1   **Climatic and geographic variation as a driver of phenotypic divergence in reproductive characters and**

2   **body sizes of invasive *Gambusia holbrooki***

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28 **Abstract**

30 Understanding the invasion success of alien species includes developing knowledge on how the biological traits

31 of their populations respond to spatial differences in environmental conditions. For invasive fishes, while the

32 influence of latitudinal and climatic gradients on their biological traits over large spatial scales are well

33 established, there is less certainty in how these vary over smaller scales. Here, we tested the influence of a

34 climatic and geographic gradient on the reproductive traits and body sizes of the invasive mosquito fish

35 *Gambusia holbrooki* across five climatic regions in Turkey. The results revealed that the environmental

36 conditions across eight sites provided two gradients: a latitudinal gradient (where northern sites were cooler with

37 less rainfall) and a coastal-inland gradient (where changes were apparent from coastal areas and into inland

38 areas). These gradients had marked effects on the traits of both sexes, in sites in coastal and southern areas, male

39 had significantly larger gonopodia and females had higher egg numbers than in inland and warmer areas, and

40 with both sexes having body sizes that were smaller. The spatial differences in the male traits were suggested as

41 being driven by differences in selection pressures at the sites that related to differences in their population

42 abundances. The results revealed that environmental conditions across a relatively small spatial scale had some

43 strong influences on the expression of specific biological traits of these mosquito fish populations, but with

44 further work needed to test how these influences affect their invasion success.

46 **Key-words:** Gonopodium, fecundity, ecological variations, climatic gradients, Turkey.

Introduction

Environmental factors strongly influence fish physiology (Jun et al. 2012; Zhang et al. 2016), with climatic regimes often creating divergent selective regimes that directly affect phenotypic traits (Royer et al. 2009). Understanding the ecological factors that drive phenotypic diversification along climatic gradients is thus important for understanding the eco-evolutionary responses of populations to their environments (Ouyang et al. 2018). Climatic gradients are usually approximated by differences in temperatures, with local thermal regimes generally having strong effects on the expression of fish life history traits, including those associated with reproduction (Pankhurst and Munday 2011).

Species of the *Gambusia* genus, which are distributed naturally in North America (Lloyd and Tomasov 1985; Lloyd 1987), are collectively known as mosquito fish and have been introduced around the world for biocontrol purposes, especially for malaria (Moyle 2002). Introductions of species such as *Gambusia holbrooki* (Girard 1859) and *Gambusia affinis* (Baird and Girard 1853) have resulted in invasive pest populations in many countries, where their invasion success has been facilitated by their high ecological plasticity in the use of different freshwater habitats (Feder et al. 1984). Populations of *Gambusia* spp. generally comprise of small bodied individuals (< 50 mm), where males tend to be the smaller sex (Doadrio 2002). Their mode of reproduction, where males inseminate ovoviviparous females using their gonopodium (a modification of the anal fin), has resulted in the genus being suitable for investigating inter- and intra-specific diversification in fish sexual phenotypes (Nelson 2006; Ouyang et al. 2018).

Studies on *Gambusia* spp. reproductive characteristics have investigated how biological and environmental selection pressures interact to influence the expression of their reproductive traits (Head et al. 2015; Riesch et al. 2018). In males, sexual selection pressures are likely to be for large male body sizes (as larger males can dominate access to females) and for males with larger gonopodia (as these males are more likely to inseminate females than smaller males with a shorter gonopodium) (Bisazza and Marin 1991; Kahn et al. 2010; Head et al. 2015). However, as smaller males are also adept at sneaking copulations with females (Pilastro et al. 1997), there is the possibility of contrasting sexual-selection pressures on male traits that are also influenced by the influence of the environmental characteristics acting on the population (Haynes and Cashner 1995; Kahn et al. 2010). For example, relatively benign environmental conditions can promote population stability (e.g. lower mortality rates,

77 relatively high abundances), with these conditions usually encountered in coastal areas of lower altitude that are

78 generally favoured by *Gambusia* spp. populations, with the converse in inland habitats at higher altitude (Pyke

79 2005; Ouyang et al. 2018). Consequently, we posit that selection pressures, driven by female mate choice, will

80 result in males having elongated gonopodia in coastal, lower latitude populations compared with inland

81 populations at higher altitudes (Kahn et al. 2010; Ouyang et al. 2018).

