1 Title

2 The ecological function of insect egg micropyles

3 **Abstract**

4 1. Insect egg micropyles are openings through the chorion allowing sperm entry for fertilisation.

5 Micropyles are diverse structures showing remarkable variation in number, spatial arrangement

6 and physical structure across extant insect orders. Despite being almost ubiquitous across

7 insects, they have received little attention. As key morphological features of an immobile life

8 stage, it is plausible that part of the diversity exhibited by micropyles is adaptive, supporting

9 other egg structures during embryo development. So, whilst egg fertilization is the primary

10 function of micropyles, they could aid embryo development and be shaped by natural, as well as

11 sexual selection.

12 2. Here I first used ancestral reconstruction to investigate micropyle presence, number and

13 variation in primitive insects. Then, I used phylogenetic comparative analyses to explore the

14 ecological function of micropyle number.

15 3. I hypothesised that micropyle number correlated with: (i) aeropyle presence facilitating oxygen

16 exchange; (ii) aquatic oviposition supporting development in water; and is influenced by (iii)

17 critical bioclimatic variables.

18 4. Across 24 hexapod orders the most likely ancestral state was one or two micropyles,

19 interspecific variation was high and intraspecific variation low. Mean micropyle number ranged

20 from zero in Entognatha, Strepsiptera and Thysanoptera to 120 in *Panstrongylus geniculatus*,

21 Hemiptera, and over 100 on average for Apidae, Hymenoptera. Micropyle number was strongly

22 positively related to: i) egg size, with larger eggs having more micropyles; ii) the presence of

23 aeropyles; iii) annual precipitation, with eggs developing in habitats with low annual

24 precipitation exhibiting fewer micropyles; and iv) negatively related to micropyle width, insect

25   eggs having fewer larger micropyles or numerous smaller ones. However, aquatic oviposition did

26   not affect micropyle number.

27   5. Overall these findings point to an adaptive ecological function of egg micropyles in addition to

28   their primary fertilisation function. This is consistent with the hypothesis that micropyles aid

29   embryo survival, and so this almost-ubiquitous trait across insects is shaped by sexual and

30   natural selection pressures during this critical life stage.

31

32   **Keywords**: aeropyles, ancestral reconstruction, micropyle width, micropyle number, variation,

33   phylogenetic comparative analysis

34

35   **Introduction**

36   Micropyles are egg openings that allow sperm entry for fertilisation, not just in insects but in a wide

37   range of taxa, including fishes, cephalopods and plants (Yanagimachi et al., 2013; Lora et al., 2019).

38   In insects, the general structure of a micropyle is that of an outer opening on the egg's surface and

39   an internal channel through the chorion (Counce, 1973; Hinton, 1981). Post-copulation and chorion

40   formation (rather than preceding eggshell formation as in birds, Polhammer, 1978; Jamieson, 2011),

41   one or multiple sperm penetrate the micropyle during the egg's passage across the spermatheca

42   (Counce, 1973). Whilst the primary function of insect micropyles is the internal fertilization of the

43   egg, other egg structures enable embryo development. Aeropyles and respiratory horns (e.g. in

44   Diptera) allow gas and water exchange by diffusion between the embryo and the outside

45   environment, whereas hydropyles are responsible for water absorption in some taxa (e.g.

46   Plecoptera, Madhavan, 1974; Hinton, 1981).

47   Micropylar diversity is considerable, showing remarkable variation in number, spatial

48   arrangement and physical structure across insect orders (Cobben, 1968; Hinton, 1981; Trougakos &

49   Margaritis, 2003). Micropyle number varies not only inter-specifically, but also intra-specifically

50   within egg clutch or between females' egg clutches, e.g. 38-58 micropyles in *Chinavia runaspis*

51   (Hemiptera: Pentatomidae, Matesco et al., 2014), and 12-61 in *Kalotermes flavicollis* (Blattodea:

52   Kalotermitidae, Roonwal & Rathore, 1975). Micropyles are usually located at the anterior pole of the

53   egg but can be located dorsally (e.g. *Bacillus rossius*, Mazzini & Scali, 1977), ventrally (e.g. *Acheta*

54   spp., *Gryllus domesticus*, Sauer, 1966; *Teleogryllus* sp., Polhammer, 1978) and to the posterior pole

55   (e.g. *Lytta viridana,* Sweeny et al., 1968; Panorpidae, Ando, 1973). Multiple micropyles are

56   commonly located in close proximity (e.g. micropylar pit in Lepidoptera) or can be co-located with

57   aeropyles on protruding stalks, e.g. in the operculum in Reduviidae, Heteroptera (Haridass, 1986).

58   Sperm-specific structures, such as storage dome-shaped chambers, (e.g. in *Brachydiplax sobrina*,

59   Odonata: Libellulidae; Andrew, 2009) and sperm guides in tagenoform (funnel-shaped) micropyles

60   (e.g. Ephemeroptera; Koss & Edmunds, 1974) are also present. Internal channels trace various paths

61   into the chorion (U-shaped in Reduviidae, Haridass, 1986, oblique in Plecoptera, Rościszewska, 1991,

62   or curved in *Bombyx mori*, Yamauchi & Yoshitake, 1984) and are variable in length (0.5 to 1.5 µm,

63   Meloidae: *Lytta viridana*, Sweeny et al., 1968; 90 µm, Bruchidae: *Acanthoscelides obtectus,* Biemont

64   et al., 1981).

