Immediate predation risk alters the relationship between potential and realised selection on male traits in the Trinidad guppy *Poecilia reticulata*

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Abstract

Predation risk perception can alter mating behaviours in males and females, but the consequences for sexual selection remain underexplored. We have previously shown that in experimental populations of Trinidadian guppies *Poecilia reticulata* the opportunity for sexual selection (i.e. the variance in male reproductive fitness) was higher following exposure to a simulated risk of predation than in a no-risk condition. We build upon this result by exploring whether imminent predation risk affects: 1) the relationship between the opportunity for sexual selection and the actual strength of selection on male traits and 2) the traits contributing to male fitness, and he shape of selection on these traits. While predation risk increased the variance in male fitness, realised selection on traits remained unaffected. Pre- and postcopulatory traits follow complex patterns of nonlinear and correlational selection in both treatments. Differences in selection gradients deviate from predictions based on evolutionary responses to predation, the most notable being stronger selection on courtship rate under predation risk. Our results demonstrate that the operation of sexual selection can be altered by perception of an imminent predation risk and reinforce the notion that both trait-based and variance-based metrics should be employed for an informative quantification.

Introduction

Non-lethal effects of predation are increasingly more often recognised as relevant drivers of changes in prey populations (1-3). Perception of predator cues can affect provisioning of offspring, ultimately determining their fitness (4-6). Other non-lethal effects consist of changes in mating behaviour in both males and females, with the potential to impact sexual selection trajectories. Changes in female choosiness and/or preference for conspicuous male traits following predator threat perception have been reported in variable field crickets *Gryllus lineaticeps* (7), lesser waxmoths *Achroia grisella* (8), and swordtails *Xiphophorus helleri* (9, 10). Exposure to predator cues is associated with a higher number of failed copulations in *Pardosa milvina* wolf spiders (11) and with a reduction in male courtship in *Schizocosa* wolf spiders (12). Predation threat perception can intensify sexual conflict if females engaged in antipredator behaviours are not able to evade unwanted mating attempts(13). For example, female water striders perceiving a risk of predation are more likely to accept matings they would otherwise avoid, particularly with large males (14, 15). Similarly, females of pygmy squid *Idiosepius paradoxus* remove fewer forcibly-inserted spermatangia in the presence of predator cues (16), with possible effects for sperm competition.

Whether or not non-lethal effects of predation do influence the operation of sexual selection (e.g. its strength, shape, targeted traits) remains underexplored (17). This is perhaps surprising, given the increasing awareness of how ecological factors affect sexual selection dynamics (18-21) and the omnipresence of predation risk in the wild. Moreover, immediate predation risk can vary over short time scales irrespective of the background predation intensity characteristic of the habitat (22, 23), therefore it is plausible that the same population experiences consecutive reproductive episodes under different levels of predation risk, with potentially different outcomes for the shape and strength of sexual selection and the traits involved.

One species in which the effects of predation risk on aspects of reproduction are extensively documented is the guppy *Poecilia reticulata*. On its native island of Trinidad, this small freshwater fish with internal fertilisation inhabits rivers and pools along a predation gradient, with consequences for ecology and life-history traits (24, 25). Males from low-predation localities reach maturation at a larger body size, are more brightly-coloured and perform courtship behaviours (sigmoid displays; SDs hereafter) at relatively high frequencies, while males from high-predation populations are smaller, duller and rely more heavily on forced copulation attempts (gonopodial thrusts; GTs hereafter)(24). In addition, predation regime indirectly affects postcopulatory traits linked to guppy sperm performance (26). Observations in the lab indicate that more colourful males with a higher courtship rate are usually preferred by females and have a higher reproductive success (25, 27). In the presence of predator cues, males reduce the frequency SDs while increasing the rate of GTs (28). This change in male mating tactic is partly mediated by a reduction in female receptiveness (29) and preference for conspicuous male colouration (30). At the same time, if given the chance to observe male behaviour in the presence of predators, females prefer bolder males (that show a higher propensity to take risks, i.e. (31)), who indeed benefit from a higher reproductive success compared to their shier counterparts (32).

We have previously shown that simulation of immediate predation risk increases the strength of sexual selection (expressed as the standardised variance in male reproductive success, I_{RS}) on guppy males (33). This was mainly driven by a higher variance in mating success, suggesting that, at least in our experimental

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88 conditions, predation risk may be associated with stronger sexual selection on male precopulatory traits. The

89 variance in male reproductive success, however, does not necessarily represent the realised selection on

90 traits, but rather an estimation of the upper limit of the strength of sexual selection (34-36), and does not

91 distinguish between contributions from male traits and random variation in reproductive success not

92 attributable to sexual selection (37).

93 Despite intense debate regarding the use of trait-based statistics (such as selection gradients) or variance-

94 based statistics (for example the opportunity of sexual selection) for quantifying sexual selection (38, 39),

95 comparisons between the two methods have been largely based on simulated datasets (40), while empirical

96 tests have yielded mixed results (41-43).

97 Here we build on our previous findings by aiming to quantify the effects of immediate predation risk on: 1) the

98 relationship between the total opportunity for sexual selection (standardised variance in reproductive

99 success, I_{RS}) and the actual strength of selection on male traits and 2) the targets and shape of selection.

