stages of 1294 amphibian species (phylogeny modified from Jetz & Pyron, 2018). Branches are coloured by the highest probability state of the most recent node based on stochastic character mapping with an all-rates-different transition model across 100 trees. Lineages discussed in the text are labelled for reference. Inset depicts estimated transitions between non-elongated (orange), horizontally elongated (pink) and vertically elongated (blue) pupils based on stochastic character mapping. The thickness of the arrows is proportional to the mean transitions estimated across 100 simulations.

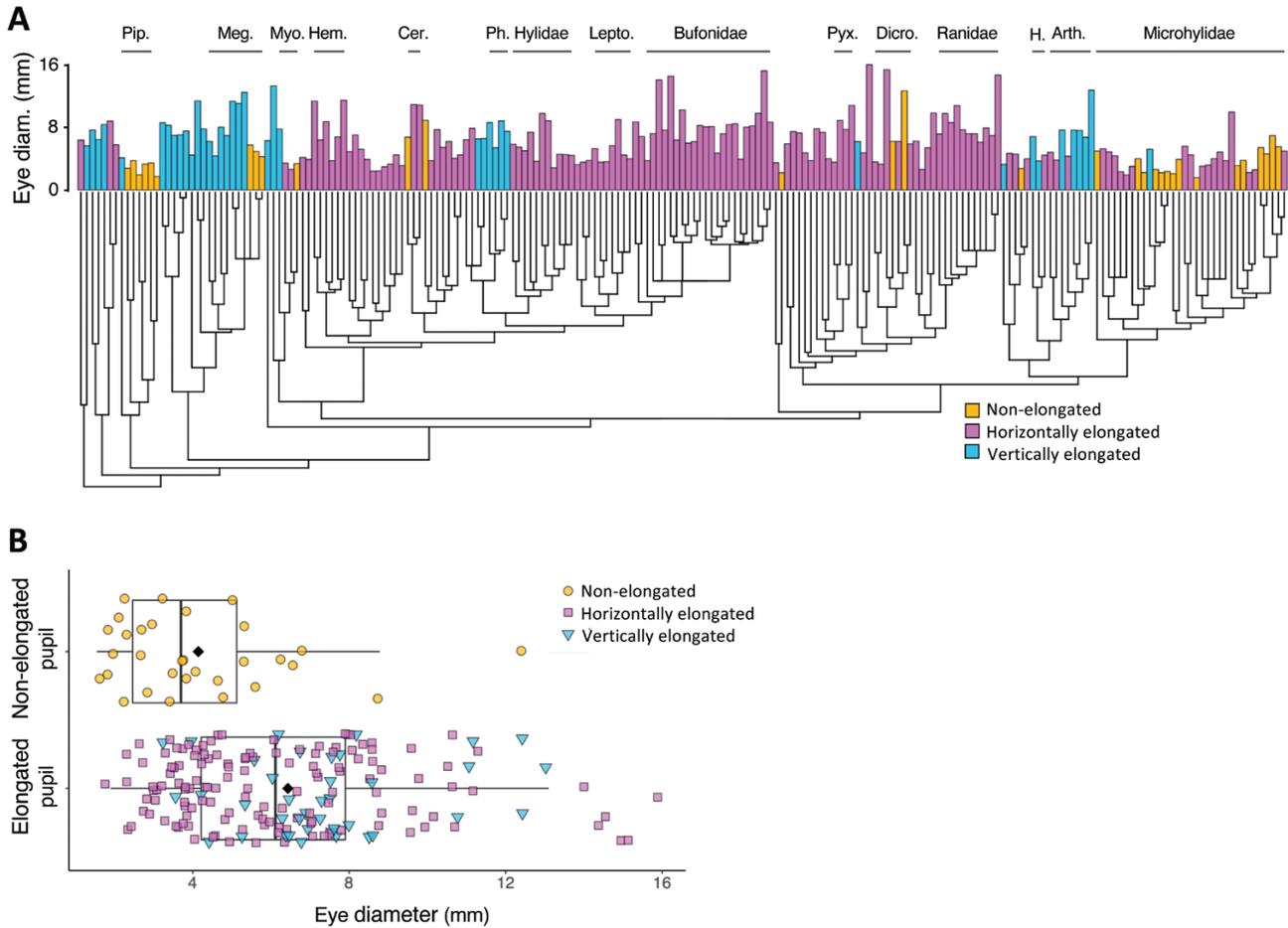


Figure 5. Eye size and pupil shape across 207 species of anuran amphibians (A). Species with elongated (horizontal or vertical) pupils have significantly larger eye diameters than those with non-elongated pupils (B). Pip. = Pipidae, Meg. = Megophryidae, Myo. = Myobatrachidae, Hem. = Hemiphractidae, Cer. = Ceratophryidae, Ph. = Phyllomedusidae, Lepto. = Leptodactylidae, Pyx. = Pyxicephalidae, Dicro. = Dicroglossidae, H. = Hyperoliidae, Arth. = Arthroleptidae.