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83 For *Gambusia* spp. females in relatively stable populations in low lying coastal areas, the relatively high levels

84 of intra-specific competition are likely to favour the production of fewer, larger progeny, but whose body sizes

85 are relatively bigger and that have strong competitive abilities (Reznick et al. 2002; Bashey 2008; Ouyang et al.

86 2018). Consequently, we posit that female *Gambusia* in populations that are inland and at higher altitude will be

87 more fecund than those in coastal populations. For both sexes, we suggest that body sizes will be smaller in

88 coastal and lower latitude (warmer) regions due to higher rates of intra-specific competition in these more

89 abundant populations (Daniels and Felley 1992), and with larger body sizes also more apparent in cooler

90 environments due to Bergmann's Rule (Blanchet et al. 2010). However, the manner in which environmental

91 conditions could affect mosquito fish trait expression could differ between the sexes, as Ouyang et al. (2018)

92 revealed that male trait divergence in *G. affinis* was most apparent along a latitudinal gradient, but in females,

93 the divergence was along a coastal-inland (continental) gradient. These variations could be even more prominent

94 in Turkey, being a country that is a peninsula, surrounded by the sea and coastal areas to North, West and South

95 (Smith and Darwall, 2006). Within the country, there are 11 recognised freshwater ecoregions (Abell et al. 2008)

96 that incorporate three major habitat types: xeric and endorheic basins, with large and small lakes and small

97 streams (Central Anatolia and Lake Van - saline and soda lakes); temperate floodplain rivers and wetlands

98 (Upper Dicle (Tigris) and Fırat (Euphrates) and Kura - Southern Caspian Drainages); and temperate coastal

99 rivers (Trakya (Thrace), Western Anatolia, Southern Anatolia, Northern Anatolia, Western Transcaucasia and

100 Asi (Orontes) (Tarkan et al. 2015).

101

102 The aim here was thus to test intra-specific differences in the reproductive characteristics of invasive *G.*

103 *holbrooki* across a climatic and geographic gradient. Using body size as a focal biological characteristic, male

104 gonopodium lengths and female fecundity as focal reproductive characteristics, and invasive populations within

105 five climatic regions of Turkey as the study area, we test whether males in populations in warmer, coastal

106 regions would have relatively large gonopodia, females would be less fecund, and both sexes would be smaller

107   in body size when compared with cooler, inland regions. It has been more than 100 years since *G. holbrooki* was

108   introduced to Turkey (Walton et al. 2012) and so it is considered that this is of sufficient time for the interaction

109   of the biological and environmental selection pressures to have acted on these traits.

110

111   **Materials and Method**s

112

113   *Sampling sites and data collection*

114   A total of 8 sampling locations were used that were located across five different geographical and pollical that

115   covered all the ecoregions present in Turkey (Aegean, Mediterranean, Black Sea, Marmara, and South-eastern

116   Anatolia regions). These covered five degrees of latitude (36 to 41 $^{\circ}$N) and nine degrees of longitude (26 to 37

117   $^{\circ}$E) (Table 1, Fig. 1). The location of Turkey is between the subtropical zone and temperate zone, and the country

118   has a variety of mountain landforms and has a relatively long coastline, which results in a wide range of climate

119   types. According to the Köppen-Geiger climate classification, the main climate categories are B (arid,

120   evaporation is greater than precipitation and there is a water deficit), C (warm temperate, winters are mild and

121   humid) and D (snow, winters are cold and humid) (Kottek et al. 2006). The sites were selected to provide

122   sufficient latitudinal, coastal-inland and climatic gradients for testing the predictions (Fig. 1; Table 1).

123

124   A total of 125 males and 80 females were collected from across all of the sites using a combination of seine and

125   hand netting during 2016 and 2017 (Fig. 1). Although *Gambusia* spp. can reproduce year-round when

126   environmental conditions are especially favorable, their breeding season in the northern hemisphere is during

127   spring and summer (Pyke, 2008). Therefore, the sampling period covered these seasons (except for one sampling

128   in autumn), ensuring high fish activity that facilitated their collection (Table 2). Following their capture, all

129   individuals were euthanized (anaesthetic overdose; phenoxyethanol) and then fixed in ethanol (96%). Once in

130   the laboratory, the fish were measured using digital callipers (standard length (SL), ± 0.01 mm) and sexed. For

131   males, the gonopodium length (GL) was then measured (± 0.05 mm; n = 125) and for females (n = 80), the

132   developmental stages of embryos were evaluated for each development stage (Haynes 1995). In subsequent

133   fecundity (F) calculations, fertilized eggs classified as Stage 3 (blastodisc embryo) and all subsequent stages

134   were considered, with the number of embryos for each female used as the fecundity estimate. To test the

135   influence of climate and geographical parameters on the fish reproductive characteristics, climatic data were

136   retrieved from Turkish State Meteorological Service's official statistics data (1927 to 2018), where the site-

137   specific climatic information retrieved were mean annual temperatures and annual precipitation (Table 1).