100 We conduct multivariate selection analyses followed by canonical rotations, focusing on male traits known to

101 contribute to pre and postmating success in the guppy (20, 31, 44-48).

102 The effect of immediate predation risk on the strength and shape of selection on male traits will be influenced

103 by female choosiness and polyandry, although the exact pattern is not easily predictable. If predation risk

104 causes a decrease in female choosiness, then mating should be more random with respect to male

105 precopulatory traits (such as body size, colouration and courtship behaviour), leading to weaker sexual

106 selection on these traits (49, 50). On the other hand, if polyandry decreases in response to predation risk (as

107 observed in our guppy population), then the potential for selection on male precopulatory traits should

108 increase (51) while the importance of postcopulatory traits for male reproductive fitness should decrease.

109 Based on observations of guppies in the wild and in the laboratory (see above), and considering the complex

110 patterns of linear and nonlinear selection identified in our population (20, 52), we can predict that the

111 combinations of traits advantaged under perceived predation risk include boldness and GTs, while in control

112 conditions they comprise orange colouration, SDs, gonopodium length, iridescence and GTs. In addition, in

113 accordance with the reduction in female polyandry observed previously (33), we expect that postcopulatory

114 traits would be less relevant for male fitness in the presence of predation compared to control conditions.

115

116 **Materials and methods**

117

118 *(a) Experiment overview*

119 As described in (33), mating trials in the presence and absence of predation risk were carried out in

120 populations consisting of six males and six virgin females (hereafter "replicates", see below). Males were

121 selected from stock tanks ensuring that, within the same experimental population, they could be individually

122 recognisable by the human observer from colour patterns. The sequence of data collection is presented in

123 supplementary Figure S1. Briefly, males were isolated individually for three days, then subject to two

124 boldness tests (see below), photographed and stripped of sperm to standardize their initial sperm reserves.

125 Five days after photography, males were subject to mating trials in the first treatment, with the second

126 treatment following six days after. We tested a total of 20 male replicates both in the presence and absence

127 of predation cues (i.e. a repeated-measure design), while the groups of females differed between treatments.
128 Fin clips for the purpose of DNA extraction were obtained from males at the end of behavioural observations
129 and from females after they produced a brood. Offspring were euthanised at 24-48h of age and preserved in
130 pure ethanol at -20°C until processing. Following data collection, all adults were released into post-
131 experimental tanks and not reused in further experiments. Predation risk simulation, observations of mating
132 behaviour and paternity assignment are the same as in (33) and also described in the supplementary
133 material.

134

135 *(b) Boldness test*

136 We measured boldness using a modified version of the open-field test. Our setup consisted of a white circular
137 arena, 40 cm in diameter filled with water to a depth of 2.5 cm. The arena contained a 3.5 cm diameter refuge
138 in the centre, manufactured from a plastic bottle cap. A 15-W neon light on each side provided illumination.
139 The plain white background and shallow water very likely generates fear in guppies, which is central to
140 boldness measurement (53). The fish was released close to the refuge and its behaviour recorded for 10
141 minutes with a Panasonic HCV180 video camera mounted 1m above the arena. Two boldness tests,
142 separated by 48h, were performed for each male. The latency to leave the refuge and the total time spent
143 underneath the refuge were scored from videos using BORIS 7.1.3 ((54),
144 http://www.boris.unito.it/pages/download.html). Both behaviours were repeatable (r ≥ 0.3 according to the
145 formula proposed by (55)). For each behaviour, we calculated the average between the two observations
146 and reduced them to a single variable using a principal component analysis. The loading factor of each
147 original variable was 0.94 and the resulting principal component, hereafter referred to as boldness, explained
148 89% of the total variance.

149

150 *(c) Morphology and sperm assays*

151 Males were anesthetised in a bath of MS-222, placed on a grid-lined slide under a dissection microscope
152 equipped with a Canon 450D camera and their left sides photographed. The ejaculates were stripped into a
153 drop of 0.9% saline solution by swinging the gonopodia (intromittent organs) back and forth and applying
154 gentle pressure to the abdomen. In this species, sperm is organised in discrete bundles (spermatozeugmata),
155 each containing ~22000 sperm cells (56). All bundles were photographed for the purpose of sperm counting.
156 Male body area, gonopodium length, area of colouration (orange and iridescent) and sperm number where
157 scored from pictures using ImageJ software (https://imagej.nih.gov/ij/download.html).
158 Three sperm bundles were placed on a multi-well slide coated with 1% polyvinyl alcohol to prevent sperm
159 cells from sticking to the glass (47) and activated by 3 µl of water containing 150 mM KCl and 2 mg/L bovine
160 serum albumin (57). Sperm velocity was measured using a CEROS sperm tracker (Hamilton-Thorne
161 Research, Beverly, MA, USA) as cells were swimming away from the dissolving bundle. The sperm tracker
162 provides a series of sperm velocity parameters of which we retained VAP (average path velocity) for further
163 analyses (26). Sperm velocity for each male was measured from 295 ± 14.2 (mean ± S.E.) cells.
164 Sperm viability was measured with a VitalTest kit (Halotech, Spain). Forty sperm bundles were placed in a
165 0.5 ml Eppendorf tube containing 40 µl saline solution and broken by vortexing for 90 seconds (58). We

166 transferred 6 µl of the resulting mixture into a 0.5 ml Eppendorf tube to which we added 0.5 µl acridine orange,

167 which stains live cells in green, and 0.5 µl propidium iodide which stains dead cells in red. Fluorescent images

168 of the sample were taken with a Leica 5000 B microscope (Leica Microsystems, Wetzlar, Germany) equipped

169 with a digital camera (DFC480; Leica Microsystems, UK). Sperm cells were counted using ImageJ software

170 and viability was calculated as the proportion of live sperm out of the total, from at least 200 cells.

