1   **Male-male interactions select for conspicuous male colouration in damselflies**

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3   Md Kawsar Khan, Marie E. Herberstein

4   Department of Biological Sciences, Macquarie University, Sydney, Australia

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7   Corresponding author:

8   Md Kawsar Khan

9   Department of Biological Sciences

10   Macquarie University, Sydney

11   NSW-2109, Australia

12   Email: Kawsar.khan@mq.edu.au

13   Phone: +612-9850-6279

14

15   Abstract

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17   Male ornamentation, such as conspicuous male colouration, can evolve through female mate

18   choice. Alternatively, in species without overt female mate preference, conspicuous male

19   colouration can evolve via intrasexual selection to resolve male-male competition or to prevent

20   costly male-male mating attempts. Here, we investigated the drivers of conspicuous male

21   colouration in an ontogenetic colour changing damselfly, *Xanthagrion erythroneurum,* where the

22   juvenile males are yellow and change colour to red upon sexual maturity. We first showed that

23   red males were chromatically and achromatically more conspicuous than the yellow males. We

24   then quantified the condition of the males and showed that red males were larger and in better

25   condition than yellow males. We tested female preference in a choice experiment where we

26   artificially manipulated male colour, and found that females did not choose mates based on male

27   colouration. We further tested whether the male colouration affected male-male interactions. We

28   presented red and yellow males in the breeding arena, and found that red males received less

29   intra- and interspecific aggression than yellow males. Our study experimentally showed, for the

30   first time, that male conspicuousness is not a target of female mate choice in damselflies. Intra-

31   and interspecific male-male interactions therefore appear to be the driver of conspicuous male

32   colouration in damselflies.

33   Key words: Ontogenetic colour change, Sexual selection, Sensory ecology, Communication and

34   signalling, Colour polymorphism, Sexual conflict, Scramble competition

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36   Introduction

37   Male armaments and ornaments, such as horns in antelopes, the long tails of peacocks, giant hind

38   legs in beetles, and conspicuous colours in birds, lizards and insects, are not thought to afford

39   survival benefits, but rather evolve through sexual selection (Balmford et al., 1992a; Balmford et

40   al., 1992b; Emlen, 2008; Parker, 2013; Petrie et al., 1991). Among ornamental traits, the function

41   and evolution of male ornamental colouration has been studied in many taxa since Darwin

42   proposed his sexual selection theory (Jones and Ratterman, 2009). Male conspicuous colouration

43   can evolve via intersexual selection through female preference (Gomez et al., 2009; Kemp,

44   2007), or via intra-sexual selection through male-male competition for mating and/or to avoid

45   male-male mating attempts (Bajer, Molnár, Török, & Herczeg, 2011; Sherratt, 2001).

46   Females are predicted to express mate choice if males vary in their ability to provide either direct

47   benefits such as parental care, nuptial gifts, higher social status, safer territory, or indirect

48   benefits such as higher immunity and better genetic alleles (Albo and Peretti, 2015; Georgiev et

49   al., 2015; Kirkpatrick and Barton, 1997; López Pilar and Martín José, 2005). In this context,

50   conspicuous colouration may function as a signal of male condition (Hill, 1991; Keyser and Hill,

51   2000) and by selecting to mate with a conspicuous males over dull males females may be

52   responding to signals of good condition (Montoya and Torres, 2015; Setchell, 2005; Vásquez

53   and Pfennig, 2007). Consequently, conspicuous male colour can evolve via female mate choice.

54   Conspicuous male colouration can evolve via male-male competition, in addition to or without

55   female preference, when males compete for limited breeding resources such as nest sites,

56   oviposition sites, or to access females for mating (Ahnesjö et al., 2001; Klug et al., 2010; Morris

57   et al., 1992; Wacker and Amundsen, 2014). Competition for breeding resources can largely

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58   determine the mating success in some species, therefore sexual selection can favour male traits to

59   acquire breeding resources (Debuse et al., 2003; Shuster and Wade, 2003). Otherwise, male-male

60   competition to access females arises when the males outnumber females, or when the frequency

61   of receptive females in the breeding territory is lower than that of males (Klug et al., 2010; Weir

62   et al., 2011). Because of a male biased sex ratio, the males compete with each other to access

63   females, and only the best quality males are assumed to secure mates (Forslund, 2000; Morris et

64   al., 1992). Conspicuous colouration can function as an honest signal of male condition, resource

65   holding potentiality and fighting ability (Ligon and McGraw, 2013; Lim and Li, 2013; Weaver et

66   al., 2017). Conspicuous males may therefore maintain larger and safer breeding territories,

67   higher social dominance, and better access to females, which result in higher mating success

68   (Korzan and Fernald, 2007; Setchell and Wickings, 2005).

69   Conspicuous male colouration is common in many damselflies (Corbet 1999; Bybee et al. 2016).

70   In territorial damselflies, conspicuousness is condition-dependent and related to males

71   maintaining territory and achieving higher mating success via female mate choice and inter- and

72   intraspecific male-male competition (Siva-Jothy, 1999; Svensson et al., 2004; Tynkkynen et al.,

73   2005). The function of conspicuous male colouration is, however, largely debated in damselflies

74   where the males do not defend territory. In these damselflies, adult male and female damselflies

75   assemble in waterbodies such as ponds, lake, streams for mating, and ovipositing. Because of the

76   high male density and limited oviposition sites, males compete to access and persist in the

77   mating territories. In such scramble scenarios, males approach females from behind for mating

78   and females cannot see the colour of males, in the first step of the mating sequence, which is

79   unlikely to be the point of mate selection (Corbet 1999; Sherratt and Forbes 2001). Subsequently,

80   however, males require the cooperation of females to lock genitalia (Fincke, 1997) and it has

81   therefore been argued that conspicuous male colour can evolve through female mate choice.

82   Male-male competition for mating is the other probable function of conspicuous male

83   colouration in damselflies with scramble mating system. In these damselflies, a large number of

84   conspecific and heterospecific males gather within a breeding arena (pond) for mating, resulting

85   in male biased aggregations (Corbet, 1999). Conspecific male-male competition arises when two

86   or more males compete over access to the same female or breeding spot. On the other hand,

87   interspecific male-male competition occurs in sympatric populations to acquire limited

88   oviposition and mating sites. These intra- and interspecies competition could induce male-male

89   interactions which can reduce male fitness (Gering, 2017). In such circumstances, conspicuous

90   male colouration can evolve to signal male condition thereby reducing intra- and interspecific

91   male-male interactions. This hypothesis, although intriguing, is yet to be experimentally tested.