amphibians, whereas scansorial lifestyle has no effects on pupil shape (Tables 2, 3). Comparing AIC scores of multivariate phylogenetic logistic regression models of pupil shape vs. habitat and/or activity pattern indicated that a model including both habitat (fossorial, aquatic or neither) and activity period (diurnal or non-diurnal) was the best fit to species data ($N = 644$, Table 2). This model indicated that both fossorial ($P = 0.02$) and aquatic ($P = 0.001$) ecologies are associated with non-elongated pupils (Table 3). Contrary to predictions, diurnal activity patterns were significantly correlated with elongated pupils ($P = 0.04$) rather than non-elongated pupils; in fact, all of the 70 primarily diurnal species studied had horizontally or vertically elongated pupils. In a separate model, we found no association between vertically elongated pupils and scansorial lifestyles across 904 species (Table 3). Finally, among 207 anuran amphibians with data for both eye size and pupil shape, species with vertically or horizontally

Table 2. Comparison of three multivariate phylogenetic logistic regression models of the effects of ecology on binary pupil shape (non-elongated or elongated). Models were fit to 644 species with complete data on habitat (aquatic, fossorial or neither) and activity period (diurnal or non-diurnal) so that they could be compared via AIC. Alpha is the phylogenetic correlation parameter estimate from phyglm

Model	Alpha	Log-lik	AIC	Δ AIC
Pupil ~ activity period	0.005	-130.2	266.4	26.5
Pupil ~ habitat	0.006	-117.6	243.2	3.3
Pupil ~ activity period + habitat	0.007	-115.0	239.9	0

elongated pupils had significantly larger eyes than species with non-elongated pupils (PGLS: $F = 4.89$, d.f. = 1 and 205, $R^2_{adj} = 0.02$, $P = 0.03$, Figure 5).

Table 3. Summary of multivariate phylogenetic logistic regression analyses for the effects of ecological traits on binary pupil shapes. Binary traits (pupil shape, scansoriality, diurnality) are described in the model by the state set to equal 1. Habitat has three discrete states (aquatic, fossorial, neither). Predictors of pupil shape are considered significant at Wald-type $P < 0.05$ (shown in bold) for the given alpha value. Alpha is the phylogenetic correlation parameter estimate from phyloglm. Coefficient estimates are shown with upper and lower bootstrap estimates in parentheses based on 1000 fitted replicates

Model	<i>N</i>	Alpha	Covariate	Coefficient	SE	z-value	<i>P</i> -value
Vertical pupil vs. scansorial	904	0.004	Non-scansorial	-2.41 (-3.26, -0.28)	1.37	-1.76	0.08
			Scansorial	0.02 (-0.18, 0.20)	0.23	0.10	0.92
Non-elongated pupil vs. habitat + diurnal	644	0.007	Neither/ non-diurnal	-2.15 (-3.49, -0.51)	0.86	-2.50	0.01
			Fossorial	2.07 (0.45, 3.69)	0.87	2.38	0.02
			Aquatic	2.93 (1.52, 4.54)	0.89	3.28	0.001
			Diurnal	-2.69 (-15.9, 0.11)	1.31	-2.05	0.04