65   Micropyles are often used diagnostically at higher taxonomic level (order, family or genus)

66   but not at species level due to their variation in number (Downey & Allyn, 1981). However, the

67   functional significance of their diversity and specifically, the variation in micropyle number is, as yet,

68   underexplored. Micropyle diversity has primarily been thought to be shaped by sexual selection,

69   with emergent examples of direct female control of fertilisation via micropyle number manipulation

70   (Yashiro & Matsuura, 2014), male-female interaction (Sun et al., 2019) and correlation with

71   promiscuity (Iossa et al., 2016). Immobile life stages, such as eggs, are critical components of an

72   organism's life cycle impacting development, fitness and survival. As a single-cell life stage, insect

73   eggs serve two main functions: to be fertilised within the female body, and to allow embryo

74   development in environments as diverse as water, air or within a live host. To overcome the

75   challenges provided by these diverse, internal and external environments, insect eggs have evolved

76   a variety of structures including a hardened chitinous chorion, micropyles and aeropyles (Hinton,

77   1981; Zeh et al., 1989; Cloudsley-Thompson, 2012). Indeed, as a critical immobile stage in an insect's

78   life cycle, it is plausible that part of the diversity exihibited by micropyles is adaptive supporting

79   other egg structures during embryo development. It is unknown however, to what extent the

80   micropyle and micropylar diversity are shaped not only by sexual selection, but also by natural

81   selection. We know for example, that in the majority of taxa observed the micropyle is left

82   uncovered after fertilisation (author, pers. obs.) and therefore purportedly an ancillary function of

83   the micropyle is aiding embryo survival. Many insect traits are shaped by ecological variables. For

84   instance, the ecology of oviposition drives the evolution of egg shape and size across all insects

85   (Church et al., 2019). Similarly, egg ecology has been hypothesised to influence micropyle number in

86   Heteroptera where aquatic families have one, as opposed to multiple micropyles in terrestrial ones,

87   possibly due to the increased oxygen need of larger embryos (Cobben, 1968). These examples

88   support the idea that ecology might influence the evolution of micropyles.

89   Previous studies have also suggested that micropyle number is a phylogenetic-related trait,

90   for example, a low micropyle number is typical of more primitive Heteroptera (Cobben, 1968).

91   Moreover, it is likely that the presence of a micropyle was acquired early in insect evolution since it

92   is present in most primitive insect orders (Trougakos & Margaritis, 2003) and has been subsequently

93   lost as an adaptation. Nonetheless currently a phylogenetically-informed comparative analysis is

94   lacking. Despite directly impacting female reproductive success, and potentially affecting egg-sperm

95   coevolution, sexual selection and conflict, insect micropyles have been investigated exclusively for

96   taxonomic and classification purposes (e.g. Downey & Allyn, 1981; Livingstone & Yacoob, 1987;

97   Becnel & Dunkle, 1990) and in developmental biology (e.g. micropyle formation, Margaritis et al.,

98   1980; Horne-Badovinac, 2020).

99   With this work, firstly, I used ancestral reconstruction to investigate micropyle presence,

100   number and variation in insects. Then, I used phylogenetic comparative analyses to examine the role

101   that natural selection has played in shaping micropyle number. Specifically, I analysed the ecological

102   function that micropyles and aeropyles play with regards to egg laying behaviour and bioclimatic

103   variables. I hypothesised that due to its ancillary function in embryo development, micropyle

104   number: (i) facilitates oxygen exchange and therefore correlates with the presence of aeropyles; (ii)

105   correlates with aquatic oviposition supporting development in water; and (iii) is influenced by critical

106   bioclimatic variables, such as annual precipitation and temperature, seasonality and extreme

107   environmental factors.

108

109   **Methods**

110   *Literature search*

111   I collated a dataset from the published literature on micropyle and egg characters: egg length and

112   width, which I used to calculate the aspect ratio (egg length/ egg width), micropyle number and

113   width and aeropyle presence/absence. I used Google Scholar and Web of Science to search for

114   keywords: "aeropyl*" AND "micropyl*" AND "insect", "micropyl*" AND "insect", "micropyl*" AND

115   "Lepidoptera", and so on for each Hexapoda order. Data on micropyle number varied extensively

116   across studies, and some authors reported accurate micropyle counts (e.g. female individual

117   variation in the number of micropyles across her egg clutches). To capture this individual variation in

118   micropyle number, I calculated the range (maximum micropyle number – minimum micropyle

119   number), in addition to average micropyle number where given. For Phthiraptera, I could only find

120   articles stating that all species within the order have two micropyles, so I did not include those data.

121   Micropyle width was sometimes reported in the text, but I also measured width from study figures

122   only when scale bars were present, using the program ImageJ v2.0.0 (http://imagej.nih.gov/ij). I

123   collated data on laying ecology from published datasets (Church et al., 2019; Régnière et al., 2019)

124   and references therein. Briefly, data on egg laying behaviour across insects differed in the taxonomic

125   level described. For each source Church et al. (2019) used the lowest recorded taxonomic level to

126   annotate taxa in the egg dataset (order, family, genus or species). Two logical variables described

127   egg laying behaviour: a) semi-aquatic or riparian whether a taxon was associated with water in the

128   egg stage, but not laid directly in water (TRUE or FALSE); b) in water, whether eggs are laid in or on

5

129   water directly (TRUE or FALSE). Taxa that lay eggs inside aquatic plants or overhanging water were

130   not counted as aquatic in any form.

131

132   **Georeferencing**

133   All georeferencing, phylogenetic and statistical analyses were conducted in RStudio (R Core Team,

134   2020, v 1.3.1056) and figures were generated with the *ggplot2* (Wickham, 2016). All datasets were

135   summarized using *dplyr* (Wickham et al., 2020). Georeferencing of species ranges was taken from

136   the location occurrences recorded in the Global Biodiversity Information Facility (GBIF,

137   https://www.gbif.org/). The GBIF is the largest digital repository of species distribution records and

138   although it has been criticised for inherent biases (Boakes et al., 2010; Beck et al., 2014), most

139   criticism is aimed at using it to model species distribution ranges. I used *rgibf* (Chamberlein et al.,

140   2021) occ_search function to search and retrieve data from GBIF on decimalLongitude and

141   decimalLatitude in addition to species, countryCode, individualCount, gbifID, family, taxonRank,

142   year, basis0fRecord, institutionCode, and *CoordinateCleaner* (Zizka et al., 2019) clean_coordinates

143   function for cleaning and cross-checking GBIF data. I selected up to 500 records for each of 582

144   species and extracted 35,501 total location records (Supplementary material, Figure S1). Records

145   location were then mapped with the *maptools* package (Bivand and Lewin-Koh, 2020). To

146   understand how climate has been shaping micropyle diversity patterns, I obtained bioclimatic

147   variables representing annual trends, seasonality and extreme or limiting environmental factors (e.g.

148   temperature of the coldest and warmest month, precipitation of the wet and dry quarters) from

149   WorldClim.org (Hijmans et al., 2005), using the getData function from *raster* (Hijmans, 2019). I chose

150   the spatial resolution of 2.5 minutes of a degree (corresponding to approximately 4.5 km at the

151   equator) as representative of the landscape-level for an insect population.