171 A summary of the phenotypic characteristics of the males used in this experiment is given in the

172 supplementary material (Table S1).

173

174 *(d) Statistical analyses*

175 We estimated the relationships between male relative fitness and phenotype using separate multivariate

176 selection analyses (59) for each predation treatment followed by canonical rotations (60). We calculated

177 fitness as the proportion of offspring sired by each male out of the total number of offspring produced within

178 each replicate. We included (*i*) body area, (*ii*) gonopodium length, (*iii*) area of orange colouration, (*iv*) area of

179 iridescent colouration, (*v*) sperm number, (*vi*) sperm velocity, (*vii*) sperm viability, (*viii*) number of sigmoid

180 displays, (*ix*) number of gonopodial thrusts and (*x*) boldness as predictor variables in both models. The sets

181 of males were repeated across the two conditions, therefore the values for boldness, morphological and

182 ejaculate traits are the same for the control and predation treatments, while sexual behaviour was measured

183 during mating trials, therefore values for SDs and GTs differed between treatments. While predation risk was

184 associated with a reduced average courtship rate (33), between-individual differences remained constant

185 (see supplementary material). We standardised response variables to a mean of one and trait values to a

186 mean of zero and standard deviation of one (59).

187 First, we conducted linear regressions including all trait estimates to obtain linear selection gradients (β). We

188 then fitted second-order regressions including all linear, quadratic and correlational terms to estimate the

189 matrices of nonlinear selection gradients (hereafter referred to as gamma matrices). Statistical packages

190 underestimate quadratic coefficients by 0.5, therefore we doubled these estimates to obtain the correct values

191 (61).

192 We compared the linear, quadratic and correlational coefficients between treatments with a Monte-Carlo

193 simulation with 10000 iterations. We compared the observed differences (predation – control) in the

194 coefficients with a random distribution of differences obtained by shuffling each male's reproductive success

195 across treatments. Significance was calculated as the proportion of iterations in which the observed

196 difference exceeded the 95% distribution in the random differences. We used a similar procedure to estimate

197 differences in standardised variance in reproductive success (I_{RS}; see also (33)), proportion of variance

198 explained by male traits (R^2 from full quadratic regressions) and total amount of variance explained by traits

199 ($I_{RS} * R^2$). We obtained standard errors of these point estimators with a bootstrap procedure based on 10000

200 samples.

201 Interpreting the size and significance of individual coefficients can underestimate the strength of nonlinear

202 selection (62). To overcome this problem, we conducted canonical rotations of the gamma matrices by

203 multiplying them with the matrices of standardised traits (60). Canonical rotations produce new axes of

204 nonlinear selection characterised by loadings of the original traits, similarly to loadings of original variables

205   on principal components obtained by PCA, and identify combinations of traits under selection beyond

206   pairwise comparisons (63, 64). The number of canonical axes obtained is equal to the number of traits

207   included in the analysis (eigenvectors M1 – M10 in each treatment; see below). Each eigenvector has an

208   associated eigenvalue (λ), equivalent to the quadratic selection coefficient along the new axis. The strength

209   of selection (curvature) along each eigenvector is given by its eigenvalue and the shape of selection by its

210   sign, with positive eigenvalues indicating disrupting selection and negative eigenvalues stabilising selection.

211   We also rotated original linear selection coefficients (β) onto the new traits in order to obtain estimates of

212   linear selection along the new axes (θ) (65). We used the permutation procedure proposed by (66) to

213   calculate the significance of each eigenvector. Analyses were conducted with R 4.0.3 (67) and PopTools 3.2

214   (68) in Microsoft Excel. We visualised fitness surfaces using the 'Tps' function of the 'fields' package in R

215   (69).

216

217   **Results**

218

219   In a previous paper (33) we demonstrated that the standardised variance in male reproductive fitness is

220   significantly higher in the predation treatment (I_{RS} = 1.073) compared to control (I_{RS} = 0.633; Figure 1. See

221   also (33)). Of this total variance, the proportion explained by traits, as estimated by the multiple regression

222   analyses, was significantly higher in the control treatment (R^2 = 0.709) compared to predation (R^2 = 0.546;

223   delta ± SE = - 0.162 ± 0.096, p = 0.026; Figure 1). By multiplying the standardised variance in reproductive

224   fitness observed in the two treatments by the proportion of variance explained by traits, we obtained an

225   estimate of the strength of overall sexual selection on the male traits considered in this study. We found that

226   sexual selection on traits was higher in the predation treatment (I_{RS} * R^2 = 0.586) compared to control (I_{RS} *

227   R^2 = 0.449), although this difference was not statistically significant (delta ± SE = 0.138 ± 0.103, p = 0.127;

228   Figure 1).

229   When reproductive fitness was analysed separately for each treatment, we did not find any significant linear

230   selection gradients (β) (Table S2). We identified significant quadratic selection on body area (disruptive) and