DISCUSSION

AMPHIBIANS EXHIBIT A HIGH DIVERSITY OF PUPIL SHAPES

In our assessment of pupil shape in nearly 1300 extant amphibian species (*c.* 15% of described species) we observed the greatest diversity among anurans. This diversity is in stark contrast to birds, turtles and teleost fishes, which all have predominantly non-elongated, circular pupils, and to crocodylians, in which pupils all constrict to a vertical slit (Douglas, 2018). Mammals and squamate reptiles, however, exhibit a wide diversity of pupil shapes, including shapes we did not observe in amphibians. For instance, many ungulates have horizontally elongated, rectangular pupil shapes (Miller & Murphy, 2016). Likewise, some geckos have scalloped edges along the pupil margin such that when the pupil constricts they are left with a vertical row of pinhole pupils (e.g. Mann, 1931). Although we did not observe this extensive scalloping in amphibians, we did see irregular pupil margins in many anuran species (in association with opercula and umbracula; e.g. brevicipitid rain frogs and centrolenid glass frogs) that could result in multiple pupil apertures if the pupil is constricted to a greater degree than we observed in available photographs. The proposed functional advantage of multiple apertures is that they enable accurate depth perception even when the pupil is constricted (Douglas, 2018). Alternatively, irregular pupil shapes may serve to conceal the eye as proposed for some bottom-dwelling fishes and for some reptiles (Walls, 1942; Douglas *et al.*, 2002; Roth *et al.*, 2009; Douglas, 2018; Youn *et al.*, 2019). Finally, it has been suggested that the shape and orientation of a constricted pupil may correspond to the shape and orientation of increased photoreceptor density in the retina (i.e. retinal streaks), but this hypothesis is not supported in the birds, mammals and fishes examined

to date (Douglas, 2018). The variation in amphibian pupil shape we documented in the present study is consistent with a recently published, independent study of pupil shape in frogs and salamanders (Cervino *et al.*, 2021), and warrants further attention with respect to the underlying musculature of the iris, latency and extent of the pupillary response, presence of multifocal lenses, and arrangement of photoreceptor cells in the retina to better understand the functional consequences of this diversity.

ONTOGENETIC CHANGES IN PUPIL SHAPE

Our sampling of larval and adult pupil shape across 92 ecologically diverse species of frog and salamander indicates that pupils are likely non-elongated and circular in most or all amphibian larvae. In addition, in many species that occupy distinct habitats before and after metamorphosis, pupil shape changes during ontogeny. In particular, species that remain in aquatic habitats as adults retain non-elongated, circular pupils, whereas species that occupy non-aquatic habitats as adults exhibit non-elongated, horizontally elongated and vertically elongated shapes. Thus, our results are consistent with other studies of the visual system in larval and adult amphibians demonstrating that eye-body scaling (Shrimpton *et al.*, 2021), lens shape (Sivak & Warburg, 1980, 1983) and whole-eye gene expression (Schott *et al.*, 2022) are decoupled when larvae and adults inhabit different light environments. Detailed examination of the iris musculature in developmental series of species that do and do not exhibit changes in pupil shape across ontogeny would provide greater insight into the key anatomical differences and onset of these changes within and among species. In addition, it is not clear whether pupils in some or all amphibian larvae have a pupillary light response. We explored this in larvae of two species (*Bufo bufo* and *Rana*

temporaria) and did not observe any changes in pupil diameter or shape when exposed to bright light after 1 h of dark adaptation (Supporting Information, Part B; Fig. S5). We propose that future studies investigate the extent of the pupillary response in a more diverse sample of amphibian larvae, including species that may experience a wider range of light environments than fully aquatic larvae (e.g. semi-terrestrial larvae).

TRANSITIONS IN PUPIL SHAPE ACROSS THE PHYLOGENY

Pupil shape is often considered an important diagnostic character in anuran systematics (e.g. [Drewes, 1984](#); [Nuin & do Val, 2005](#); [Rödel *et al.*, 2009](#); [Menzies & Riyanto, 2015](#)), which relies on its stability within a taxon of interest. Correspondingly, the orientation of the pupil (non-elongated, horizontally elongated, vertically elongated) is largely conserved within several families that we sampled extensively (e.g. *Bufo*nidae, Hylidae, Phyllomedusidae, Ranidae). Furthermore, pupil shape is conserved within (and divergent among) genera in some families (e.g. *Afraxalus* and *Hyperolius* in the family Hyperoliidae). Yet, we also found that some genera (e.g. *Nyctibatrachus*, *Telmatobius*) exhibited diversity in pupil shape among closely related species. A recently published study that investigated transitions in pupil shape across frogs and salamanders, but with seven shape categories, also found strong support for the evolutionary lability of this trait ([Cervino *et al.*, 2021](#)). Thus, pupil shape appears to be an evolutionarily labile trait at both deep and recent timescales across amphibians, suggesting that it may not be a reliable character for systematics at some taxonomic levels and in some lineages. Pupil shape also varies among closely related species in elapid snakes ([Brischoux *et al.*, 2010](#)), and in felids and canids ([Banks *et al.*, 2015](#)), likely reflecting the diverse visual environments these tetrapod groups occupy.