152

153   **Phylogeny**

154   To build the insect phylogenetic tree, I used the R package *rotl* (Michonneau et al., 2016) to interact

155   with the Open Tree of Life (OTL, Hinchliff et al., 2015), matching species names in my dataset to OTL

156   taxonomic names using the function tol_induced_subtree to retrieve phylogenetic relationships and

157   produce a phylo object. I then pruned phylogenies using drop.tip() so that they only contained the

158   species needed for each analysis. I verified taxonomic synonyms using the GBIF and the National

159   Center for Biotechnology Information Taxonomy Browser

160   (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi).

161

162   **Ancestral reconstruction**

163   In some insect orders micropyles are generally absent (e.g. Collembola). Therefore, I initially

164   categorised micropyle as a binary trait (presence or absence) to analyse ancestral reconstruction of

165   micropyle number. Following this, I reconstructed ancestral states restricting the dataset to those

166   species that have micropyles. Micropyle number was treated as a discrete character varying

167   between 1 and 120 (treating micropyle number as a continuous variable did not affect the results,

168   Figure S1). Finally, to reconstruct the ancestral character state for intraspecific variation (micropyle

169   range), I treated intraspecific variation as a discrete character, i.e. presence or absence of

170   intraspecific variation in micropyle number. The absence of intraspecific variation included those

171   species without a micropyle. I analysed presence/absence of intraspecific variation across orders

172   similarly to what described below for micropyle number. I fit a single-rate model and reconstructed

173   ancestral states at internal nodes in the tree using the fitER function in *phytools* (Revell, 2012) to

174   obtain empirical Bayesian posterior probabilities. I then generated stochastic character maps

175   sampling node states and discrete character evolutionary histories from their joint Bayesian

176   posterior distribution (Huelsenbeck et al., 2004). I generated 100 possible histories of the transitions

177   between presence or absence of micropyles on the insect phylogeny tree and 10 on each tree of the

178   posterior distribution, using make.simmap in *phytools*. In this way, I obtained a probability

179   distribution on the number of changes of each type on the tree.

180

Phylogenetic comparative analysis

To account for different sample sizes among variables and maximise the use of the dataset, I run

separate phylogenetically-controlled models for each key group of explanatory variables in relation

to micropyle number. The first set of variables I analysed were to correct for the allometric effect of

body size. Egg width and length (mm) across the species collated were highly correlated (Spearman's

correlation test, rho =0.567, p<0.005, n=434). To avoid collinearity, I used egg length in subsequent

analyses because this corresponded to the largest dataset (N species egg length = 447 vs N species

egg width = 437). I also analysed the relationship between micropyle number and the aspect ratio,

(egg length mm)/(egg width mm), a dimensionless measure. Insect diversity shows strong latitudinal

gradients, a spatial pattern common across biodiversity (butterflies, Cardillo, 1999; cross-taxa

comparison, Willig et al., 2003; Hillebrand, 2004). To investigate for a latitudinal effect on the

number of micropyles, I used bioclimatic variables matching the distribution of the species in the

dataset. I selected three pairs of bioclimatic variables representative of annual trends (mean annual

temperature, bio1, and annual precipitation, bio12), seasonality (temperature seasonality bio4, and

precipitation seasonality, bio15) and limiting environmental factors (temperature of the driest

quarter, bio9, and precipitation of the driest quarter, bio17) for a total of 412 species across 23

orders. The last set of variables relates to ecological traits: i) the presence or absence of aeropyles

(binary trait) which I used as a broad proxy for deposition environment, assuming that where

oviposition occurs in hot and arid habitats, aeropyles should be absent and micropyles reduced in

number; ii) and aquatic oviposition (logical traits, semiaquatic/riparian and submerged oviposition),

which I hypothesized would constrain micropyle number. Data on the aquatic laying behaviour of

106 species across 8 insect orders were gathered from published datasets (Linley et al., 1994; Church

et al., 2019).

I checked assumptions for data distribution and appropriate error distributions (Crawley,

2012). Plotting the distribution of micropyle number revealed two problems. First, toward the left-

206   hand side of the distribution, micropyle number was over-dispersed, with a great majority of species

207   exhibiting 0 or 1 micropyle. Second, the tail of the distribution revealed underdispersion. The use of

208   Poisson and negative binomial distributions did not ameliorate the problem, neither did Markov

209   Chain Monte Carlo simulations. To correct for the effect of phylogenetic relatedness among species

210   and non-independence, I used phylogenetic generalized least square models (PGLS) with the gls

211   function in *geiger* (Pennell et al., 2014), the maximum likelihood method and Brownian correlation. I

212   log-transformed micropyle number to better fit assumptions of normality of residuals.

213

214   **Results**

215   **Micropyle number across Hexapoda**

216   I collated data for micropyle number in 612 species, across 24 hexapod orders (21 Insecta, and the

217   Entognatha: Collembola, Diplura and Protura, 24/31 or 77% of hexapod orders, Misof et al., 2014)

218   and 132 families (128 Insecta, 2 Collembola families, Entomobryidae and Hypogastruridae, the

219   Campodeidae, Diplura, and the Eosentomidae, Protura). The geographic distribution of the species

220   for which micropyle number was collated, is shown in Figure S2. Mean micropyle number varied

221   substantially across families (overall mean $\pm$ standard deviation, 7.89 $\pm$ 14.12 micropyles, median

222   3.00, range 0-120, N=612), with the maximum number found in Hemiptera (120 micropyles in

223   *Panstrongylus geniculatus*, Reduviidae: Triatominae) and Hymenoptera (116 in *Apis mellifera*,

224   Apidae) and micropyles absent in Collembola, Diplura, Protura, Strepsiptera and Thysanoptera

225   (Figure 1). Micropyle width varied comparatively less (overall mean $\pm$ S.D., 3.12 $\pm$ 2.59 μm, median

226   2.26 μm, range 0.20-11.8 μm, N=228; order specific data can be seen in Figure 1). Within this

227   dataset, aeropyles were present in 305 species, absent in 91 and the remaining 339 species were

