1   **Diversity and evolution of amphibian pupil shapes**

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3   Kate N. Thomas[1]*, Caitlyn Rich[2]*, Rachel Quock[2, 3]*, Jeffrey W. Streicher[1], David J. Gower[1], Ryan

4   K. Schott[4,5], Matthew K. Fujita[6], Rayna C. Bell[2,5]

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6   [1] Department of Life Sciences, The Natural History Museum, London SW7 5BD, UK

7   [2] Department of Herpetology, California Academy of Sciences, San Francisco, CA 94118, USA

8   [3] Department of Biology, San Francisco State University, San Francisco, CA 94132, USA

9   [4] Department of Biology, York University, Toronto M3J 1P3, Canada

10   [5] Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian

11   Institution, Washington, DC 20560-0162, USA

12   [6] Department of Biology, Amphibian and Reptile Diversity Research Center, The University of

13   Texas at Arlington, Arlington, TX 76019, USA

14   *These authors contributed equally

15

16   Corresponding authors:
17   Kate Thomas kate.thomas@nhm.ac.uk
18   Department of Life Sciences
19   The Natural History Museum
20   London SW7 5BD, UK
21
22   Rayna Bell rbell@calacademy.org
23   Department of Herpetology
24   California Academy of Sciences
25   San Francisco, CA 94118, USA
26

27   Running title: evolution of amphibian pupil shape

28   **Abstract**

29   Pupil constriction has important functional consequences for animal vision, yet the

30   evolutionary mechanisms underlying diverse pupil sizes and shapes, often among animals that

31   occupy optically similar environments, are poorly understood. We aimed to quantify the

32   diversity and evolution of pupil shapes among amphibians and test for potential correlations to

33   ecology based on functional hypotheses. Using photographs, we surveyed pupil shape and the

34   orientation of the constricted pupil across adults of 1293 amphibian species, 72 families, and 3

35   orders, and additionally for larval life stages for all families of frogs and salamanders with a

36   biphasic ontogeny. Pupil shape is exceptionally diverse in amphibians with evolutionary

37   transitions throughout the amphibian tree of life. For amphibians with a biphasic life history,

38   we found that pupils change in many species that occupy distinct habitats before and after

39   metamorphosis. Finally, we found that non-elongated (round or diamond) constricted pupils

40   were correlated with species inhabiting consistently dim light environments (burrowing and

41   aquatic species) and that elongated pupils (vertical and horizontal) were more common in

42   species with larger absolute eye sizes. We propose that amphibians provide a valuable group

43   within which to explore the anatomical, physiological, optical, and ecological mechanisms

44   underlying the evolution of pupil shape.

45

46   **Key words:** activity period - Anura - aquatic - Caudata - fossorial - Gymnophiona - optics -

47   scansorial - vision

48 **Introduction**

49   The ability to detect light and form images is an important sensory modality for most

50 animals. Almost all animal phyla have evolved light-sensitive organs, ranging from eye-spots

51 that simply detect the presence or absence of light to compound eyes that detect fast

52 movement, providing a wide field of view and allowing images to be formed (Halder *et al.*,

53 1995). Vertebrates and cephalopods have camera-type eyes, in which the aperture of the iris

54 (the pupil) modulates the amount of light that enters the eye. In most species, iris muscles

55 contract or relax to dilate or constrict the pupil in response to ambient light levels. This action

56 dynamically changes the size of the aperture, enabling the individual to adjust the sensitivity

57 and resolution of their visual system to current conditions, termed the pupillary light response

58 (e.g., Douglas, 2018). The configuration of iris musculature determines the extent of

59 constriction and the shape of the constricted pupil, ranging from the round and fixed pupils of

60 most teleost fishes to the highly dynamic and complex pupil shapes of cephalopods (Douglas,

61 2018). The shape of the constricted pupil, in combination with other optical properties of the

62 eye, will also reduce spherical aberration and can determine which wavelengths of light are

63 focused on the retina (Kröger *et al.*, 1999; Malmström & Kröger, 2006; Roth *et al.*, 2009).

64 Despite the clear functional consequences of pupil constriction for animal vision, the

65 evolutionary mechanisms underlying diverse pupil sizes and shapes, often among animals that

66 occupy optically similar environments, are poorly understood.

67   Pupil diversity in vertebrates includes non-elongated shapes (e.g., circular) and vertically

68 or horizontally elongated shapes (Douglas, 2018). Some vertebrate groups exhibit little

69 variation in constricted pupil shape: birds, turtles, and teleost fishes all have predominantly

70   non-elongated, round constricted pupils, and all crocodilian pupils constrict to a vertical slit

71   (reviewed in Douglas, 2018). By contrast, within mammals, squamates (lizards and snakes), and

72   amphibians (frogs, salamanders, and caecilians) constricted pupils include all three orientations

73   (Douglas, 2018), which may reflect the greater diversity of light environments these lineages

74   occupy and their corresponding visual ecologies. For instance, vertical pupil constriction in

75   elapid snakes (cobras, mambas and marine snakes) is correlated with diel activity and foraging

76   mode: the constricted pupils of nocturnal species that are ambush predators are vertical,

77   whereas those of diurnal species that are active foragers are circular (Brishoux *et al.*, 2010). In

78   mammals, pupil constriction is also correlated with activity period, with horizontally elongated

79   and non-elongated pupils occurring in diurnal species and vertically elongated or slit pupils in

80   nocturnal and crepuscular species (Mann, 1931). Elongated, slit-like pupil constrictions are also

81   hypothesized to enhance vision in particular orientations but with conflicting evidence. For

82   instance, vertically elongated pupils have been proposed to increase depth of field in a

83   horizontal plane (e.g., Brishoux *et al.*, 2010) or alternatively in a vertical plane (e.g., Hart *et al.*,

84   2006; Banks *et al.*, 2015). These hypotheses, however, have been explored in only a relatively

85   small subset of the phylogenetic and ecological diversity of vertebrates. Here, we aim to

86   quantify the diversity and evolution of pupil shapes among amphibians and test for potential

87   correlations to ecology based on functional hypotheses.