138   Altitude and distance to the sea were obtained from Google Earth (http://earth.google.com/).

139

140   *Data analyses*

141   The initial analytical step was to use principal component analysis (PCA) to condense the various climatic data

142   (as precipitation and temperature, where the latter was used as a surrogate of latitude and longitude;

143   Cucherousset et al. 2009) and geographic data (distance to the sea and altitude) into two climate-related principal

144   components (PCs; see Results). The scores on these two PCs were then used as covariates in subsequent analyses

145   that tested for phenotypic divergence along these climatic and geographical gradients. Then, the relationships

146   between GL and male SL, and fecundity and female SL, were tested using regression analyses, before

147   differences in SL between sexes and amongst the sampling locations were tested using permutational univariate

148   analysis of variance (PERANOVA). For assessing the extent of divergence (i.e. in SL, GL and fecundity) along

149   climatic gradients, permutational multivariate analysis of variance (PERMANOVA) was used, where SL was

150   specifically used as an interaction term to ensure that its allometric effect is accounted for in the test. All

151   permutational analyses were conducted using PERMANOVA+ v1.0.1 for PRIMER version 6.1.11 (PRIMER-E

152   Ltd, Plymouth, UK) (Anderson et al. 2008). This used a Euclidean distance measure following normalization of

153   the data, with a two-way design employed where PC1 and PC2 were both used as fixed factors. These were used

154   to obtain a distance matrix, which was subjected to 9999 permutations of the raw data and tested for

155   significance. Sampling locations were produced using the ESRI (2011).

156

157   **Results**

158

159   Across all samples, male SLs were 16.59 to 26.16 mm (mean 21.49 ± 0.19 mm) and female SLs were 21.6 to

160   45.5 mm (mean 31.21 ± 0.43 mm) (Table 2). The mean lengths of both male and female *G. holbrooki* did not

161   differ significantly between the locations (PERANOVA: $F^{\#} = 0.82$ and 2.32, respectively; both $P > 0.05$).

162   Gonopodium lengths varied between 5.38 and 9.09 mm (mean 7.18 ± 0.06), while female fecundity (as the

163   number of eggs) was 8 to 83 (mean 29.3 ± 1.5). Gonopodium length significantly increased as male body length

164   increased ($F = 184.63$, $P < 0.0001$, $r^2 = 0.60$), with female fecundity also increasing significantly with body

165   length ($F = 14.09$, $P < 0.001$, $r^2 = 0.15$).

166   The PCA of the climatic and geographical parameters resulted in two PCs that explained 88.4% of their variation

167   (Table 3). PC1 described the gradient from northern towards southern sites (latitudinal variation), whereby

168   northern sites showed lower mean annual temperatures and lower annual precipitation rates. PC2 described

169   gradual longitudinal changes from coastal towards inland sites (longitudinal variation; Table 3).

170

171   The climatic (PC1) and geographic (PC2) gradients had significant effects on all of the examined variables

172   (PERMANOVA: male SL: $F^{\#}$ = 19.01, $P^{\#}$ = 0.0001; female SL: $F^{\#}$ = 32.38, $P^{\#}$ = 0.0001; GL: $F^{\#}$ = 10.08, $P^{\#}$ =

173   0.0001; Fecundity: $F^{\#}$ = 10.46, $P^{\#}$ = 0.0001), with no significant PC1 x PC2 interactions in all analyses. These

174   analyses revealed that the effects of PC1 and PC2 on body lengths of both sexes were for smaller SLs in

175   southern (warmer) versus northern (cooler) populations, and a decrease in length as distance from the coast

176   increased (Figs. 2 and 3). PC1 and PC2 also had strong effects on female fecundity, with fish in more coastal

177   waters and southern populations having higher egg numbers than those in inland populations and northern

178   populations (Figs. 2 and 3). This was also the case for gonopodium lengths where, with the effect of SL

179   accounted for, males from the inland and southern populations generally had shorter gonopodia (Fig. 3).