231   GTs (stabilising) in the control treatment and on SDs (disruptive) in the predation treatment. In the control

232   treatment, all male traits apart from sexual behaviour were involved in significant correlational selection

233   (Table S2), whereas a single negative correlational gradient, between sperm number and boldness, was

234   significant in the predation treatment (Table S2). When we compared the multiple regression coefficients

235   between treatments, we identified significant differences in two linear coefficients (GTs and boldness), two

236   quadratic coefficients (sperm velocity and SDs) and four correlational coefficients associated with sperm

237   number in combination with body area, iridescence, sperm velocity and SDs, respectively. Among these, SDs

238   showed the most pronounced difference between treatments (Table S3). In summary, we identified different

239   predictors of fitness in the two treatments whose coefficients, in turn, are associated with significant between-

240   treatment differences. Thus, in the predation treatment, high and low frequencies of SDs predict high fitness

241   (i.e. disruptive selection), whereas reproductive success in the control treatment is associated with a positive

242   correlation between body area and sperm number and a negative corelation between area of iridescence

243   and sperm number. Canonical rotations produced ten new axes of selection in each treatment (Table 1). A

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244 curvature different from 0 (given by the lambda value) indicates significant selection along the respective
245 axis. This was the case for five axes in each treatment (M1, M2, M8, M9, M10). Note that the eigenvectors
246 in each treatment are obtained from separate canonical rotations and are not equivalent, so M1 from the
247 control treatment represents a different axis in canonical space than M1 from the predation treatment. For
248 simplicity, we restrict our discussion to the strongest (highest absolute lambda value) and most significant
249 (lowest p value) two axes in each treatment (70). Thus, the highest lambda values in the control treatment
250 corresponded to M1 and M10, which described disruptive and stabilising selection, respectively. Axis M1 was
251 primarily loaded by body area (positive) and sperm number (negative), while axis M10 was mainly loaded by
252 area of iridescence (positive) and sperm viability (negative). The fitness surface defined by these two axes
253 (Figure 3) reveals peaks at extreme values of M1 and average values of M10. The highest peak is associated
254 with small body area and high sperm count in combination with intermediate values for area of iridescence
255 and sperm viability, whereas the lower peak corresponds to males with large body area, low sperm count,
256 and again intermediate values of sperm viability and iridescent colouration (Figure 3). The most significant
257 axes in the predation treatment (with the highest associated lambda values) were M1 and M10, describing
258 disruptive and stabilising selection, respectively (Figure 3). Axis M1 was mainly loaded by SDs (negative)
259 and body area (positive) and axis M10 was primarily described by sperm number (positive) followed by GTs
260 (positive) and boldness (positive). The surface built by these vectors indicates that most successful
261 phenotypes concentrate around extreme negative values of M1 and intermediate values of M10. These males
262 are small, perform SDs at high frequencies and have intermediate values for sperm count, gonopodial thrusts
263 and boldness. A secondary area of high relative fitness, at the positive end of M1 and average values of M10,
264 is associated with intermediate to low sperm count, GT frequency and boldness and also large body area
265 and low SD frequency (Figure 3).

266 In summary, in the control treatment sexual selection is nonlinear, in accordance with previous results (20,
267 52): the fitness surface has a nearly symmetrical saddle shape, with the two peaks at extreme values of M1
268 of comparable height, suggesting that the alternative phenotypes benefit from similar reproductive success
269 (Figure 3). In contrast, under predation risk sexual selection tends to be more sloped, as the surface built by
270 the two main axes of selection identifies a phenotype (negative extreme of the M1 axis) which represents a
271 main fitness peak, higher than the one at the positive end, suggesting that relatively small males performing
272 a high frequency of SDs are better favoured compared to males showing the reverse combination of traits
273 (Figure 3).

274

275 **Discussion**

276

277 In a previous study (33) we demonstrated that the perception of an imminent predation risk increases the
278 opportunity for sexual selection, as estimated from the standardized variance in male reproductive success
279 (35, 38). This result was due to an increased variance in male mating success and a reduced polyandry (as
280 derived from the number of sires per brood), confirming that polyandry is negatively associated with variance
281 in male reproductive success (51). In the present study, we used the data on male reproductive success to
282 test whether sexual selection differed in strength and shape in response to predation risk. Specifically, we

283 aimed to explore: 1) whether the greater opportunity for (sexual) selection in the presence of predation risk

284 resulted in stronger overall selection on male traits and 2) whether the perception of imminent predation risk

285 affected the importance of male traits and combinations of traits for reproductive success.

286 Our results suggest that, despite the larger I_{RS} under imminent predation risk, the overall strength of sexual

287 selection on male traits (expressed as the proportion of the variance explained by traits multiplied by the total

288 variance) did not differ significantly between treatments. This was because in the predation treatment, male

289 traits explained a lower proportion of the total variance in reproductive success compared to the control

290 treatment (Figure 1). Therefore, although predation risk nearly doubled the opportunity for sexual selection,

291 there was no corresponding increase in the strength of sexual selection on male traits. A widespread limitation

292 of studies aiming to quantify selection in small experimental populations (as in the current work) consists of

293 the noise generated by random variation in trait values. Here we overcame this issue by using a repeated

294 measures design (71), therefore our quantification of sexual selection indices under different levels of

295 predation is particularly informative. Our results provide reliable experimental evidence for the theoretical

296 notion that the opportunity for sexual selection (and selection in general) does not necessarily equal realised

297 sexual selection (38, 39, 72).