88   Amphibians are a speciose (>8,300 extant species: AmphibiaWeb, 2021), diverse, and

89   ecologically rich radiation with repeated evolutionary transitions in activity period and habitat

90   that influence the light environments in which they are active, and have evolved, in. Although

91   pupil shape has been studied in the context of species identification and systematics in some

92   groups (e.g., Drewes, 1984; Glaw & Vences, 1997; Nuin & do Val, 2005; Rödel *et al.*, 2009;

93   Menzies & Riyanto, 2015), the evolutionary lability and functional consequences of different

94   pupil shapes in amphibians are poorly understood. The limbless caecilian amphibians (order

95   Gymnophiona; >200 extant species) are predominantly fossorial with greatly reduced visual

96   systems, including eyes covered by skin and/or bone in many lineages (Mohun *et al.*, 2010;

97   Walls, 1942; Wake, 1985; Wilkinson, 1997). In even the most extensively developed eye of

98   extant caecilians, the iris musculature is rudimentary (Mohun & Wilkinson, 2015) or absent

99   (Himstedt, 1995), making changes in pupil size and shape unlikely (Douglas, 2018);

100   consequently, in this study we mostly focus on frogs (order Anura, >7300 extant species) and

101   salamanders (order Caudata, >700 extant species). A recent study characterized variation in

102   absolute and relative eye size across all anuran families, and determined that frogs generally

103   have large eyes relative to other vertebrates and that variation in adult eye size is associated

104   with differences in habitat, activity period, and breeding ecology (Thomas *et al.*, 2020).

105   Variation in salamander eye size has not yet been quantified, but this lineage is also ecologically

106   diverse with fully aquatic, arboreal, and fossorial species that likely differ substantially in visual

107   ecology. Both frogs and salamanders are considered visual predators, and behavioral studies in

108   both groups indicate that visual signals and coloration can play an important role in

109   intraspecific communication (Jarger & Forester, 1993; Haddad & Giaretta, 1999; Hödel &

110   Amezquita, 2001; Starnberger *et al.*, 2014; Yovanovich *et al.*, 2017). Likewise, both groups

111   include species that are primarily diurnal, primarily nocturnal, or that are active under a range

112   of light conditions (Anderson & Wiens, 2017). Consequently, both visual acuity and color

113   discrimination may be important for many amphibian species in bright and/or dim light

114   conditions (e.g., Toledo *et al.*, 2007; Robertson & Greene, 2017). Furthermore, species that are

115   active in both bright and dim light, and/or that have particularly large eyes, may rely on a large

116   pupillary range to optimise visual performance relative to their surroundings because slit pupils

117   allow a larger range of contraction (Walls, 1942).

118   Many amphibians have a biphasic ontogeny with an aquatic larval stage (termed

119   tadpoles in frogs) and terrestrial adult life stages (e.g., McDiarmid & Altig, 1999), whereas

120   others retain aquatic lifestyles as adults, have semi-terrestrial larvae, or develop without a

121   larval life stage (termed direct development). During amphibian metamorphosis, dramatic

122   morphological and physiological changes occur, including alterations to the visual system

123   (Hoskins, 1990). Changes in eye-body scaling (Shrimpton *et al.*, 2021) and lens shape (Sivak &

124   Warburg, 1980; Sivak & Warburg, 1983) across ontogeny in frogs suggest that several structural

125   aspects of the visual system adapt to both tadpole and adult visual requirements. Likewise,

126   whole-eye differential expression of aquatic tadpoles versus terrestrial juvenile frogs (Schott *et*

127   *al.*, *in review*) demonstrates changes in a suite of visual genes related to eye and retinal

128   development, light detection, lens crystallins, and phototransduction, indicating substantial

129   decoupling between life stages at the level of gene expression. The biphasic ontogeny and shift

130   between aquatic larval and terrestrial adult habitats in many amphibians is unique among

131   tetrapods and thus presents the opportunity to investigate whether pupil shape is adaptively

132   decoupled between life stages.

133   Here we survey and classify constricted pupil shape and orientation across adults of

134   1293 amphibian species, 72 families, and 3 orders, and additionally for larval life stages for all

135   families of frogs and salamanders with a biphasic ontogeny. We first test the hypothesis that

136   pupil shape changes across biphasic ontogeny in species that occupy distinct habitats before

137   and after metamorphosis. Second, we identify evolutionary lineages with extensive pupil shape

138   variation and quantify transition rates in pupil shape across the phylogeny. Finally, we test

139   whether pupil shape exhibits correlated evolution with traits relevant to amphibian visual

140   ecology. Specifically, we test whether (1) non-elongated pupils are correlated with inhabiting

141   consistently dim light (aquatic or fossorial habitats) environments, (2) elongated pupils are

142   associated with nocturnal and crepuscular activity, (3) vertically elongated pupils are correlated

143   with navigating complex vertical (arboreal) habitats, and (4) elongated pupils are more common

144   in species with large absolute eye size.

145

146   **Methods**

147   ***Species sampling and pupil classification***

148   To assess the diversity of pupil shapes across amphibians, we searched online photo

149   databases (primarily CalPhotos, https://calphotos.berkeley.edu) for images in which the eye

150   and partially or fully constricted pupil shape was visible. Dilated pupils are circular, therefore

151   we assumed pupils that were elongated in photos were at least partially constricted. For non-

152   elongated pupils it is more challenging to determine whether the pupil is constricted from a

153   photo but we relied on pupil size relative to eye size. We aimed to sample at least one species

154   per family or sub-family of all currently recognized amphibian orders with externally visible

155   eyes (72 families; Frost, 2021). When suitable images for a target family or species were not

156   available on CalPhotos, we searched for photographs on other user-upload sites (e.g., Flickr),

157   field guides, and primary literature. In a few instances, we relied on our personal photographs

158   and field notes. Constricted pupil shapes (round, diamond, almond/oval, slit, triangle, tear) and

159   orientations (non-elongated, horizontally elongated, vertically elongated) for each species were

160   independently classified and reviewed by at least two observers (examples of shapes and axes

161   of constrictions are in Figure 1, S1). Any discrepancies were flagged and resolved with the input

162   of additional observers and photographs when available, or removed from the dataset when

163   uncertainty remained. Larval frogs and salamanders, and adult caecilians, apparently lack or

164   have a very weak pupillary response (Douglas, 2018) and thus our scoring in these instances are