180

181   **Discussion**

182

183   The results revealed the importance of environmental conditions in determining aspects of the reproductive traits

184   of *G. holbrooki* across the study sites. The environmental conditions across the sites provided two gradients: a

185   latitudinal gradient, where the northern sites were cooler with less rainfall, and a coastal-inland gradient, where

186   changes were apparent from coastal areas and into inland areas. We tested whether males in coastal and southern

187   areas had relatively large gonopodia (which the results were consistent with), females were less fecund (which

188   the results were not consistent with), and both sexes had smaller body sizes (which the results were consistent

189   with).

190

191   The influence of abiotic gradients influencing the reproductive traits of *Gambusia* spp. are increasingly apparent

192   with, for example, Ouyang et al. (2018) revealing that in *G. affinis*, there was a general pattern of climatic

193   gradients driving some phenotypic variation in naturally selected traits, but with some sexually selected traits

194   also showing some variation, such as males in more southern populations having wider gonopodia. They argued

195   that the influence of climate was acting not just directly via only temperature regimes, but also indirectly by

196   acting on population dynamics, where the effects of differences in inter-specific competition, resulting from

197   variation in extrinsic overwinter mortality rates, altered the selective landscape along climatic gradients (Ouyang

198   et al. 2018). Indeed, the influence of environmental variables on *Gambusia* spp. traits is often suggested as being

199   mediated though their effects on population abundances. For example, stable environmental conditions enable

200   *Gambusia* spp. to develop high population abundances as conditions, such as higher winter temperatures, result

201   in lower extrinsic mortality rates (Pyke 2005). In our study, these conditions appeared to be provided in sites in

202   more southern latitudes and that were closer to the coast, and they were driving selection pressures that produced

203   males with elongated gonopodia (Kahn et al. 2010; Ouyang et al. 2018). However, our sampling methods,

204   involving a combination of hand and seine nets, coupled with site characteristics that inhibited the capture of

205   juvenile *G. holbrooki*, meant that our sample collection was primarily qualitative. While these factors prevented

206   the robust estimation of population abundance at each site (including relative measures such as catch per unit

207   effort), observations did suggest that there were substantially higher abundances in the southern and coastal

208   populations compared with elsewhere (I. Kurtul pers. obs.).

209

210   Across a range of studies on *Gambusia* spp. reproductive traits, it is apparent that a range of factors can act on

211   gonopodium size. For example, in *G. affinis* and *Gambusia hubbsi*, males within predator-free populations had

212   relatively large gonopodia compared with males in predator presence (Langerhans et al. 2005). Directional

213   selection has been apparent in *G. holbrooki* males towards smaller body sizes, larger residual gonopodium length

214   and higher heterozygosity, resulting in individuals with this suite of traits having relatively high reproductive

215   success (Head et al. 2017). In live-bearing fishes, male gonopodium length also shows an equality versus

216   efficiency trade-off between attracting females and avoiding predation (Langerhans et al. 2005). Therefore,

217   despite our demonstration that gonopodium length was influenced by climatic and geographic parameters, and

218   with this independent of differences in body size (as its effects on gonopodium length was controlled for as an

219   interaction in the PERMANOVA test), our lack of additional information on the biological components of the

220   sites (e.g. predator presence, extent of intra-specific male competition) means we cannot further decouple the

221   extent to which the environmental variables were acting directly versus indirectly on the *G. holbrooki* traits.

222

223   The reproductive features of *Gambusia* spp. enable their populations to rapidly increase in abundance in optimal

224   habitats, and thus they have to the ability to develop invasive populations relatively quickly in these habitats

225   (Pyke 2005, 2008). For example, relatively high salinity can be an important factor influencing the population

226 dynamics of Cyprinodontiform species, particularly in larval and juvenile stages (Bohlen 1999, Oliva-Paterna et

227 al. 2009). For *G. holbrooki*, their ability to adapt to lotic systems with high and differing salinity levels was an

228 important aspect of their invasion in some Spanish streams, where population abundances, hepatic condition and

229 female somatic condition were reported as higher for a population in hyper-saline conditions versus more meso-

230 saline conditions, although short-term increases in salinity did decrease abundance (Ruiz-Navarro et al. 2011).

231 However, lower salinity levels were also reported to induce higher reproductive effort of males and larger

232 females, and resulted in higher survival and recruitment rates, suggesting some complexity in the interaction of

233 salinity with *Gambusia* spp. population dynamics (Ruiz-Navarro et al. 2013). Nevertheless, the expression of

234 larger male gonopodia and smaller body sizes of both sexes in our coastal areas might again be related to higher

235 population abundances that were enabled by the likelihood that the abiotic characteristics of these waters

236 included relatively high salinity levels versus the populations further inland.