228   data deficient.
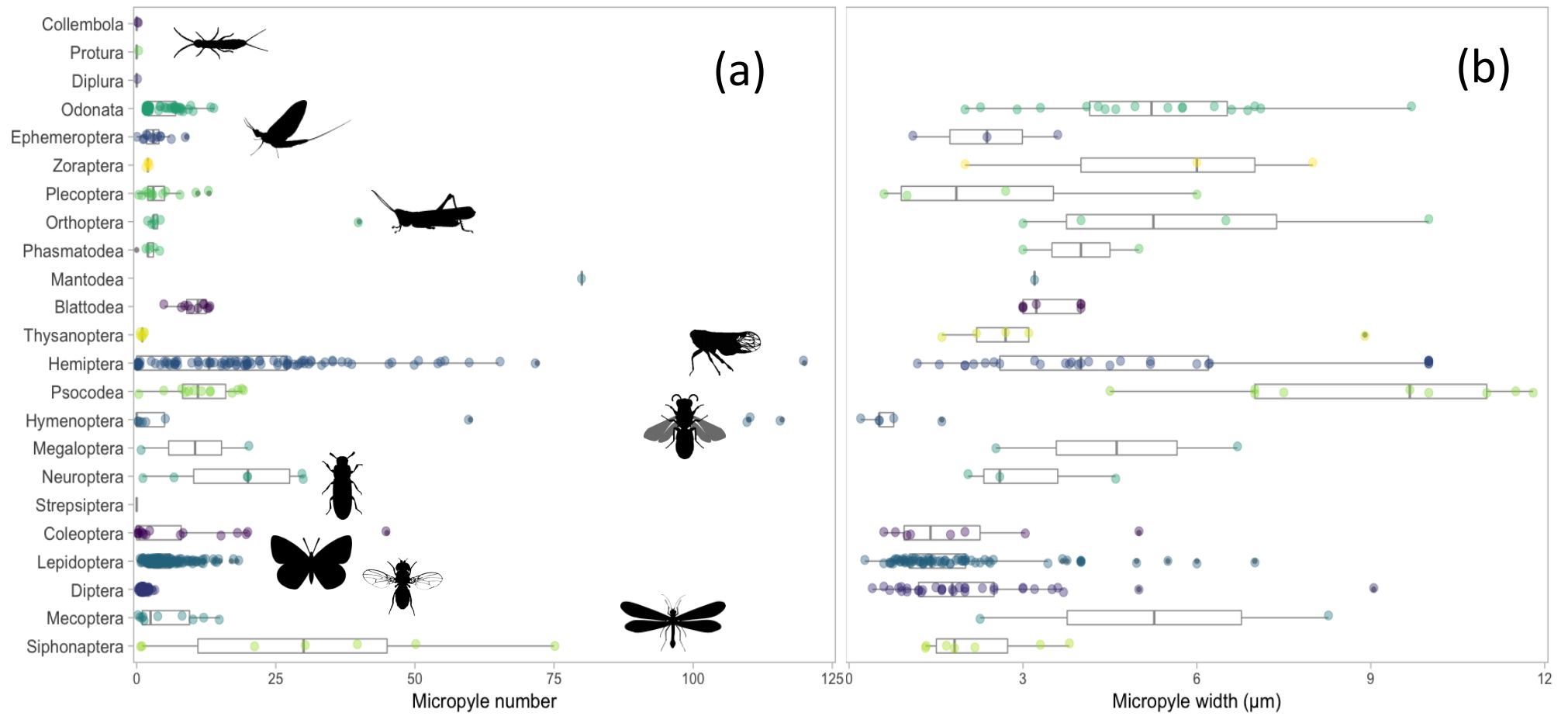
229
230

231   Figure 1. Boxplots of mean micropyle number (a) and mean micropyle width (μm, b) across Hexapoda orders. Each point represents the mean micropyle for

232   an individual species. On the far left-hand side the insect orders covered are shown. Silhouettes from http://www.phylopic.org available under a Public

233   Domain license (Broussard, 2020; Campos de Domenico, 2020; Gagalova, 2020; PhyloPic, 2020; Starr, 2020; Schomburg, 2020).

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235

Ancestral state inference

Phylogenetic relationships were obtained for 582 species across all 24 hexapod orders. When

analysing micropyle presence or absence, stochastic character mapping estimated the number of

changes of each type to be 23.53 on average. Changes from micropyle absence to presence occurred

on average 1.88 times and from presence to absence occurred 21.64 times. The model estimated

the proportion of time spent in each state, and the posterior probabilities that each internal node is

in each state (absence 0.13 and presence 0.87). Figure 2 illustrates the ancestral states at internal

nodes of the insect tree as relative Bayesian posterior probabilities associated with

presence/absence of micropyles.
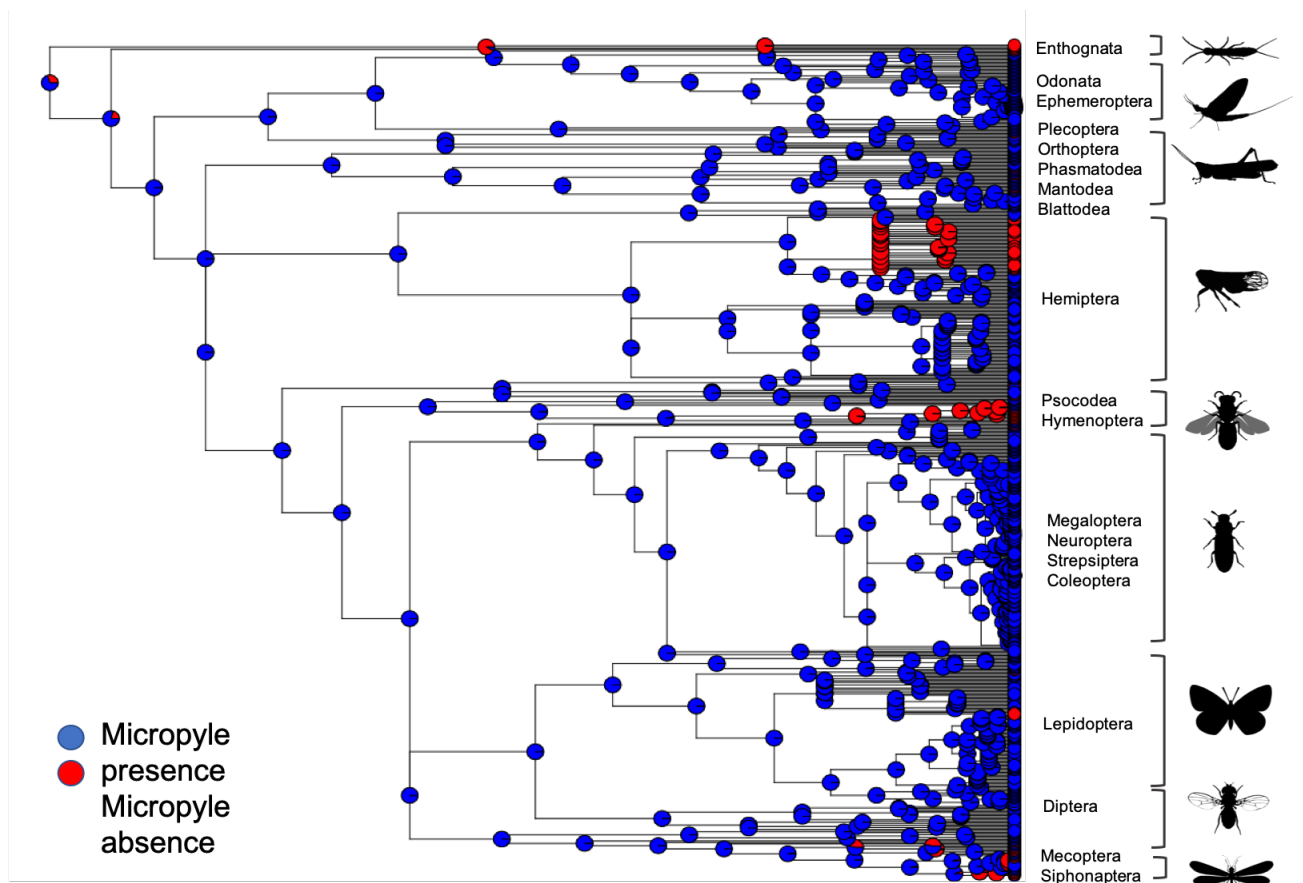
Figure 2. Ancestral state estimation of presence/absence of micropyle in hexapods. Pie charts at