165   likely of permanently (or near-permanently) fixed pupil shapes that do not clearly correspond

166   with either the fully dilated or fully constricted pupils of adult frogs and salamanders. Likewise,

167   we note that almond or oval shapes in both horizontally and vertically elongated pupils may

168   further constrict to a narrow slit under brighter light conditions. Because we relied on

169   photographs to classify pupil shapes rather than experimentally assessing pupillary response,

170   our determination of "almond or oval" versus "slit" pupil shapes were limited by the available

171   photographs. However, our approach provides a more comprehensive survey of pupil

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

173   Once we had surveyed representatives of each family or sub-family, we expanded our

174   sampling to encompass species that were the focus of recent studies on anuran visual biology

175   (e.g., Thomas *et al.*, 2020; Thomas *et al.*, *in review*; Shrimpton *et al.*, 2021) to maximize overlap

176   with existing datasets. Preliminary assessments of this diversity suggested that pupil shape was

177   diverse and/or evolutionarily labile in particular lineages and thus we elected to sample these

178   groups in more depth. This included families in the Afrobatrachia radiation (Arthroleptidae,

179   Brevicipitidae, Hemisotidae, Hyperoliidae), and the families or super-families Hylidae,

180   Microhylidae, and Myobatrachidae. For families with extensive ecological diversity as adults

181   (i.e., fully aquatic, semi-aquatic, ground-dwelling, arboreal, fossorial) we aimed to sample

182   representative species of this diversity. Our final dataset included pupil shape observations for

183   1241 species of Anura (56 families), 43 species of Caudata (10 families), and 9 species of

184   Gymnophiona (6 families). Pupil shape and associated references are given in the

185   supplementary data files.

186

187   ***Phylogeny***

188   We used the phylogenetic hypothesis of Jetz & Pyron (2018) for visualizing trait

189   distributions and modeling trait evolution across species. This phylogeny used a molecular

190   backbone as well as taxonomic information to include proposed relationships among 7238

191   amphibian species. We matched the phylogeny to our dataset and performed all subsequent

192   analyses using R v.4.1.0 (R Core Team, 2021) in RStudio v.1.4.1717 (RStudio Team, 2021). We

193   used the R package AmphiNom v.1.0.1 (Liedtke, 2019) to match tip labels in the phylogeny to

194   species names in our dataset by converting both to the taxonomy of Frost (2021) and manually

195   checking and matching any species with multiple synonyms. For 46 species in our dataset not

196   represented in the phylogeny, we used published literature to find the closest sister taxa that

197   were represented in the tree (Table S1) and then added the missing species to the node

198   representing the most recent common ancestor of these taxa using the getSisters, findMRCA,

199   and bind.tip functions in phytools v.0.7.70 (Revell, 2012). Finally, we pruned the phylogeny to

200   the 1293 species in our dataset using drop.tip in ape v.5.4.1 (Paradis *et al*., 2004; Paradis &

201   Schliep, 2019) and randomly resolved polytomies with the bifurcr function in the PDcalc

202   v.0.4.3.900 package (Nipperess & Wilson, 2021).

203

204   ***Adult habitat and activity period classification***

205   Adult ecology was categorized into binary states for activity pattern and different

206   aspects of habitat using peer-reviewed literature, online natural-history resources, field guides,

207   and field observations (see Supplemental References): 1) primarily diurnal or non-diurnal, 2)

208   aquatic or non-aquatic, 3) fossorial or non-fossorial, and 4) scansorial or non-scansorial (see

209   Supplemental data). Categorizations were simplified versions of those used by Thomas *et al.*

210   (2020). Species were classed as primarily diurnal if adults are primarily active in daylight above

211   ground; arrhythmic, cathemeral, crepuscular, and nocturnal species were all classified as non-

212   diurnal. Species in which adults are primarily visually active underwater were categorized as

213   aquatic. Species were classified as fossorial if adults are active underground, typically in soil (as

214   opposed to only aestivating or sheltering underground). Finally, species in which adults climb

215   up off the ground in vegetation were classified as scansorial.

216

217   ***Pupil shape across biphasic ontogeny***

218   To assess variation in pupil shape among larval frogs and salamanders, we searched

219   through field guides, primary literature, and online photograph databases (e.g., CalPhotos,

220   Flickr) and categorized pupils as described above. We classified larval pupil shape and

221   orientation for at least one species in every family that has species with a larval life stage,

222   including representative species with different larval habitats (i.e., semiterrestrial, phytotelm,

223   pond, and stream-dwelling). To identify which lineages exhibit changes in pupil shape between

224   larval and adult life stages, we classified pupil shape in adults for all species for which we

225   determined larval pupil shape. As with larval habitat diversity, we also aimed to maximize adult

226   habitat diversity in this paired sampling (i.e., fully aquatic, semi-aquatic, ground-dwelling,

227   arboreal, fossorial). Both larval and adult habitat classifications were determined based on field

228   guides, primary literature, and expert knowledge. To visualize variation in an evolutionary

229   context, we mapped tadpole and adult pupil shapes and habitats on the modified Jetz & Pyron

230   (2018) phylogeny using ape (Paradis *et al*., 2004; Paradis & Schliep, 2019). Pupil shape, habitat

231   classifications, and associated references are listed in the supplementary data files.

232

233   ***Evolutionary transitions of pupil orientation across the amphibian phylogeny***

234   To gain insights into the evolutionary history and lability of adult pupil shape across the

235   amphibian phylogeny, we implemented stochastic character mapping (Bollback, 2006) for the

236   three categories of pupil orientation (non-elongated, vertically elongated, horizontally

237   elongated). We used the fitDiscrete function in phytools v 0.7.70 (Revell, 2012) to fit equal-

238   rates, symmetrical-rates, and all-rates-different models of character evolution. To select the

239   "best" model, we compared AICc scores and AIC weights and then used make.simmap with the

240   best-fit transition model (all-rates-different) to simulate character evolution across 100 trees.

241   We plotted the phylogeny with branches colored based on the highest likelihood state of the

242   node it originated from, and summarized mean pairwise transitions between each set of states

243   across the 100 simulations.