92   Here we tested the causative agents of the conspicuous male colouration in *Xanthagrion*

93   *erythroneurum* damselflies. *Xanthagrion erythroneurum* exhibits ontogenetic colour change,

94   where the males change colour from yellow to red, about a week after their emergence (Khan

95   and Herberstein, 2020). First, we tested if red males are more conspicuous than yellow males for

96   damselfly vision. We then assessed whether the conspicuous colour if conspicuous colouration

97   signals male condition in this species. Finally, we experimentally tested whether female mate

98   choice or male-male interactions select for the conspicuous red colouration in males. We

99   predicted the females would prefer red males over yellow males if the conspicuous red

100   colouration evolves through female mate choice. On the other hand, male-male interactions

101   select for conspicuous male colouration we predicted that red males would receive less

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102 aggression less conspecific and heterospecific aggression than yellow males in the breeding

103 arena.

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105 Methods and Materials

106 Study Species

107 *Xanthagrion erythoneurum* is a medium-sized damselfly (21-23mm) belonging to the

108 Coenagrionidae family (Zygoptera: Odonata). This species is widely distributed throughout

109 Australia and commonly found in stagnant freshwater reservoirs such as ponds, marshes, lakes

110 and dams. The adult males can be distinguished from other sympatric damselflies by their red

111 face, red thorax, the red colouration of the first and second abdominal segments and the blue

112 bands on the eighth and ninth abdominal segments (Khan and Herberstein, 2019; Theischinger

113 and Hawking, 2016). In the Sydney region, this species starts emerging in September and is seen

114 in flight until June (Khan and Herberstein, 2020). During this whole period, this species remains

115 reproductively active (Khan and Herberstein, 2020).

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117 Field site

118 We collected the damselflies from and carried out experiments at a pond located on the North

119 Ryde campus of Macquarie University, Sydney, Australia (33.772 S, 151.114 E). In this pond,

120 *Xanthagrion erythroneurum* cooccurs with other damselflies including *Ischnura heterosticta,*

121 *Austroagrion watsoni, Austrolestes annulosus, Diplacodes melanopsis* and *Orthetrum*

122 *caledonicum.* The sympatric species *Ischnura heterosticta* and *Austroagrion watsoni,* like

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123   *Xanthagrion erythroneurum,* do not defend a territory but searches for females in the ponds

124   which looks more like a scramble competition for mating. All three species share shoreline

125   vegetations at ponds for perching and mating and submerged vegetations for ovipositing.

126

127   Reflective spectrometry

128   We measured the reflective spectra of the collected males and leaves of the vegetation

129   surrounding the pond to quantify the visual background using a JAZ EL-200 portable

130   spectrophotometer (Ocean Optics, USA) with a PX-2 pulsed light source. We measured all

131   spectra in a dark room by setting the spectrophotometer to a constant boxcar width and

132   integration time settings of 10 and 20 milliseconds respectively, and to average five scans. We

133   measured the reflectance relative to a white standard (Ocean Optics, USA) to standardize the

134   measurements. We first immobilized the damselflies by placing them in a refrigerator at 4^{0}C for

135   five minutes. Then, we set the probe of the spectrophotometer perpendicular to the cuticular

136   surface of the metathorax from a fixed working distance of two millimetres. We took three

137   reflectance spectra of each male and each background leaf from 300 nm to 700 nm and

138   subsequently averaged those three measurements. We processed the reflectance spectra with

139   OceanOptics Spectrasuite software (ver. 1.6.0_11) and eventually binned to one nm wavelength

140   intervals before minor LOESS smoothing ($\alpha = 0.35$). We performed the spectral processing using

141   the package 'pavo' v 2.1 (Maia et al., 2019) in R v 3.5.2 (R core team 2018).

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143 Visual modelling

144 We calculated the chromatic and achromatic contrast of the red and yellow males against their

145 background using the receptor noise model (Vorobyev and Osorio, 1998; Vorobyev et al., 2001).

146 This model calculates the detectability between two colours in just noticeable difference (JND)

147 units where one JND means that the receiver can distinguish between the colours (Vorobyev et

148 al., 2001). The receptor noise model has previously been applied in behavioural studies to predict

149 colour discriminability in various taxa including odonates (Barry et al., 2015; Huang et al., 2014;

150 Khan and Herberstein, 2019).

151 We aimed to determine how the colour and luminescence of the red and yellow males are

152 perceived by the receiver i.e. conspecific and heterospecific damselflies. The visual system of *X.*

153 *erythroneurm* is not known. We however, know that damselflies of the Coenagrionidae family

154 can have either trichromatic or tertachromatic visual system (Henze et al., 2013; Huang et al.,

155 2014). We therefore applied both systems to calculate the chromatic and achromatic contrast of

156 the red and yellow males against their backgrounds (Khan and Herberstein, 2019). For

157 trichromatic visual modelling, we applied the photoreceptor sensitivities of *Ischnura*

158 *heterosticta*, while the photoreceptor sensitivities of *Ischnura elegans* were used for

159 tetrachromatic modelling (Henze et al., 2013; Huang et al., 2014). We used the photoreceptor

160 sensitivities of these two species as they are the closest related species in the phylogenetic tree of

161 our study system whose visual system is known.

162 We calculated the quantum catches of the photoreceptors by following the methods of Vorobyev

163 and Osorio (1998) (Supplementary method S1). We used standard daylight (D65) as the ambient

164 light spectrum. We then log-transformed the quantum catches according to the Weber-Fencher

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165   law (Vorobyev et al., 2001). Finally, we calculated the chromatic and achromatic contrast of red

166   and yellow males against their background as a function of the log-transformed quantum catches

167   weighted by the noise of each photoreceptor (Vorobyev and Osorio, 1998). We performed the

168   visual modelling in R v 3.5.2 (R core team, 2018) using the package pavo v 2.1 (Maia et al.,

169   2019).