298 There are multiple non-mutually exclusive explanations for the observed relationships between the sexual

299 selection metrics we computed in the two treatments. Here we discuss three. First, our result may be

300 explained by the reduced female mating rate under predation risk and the consequent reduction in the

301 contribution of traits under postcopulatory selection towards the variance in male reproductive success (33).

302 Second, female mate assessment could be less accurate under predation risk. Guppies are an extreme

303 example of multiple male ornaments under simultaneous selection by female choice (20, 52). Evaluating

304 complex phenotypes requires time and cognitive effort (73, 74) that may be limited under an imminent

305 predation threat. Therefore, assuming a theoretically preferred male phenotype, "errors" in mate choice could

306 occur more frequently in these conditions. Stochasticity in female choice should reduce the variance in male

307 reproductive success (if female mate choice was purely stochastic the variance in male reproductive success

308 should tend to zero), which contrasts with our observation that predation risk was associated with an

309 increased variance in both male mating and reproductive success (33). A higher variance in male mating

310 success, however, may arise due to a higher importance of mate choice copying, which has been

311 documented in female guppies both in the presence and absence of predator cues (30, 75). In this scenario,

312 the initial choice of the first mating female in each replicate may benefit the first male to mate, irrespective of

313 his phenotype, as suggested by previous results from our population (Morbiato, Cattelan, Pilastro,

314 unpublished data). Thus, the variance in male mating and reproductive success under predation risk would

315 increase (as observed) without affecting the overall strength of sexual selection on male traits, if females tend

316 to copy the choice of other females more frequently under predation risk. Third, the higher portion of

317 unexplained variance in the presence of predation could be a by-product of traits we did not quantify

318 becoming more important for male fitness under these circumstances. Since it is difficult to know whether

319 analyses of the type conducted here capture all components of male reproductive phenotype, this explanation

320 cannot be ruled out.

321 Our second aim was to test whether predation risk influences the traits that contribute towards male fitness

322 and/or the shape of selection on these traits. Previous analyses (33) indicate that, under predation risk,

323 selection on postcopulatory traits such as sperm number, velocity, and viability should be weaker and

324 selection on precopulatory traits should be stronger. We did find significant differences between the selection

325 gradients under the two conditions (Table S3), but our results partly deviated from this prediction. One

326 important consideration is that the values for morphological traits are the same in both treatments, as they

327 are unlikely to vary substantially over the duration of the experiment (Figure S1) and are not influenced by

328 predation risk. In addition, our interpretation of the relationship between boldness and fitness is based on the

329 assumption that our boldness estimate in standard conditions reflects male propensity to take risks in other

330 contexts, including mating trials. In contrast, male sexual behaviour was recorded during mating trials and is

331 significantly affected by predation risk (33). It is therefore not surprising that the largest difference in selection

332 gradients involved sexual behaviour, although not in the expected direction (Table S3).

333 In agreement with previous work on the same population of guppies in conditions similar to our control

334 treatment (20, 52), we found that sexual selection was largely correlational and non-linear. While we found

335 no significant linear β regression coefficients or $\boldsymbol{\theta}$ coefficients on the M vectors in either treatment (Table 1

336 and Table S2), comparisons between treatments revealed that selection on GTs and boldness was more

337 strongly linear in the predation treatment, and opposite in direction, compared with control (Table S3). At the

338 same time, males with high and low values for body area and intermediate values of GTs were advantaged

339 in the control treatment. In addition, eight combinations of traits were under correlational selection. In

340 agreement with our expectation that postcopulatory traits should be more important for male reproductive

341 success in the absence of predation, all ejaculate traits contributed towards male fitness in the control

342 treatment, in combinations with morphological traits or boldness (Table S1). Canonical rotations confirmed

343 these patterns: phenotypes under strongest selection in the control treatment were characterized by

344 intermediate values for area of iridescence and sperm viability (M10) and either large body area and low

345 sperm number, or small body area and large sperm number (M1, Figure 2).

346 Extreme (high and low) frequencies of SDs were advantaged under predation risk. We also identified negative

347 correlational selection between boldness and sperm number in the same treatment (Table S2), indicating

348 that bolder males with low sperm count or shy males with high sperm reserves had a higher reproductive

349 success. Canonical rotations confirmed disruptive selection on SDs under predation risk: axis M1 was loaded

350 positively by body area and negatively by SDs, with the highest relative fitness concentrated around the

351 negative extreme (Figure 3). The most advantageous phenotype in the presence of predation risk consisted

352 of small body area, high SD frequency and intermediate sperm number, boldness and GT frequency (Figure

353 3).