244

245   *Effects of species ecology on pupil orientation*

246   We implemented multivariate phylogenetic logistic regression in the R package phylolm

247   (Paradis & Claude, 2002; Ives & Garland, 2010; Tung Ho & Ane, 2014) to examine the

248   correlation structure among binary states for pupil orientation and ecology, using the general

249   model format of pupil elongation ~ ecology. We tested three predictions in three separate

250   models. First, we tested whether non-elongated pupils are associated with diurnal activity

251   patterns (pupils: 0 = elongated, 1 = non-elongated; ecology: 0 = non-diurnal, 1 = diurnal).

252   Second, we tested whether vertically elongated pupils are associated with scansoriality (pupils:

253   0 = non-vertical, 1 = vertical; ecology: 0 = non-scansorial, 1 = scansorial). Third, we tested

254   whether non-elongated pupils are associated with fossorial and aquatic habitats (pupils: 0 =

255   elongated, 1 = non-elongated; ecology: 0 = not fossorial or aquatic, 1 = fossorial or aquatic). We

256   used the logistic_MPLE method, which maximizes the penalized likelihood of the logistic

257   regression, and ran 1000 bootstrap replicates to estimate coefficients.

258   To test the prediction that species with large eyes would benefit from having a large

259   pupillary range facilitated by elongated pupils, we tested for a correlation between eye size

260   (transverse eye diameter) and elongated (horizontal or vertical) constricted pupils using a

261   phylogenetic least squares regression in caper v.1.0.1 (Orme *et al.*, 2018). Eye size data for 207

262   anuran species representing 54 families were gathered from a previous study (Thomas *et al.*,

263   2020a; 2020b) and matched to our pupil dataset. We used phytools v.0.7.80 (Revell 2012) and

264   ggplot2 v.3.3.3 (Wickham 2016) to visualize the data.

265

266   **Data availability statement**

267   The datasets supporting this article are available from the Dryad Digital Repository:

268   [provided upon acceptance, currently Supplemental Data], and code to replicate analyses and

269   generate figures is available on GitHub ([provided upon acceptance]).

270

271   **Results**

272   ***Pupil shape diversity across amphibians***

273   We examined pupil shape in nine species of Gymnophiona that occupy aquatic or

274   fossorial habitats, all of which had non-elongated, circular pupils (Figure 2, S1). Pupil shape was

275   more diverse across the 43 species of Caudata we classified, with non-elongated (circular) and

276   horizontally elongated (almond/oval, slit, upside-down triangle) pupil shapes (Figure 2, S1). Our

277   sampling of salamanders included a greater diversity of habitats than the caecilians (aquatic,

278   fossorial, and scansorial) and also included diurnal species. Finally, we observed the greatest

279   diversity of pupil shape in the 1241 species of Anura we examined, including non-elongated

280   (circular, diamond), horizontally elongated (almond/oval, diamond, slit, upside-down triangle),

281   and vertically elongated (almond/oval, diamond, sideways triangle, slit, upside-down teardrop)

282   constricted pupils (Figure 1, 2). Our sampling of frogs included the greatest diversity of species

283   and ecologies (i.e., fully aquatic, semi-aquatic, ground-dwelling, arboreal, fossorial) and we

284   sampled representative species of this diversity within families. Pupil shape was notably diverse

285   in the Afrobatrachia radiation (Arthroleptidae, Brevicipitidae, Hemisotidae, Hyperoliidae) and

286   the families or super-families Hylidae, Microhylidae, and Myobatrachidae with all three major

287   axes of pupil constriction represented in each of these lineages (Figure 2, S2). By contrast, other

288   speciose and ecologically diverse lineages, such as Bufonidae, all exhibited horizontal

289 almond/oval pupil shapes (Figure 2, S2). Adults of the fully-aquatic clawed frogs (Pipidae), giant

290 salamanders (Cryptobranchidae), sirens (Sirenidae), amphiumas (Amphiumidae), and torrent

291 salamanders (Rhyacotritonidae) all exhibited round pupils (Figure 2, S2) as did the fossorial

292 frogs in Rhinophrynidae and Nasikabatrachidae (Figure 2, S2).

293

294 ***Pupil shape across biphasic ontogeny***

295 Larval pupil shape was circular in all 92 species of frog and salamander that we surveyed

296 regardless of their habitat (Figure 1, 3). For instance, the larvae of the Reed Frog *Hyperolius*

297 *thomensis* develop in phytotelma (small pools of murky water that collect in tree cavities) and

298 have non-elongated, circular pupils like those of the larvae of congeners *H. endjami*, which

299 develop in ponds and streams (Figure S2, Supplementary data files). Likewise, semi-terrestrial

300 tadpoles that develop in the splash zones of waterfalls (e.g., Rock River Frog *Thoropa miliaris*),

301 in terrestrial nests (e.g., Nurse Frog *Allobates magnussoni*), or in dorsal pouches (e.g., Marsupial

302 Frog *Gastrotheca piperata*) all have circular pupils. The only exception was the fossorial

303 tadpoles of the Dancing Frog *Micrixalus herrei,* which hide within the gravel of streambeds, and

304 appear to have skin-covered eyes as larvae but fully-developed eyes with horizontal pupils as

305 adults (Senevirathne *et al.*, 2016). In the 10 species (3 Anura and 7 Caudata) in our dataset that

306 inhabit aquatic habitats as both larvae and adults, pupil shape remained circular in adults

307 (Figure 3, S2). In the species that transition from an aquatic larval stage to a fossorial,

308 scansorial, or ground-dwelling adult life stage, we observed all three axes of constricted pupil in

309 adults (Figure 3, S2). Collectively, these observations indicate that pupil shape changes across

310   biphasic ontogeny in many frog species that occupy distinct habitats before and after

311   metamorphosis.