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171   Male condition

172   We calculated body length, body mass, lipid and protein content to determine the condition of

173   the males (Castaños et al., 2017). We captured *X. erythroneurum* males from the field using an

174   insect sweep net (Khan, 2015; Khan, 2018) and brought them back to the Behavioural Ecology

175   Laboratory at Macquarie University for morphometric measurements. We took measurements of

176   the damselflies within two hours after collecting them. We weighed the body mass of the live

177   damselflies on a balance (Mettler toledo, accuracy 0. 01 mg). Next, we immobilized the

178   damselflies by cooling them in a refrigerator at 4^0C for five minutes. We then positioned the

179   damselflies laterally and took digital photographs using a Canon 600D camera mounted with

180   Canon EF 55-250 lens. We measured the total body length of the damselflies from the digital

181   photographs using the ImageJ software (Schneider et al., 2012).

182

183   Lipid Quantification

184   We measured the lipid content of the damselflies by the gravimetric method (Barry and Wilder,

185   2013). First, we euthanised the damselflies by placing them in a -30°C freezer for 10 minutes,

186   then we dried the damselflies at 60^0C for 48 hours and then weighed their dried body mass. We

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187   then submerged the dried damselflies in chloroform. After 24 hours, we discarded the

188   chloroform, and replaced it with fresh chloroform for another 24 hours. The chloroform was then

189   discarded and the damselflies were air-dried under a fume hood at room temperature for another

190   24 hours. We further dried the damselflies for another 24 hours at 60^0C and later reweighed the

191   dried damselflies. The lipid content of each damselfly was calculated as the difference between

192   the body mass of the damselfly before and after chloroform extraction.

193

194   Protein extraction and quantification

195   We extracted the soluble protein from the damselflies using 0.1 M NaOH (Sigma-Aldrich) as a

196   lysis buffer. First, we finely ground the dried damselflies with a polypropylene pestle and added

197   0.1 N NaOH (100μl per 1mg of insect weight). We then vortexed the lysate, sonicated it for 30

198   minutes in a water bath, and then heated at 90^0C for 15 minutes. Finally, we centrifuged the

199   lysate at 13000 rpm for 10 minutes, discarded the undigested tissues that precipitated, and

200   collected the clarified lysate from the supernatant.

201   We quantified the protein content in the clarified lysates of the damselflies using Pierce™ BCA

202   protein assay kit (Thermo Fisher Scientific). We used the Bovine Serum Albumin (BSA)

203   supplied with the protein assay kit for preparing the standard solution. We used a linear range of

204   standard BSA protein concentrations from 1.35 mg to 0.05 mg for making the standard

205   absorbance curve. We used 0.1 M NaOH (Sigma-Aldrich) as a diluent for the standard solution

206   preparation, for the damselfly lysates preparation and also for the blank control. We took 25 μL

207   of standard BSA for standard solution preparation and 25 μL damselfly lysates for sample

208   protein quantification and then added 0.1 M NaOH (Sigma-Aldrich) to make the final volume

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209   200 µL. We added the standard and sample solution in triplicates in a in 96 well flat-bottomed

210   plate and incubated at 37 °C for 30 min. We took the absorbance of the incubated plates at 562

211   nm using a FLUOstar OPTIMA microplate reader (BMG Labtech). We quantified the relative

212   protein quantity of the lysates using the standard absorbance curve.

213

214   Female mate choice experiment

215   We experimentally tested if females prefer mating with red or yellow males. The yellow males

216   are sexually immature and unable to mate (Khan and Herberstein, 2019). We, therefore, painted

217   red males with yellow colour to determine the effect of male yellow colour on female mate

218   choice. We performed the female mate choice trials by restraining a female in an insect mating

219   cage (58cm × 32cm × 34cm) with a natural red male and a red male that was painted yellow. We

220   manipulated the thorax of the damselflies by painting yellow over red using non-toxic Tim &

221   Tess^{TM} poster paint (Khan and Herberstein, 2019). We also painted red over the natural red to

222   control for the effect of paintings. We took spectra of the painted damselflies to approximate

223   their natural colour (supplementary figure S1).

224   We placed the mating cage approximately three meters away from the pond — the natural

225   habitat of the damselflies. We recorded the sexual interactions of the damselflies while sitting

226   one meter away from the cages. We counted the number of tandems formed by the control red

227   males and the manipulated yellow males. The tandem is the first step of damselfly mating where

228   a male becomes physically connected to a female by his cerci. A tandem event can dissociate if

229   the female does not cooperate, or it can form a wheel if the female cooperates. When a tandem

230   was formed, we continued the trials until the tandem disassociated or formed a wheel. We

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231   recorded the duration of tandems when disassociated and when forming a wheel. When a wheel

232   was formed, we recorded the duration of the wheel before disassociation.

233   We performed the female mate choice trials on sunny days between 10:00 hrs and 16:00 hrs

234   when mating usually occurs in the field (Khan and Herberstein, 2019). We performed each trial

235   for 30 minutes with two new males and a female; no damselflies were reused. Paint was washed

236   off the damselflies after every trial, and the damselflies were released at the end of the day. The

237   aim of the experiment was to determine female mate choice between the red and yellow males,

238   so in the analysis we included trials where a female choose to form a tandem or wheel with one

239   of the males.

240

241   Male-male interactions experiment

242   We conducted three sets of experiments to determine the effect of male colour on male-male

243   interactions by tethering the experimental males. In the first experiment, we tethered a naturally

244   occurring red male and a naturally occurring yellow male and determined the male-male

245   interactions received by the red and yellow males. To determine whether the incurred

246   interactions are the effect of colour or other developmental changes, we painted a red male

247   yellow and tethered it with a red male and determined any male-male interactions. In the third

248   experiment, we altered the colour of yellow males by painting them red and presented the

249   naturally occurring yellow males with the red-painted yellow males and determined any male-

250   male interactions. If body colour determines the interactions at the pond, we expect the yellow

251   painted red males will receive less aggression than the red males. Similarly, the red-painted

252   yellow males will receive less aggression than yellow males.