internal nodes represent the relative Bayesian posterior probabilities associated with the ancestral

249   state being presence (in blue) and absence (in red) based on a single stochastic character map out of

250   100.

251

252   Ancestral state reconstruction for the number of micropyles across species that exhibit micropyles

253   (species with no micropyles excluded) revealed that the commonest states were 1 and 2 micropyles

254   with the estimated proportion of time spent in these two states being 0.26 and 0.10 respectively.

255   The probability of the first six states, from 1 to 6 micropyles, was 0.51. All of the other possible

256   states combined (7-120) had a probability of 0.49. Simulated trees had 415.1 average changes

257   between states. Ancestral character estimation of 100 sample character histories from the posterior

258   probability distribution for intraspecific variation, estimated the average number of changes

259   between presence and absence of intraspecific variation to be 155.5, and the posterior probabilities

260   that each internal node is in each state, to be 0.55 for the absence of intraspecific variation in

261   micropyle number and 0.44 for its presence (Figure 3).

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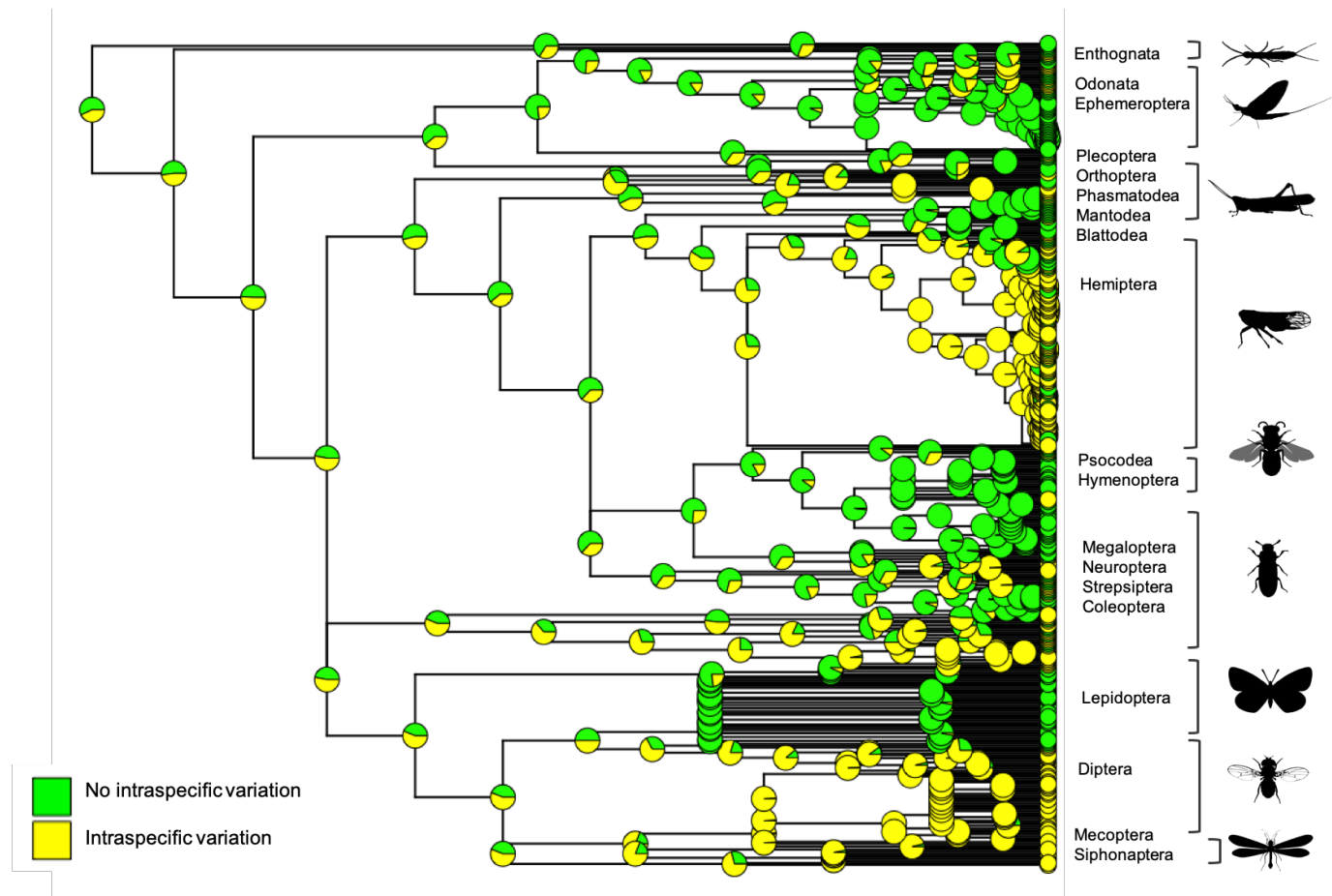
Figure 3. Ancestral state estimation of presence/absence of intraspecific variation in micropyle number across hexapod orders. Pie charts at internal nodes represent the relative Bayesian posterior probabilities associated with the ancestral state being presence (in yellow) and absence (in green) based on a single stochastic character map out of 100. Absence of intraspecific variation includes species with no micropyle.