312

Evolutionary transitions pupil orientation across the amphibian phylogeny

314   The "all-rates-different" model of character evolution was by far the best fit to our data

315   for pupil orientation (Table 1) and we found high transition rates across the phylogeny (average

316   of 84.56 changes between states across 100 total simulations) demonstrating the high

317   evolutionary lability of this trait. The majority of transitions occurred from non-elongated to

318   horizontally elongated pupils, whereas transitions from horizontally elongated to vertically

319   elongated pupils were the least common (Figure 4). Many of the evolutionary transitions were

320   concentrated within the Afrobatrachia radiation (Arthroleptidae, Brevicipitidae, Hemisotidae,

321   Hyperoliidae) and the families or super-families Microhylidae and Myobatrachidae.

322

Correlated evolution of species ecology and pupil orientation

324   Tests for correlation between the orientation of pupil constriction and ecology indicated

325   that fossorial and aquatic ecologies are associated with non-elongated pupils in amphibians,

326   while diurnality and scansoriality have no effects on pupil orientation (Table 2). Multivariate

327   phylogenetic logistic regression found no significant association between non-elongated pupils

328   and diurnal activity patterns across the 648 species in our activity pattern dataset; in fact, all of

329   the 72 primarily diurnal species studied had horizontally or vertically elongated pupils. Likewise,

330   we found no association between vertically elongated pupils and scansorial behaviors across

331   904 species. However, fossorial or aquatic habitats were a significant predictor of non-

332   elongated pupils among 902 species tested (Table 1). Finally, among 207 anuran amphibians

333   with data for both eye size and pupil shape, species with vertically or horizontally elongated

334   pupils had significantly larger eyes than species with non-elongated pupils (PGLS: F = 5.24, df =

335   1 and 205, R^2_{adj} = 0.02, p = 0.02).

336

337   **Discussion**

338   ***Amphibians exhibit exceptional diversity of pupil shapes***

339   In our assessment of pupil shape in nearly 1300 extant amphibian species (ca. 15% of

340   described species) we observed great diversity among anurans including multiple shapes (e.g.,

341   circle, diamond, triangle, slit, teardrop) within each of the major axes of constriction (non-

342   elongated, horizontally elongated, and vertically elongated). This diversity is in stark contrast to

343   birds, turtles, and teleost fishes, which all have predominantly non-elongated, round

344   constricted pupils and to crocodilians, in which pupils all constrict to a vertical slit (Douglas,

345   2018). Mammals and squamate reptiles, however, exhibit a wide diversity of pupil shapes,

346   including shapes we have not observed in amphibians. For instance, many ungulates have

347   horizontally constricting, rectangular pupil shapes (Miller & Murphy, 2016) that we did not

348   observe in amphibians. Likewise, some geckos have scalloped edges along the pupil margin

349   such that when the pupil constricts to a slit they are left with a vertical row of pinhole pupils

350   (Mann, 1931). While we did not observe this extensive scalloping in amphibians, we did see

351   irregular pupil margins in many anuran species (in association with opercula, elygia and

352   umbracula e.g., brevicipetid Rain Frogs, centrolenid Glass Frogs) that could result in multiple

353   pupil apertures if the pupil is further constricted than what we observed in available

354   photographs. The proposed functional advantage of multiple apertures is that they enable

355   accurate depth perception even when the pupil is constricted (Douglas, 2018). Alternatively,

356   irregular pupil shapes may serve to conceal the eye as proposed for some bottom-dwelling

357   fishes and for some reptiles (Walls, 1942; Douglas *et al.*, 2002; Roth *et al.*, 2009; Douglas, 2018;

358   Youn *et al.*, 2019). Finally, it has been suggested that pupil constriction matches the shape and

359   location of increased photoreceptor density in the retina (i.e., retinal streaks) but this

360   hypothesis is not supported in the birds, mammals, and fishes examined to date (Douglas,

361   2018). The variation in amphibian pupil shape we documented in the present study, particularly

362   in frogs, warrants further attention with respect to the underlying musculature of the iris,

363   latency and extent of the pupillary response, properties of the lens, and the arrangement of

364   photoreceptor cells in the retina to better understand the functional consequences of this

365   diversity.

366

367   ***Ontogenetic changes in pupil shape***

368   Our sampling of larval and adult pupil shape and constricted pupil orientation across 92

369   ecologically diverse species of frog and salamander indicates that pupils are likely non-

370   elongated and round in most or all amphibian larvae. In addition, in many species that occupy

371   distinct habitats before and after metamorphosis pupil shape changes during ontogeny. In

372   particular, species that remain in aquatic habitats as adults retain non-elongated, round pupils

373   whereas many species that occupy non-aquatic habitats as adults exhibit all three major axes of

374   pupil constriction. Thus, our results are consistent with other studies of the visual system in

375   larval and adult amphibians demonstrating that eye-body scaling (Shrimpton *et al.*, 2021), lens

376   shape (Sivak & Warburg, 1980; Sivak & Warburg, 1983), and whole-eye gene expression (Schott

377   *et al.*, *in review*) are decoupled when larvae and adults inhabit different light environments.

378   Detailed examination of the iris musculature in developmental series of species that do and do

379   not exhibit changes in pupil shape across ontogeny would provide greater insight into the key

380   anatomical differences and onset of these changes within and among species. In addition, it is

381   not clear whether pupils in some or all amphibian larvae have a pupillary light response. We

382   explored this in larvae of two species (*Bufo bufo* and *Rana temporaria*) and did not observe any

383   changes in pupil diameter or shape when exposed to bright light after 1 hour of dark adaptation

384   (KNT, JWS, pers. obs.). We propose that future studies investigate the extent of pupillary

385   response in a more diverse sample of amphibian larvae, including species that may experience

386   a wider range of light environments than fully aquatic larvae (e.g., semi-terrestrial larvae).

387

388   ***Transitions in pupil orientations across the phylogeny***

389   Pupil shape is often considered an important diagnostic character for anuran

390   systematics (e.g., Drewes, 1984; Nuin & do Val, 2005; Rödel *et al.*, 2009; Menzies & Riyanto,

391   2015), and the orientation of pupil shape (non-elongated, horizontally elongated, vertically

392   elongated) is largely conserved within several families that we extensively sampled (e.g.,

393   Bufonidae, Hylidae, Phyllomedusidae, Ranidae). Furthermore, pupil shape is conserved within

394   (and divergent between) genera in some families (e.g., *Afrixalus* and *Hyperolius* in the family

395   Hyperoliidae). Yet, we also found that some genera exhibited diversity in pupil orientation and

396   shape among closely related species (e.g., *Nyctibatrachus*, *Telmatobius*). Thus, pupil shape

397   appears to be an evolutionarily labile trait at both deep and recent timescales across the

398   amphibia suggesting that this trait may not be a reliable character for systematics at some

399   taxonomic levels in some lineages. Pupil shape also varies among closely related species in

400   elapid snakes (Brischoux *et al.*, 2010), and in felids and canids (Banks *et al.*, 2015), likely

401   reflecting the diverse visual environments these tetrapod groups occupy.