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253   We applied a modified damsel-on-a-dowel technique (Fincke, Fargevieille, & Schultz, 2007) to

254   determine the male-male interaction of the red and yellow males in their natural habitat. We

255   glued a live yellow male and a red male 20 cm apart from each other on a dowel using UHU^{TM}

256   glue. The damselflies were glued in perching positions, with their legs attached to the dowel. The

257   dowel was then placed at the edge of the pond. In this pond, *X. erythoneurum* coexists with two

258   heterospecific damselfly species of the Coenagrionidae family: *Ishnura heterosticta and*

259   *Austroagrion watsoni*, with whom they share mating and oviposition sites. We measured the

260   aggressive and non-aggressive responses received by the red and yellow *X. erythoroneurum*

261   males from conspecific males and heterospecific (*Ishnura heterosticta*) males. We observed the

262   responses by sitting approximately one meter away from the dowel, which allowed us to observe

263   the focal damselflies clearly without disturbing regular movements of the approaching

264   damselflies. An approaching damselfly can detect the focal damselfly when it passes within 10

265   cm of the focal damselfly (Fincke, 2015) and can either show aggression or non-aggression when

266   it passes. When an intruder male passed within 10 cm left or right of the focal male without any

267   physical contact, we counted it as a non-aggressive interaction. On the other hand, when the

268   intruder male bit the experimental male, we counted it as an aggressive interaction (Fincke et al.,

269   2007). Finally, if the intruder male tried to form a clasp (grab the focal male and tried to move its

270   cerci to the prothorax of the focal male) or formed a tandem (intruder male physically connected

271   with the focal male), we counted it as a mating attempt.

272   We conducted each trial for 10 minutes by placing the tethered damselflies in different locations

273   around the lake. We used the same pair for three consecutive trials unless the focal damselflies

274   were predated by sympatric odonates after one or two trials. We conducted all our experiments

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275   on sunny days between 10:00 hrs and 1600 hrs when damselfly density and interactions are high

276   (Khan and Herberstein, 2019). We counted aggressive and non-aggressive responses received by

277   the focal males from approaching conspecific and heterospecific males. We manipulated

278   damselfly colour following the same procedure as described in the female mate choice

279   experiment. After finishing the experiment, we unglued the damselflies by hand, washed off the

280   paint, and released them at the end of the day.

281

282   Statistical analyses

283   We applied Shapiro-Wilk tests to determine normality and F-tests to compare the variance of the

284   data. Two-sample t-tests were applied to compare the chromatic and achromatic contrast of the

285   males against their background. We applied Two-sample t-tests to analyse the total length, and

286   Welch Two Sample t-test to analyse body mass, protein content and lipid content of red males

287   and yellow males – Bonferroni corrections were applied to adjust the p-values.

288   We applied Generalized linear models (GLMs) to determine whether females are more likely to

289   form tandems and wheels with red males than yellow painted red males. We fitted GLMs with

290   the numbers of tandems and wheels as a response variable and male colour (red or painted

291   yellow) as covariates. We applied Cox regression models to analyse tandem duration and wheel

292   duration of the red and yellow painted red males. Tandems that transitioned to wheels were

293   analysed separately from tandems that dissociated before forming wheels.

294   To analyse the aggression received by red and yellow males in male-male interaction

295   experiments, we applied generalized linear mixed models (GLMMs) with aggression or non-

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296   aggression as a response variable, focal male (red male and yellow) and intruder male

297   (conspecific male and heterospecific male) as fixed effects and the pair identity as a random

298   effect. For the experiments involving natural red and yellow males and natural red males with a

299   red male painted yellow we applied binomial distribution. On the other hand, we applied a quasi-

300   binomial distribution (to account for the over-dispersion) for the experiment in which a natural

301   yellow male with a yellow male painted red were used as focal male. For each analysis, we used

302   the full model (glmer (attack, passby) ~ focal male*intruder male + (1|id)) by including

303   interactions between fixed effects and pair identity as random effect. All the analyses were

304   conducted in R v 3.5.2 (R core team, 2018) using the 'survival' (Therneau and Lumley, 2019),

305   'lme4' (Bates et al., 2019), and 'MuMIn' (Bartoń, 2019) packages.

306

307   Results

308   Damselfly spectra

309   The reflectance spectra of the yellow males showed peaks between 588 nm and 700 nm whereas

310   the red males showed peaks between 657 nm and 700 nm (Figure 1a). The reflectance spectra of

311   the background showed a Gaussian peak between 551-554 nm (Figure 1a).

312

313   Visual modelling

314   The chromatic and achromatic contrast between the red and yellow males and the background

315   was more than one JND, suggesting that the males are discriminable against their background by

316   both trichromatic (Figure 1b-1c) and tetrachromatic (Figure 1d-1e) visual systems (Figure 1b-

317   1e). The red males showed both higher chromatic contrast (Mann-Whitney U test: $W = 0$, p

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318   <0.0001) and achromatic contrast (Welch two sample t-test: t = -2.07, df = 27.32, p <0.05) than

319   the yellow males in trichromatic damselfly vision (Figure 1b-1c). Similarly, in tetrachromatic

320   damselfly vision, the red males showed higher chromatic contrast (Two sample t-test: t = -11.81,

321   df = 31, p <0.0001) and achromatic contrast (Two sample t-test: t = -2.49, df = 30, p <0.05) than

322   the yellow males (Figure 1d-1e).

323

324   Male condition

325   The red males were longer in total length (Two sample t-test: t = -5.13, df = 75, p <0.0001) and

326   their body mass was heavier (Welch two sample t-test: t = -16.65, df = 70.39, p <0.0001) than the

327   yellow males (Figure 2a-2b). Furthermore, lipid content of the red males was significantly higher

328   (Mann-Whitney U test: W = 21, p <0.0001) than in yellow males (Figure 2c). Similarly, protein

329   content of the red males was significantly higher (Welch two sample t-test: t = -8.69, df = 28.25,

330   p <0.0001) than in yellow males (Figure 2d).

331

332   Female mate choice

333   In total, 60 tandems were formed during the female mate choice experiment. The number of

334   tandem formations did not differ significantly (GLM: χ^2 = 0.46, df = 1, p = 0.49) between the red

335   males (n = 32) and the yellow painted red males (n = 28) (Figure 3a). Over half (51.7%) of the

336   males failed to form a wheel after forming a tandem. The number of males proceeding from

337   tandem to wheel did not differ significantly (GLM: χ^2 = 1.63, df = 1, p = 0.20) between the red

338   males (n = 13) and the yellow painted red males (n = 16) (Figure 3b). When the tandems

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339   dissociated before forming wheels, the tandem duration was not affected by male colour (Cox

340   regression: $\chi2 = 1$, $df = 1$, $p = 1$) (Figure 3c). Similarly, the time to wheel formation did not differ

341   significantly (Cox regression: $\chi2 = 0.62$, $df = 1$, $p = 0.6$) between the red or yellow painted red

342   males (Figure 3d).   Finally, the duration of the wheel before disassociation did not differ

343   significantly (Cox regression: $\chi2 = 0.06$, $df= 1$, $p = 0.06$) between the red males ($n = 13$) and the

344   yellow painted red males ($n = 16$) (Figure 3e).