Allometric relationship between egg size and micropyle number

There was a positive relationship between micropyle number and egg width and egg length as wider or longer eggs had more micropyles (tested separately, see Methods) but no significant relationship with aspect ratio (Table 1). Intraspecific variation in micropyle number was low, with the majority of species showing no intraspecific variation (55%, 236 of 428, Figure S3). Micropyle width was negatively related to micropyle number, so the greater micropyle number, the smaller the width of individual micropyles (Figure 4a, pgls: $\beta \pm$ s.e. = -0.157 $\pm$ 0.062, t = -2.546, p = 0.011, N = 211).

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277

278 Table 1. Phylogenetic generalized least square models (PGLS) of micropyle number in relation to egg

279 size (tested separately), egg length and aspect ratio controlling for phylogenetic effects (λ = 1).

Trait	Predictor	$\beta \pm$ s.e.	t	p
Micropyle number	Intercept	1.242 ± 0.563	2.20	0.028
(N=437 species)	Egg width (mm)	0.239 ± 0.070	3.399	**<0.001**
Micropyle number	Intercept	1.179 ± 0.564	2.091	0.037
(N=447 species)	Egg length (mm)	0.245 ± 0.069	3.572	**<0.001**
Micropyle number	Intercept	0.970 ± 0.567	1.709	0.088
(N=432 species)	Aspect ratio	0.070 ± 0.072	0.977	0.329

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281

282 **The influence of bioclimatic variables on micropyle number**

283 I ran the models investigating the influence of bioclimatic variables on micropyle number with and

284 without egg length after correcting for phylogenetic effects. Here I report the results without egg

285 length because removing egg length increased the sample size by 1/3 from 202 to 274 species.

286 Micropyle number was positively associated with annual precipitation (Figure 4b) but no other

287 bioclimatic variable (Table 2). In each of the three models, micropyle number was strongly positively

288 correlated with egg length, in concordance with the previous analyses on egg dimensions (Table S1)

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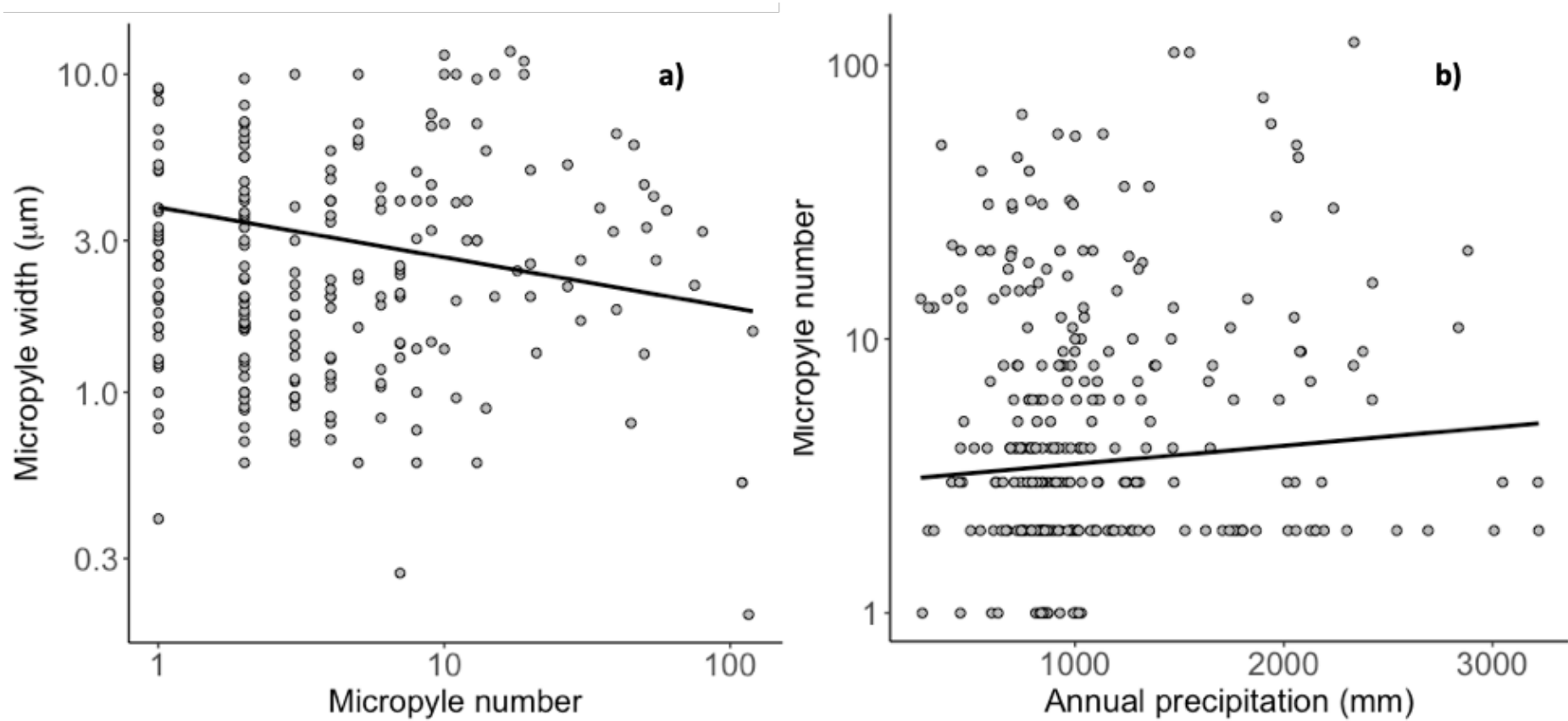
Figure 4. a) The relationship between average micropyle width (μm) and micropyle number before correcting for phylogenetic effects. Each point

represents average micropyle width for a species (N= 211). b) The relationship between micropyle number and annual precipitation (mm) for the average

species location occurrence on GBIF (see Methods), before correcting for phylogenetic effects. Each point represents the number of micropyles for a

species (N= 274). Trendline derived from the pgls models.