402   Ancestral character state reconstructions infer that the ancestral state for caecilians and

403   salamanders was a non-elongated pupil whereas for frogs, vertically elongated pupils were the

404   ancestral state. This result is consistent with the observation that teleost fishes exhibit

405   predominantly non-elongated, circular constricted pupils (Douglas, 2018), and suggests that

406   horizontal pupils evolved independently, and repeatedly, within salamanders and frogs.

407   Elongated pupils are associated to some extent with multifocal lenses in which the lens has

408   concentric zones of different focal lengths that enable the animal to correct for chromatic

409   aberration or distortion (Malmström & Kröger, 2006). Consequently, an elongated pupil shape,

410   which utilizes the whole lens diameter, enables the animal to use the full refractive range of the

411   lens while regulating the total amount of light that enters the eye, thus providing crisp color

412   vision in dim light (when the pupil is dilated and circular) and in bright light (when the pupil is

413   constricted and elongated). The presence of elongated pupils in several anuran lineages, and in

414   plethodontid and salamandrid salamanders, suggests they may have multifocal lenses and rely

415   on color vision in a range of light environments (Malmström & Kröger, 2006), though multifocal

416   lenses are also present in birds, which have circular pupils (Lind *et al.*, 2008). Radiations like

417   Afrobatrachia, which exhibit multiple transitions in the orientation of pupil elongation, diurnal

418   and nocturnal activity periods, and include colorful and sexually dichromatic species (Portik *et*

419   *al.*, 2019), may be particularly fruitful for investigating the optical and evolutionary

420   consequences of pupil elongation.

421

422   ***Ecological correlates of pupil orientation in amphibians and other vertebrates***

423   Animals that are active in a wide range of light levels, either because they are active

424   both at nighttime and during the day or because they move between aquatic and terrestrial

425   environments, tend to have a large pupillary range (Douglas, 2018). Pupils that are elongated

426   (either vertically or horizontally) when constricted have a greater dynamic range than pupils

427   that maintain a circular shape when constricted and are advantageous for species that rely on

428   vision under a range of light conditions (Hart *et al.*, 2006). Correspondingly, there was a

429   significant correlation between non-elongated pupil constriction and amphibian species

430   inhabiting consistently dimmer light environments (fully aquatic and fossorial lifestyles).

431   Likewise, species with smaller absolute eye sizes tend to have non-elongated constricted pupils

432   whereas those with larger absolute eye sizes have elongated pupils. However, there was no

433   significant association between pupil elongation and activity period as proposed in other

434   vertebrates such as snakes (Brishoux *et al.*, 2010) and mammals (Mann, 1931). The vast

435   majority of species in our dataset had elongated pupils, regardless of activity period and thus

436   maintaining a greater range of pupil constriction is likely advantageous across most amphibian

437   species.

438   Vertically elongated pupils are proposed to provide greater astigmatic depth of field in

439   vertical planes (Banks, 2015), which could provide better spatial resolution for navigating

440   complex vertical environments. However, we did not find a correlation between vertical pupils

441   and scansorial behavior. Furthermore, horizontally elongated pupil constriction is prevalent

442   across diverse families of largely arboreal species including hylid treefrogs and hyperoliid reed

443   frogs. An alternative hypothesis for the functional consequences of vertically versus

444   horizontally elongated pupils points to the visual ecology of predator versus prey species

445   (Banks, 2015). In particular, vertical pupils are proposed to provide greater depth of field for

446   ambush predators without the use of motion parallax movements whereas horizontally

447   elongated pupils are proposed to improve image quality and provide greater field of view for

448   detecting potential predators (Banks, 2015). Future studies of feeding ecology and predator

449   avoidance in closely related species that differ in orientation of pupil shape may shed light on

450   the functional consequences of vertically versus horizontally elongated pupils.

451

452   **Conclusion**

453   Pupil shape is diverse in amphibians, especially in anurans, with evolutionary transitions

454   throughout the amphibian tree of life. For amphibians with a biphasic life history, pupil shape

455   changes in many species that occupy distinct habitats before and after metamorphosis, with all

456   larvae having circular pupils. Furthermore, non-elongated pupils were correlated with species

457   inhabiting consistently dim light environments (burrowing and aquatic species) and elongated

458   pupils (vertical and horizontal) were more common in species with larger absolute eye sizes.

459   We did not, however, find support for diurnal species having non-elongated pupil constriction

460   or for species navigating complex vertical habitats (arboreal and scansorial) having vertically

461   elongated pupils. Amphibians provide an exciting group for future research exploring the

462   anatomical, physiological, optical, and ecological mechanisms underlying the evolution of pupil

463   diversity.

464

465   **Acknowledgements**

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613

614 **Figures & Tables**

615 **Table 1:** Comparison of three Mk models of discrete character evolution for pupil constriction

616 (non-elongated, vertically elongated, horizontally elongated) across sampled amphibian species

617 (n = 1293). Models include an equal rates model with one transition rate parameter, a

618 symmetric rates model with 3 transition rate parameters, and an all rates different model with

619 6 transition rate parameters.

620

Model	log-lik	AICc	ΔAICc	AIC weight
equal rates	-342.6	687.2	44.6	0
symmetric rates	-339.7	685.4	41.9	0
all rates different	-316.5	645.0	0	1

621
622

623 **Table 2:** Summary of multivariate phylogenetic logistic regression analyses for the effects of

624 binary ecological traits on binary pupil constriction orientation. Bolded predictors of pupil

625 constriction are significant at Wald-type P < 0.05 for the given alpha value. Alpha is the

626 phylogenetic correlation parameter estimate from phyloglm. Coefficient estimates are shown

627 with upper and lower bootstrap estimates in parentheses based on 1000 fitted replicates.