354 Our results regarding the traits under selection only partly reflect the expected patterns. We did not find a

355 relationship between GTs and male fitness in either treatment, despite a significant difference (yet in the

356 unexpected direction) in linear gradients (Table S3). We did not observe any successful coercive mating but

357 note that observations only covered 50% of the duration of the trials (33), thus we cannot exclude that forced

358 copulations occurred. Even so, their contribution to male reproductive success was most likely limited, given

359 the low insemination success of this mating tactic (76, 77) and considering that females were virgin and

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therefore expected to be sexually receptive (i.e. to mate cooperatively more often). This was necessary in order to avoid the production of offspring from previously stored sperm that would have biased our measures of male reproductive success, but it has to be noted that in the wild, virgin females are a minority and their mating behaviour may not be representative to that of the population at large (27). In addition, the stronger positive correlation between SDs and reproductive success in the same treatment is surprising, considering that on average males reduced their courtship effort in the presence of predator cues (33). This observation, coupled with the lower polyandry, suggests that cooperative female mating rate is a key determinant of the strength of sexual selection on male traits while sexual conflict plays a minimal role, at least in our population under these experimental conditions.

In conclusion, our study demonstrates that, although imminent predation risk was associated with a higher opportunity for sexual selection and a stronger association between male mating and reproductive success (33), sexual selection on male reproductive phenotype is not significantly stronger and largely similar in shape to that observed in control conditions. The most notable difference in the operation of sexual selection regards the increased relevance of courtship rate under predation risk compared to the control treatment. This is particularly instructive, in our opinion, because it highlights a situation in which males respond to the presence of predator cues by reducing, on average, the frequency of the behaviour (33), yet its importance for male reproductive success increases. Our results therefore demonstrate that non-lethal effects of predation can influence sexual selection trajectories, but in ways that can neither be deduced from lethal effects (e.g. selection against more conspicuous male phenotypes), nor predicted by behavioural responses of males and females to the perception of an imminent predation risk. Finally, our results confirm that, on its own, the variance in male reproductive success is not a sufficiently informative predictor of the strength of sexual selection, at least in polyandrous species (38, 39, 72).

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Figures and tables

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Figure 1. Differences between treatments in standardised variance in fitness (opportunity for selection, I_{RS}), amount of standardised variance explained by male traits (I_{RS} * R^2 from second-order regressions), and proportion of variance (R^2 from second-order regressions) explained by male traits (white bar). Asterisks indicate significant values ($p < 0.05$).
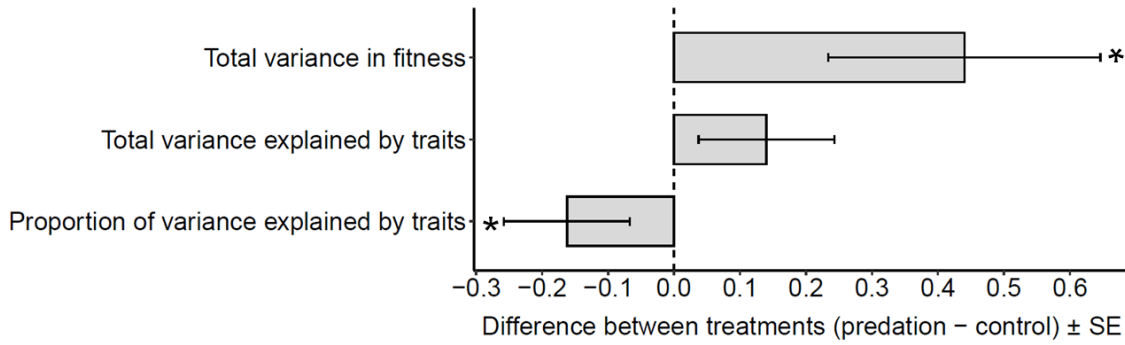
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Figure 2. Fitness surface (A) and two-dimensional contour plot (B) illustrating the relationships between relative fitness and major axes of selection in the control treatment. Axis M1 represents disruptive selection and M10 stabilising selection.