345

346   Male-male interactions

347   The yellow males received significantly higher aggressive responses than red males from

348   conspecific males (estimate: 4.48 ± 0.38, $z = 11.74$, $p < 0.001$) in the male-male competition

349   experiment when natural red and yellow males were presented to intruder males (Figure 4a).

350   Also, there was a significant interaction between focal males and intruder males (estimate: -1.59

351    $\pm$ 0.67, z = -2.37, p < 0.05) showing that the probability of receiving aggression from

352   heterospecific males was higher in yellow males than red males (Figure 4b; supplementary table

353   2). Similarly, when yellow painted red males were presented with natural red males, yellow

354   painted red males received significantly higher aggression than natural red males from

355   conspecific and heterospecific males (Figure 4c- d; supplementary Table 3-4). Furthermore,

356   when yellow males were painted red, and presented with the natural yellow males, the natural

357   yellow males received higher aggression from conspecific and heterospecific males than the red

358   painted yellow males (Figure 4e-f; supplementary table 5-6).

17

359   Discussion

360   Conspicuous male colouration can evolve through female mate choice, male-male competition

361   for mating, to reduce male-male mating attempts or through a combination of all three (Clutton-

362   Brock, 2007; Sherratt & Forbes, 2001). We investigated the drivers of conspicuous male

363   colouration in *Xanthagrion erythroneurum* damselflies. We found that red males were

364   chromatically and achromatically more consipicuous than yellow males. We further showed that

365   red males were in better nutritional and physiological condition than yellow males. Next, we

366   experimentally tested female preferences for male colouration and found that the females did not

367   prefer red males over yellow males when given a choice. Finally, we tested male-male

368   interactions in the breeding arena and found that yellow males received more aggression than red

369   males from conspecific and heterospecific males.

370   The female mate choice experiments showed that the number of tandems did not differ between

371   red and yellow males. In the cage experiment, females cannot avoid tandem as they cannot fly

372   away from the approaching males. A female, however, can reject a mate by dissociating from

373   the tandem (Khan and Herberstein, 2019). In support of that, we found that the females rejected

374   51.7% of mating attempts in our trials. The rejection rate, however, was not different between

375   the red and the yellow males. A female can further express refusal by delaying the wheel

376   formation, or by dissociating from the wheel quickly before sperm transfer (Khan and

377   Herberstein, 2019). If females preferred red males, we would have expected 1) tandem durations

378   before dissociation are longer for red males, 2) red males attain wheel more quickly, and 3) the

379   wheel durations are longer for red males. We, however, did not find significant differences

380   between the red and the yellow males in any of these choice indicators. Females are unlikely to

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381   detect the coloration of the males as they approach from behind to form a tandem. The females

382   probably use tactile cues and clasping strength rather than colour to estimate male quality

383   (Barnard and Masly, 2018; Barnard et al., 2017). Taken together, our results strongly suggest

384   that female preferences are not selective agents of male colouration. Cryptic female choice,

385   however, could select for male conspicuous colouration, and further studies are required for test

386   this possibly.

387   Conspicuous male colouration can evolve by intrasexual selection if conspicuousness increases

388   male mating success by reducing conspecific aggression in the breeding arena. Thus, we

389   predicted that red males would receive less aggression than yellow males. Our results confirmed

390   this prediction: yellow males (whether natural or painted) received more overall aggression than

391   painted or natural red males. The red males received less aggression probably because the red

392   colour signals male quality and competitive ability, thereby, serving as a status badge to resolve

393   costly disputes without direct physical contact. Our findings are consistent with previous

394   findings suggesting that red colouration is a signal of male condition and dominance, and

395   functions to intimidate rivals in lizards (Healey et al., 2007; Whiting et al., 2006), fishes

396   (Dijkstra et al., 2005), birds (Pryke and Griffith, 2006) and primates (Setchell and Wickings,

397   2005). Furthermore, we showed that red painted yellow males received more aggression than

398   control yellow males supporting the hypothesis that red is inherently intimidating to rivals (Baird

399   et al., 2013; Barlow, 1983; Pryke, 2009; Rowland et al., 1995) even when additional phenotypic

400   information (e.g. size) was available.

401   Interspecific interactions can be a significant evolutionary force to shape traits in sympatric

402   species (Tynkkynen et al., 2004; Tynkkynen et al., 2005). Here, we showed that the natural and

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403 painted red males received less heterospecific aggression than the natural and painted yellow

404 males. The heterospecific aggression can occur due to an interspecific recognition error where

405 males are phenotypically similar or because of male competition for common resources. Our

406 study species, Xanthagrion erythroneurum with a red thorax and *Ischnura heterosticta* with a

407 blue thorax are phenotypically dissimilar, therefore recognition error is probably an unlikely

408 mechanism for the observed interspecific aggression. On the other hand, both species assemble

409 at the pond for breeding, share the same perching sites for mating, foraging, and resting in

410 between mate searching, and also share the same oviposition sites suggesting interspecific

411 competition for breeding resources are a possible mechanism. Our findings support the idea that

412 conspicuous colouration can reduce interspecific aggression (Drury and Grether, 2014) to

413 acquire shared breeding resources (Lipshutz, 2018; Peiman and Robinson, 2010). In breeding

414 ponds where damselflies mate, a large number of conspecific and heterospecific males aggregate

415 and compete for limited mating and oviposition sites (Corbet, 1999). This suggests that, in *X.*

416 *erythroneurum*, and probably also in other damselflies, the conspicuous colouration has evolved

417 to reduce intra- and interspecific male-male interactions.

418 Conspicuous male colouration can also evolve through intrasexual selection to avoid costly

419 male-male tandem attempts (Sherratt, 2001). This anti-harassment aposematic hypothesis has

420 been supported in moor frogs showing that the males attain conspicuous colouration upon

421 reproductive maturity to avoid male-male amplexus formation (Sztatecsny et al., 2012).

422 Similarly, sexually dimorphic abdominal blue bands in non-territorial damselflies reduce

423 intraspecific male-male tandem formation (Beatty et al., 2015; Khan and Herberstein, 2019). In

424 the male-male competition experiment of this study, while presenting the males, we found that

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425   conspecific males formed tandems with 11 out of 20 experimental yellow males, but never with

426   the red males (data not shown). This preliminary result suggests that the conspicuous red

427   colouration might also function to avoid male-male tandem formation in this species. Further

428   behavioural choice experiments to test conspecific male mate choice between the red and yellow

429   morphs are needed to better understand the anti-harassment signal of the red colour.