628

Model	$N_{species}$	Alpha	Intercept	Predictor Coefficient
Non-elongated pupil ~			-1.52	-0.07
Diurnal activity	648	0.004	(-2.57, -0.27)	(-0.26, -0.62)
Vertical pupil ~			-2.40	0.004
Scansorial habitat	904	0.005	(-3.15, -0.30)	(-0.17, 0.14)
Non-elongated pupil ~			**-2.06**	**2.23**
Aquatic/fossorial habitat	902	0.005	**(-3.39, -1.25)**	**(1.50, 3.39)**

629

Figure 1: Examples of horizontally elongated, non-elongated, and vertically elongated adult and larval anuran pupil constriction shapes. We note that almond or oval pupil shapes may further constrict to a slit under bright light conditions. Photo 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), *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).
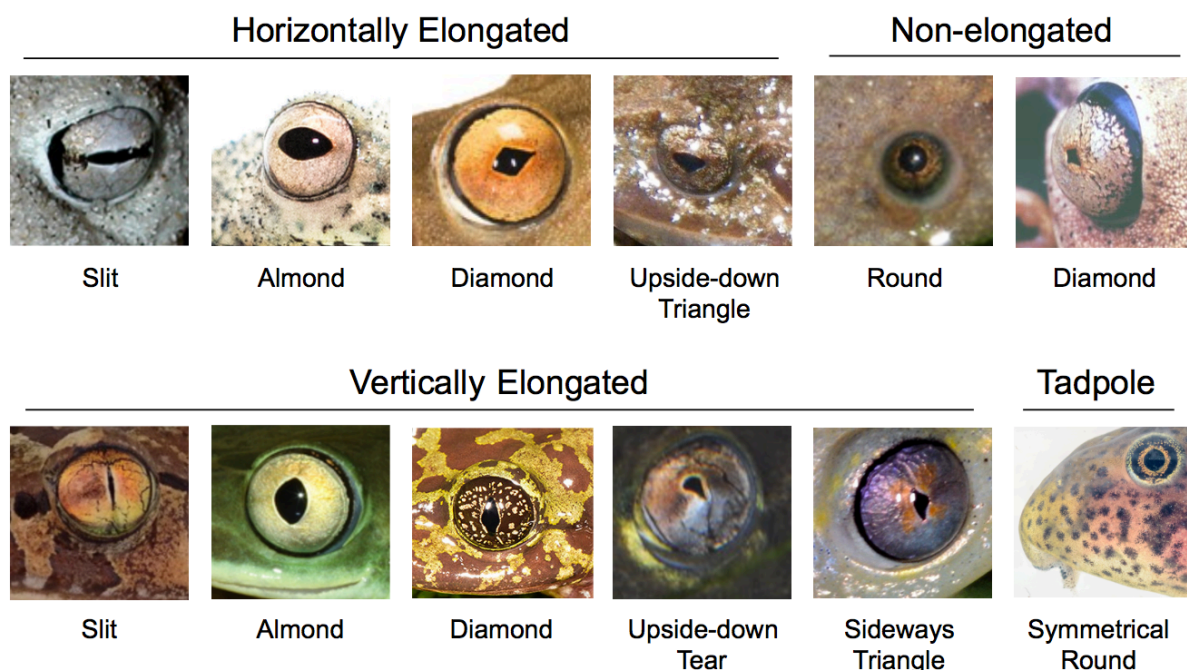
Figure 2: A phylogeny of all amphibian families that have developed eyes, showing the pupil shapes and constrictions we found within that family. We note that almond or oval pupil shapes may further constrict to a slit under bright light conditions but this was not always possible to assess from the available photographs. The phylogeny is modified from Jetz & Pyron (2018) and the complete dataset is shown in Figure S2.
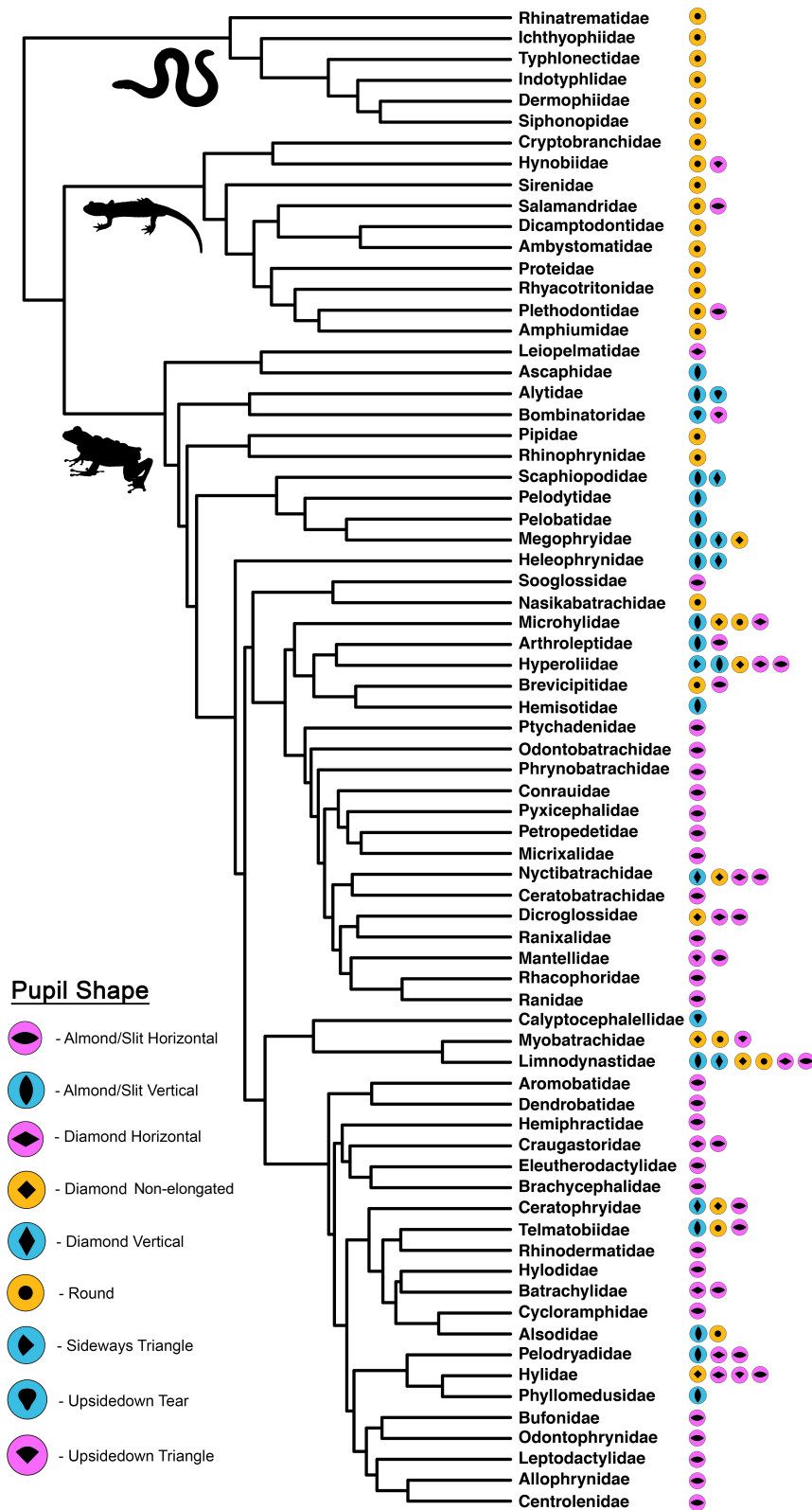
Figure 3: A phylogeny of larval and adult species pairs in our dataset (including representative