Ancestral character-state reconstructions infer that the ancestral state for caecilians and salamanders was a non-elongated pupil, whereas for frogs, vertically elongated pupils were the ancestral state. This result is in contrast to a recent study, which found support for vertical pupils as the ancestral state for salamanders and frogs ([Cervino *et al.*, 2021](#)). This discrepancy largely results from differences in how each study categorized three cryptobranchid salamanders that we consider to have circular pupils (see Supporting Information Part C for details), though may also be due to a greater number of pupil shape categories (vertical, horizontal, rhomboidal, triangular, circular, fan and inverted fan) modeled in [Cervino *et al.* \(2021\)](#) and sparser taxonomic sampling outside of Anura [[Cervino *et al.* \(2021\)](#) did not include caecilians and classified

fewer salamanders]. Our results, however, suggest that elongated pupils evolved independently, and repeatedly, within salamanders and frogs. Elongated pupils are associated to some extent with multifocal lenses in vertebrates (including amphibians) in which the lens has concentric zones of different focal lengths that enable the animal to correct for chromatic aberration ([Kröger *et al.*, 1999](#); [Malmström & Kröger, 2006](#)). Consequently, an elongated pupil, which utilizes the whole lens diameter, enables the animal to use the full refractive range of the lens while regulating the total amount of light that reaches the retina, thus providing a sharp image across various wavelengths both in dim light (when the pupil is dilated and circular) and in bright light (when the pupil is constricted and elongated). The presence of elongated pupils in several anuran lineages, and in plethodontid and salamandrid salamanders, suggests they too may have multifocal lenses to minimize chromatic aberration in a range of light environments ([Malmström & Kröger, 2006](#)), though multifocal lenses are also present in birds, which have circular pupils ([Lind *et al.*, 2008](#)). Radiations like Afrobatrachia, which exhibit multiple transitions in pupil shape, have diurnal and nocturnal activity periods, and include colourful and sexually dichromatic species ([Portik *et al.*, 2019](#)), may be particularly fruitful for investigating the optical and evolutionary consequences of pupil elongation and whether it is associated with multifocal lenses.

ECOLOGICAL CORRELATES OF PUPIL SHAPE IN AMPHIBIANS AND OTHER VERTEBRATES

Animals that operate in a wide range of light levels, either because they are active both at night-time and during the day or because they move between aquatic and terrestrial environments, tend to have large pupillary ranges ([Douglas, 2018](#)). Pupils that are elongated (either vertically or horizontally) can constrict to a greater extent than pupils that maintain a circular shape when constricted due to the greater reduction in pupil area that can be achieved when the radial sphincter muscles forming the elongated pupil close the pupillary aperture with a 'scissor-like' action ([Walls, 1942](#); [Douglas, 2018](#)). The circular sphincter muscles around a circular pupil constrict the pupil to a lesser degree due to spatial constraints. Thus, elongated pupils are advantageous for species that rely on vision under a range of light conditions ([Hart *et al.*, 2006](#)). Correspondingly, there was a significant correlation between non-elongated pupils and amphibian species with fossorial lifestyles where individuals are active in a consistently dim environment and thus would not benefit from the extended range of constriction afforded by an elongated pupil.

Based on patterns observed in snakes (Brischoux *et al.*, 2010) and mammals (Mann, 1931), we predicted that amphibians active primarily during the day would be more likely to have non-elongated pupils than crepuscular, nocturnal or arrhythmic species. In contrast, we found that diurnal activity is associated with elongated pupils in amphibians. This finding is also different to previous studies that found no association between pupil shape and activity period in amphibians (Yovanovich *et al.*, 2020; Cervino *et al.*, 2021). The vast majority of species in our dataset had elongated pupils, regardless of activity period, and thus maintaining a greater range of pupil constriction is likely advantageous across most amphibian species.