430

431   Our study combined colour vision modelling with laboratory and behavioural experiments to

432   explain the function of the conspicuous colouration in damselflies with a scramble mating

433   system. We showed that the conspicuous red colour in *X. erythroneurum* damselflies signal male

434   condition. We further demonstrated that the conspicuous colouration is not a target of female

435   mate choice but reduces inter- and intraspecific male aggression in the breeding arena. Our

436   findings suggest that conspicuous male colouration can evolve tto reduce costly male-male

437   interactions. Because our study presents clear intra-sexual advantage of red males, the question

438   of why yellow males persist in the population remains elusive. Further studies are needed to

439   explain if being yellow is a resource limited constrain or it is an adaptation to reduce the foraging

440   and predation risks associated with a red colour.

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443   Figure legends:

444   Figure 1: a) Reflectance spectra (mean ± standard deviation) of the red males ($n = 17$), yellow

445   males ($n = 17$) and background leaves ($n = 30$); b) chromatic and c) achromatic contrast of the

446   red and yellow males against their background in trichromatic damselfly vision; d) chromatic

447   and e) achromatic contrast of the red and yellow males in tetrachromatic damselfly vision.

448   Boxplots depict the median, the 25th and 75th percentile with the whiskers extended to the

449   minimum and maximum data points. Outliers that were > 1.5 times the interquartile range. (*

450   indicates p <0.05).

451   Figure 2: a) Total length of the yellow males ($n = 31$); b) and red males ($n = 46$); b) body mass of

452   the yellow males ($n = 27$) and red males ($n = 46$); c) lipid content of the yellow males ($n = 19$),

453   and red males ($n = 19$), and d) protein content of the red males ($n = 19$) and yellow males ($n = $

454   19). Boxplots depict the median, the 25th and 75th percentile with the whiskers extended to the

455   minimum and maximum data points. Outliers that were > 1.5 times the interquartile range were

456   excluded. (* indicates p <0.0001).

457   Figure 3: a) Number of tandems formed by red males ($n = 32$), and yellow males ($n = 28$); b)

458   percentage of the tandems involving red males ($n = 13$) and yellow males ($n = 16$) that ended in

459   wheel formation; c) duration of red and yellow male tandems that ended in dissociation rather

460   than wheel formation; d) duration of red and yellow male tandems that ended in wheel

461   formation, and e) wheel duration of red and yellow males Boxplots depict the median, the 25th

462   and 75th percentile with the whiskers extended to the minimum and maximum data points.

463   Outliers that were > 1.5 times the interquartile range were excluded.

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464   Figure 4: Aggressions (number of attacks/number of approaches) received by natural red and

465   natural yellow males ($n = 20$) from a) conspecific males and b) heterospecific males; c)

466   aggressions received by natural red males, and yellow painted red males ($n = 20$) from

467   conspecific males and d) heterospecific males; e) aggressions received by natural yellow males

468   and red painted yellow males ($n = 18$) from conspecific males, and f) heterospecific males.

469   Boxplots depict the median, the 25th and 75th percentile with the whiskers extended to the

470   minimum and maximum data points. Dots are data points; outliers that were > 1.5 times the

471   interquartile range were excluded.

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478   Data accessibility: All data will be uploaded in Figshare upon acceptance for publication

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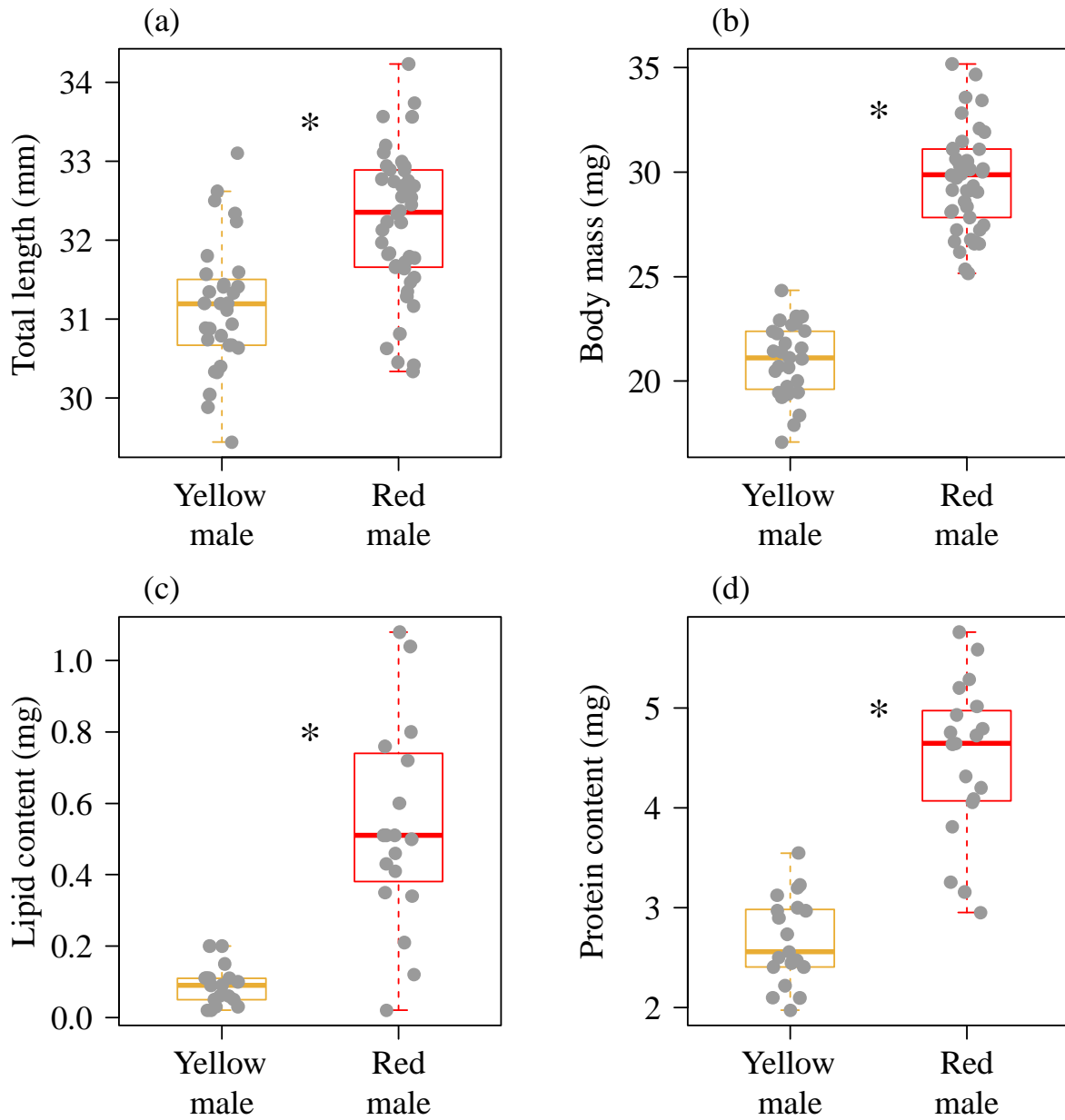638   displacement in the damselfly Calopteryx splendens. *J. Evol. Biol.* **17**, 759–767.