237

238 While investment in most of male somatic growth ceases after reaching maturity (i.e. with the full development

239 of gonopodium, but see Hughes 1986), female individuals continue to grow (Vargas and Sostoa 1996; Trendall

240 1983). With females being ovoviviparous, *Gambusia* spp. have pregnancies of 2 to 3 weeks and can,

241 theoretically, produce offspring every 3 to 4 weeks (Turner 1941). Furthermore, older and larger females might

242 give birth more than once during a breeding season (Pyke 2005). The number of offspring produced within a

243 single pregnancy can also be highly variable, ranging between 5 and 100 (Brown-Peterson and Peterson 1990).

244 One of the most important assumptions for understanding life history strategies is that each individual uses a

245 limited amount of energy and has to allocate that energy between activities such as somatic growth and

246 reproduction (Nikolskii 1963). Life-history theory suggests that females can produce more offspring at higher

247 latitudes, but with a trade-off that these females might have to invest more into their reproductive effort (Reznick

248 et al. 2002), suggesting higher fecundity in females with increased latitude. This was not, however, evident in

249 our results, where despite having smaller body sizes in more southern populations, the fecundity of these females

250 was relatively high, although the reasons for this were not clear. Also, it should be noted that a low sample size,

251 as was the case for fecundity in one of our sampling sites (Lake Kazan, n = 3) can result in patterns that are not

252 representative of the actual population mean for the analyzed trait(s).

253

254 It was thus apparent that there was some strong divergence in the expression of our tested mosquitofish

255 reproductive traits, with smaller body sizes being selected for in both species in the sites located in coastal and

256 southern areas, a finding detected in other recent studies where populations in more seasonal and colder areas

257 were larger in body size, invested more in reproduction and produced smaller offspring (Riesch et al. 2018). In

258 these harsher environments, the reproductive metrics of mosquitofish, such as multiple paternity within broods,

259 have also increased, with it posited that these higher rates of multiple paternity increase their invasive potential

260 at the northern edges of their distribution (Gao et al. 2019). Importantly, a common garden experiment by Santi

261 et al. (2020) indicated that male body size and offspring fat content were the only phenotypic traits that were

262 heritable between generations, suggesting that high trait plasticity is important for facilitating range expansions

263 across large spatial areas, especially where there are considerable differences in the selection pressures.

264 Although it is currently unclear as to the extent to which these spatial differences in the trait expression of

265 *Gambusia* spp. influence their invasion success, studies on the distribution of *G holbrooki* in Turkey have

266 revealed their distribution has been restricted to southern and western areas, with most populations being inland

267 (Yoğurtçuoğlu and Ekmekçi 2018), although this pattern is probably biased by most studies being completed in

268 western regions (e.g. Ekmekçi et al. 2013; Kurtul and Sarı 2020). Low winter temperatures in central and eastern

269 Anatolia might also be restricting the species' eastward invasion through increasing overwintering mortality

270 rates (Ouyang et al. 2018), although trait plasticity (such as larger males and producing more but smaller

271 offspring) might be able to overcome this constraint (Riesch et al. 2018).

272

273 In summary, our results indicated that environmental and climatic gradients strongly influence the expression of

274 reproductive traits and body sizes of *G. holbrooki*. As we were unable to decouple the direct versus indirect

275 effects of the environmental gradients then we suggest that future studies should focus on testing how these

276 effects act on these invasive populations, including the extent to which the effects are related to differences in

277 their population abundances.

278

286

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Table 2. Number of specimens, minimum, maximum, and mean standard length, and fecundity with standard error of *Gambusia holbrooki* sampled from different regions across Turkey between April of 2016 and November of 2017.