species for all amphibian families that have a larval life stage with developed eyes) with

observed pupil shapes and constrictions. The phylogeny is modified from Jetz & Pyron (2018)

and the complete dataset is shown in Figure S2.

Figure 4: Distribution of non-elongated and elongated pupil constriction (or shape for Gymnophiona) in adult life stages of 1293 amphibian species (phylogeny modified from Jetz & Pyron 2018). Branches are colored 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 labeled for reference. Inset depicts estimated transitions between non-elongated, horizontally elongated, and vertically elongated pupils based on stochastic character mapping. The thickness of the arrows is proportional to the mean transitions estimated across 100 simulations.
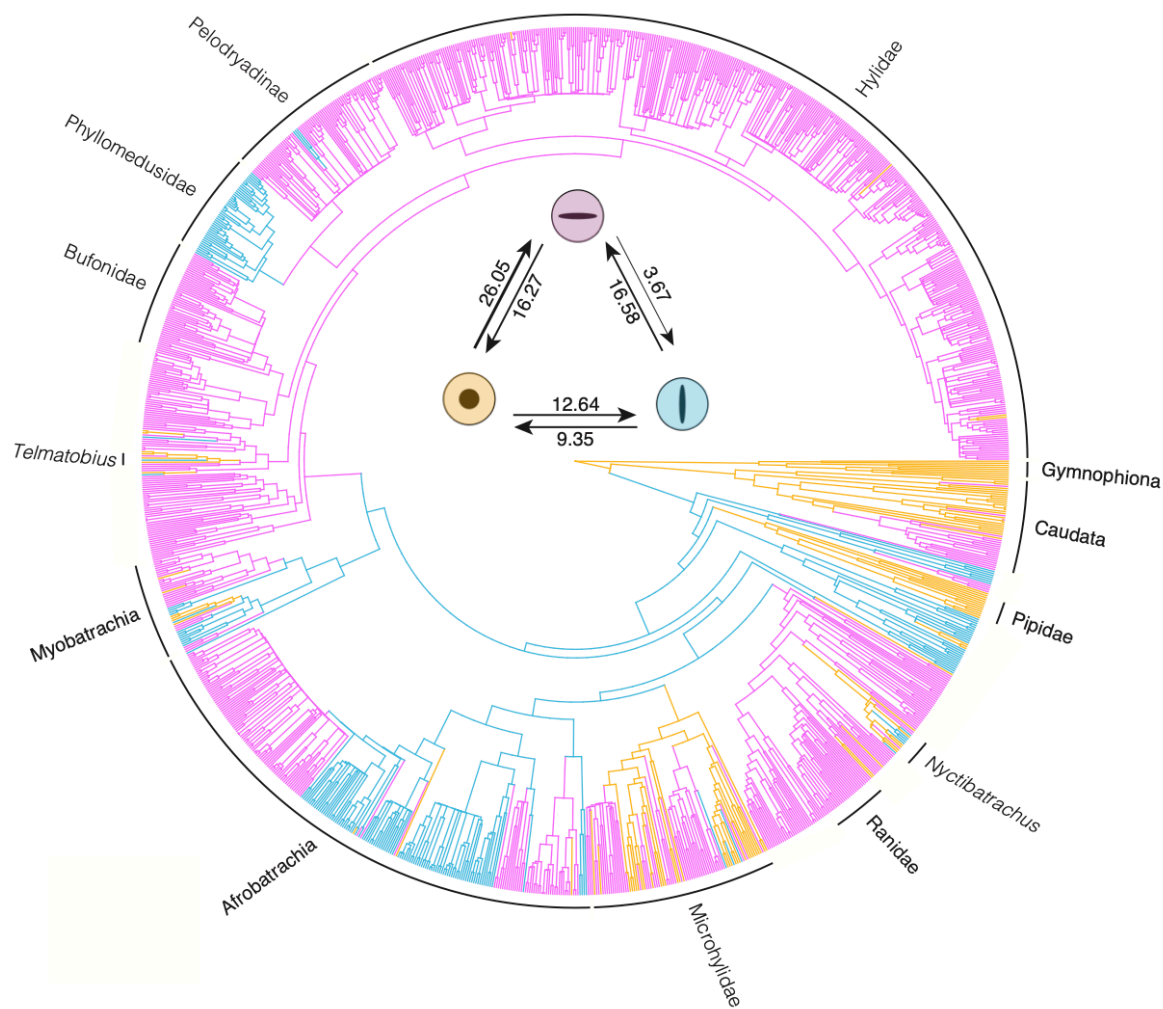
Figure 5: Eye size and the orientation of pupil constriction across 207 species of anuran amphibians (A). Species with elongated (horizontal or vertical) pupils have significantly larger eye diameters than those with non-elongated constricted pupils (B). Pip. = Pipidae, Meg. = Megophryidae, Myo. = Myobatrachidae, Hem. = Hemiphractidae, Cer. = Ceratophryidae, Ph. = Phyllomedusidae, Lepto. = Leptodactylidae, Pyx. = Pyxicephalidae, Dicro. = Dicroglossidae, H. = Hyperoliidae, Arth. = Arthroleptidae.