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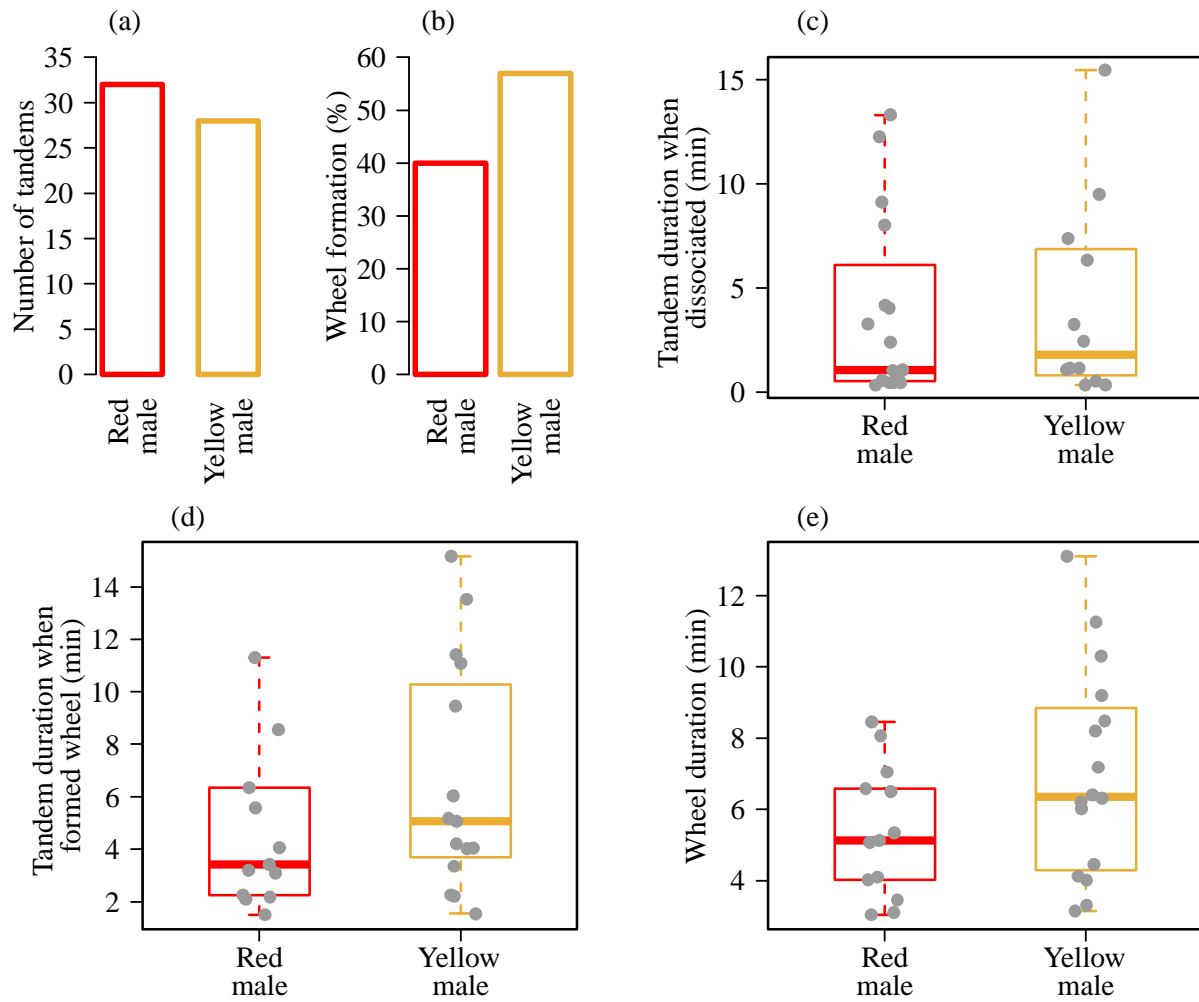
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660   Figure 1