Location	n_{male}	M-M SL_{male} (cm)	$MSL_{male}\pm(SE)$	n_{female}	M-M SL_{female} (cm)	$MSL_{female}\pm(SE)$	M-M Fecundity	MF$\pm$(SE)	BE/n_{female}	Date of sampling
Lake Akgöl	11	16.59-19.88	18.34±0.294	12	26.50-34.75	29.43±0.603	20-62	34.42±3.18	1	23.05.2016
Lake Azap	11	18.32-22.90	19.81±0.563	13	29.62-35.49	32.67±0.561	25-83	45.92±4.01	1	25.05.2016
Lake Kazan	9	19.13-25.51	22.19±0.702	3	26.12-29.99	28.42±1.177	15-17	15.67±0.67	0.5	05.04.2016
Karpuz River	10	20.77-25.95	23.00±0.517	21	26.44-34.61	30.48±0.495	12-41	22.67±1.31	0.6	14.05.2016
Miliç River	19	17.00-21.33	19.06±0.278	13	22.88-28.72	26.50±0.573	12-42	20.77±2.34	0.0	04.08.2017
Güllapoğlu Reservoir	15	19.54-23.46	21.65±0.291	NP	NP	NP	NP	NP	NP	01.11.2017
Narlı River	18	20.29-26.16	23.02±0.401	8	35.66-40.39	37.94±0.655	8-46	24.5±5.38	0.4	08.06.2016
Sünbaş River	32	18.13-24.69	21.90±0.284	10	31.80-36.73	34.54±0.505	18-57	34.6±3.12	0.5	18.05.2016

M-M: Minimum-Maximum, MSL: Mean standard length, MF: Mean fecundity, BE: blastodisc embryo (fertilized eggs classified as Stage 3), SE: Standard error, NP: No pregnancy, n: number of specimens.

Table 1. Mean annual temperature (Ta), Annual precipitation (Ap), Altitude (Al) and Distance to the Sea (DS) of analysed locations in Turkey.

No	Location	City and Region	T_A°C	Ap (mmhg)	Al (h)	DS (km)	Latitude	Longitude
1	Narlı River	Uşak (Aegean)	12.5	547.3	550	156.00	38°19′16″	29°06′15″
2	Lake Akgöl	Muğla (Aegean)	15.0	1195.6	1	0.02	36°41′50″	29°02′08″
3	Lake Azap	Aydın (Aegean)	17.7	647.0	3	19.55	37°35′19″	27°26′25″
4	Lake Kazan	İzmir (Aegean)	17.9	695.4	3	2.40	37°59′12″	27˚16′30″
5	Karpuz River	Antalya (Mediterranean)	18.8	1058.3	1	0.50	36°43′01″	31°33′09″
6	Sünbaş River	Adana (Mediterranean)	19.1	647.5	38	51.00	37°23′51″	35°55′11″
7	Miliç River	Samsun (Black Sea)	14.4	1038.4	4	0.50	41°11′06″	37°01′47″
8	Güllapoğlu R.	Edirne (Marmara)	13.8	608.1	41	105	41°38′19″	26°37′11″

R: Reservoir.

Table 3. Results of a correlation matrix-based principal component analysis (PCA). Input variables were climatic (temperature and precipitation) and geographic (distance to the sea and altitude) variables. Axis loadings $\geq |0.5|$ are highlighted in bold.

	Principal components	
	1	**2**
Eigen-value	0.879	2.66
Variance explained	22.0	66.5
Mean annual temperature	**-0.708**	0.433
Annual precipitation	**0.701**	0.439
Altitude	0.062	**-0.546**
Distance to the sea	-0.057	**-0.568**

Figure captions

Fig. 1. Inset: location of Turkey in relation to world regions; main: locations of sampling sites across Turkey (as site, where site details are available in Table 1).

Fig. 2. Relationships between the main effect of climatic variables (mean annual temperature and annual precipitation) on body size (standard length (SL); a: male, b: female), male gonopodium length (c) (GL, mm) and female fecundity (d). Dashed lines denote the significant relationship between the variables according to linear regression, where a: $R^2 = 0.13$; $F = 18.31$, $P < 0.01$; b: $R^2 = 0.12$, $F = 13.62$, $P < 0.01$; c: $R^2 = 0.02$, $F = 2.11$, $P > 0.05$; d: $R^2 = 0.06$, $F = 0.02$, $P > 0.05$.

Fig. 3. Relationships between the main effect of geographic variables (altitude, distance to the sea) on body size (standard length (SL); a: male, b: female), male gonopodium length (c) (GL, mm) and female fecundity (d). Dashed lines denote the significant relationship between the variables according to linear regression, where a: $R^2 = 0.15$, $F = 21.50$, $P < 0.01$; b: $R^2 = 0.13$, $F = 15.76$, $P < 0.01$; c: $R^2 = 0.19$, $F = 27.72$, $P < 0.01$; d: $R^2 = 0.05$, $F = 0.28$, $P > 0.05$.