In agreement with a recent study on frog and salamander pupil shape (Cervino *et al.*, 2021), we observed a correlation between non-elongated (mostly circular) pupils and species occupying aquatic habitats. It is possible that some aquatic species, like fossorial species, experience a diminished range of light levels relative to those inhabiting terrestrial habitats. Freshwaters often have high attenuation of light due to scattering and absorption caused by high concentrations of organic matter or suspended particulates from the surrounding land (Levine & MacNichol, 1982; Costa *et al.*, 2013; Fouilloux *et al.*, 2022). In such environments, even quite shallow depths can be considered 'dim' and thus frogs in these murky waters would likely not benefit from a greater dynamic range afforded by an elongated pupil. However, aquatic amphibians inhabiting highly transparent waters or well-illuminated surface waters should experience similar light conditions to terrestrial species. Therefore, aquatic amphibians may have circular pupils for reasons other than a limited light range. It is noteworthy that teleost fishes also have circular pupils that in most species are immobile (Douglas, 2018), and that all of the tadpoles we examined in this study also had circular pupils that did not appear to show a rapid or substantial pupillary response. Aquatic amphibians and teleosts have more spherical lenses than terrestrial vertebrates (Walls, 1942; Sivak *et al.*, 1985), and perhaps a spherical lens shape places physical constraints on the iris by protruding through the pupil, limiting pupil constriction in aquatic vertebrates. Further, both aquatic and fossorial frogs typically have small eyes (Thomas *et al.*, 2020a), which may be affected more than large eyes by the loss in sensitivity resulting from the greater reduction in aperture caused by an elongated pupil. Indeed, we found that species with smaller eyes tend to have non-elongated constricted pupils, whereas those with larger absolute eye sizes are more likely to have elongated constricted pupils.

It has been suggested that vertically elongated pupils provide greater astigmatic depth of field in vertical planes (Banks, 2015), which could provide better spatial resolution for navigating complex vertical environments. However, we, in agreement with Cervino *et al.* (2021), did not find a correlation between vertical pupils and scansorial lifestyles in amphibians. Furthermore, horizontally elongated pupil constriction is prevalent across diverse families of largely arboreal species including hylid treefrogs and hyperoliid reed frogs. Alternatively, vertical pupils may provide greater depth of field for ambush predators without the use of motion parallax movements, and horizontally elongated pupils may improve image quality and provide greater field of view for detecting potential predators (Banks, 2015). Future studies of feeding ecology and predator avoidance in closely related species that differ in pupil shape may shed light on the functional consequences of vertically vs. horizontally elongated pupils.

CONCLUSION

Pupil shape is diverse in amphibians, especially in anurans, with evolutionary transitions throughout much of the amphibian tree of life. For amphibians with a biphasic life history, pupil shape changes in many species that occupy distinct habitats before and after metamorphosis, with all larvae having circular pupils. Furthermore, non-elongated pupils were correlated with fossorial and aquatic lifestyles, and elongated pupils (vertical and horizontal) were more common in species with larger absolute eye sizes. We did not find support for diurnal species having non-elongated pupils or for species navigating complex vertical habitats (arboreal and scansorial) having vertically elongated pupils. Amphibians provide an exciting group for future research exploring the anatomical, physiological, optical and ecological mechanisms underlying the evolution of pupil diversity.

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DATA AVAILABILITY

The datasets supporting this article are available from the Natural History Museum (London, UK) Data Portal: doi: 10.5519/4q5uvvpa (Thomas *et al.*, 2022b), and the code to replicate the analyses and generate the figures is available on GitHub: <https://github.com/knthomas/amphibian-pupils>.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Part A. Categorization of amphibian pupil shapes.

Figure S1. Diagrams of constricted pupil shapes observed in amphibians.

Table S1. Frequency of pupil shapes observed across adult amphibians.

Figure S2. Examples of pupil shapes in salamanders (Caudata).

Part B. The pupillary light response of amphibians.

Figure S3. The pupils of three unrestrained anurans in darkness and after exposure to bright illumination.

Table S2. Area of a fully dilated and a constricted pupil in three amphibian species relative to the area bounded by the outer edges of the visible iris.

Figure S4. Pupil light response of an unrestrained *Xenopus laevis* and *Rana temporaria*.

Figure S5. The pupils of two anuran larvae after exposure to bright illumination.

Part C. Comparison of amphibian pupil shape categorization across studies.

Part D. Species additions to the phylogeny.

Table S3. Sister taxa used to graft species onto the phylogeny.

Part E. Phylogeny and dataset used in analyses.

Figure S6. Phylogeny, pupil shape and ecology of all amphibian species sampled.

Part F. References used for pupil shape and ecology classification.