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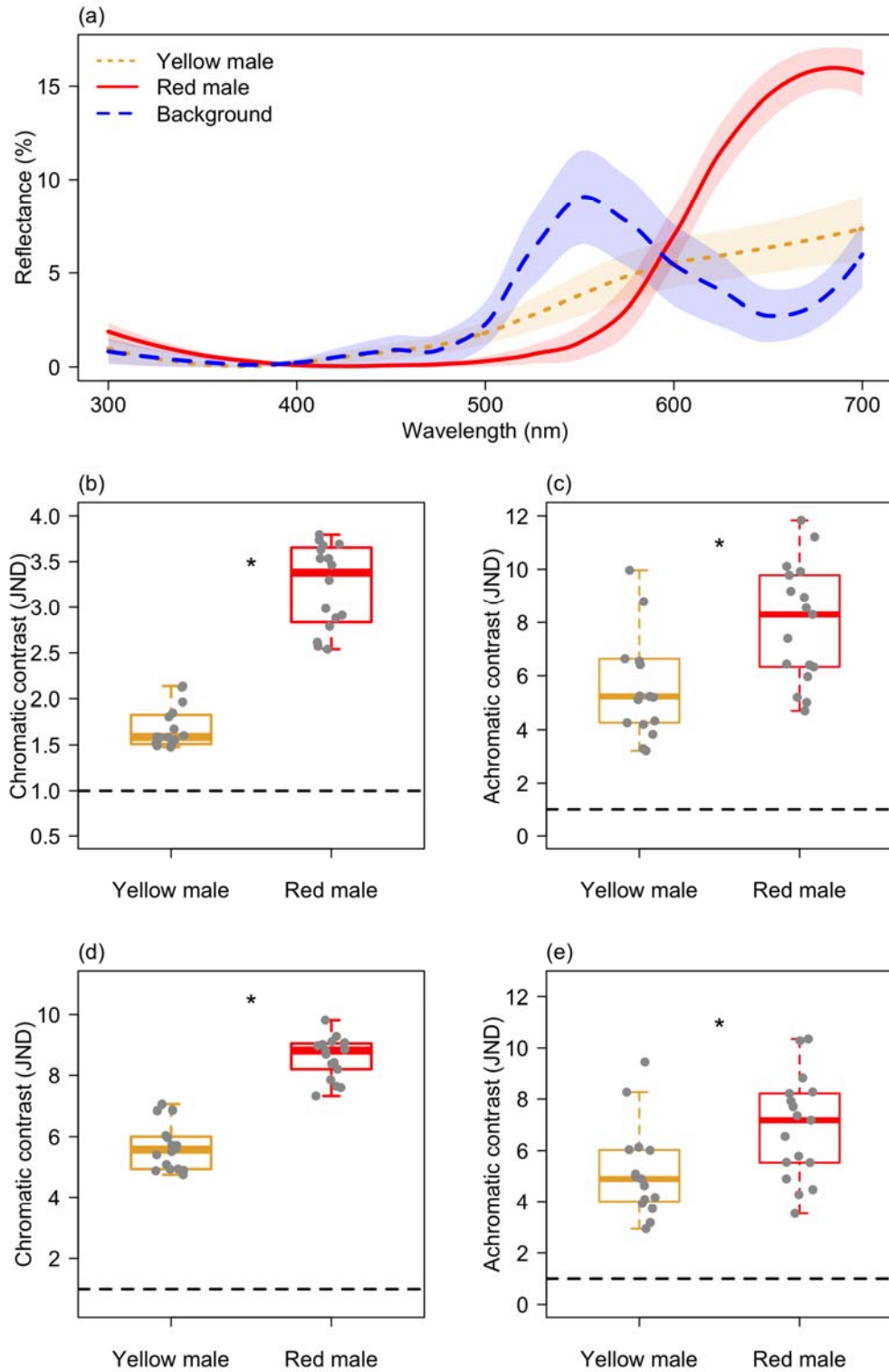
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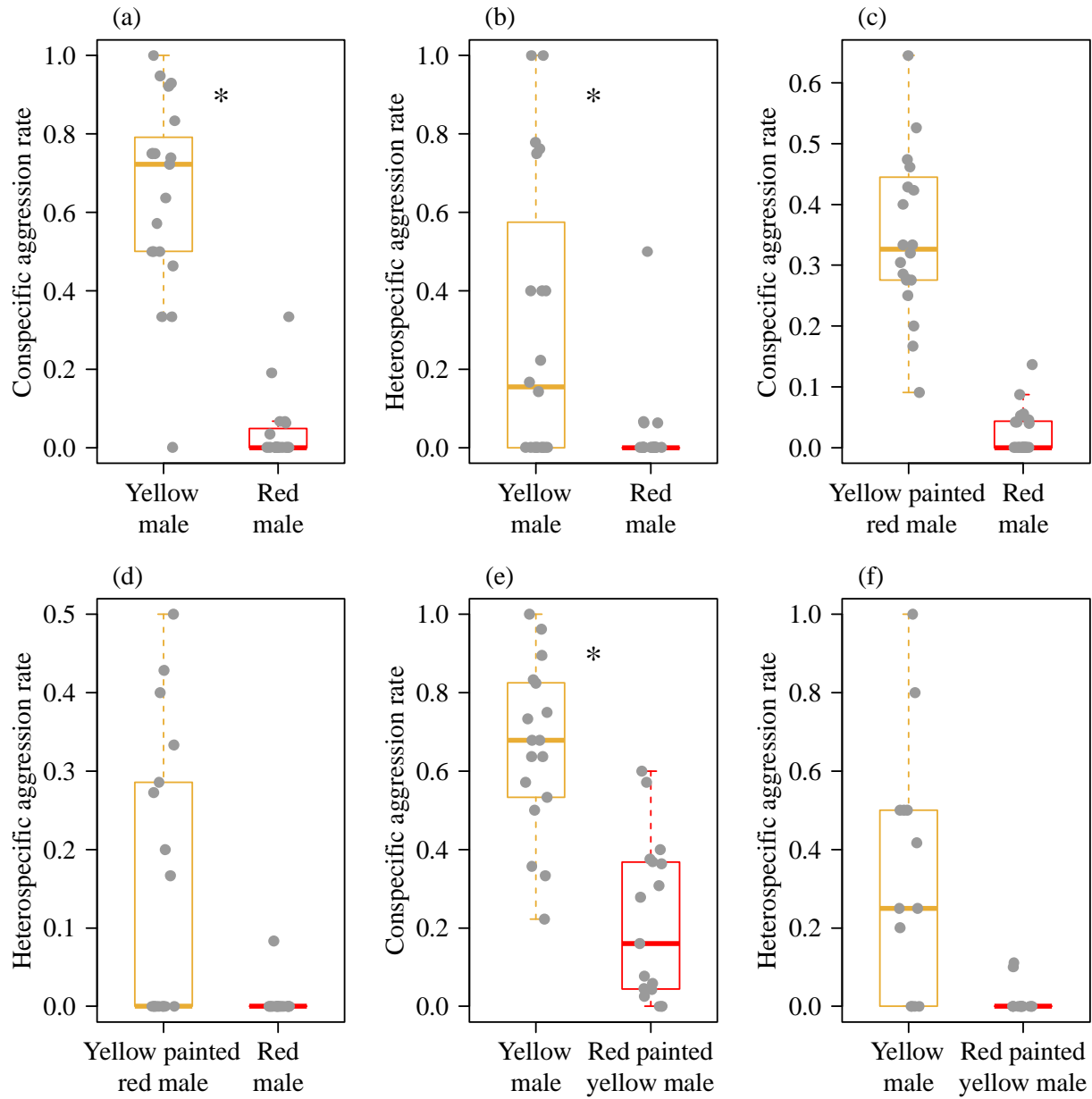667   Figure 3:

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