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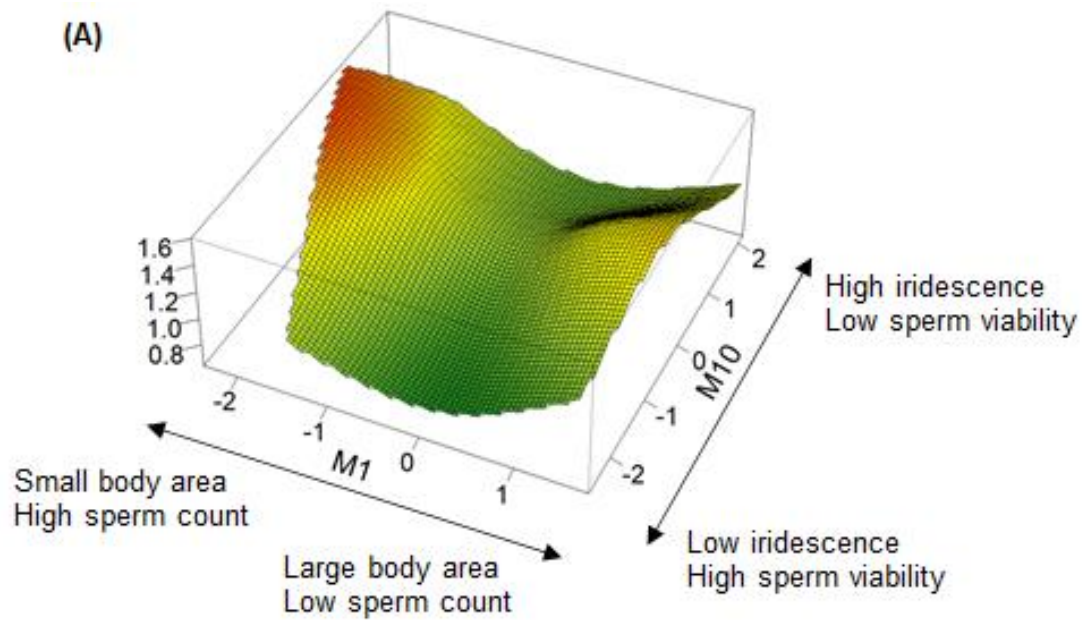
Figure 3. Fitness surface (A) and two-dimensional contour plot (B) illustrating the relationships between relative fitness and major axes of selection in the predation treatment. Axis M1 represents disruptive selection and M10 stabilising selection.
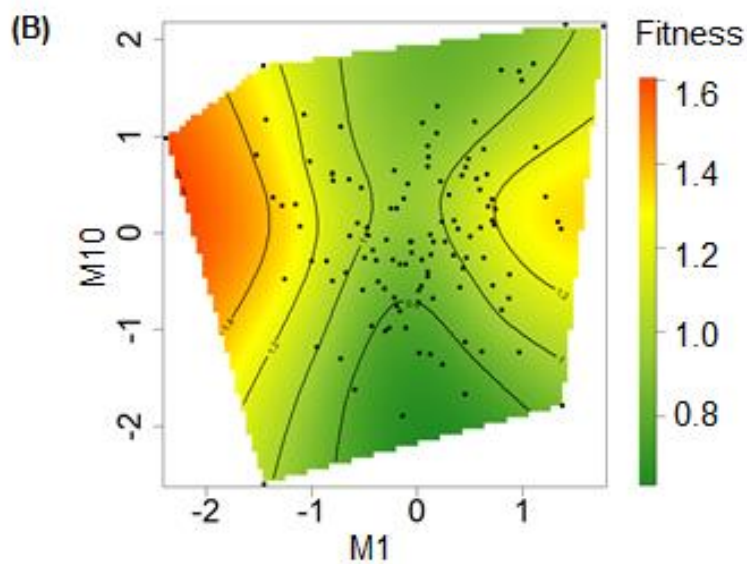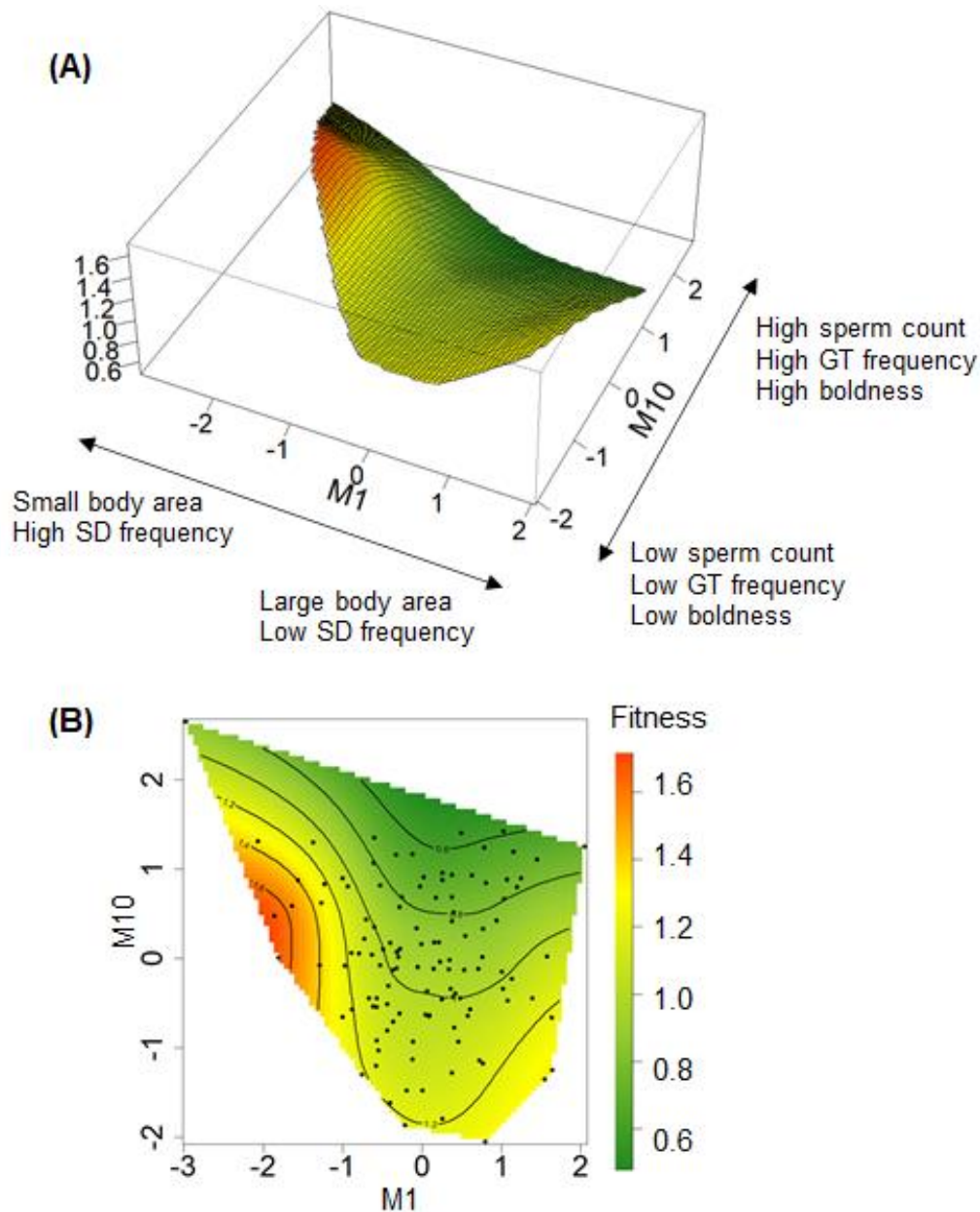
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Table 1. Eigenvectors obtained by canonical rotations of the gamma matrices and estimates of linear (theta) and nonlinear (lambda) selection gradients along each axis (M1-M10) in each predation treatment. Trait loadings on each eigenvector can be interpreted similarly to those obtained by a principal component analysis. The strength of selection (curvature of the surface) is given by eigenvalues and the shape by their signs (positive=disruptive; negative=stabilising). Significant lambda values ($p < 0.05$) are indicated in bold. *P* values obtained with permutation tests (5000 iterations) following (66).