294

295

296 Table 2. Phylogenetic generalized least square models (PGLS) of micropyle number (N=281 species)

297 in relation to bioclimatic variables controlling for phylogenetic effects (λ = 1).

Trait	Predictor	$\beta \pm$ s.e.	t	p
Micropyle number	Intercept	1.111 ± 0.637	1.745	0.082
	Annual precipitation (mm)	0.000 ± 0.000	2.223	**0.027**
	Annual mean temperature (C°)	-0.000 ± 0.000	-0.267	0.790
Micropyle number	Intercept	1.360 ± 0.652	2.084	0.038
	Temperature seasonality*(C°)	-0.000 ± 0.000	-1.548	0.122
	Precipitation seasonality	-0.000 ± 0.002	-0.047	0.962
Micropyle number	Intercept	1.109 ± 0.638	1.738	0.083
	Mean temperature driest quarter	0.000 ± 0.000	0.704	0.482
	Precipitation of driest quarter	0.000 ± 0.000	0.991	0.322

298 * Standard deviation x 100

299

300 **Ecology: egg laying behaviour and micropyle number**

301 The number of micropyles was strongly positively related to the presence of aeropyles (pgls: $\beta \pm$ s.e.

302 = 0.334 ± 0.119, t = 2.797, p = 0.006, N = 245, Figure 5). Eggs of species that lay in water did not have

303 significantly fewer micropyles than eggs of those species that lay eggs in riparian habitats, and there

304 was no relationship between the number of micropyles and either of those variables. However, after

305 correcting for phylogeny, I had data for all variables for only 66 species, so the power of this analysis

306 is likely to be low. In particular, I had data for only 4 species with riparian egg laying behaviour

307 (Figure S4). Moreover, I could not investigate the interaction between the number of micropyles,

308 presence of aeropyles and aquatic oviposition as I only had data for all variables for just 28 species.

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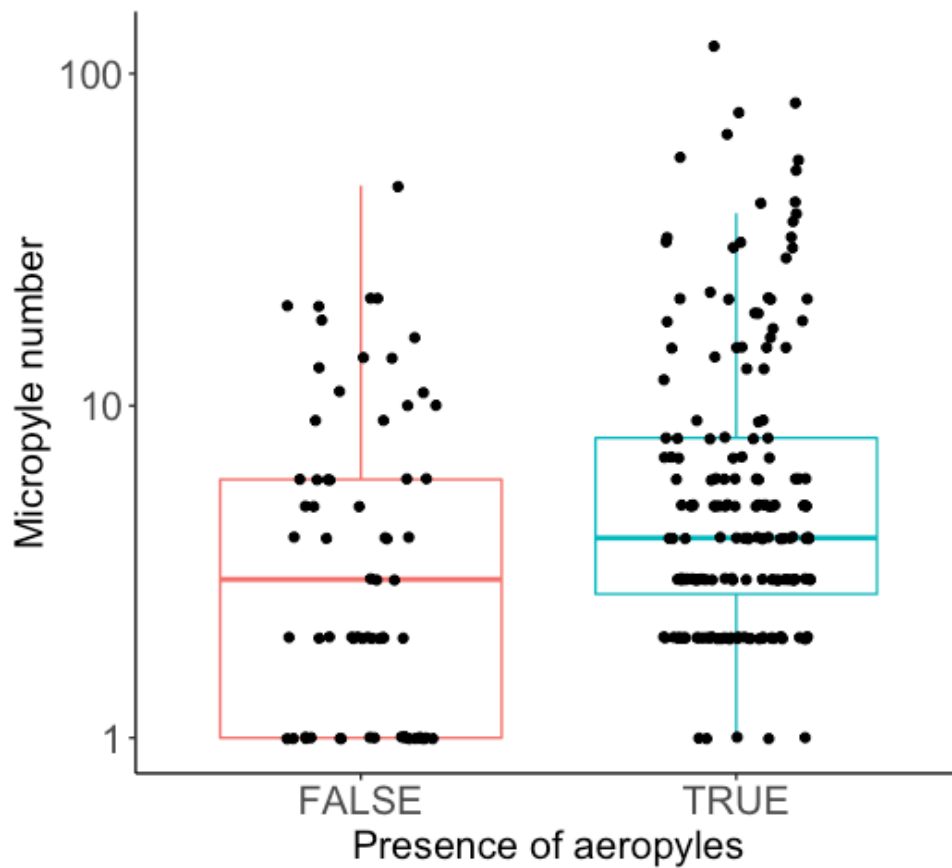
Figure 5. Boxplots of the number of micropyles on hexapod eggs in relation to the presence or

absence of aeropyles.

Discussion

This phylogenetic comparative analysis investigates the variation in micropyle number across eggs of

24 hexapod orders: 21 Insecta, and Entognatha (Collembola, Diplura and Protura), spanning all

continents except Antarctica (Figure S2) and including representative species of the most speciose

orders, such as Coleoptera, Lepidoptera, Diptera and Hymenoptera (Forbes et al., 2018). Mean

micropyle number ranged from zero in Entognatha, Strepsiptera and Thysanoptera to 120 in

Panstrongylus geniculatus, Hemiptera, and over 100 on average for Apidae, Hymenoptera. Ancestral

reconstruction of micropyle presence or absence showed that the most likely ancestral state was the

presence of 1 or 2 micropyles in insects and the absence of micropyles in more primitive related

orders, such as Entognatha. During the course of insect evolution, micropyles were more likely to be

324 lost (on average 21.6 times) than to be gained (1.9 times). Across orders the mean time spent with

325 presence of micropyles (87%) was considerably more likely than the absence (13%). Stochastic

326 mapping estimated the majority of time (51%) was spent in the first six states (micropyle number

327 varying from 1 to 6), with all other states combined the remainder. The present analysis confirms

328 previous hypotheses that the presence of a micropyle is the ancestral character state in insects

329 (Cobben, 1968; Trougakos & Margaritis, 2003).