Control

	Theta (P value)	Lambda (P value)	Body area	Gonopodium	Orange	Iridescent	Sperm number	Sperm velocity	Sperm viability	SD	GT	Boldness
M1	-0.038 (0.739)	**2.000 (<0.010)**	0.694	-0.020	-0.171	-0.277	-0.536	-0.157	-0.284	-0.071	0.079	0.093
M2	-0.068 (0.414)	**0.854 (0.020)**	-0.040	-0.071	-0.416	-0.281	0.212	0.412	-0.382	0.116	0.129	-0.593
M3	0.102 (0.171)	0.342 (0.086)	0.578	-0.230	0.377	0.140	0.504	-0.174	0.009	0.186	-0.208	-0.298
M4	-0.046 (0.533)	0.204 (0.296)	-0.186	-0.670	0.286	-0.429	-0.260	0.212	0.110	0.253	-0.235	0.078
M5	0.002 (0.992)	-0.077 (0.690)	-0.360	-0.196	0.052	-0.133	0.062	-0.712	-0.496	-0.193	-0.045	-0.126
M6	-0.019 (0.788)	-0.183 (0.216)	0.089	-0.286	-0.291	0.083	0.108	0.160	0.102	-0.738	-0.474	0.030
M7	0.046 (0.583)	-0.338 (0.128)	-0.006	0.210	-0.243	-0.028	0.193	0.055	-0.369	0.400	-0.595	0.453
M8	0.028 (0.744)	**-0.585 (<0.010)**	0.040	-0.428	-0.640	0.190	0.098	-0.323	0.329	0.314	0.193	0.110
M9	0.094 (0.353)	**-0.996 (<0.010)**	0.095	-0.011	0.033	-0.544	0.533	0.031	0.030	-0.199	0.387	0.468
M10	0.069 (0.445)	**-1.096 (<0.010)**	0.017	-0.381	0.131	0.531	0.009	0.300	-0.509	-0.048	0.333	0.304

Predation

	Theta (*P* value)	Lambda (*P* value)	Body area	Gonopodium	Orange	Iridescent	Sperm number	Sperm velocity	Sperm viability	SD	GT	Boldness
M1	-0.159 (0.246)	**1.501 (0.010)**	0.405	-0.124	0.070	-0.100	0.222	-0.386	0.040	-0.774	0.076	-0.026
M2	0.15 (0.253)	**1.412 (0.014)**	0.405	-0.254	-0.519	-0.248	0.041	-0.226	-0.184	0.307	-0.339	0.380
M3	0.065 (0.584)	0.679 (0.206)	0.033	-0.112	0.427	0.598	0.213	-0.326	-0.446	0.171	-0.246	0.073
M4	-0.111 (0.309)	0.220 (0.597)	0.021	0.245	0.231	0.076	-0.556	-0.341	0.287	-0.002	0.105	0.601
M5	-0.041 (0.66)	0.036 (0.910)	-0.457	-0.396	-0.167	0.005	-0.286	0.164	-0.505	-0.380	0.114	0.291
M6	-0.009 (0.946)	-0.034 (0.898)	0.495	-0.466	0.169	0.298	-0.085	0.547	0.205	0.038	0.203	0.170
M7	0.023 (0.819)	-0.247 (0.202)	-0.048	0.323	0.092	0.006	0.193	0.446	0.077	-0.308	-0.656	0.343
M8	0.048 (0.629)	**-0.524 (0.040)**	-0.238	-0.534	0.503	-0.476	0.009	-0.116	0.250	0.114	-0.296	-0.031
M9	-0.061 (0.631)	**-0.913 (0.032)**	-0.389	-0.256	-0.365	0.411	0.350	-0.179	0.546	-0.036	0.005	0.167
M10	-0.173 (0.127)	**-1.715 (<0.010)**	-0.074	0.132	0.196	-0.283	0.588	0.077	-0.139	0.149	0.486	0.479