330   I hypothesised that an ancillary, ecological function of micropyles is aiding the development

331 of the embryo post-oviposition and this comparative analysis supports it, albeit not completely. The

332 number of micropyles across orders was strongly positively correlated to the presence of aeropyles,

333 as well as egg size. Additionally, micropyle number also strongly negatively correlated with

334 micropyle width, insect eggs having fewer larger micropyles or numerous smaller ones. This is

335 compatible with an ecological function of micropyles, as larger eggs have additional needs for

336 oxygen and water exchange. The finding that micropyle number was also positively correlated with

337 annual precipitation also fits with this hypothesis: for eggs developing in habitats with low annual

338 precipitation, water retention becomes a limiting factor and the presence of fewer pores on the

339 eggshell may facilitate this. Because of their size, eggs are isothermal with their surrounding

340 environment, and therefore embryo temperature depends on maternal selection of microhabitats

341 (Potter et al., 2009). Insects use a variety of strategies to avoid egg desiccation, such as strategic

342 oviposition site at the individual egg level, and egg clustering, with clutch layering and density

343 promoting survival (Clark & Faeth, 1998) as well as egg colouration (Farnesi et al., 2017). In hot and

344 arid conditions, leaf microclimate of plant hosts buffers leaf-associated insect eggs from extreme

345 heat (Potter et al., 2009). It has been proposed that harsh environmental conditions could have

346 driven the evolution of insect parental care, for example parental egg attendance as an alternative

347 route to the development of a more resistant egg shell (Wong et al., 2013). In this analysis

348 bioclimatic variables linked to the driest quarter did not correlate to the number of micropyles,

349 however I did not measure the length of time spent in the egg stage. This is an important

350 component for overall survival to the subsequent life stage (Pritchard et al., 1996), and could be

351 included in future analyses.

352   For those taxa egg-laying in aquatic environments, I predicted that eggs laid in riparian

353 habitats would have more micropyles than those laid in water, but the data did not support this

354 hypothesis. The dataset on aquatic egg laying was smaller than the other datasets, and this will have

355 likely influenced the power of detection of this analysis. On the other hand, many confounding

356 variables may be at play in aquatic habitats making this trend less clear. For example, aquatic insects

357 generally overwinter as larvae, such as most Trichoptera, Megaloptera, Ephemeroptera, Odonata

358 and Plecoptera, where larvae can select appropriate overwintering sites by burrowing in a substrate

359 (Danks, 1978). Other taxa overwinter mainly in the egg stage, for example the taxa previously

360 grouped under Homoptera including Hemiptera (25-75%) and Diptera (50%), while Coleoptera

361 (2.5%) and Lepidoptera (11.7%) do not (Leather et al., 1995). Even though oviposition environment

362 and egg defences are relatively well studied (Hinton, 1981; Hilker & Meiners, 2008), there is much

363 we still do not know about insect eggs. Other egg traits, such as egg colouration and camouflage

364 (Guerra-Grenier, 2019) but also chorion sculpturing and architecture, are likely to play a role in

365 influencing the egg immediate microenvironment through photoprotection, air flow, water and gas

366 exchange. Such traits are likely key determinants of survival in this life stage (Downes et al., 2021)

367 and yet they are under-researched.

368   The number of micropyles in insect eggs is likely a trade-off between sexual and natural

369 selection pressures. For example, in *Reticulitermes speratus* (Blattodea: Rhinotermitidae), queens of

370 different ages, variation in the number of micropyles marks a switch from asexual to sexual

371 reproduction and therefore, this variation represents a mechanism for direct control of fertilisation

372 by females (Yashiro & Matsuura, 2014). Similarly, in *Harmonia axyridis* (Coleoptera: Coccinellidae)

373 female control of micropyle number depends on the stimulus provided by copulation, as well as

374 female-male interaction (Sun et al., 2019). Furthermore in Lepidoptera, micropyle number is

375 positively related to the degree of female promiscuity (Iossa et al., 2016). These examples show a

376   potential role of cryptic female choice and direct female control of reproduction, at least in some

377   insect taxa and therefore shed some light on the reproductive function of the micropyle. However,

378   there is also some evidence that variation in micropyle number is linked to aging, which could

379   represent a plausible mechanism for a decline in female fertility with age. This could be adaptive or

380   non-adaptive and linked to senescence. This is intriguing as plasticity in progeny size among females

381   within populations, and among progeny produced by a single female, has remained elusive to

382   explain (Fox & Czesak, 2000). For instance, in young female Reduviidae there are as many as 31

383   micropyles but in older females this number is reduced and may account for the higher number of

384   unfertilised eggs (Beament, 1947). To the best of my knoweldge, how sperm gain entry into the egg

385   in the absence of a micropyle, is not known. The vast majority of insect taxa studied possess a

386   micropyle to allow egg fertilisation, yet the variation in this character is underappreciated, including

387   in disciplines which have used this trait extensively. Micropyle formation is well-described in the

388   developmental biology literature (e.g. Ando, 1973; Yamauchi & Yoshitake, 1984; Wenzel et al., 1990)

389   and yet many open questions remain about micropyle morphogenesis (Horne-Badovinac, 2020).

390   Similarly, understanding variation in this egg character will improve our understanding of a critical

391   life stage in insect development, which in turn, may inform future modelling of insect trends under

392   climate change (e.g. MacLean et al., 2016; Gonzales-Tokman, 2020).

393

394   **Conclusions**

395   In conclusion, in this phylogenetic comparative analysis across extant Hexapoda, I show that

396   the most likely ancestral state for insects was the presence of one or two micropyles. Across insects,

397   interspecific variation was high and intraspecific variation was low, with 55% of species showing no

398   variation. Micropyle number was strongly positively related to egg size and the presence of

399   aeropyles, and negatively related to micropyle width. In addition, micropyle number was positively

400   related to annual precipitation. However, I did not find support for the hypothesis that in aquatic

401   taxa, eggs laid in riparian habitats have more micropyles than those laid in water. Overall these

findings support the hypothesis that in addition to their primary fertilisation function, insect

micropyles also have an adaptive ecological function tailored to the specific micro-climatic

conditions in the immediate egg micro-environment, ultimately aiding embryo survival. Further

studies are needed to increase our understanding of the sexual and natural selection pressures that

have shaped an almost-ubiquitous trait across insects in a critical life stage.

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