Functional significance of conspicuous colouration in ontogenetic colour changing damselflies

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Maa, you are so far away…

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38 **Table of contents**

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Summary

Conspicuous animal colouration is predicted to evolve via sexual selection either to increase mating frequency or to reduce unprofitable mating harassment. The selective agents of conspicuous colouration can vary between the sexes and at different developmental stages. The function of conspicuous colouration is well studied in territorial mating systems but poorly understood in non-territorial mating systems. Here, I aim to study the functional significance of males and females conspicuous colouration at different developmental stages in non-territorial damselflies. In ontogenetic colour changing animals, individuals change colour during adulthood but the causes and consequences of conspicuous colouration at different life stages are often unclear. I studied the functions of male conspicuousness in *Xanthagrion erythroneurum* damselflies. In this species, males but not females carry conspicuous blue bands on the terminal abdominal segments and thoracic colouration of males change from yellow to red during ontogenesis. I performed mating experiments with males before and after colour change and showed that yellow males are sexually immature and attain conspicuous red colouration upon sexual maturity. Then, I showed that male conspicuous colouration (blue abdominal bands and red thoracic colouration) do not increase mating success via female mate choice, but reduce male-male mating attempts and male aggression in breeding territories. By reducing male aggression, conspicuous males can persist in breeding territories, ultimately increasing their mating success. I investigated the conspicuous female colouration in *Agriocnemis femina* damselfly, where females change colour from conspicuous red to green upon sexual maturity. I showed that males avoid mating with sexually immature red females and preferred green females that are

larger and carry eggs. The juvenile females signal their sexual unprofitability with conspicuous colouration, thereby reducing sexual harassment in the pre-reproductive stages. In conclusion, my thesis provides evidence for the selective benefits of male and females conspicuous colouration in non-territorial mating systems.

Declaration

I declare that that this thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by co-authors that I have included in my thesis. The content of my thesis is the result of work I have carried out during my higher degree candidature, and does not include work that has been submitted to qualify for the award of any other degree or diploma in any university or institution.

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Introduction

Animal ornaments and armaments, such as horns in antelopes, the long tails of peacocks, giant hind legs in beetles, and conspicuous colours in birds, lizards and insects, are not thought to afford survival benefits, but instead evolve through sexual selection (Balmford, Albon, & Blakeman, 1992; Balmford, Rosser, & Albon, 1992; Darwin, 1871; Emlen, 2008; Parker, 2013; Petrie, Tim, & Carolyn, 1991). In males, conspicuous colouration is thought to evolve via intersexual selection through female preference, or via intra-sexual selection through male-male competition for mating and/or to avoid male-male mating attempts (Clutton-Brock, 2007). Selection via female choice and male-male competition assumes that conspicuous colouration honest signals of male condition (Hill, 1991; Keyser & Hill, 2000), resulting in higher social status, safer territory, better immunity, and better parental caring ability (Albo & Peretti, 2015; Georgiev, Muehlenbein, Prall, Emery Thompson, & Maestripieri, 2015; Kirkpatrick & Barton, 1997; López & José, 2005). In this context, by preferring to mate with a conspicuous rather than dull male, a female may respond to signals of good condition or good genes (Montoya & Torres, 2015; Setchell, 2005; Vásquez & Pfennig, 2007). For example, female house finches (*Carpodacus mexicanus*) prefer males with brighter plumage colouration that signal parental care and genetic quality, whereas female guppies (*Poecilia reticulata*) prefer conspicuous males that signal boldness (Godin & Dugatkin, 1996; Hill, 1991)

Conspicuous male colouration can evolve via male-male competition in systems where males compete for mates or limited breeding resources such as nest sites or oviposition sites (Ahnesjö, Kvarnemo, & Merilaita, 2001; Klug, Lindström, & Kokko, 2010; Morris,

160 Batra, & Ryan, 1992; Wacker & Amundsen, 2014). In this case, conspicuous colouration
161 can signal resource holding potentiality and fighting ability (Ligon & McGraw, 2013;
162 Lim & Li, 2013; Weaver, Koch, & Hill, 2017). For example, the ultraviolet-green
163 iridescence of *Cosmophasis umbratical* spider signals males' resource holding
164 potentiality (Lim & Li, 2013). Likewise, the orange head colouration of the tiger
165 damselfly (*Tigriagrion aurantinigrum*) signals male fighting ability, and males with
166 brighter patches win contests over territories. Male conspicuous colouration thus
167 enhances mating success by reducing male-male competition for mates (Korzan &
168 Fernald, 2007; Setchell & Wickings, 2005).

169 Conspicuous female colouration is thought to evolve due to genetic correlation with the
170 male or via direct sexual selection by males. For example, competition for mates can
171 select for conspicuous female colouration because mating provides either direct or
172 indirect benefits to females (Amundsen, 2000; Rosvall, 2011) or because male
173 availability is limited (Kokko & Mappes, 2005). For example, male two-spotted gobies
174 (*Gobiusculus flavescens*) choose bright yellow orange coloured females in a mating
175 context (Amundsen & Forsgren, 2001; Griggio, Devigili, Hoi, & Pilastro, 2009). On the
176 other hand, bright colour in female pipefish (*Syngnathus typhle*) intimidates competitor
177 females, thereby enhancing the likelihood of securing a mate (Berglund & Rosenqvist,
178 2009).

179 Despite the adaptive significance of conspicuous animal colouration, colour variation
180 persists because of ecological differences, genetic variation or through developmental
181 constraints. For example, *Megalagrion calliphya* damselflies exhibit monomorphic
182 female colouration at higher and lower elevations, and female dichromatism in mid
183 elevations (Cooper, 2010). On the other hand, colour polymorphism in the damselflies

184 *Mnais costalis*, *Ischnura elegans*, *Ischnura senegalensis*, and *Ischnura graellsii* occurs
185 because of genetic variation (Sánchez-Guillén et al., 2018; Tsubaki, 2003). Furthermore,
186 in other odonates, male dichromatism in *Crocothemis servilla, Sympetrum darwinianum*
187 and female dichromatism *Ischnura heterosticta* is the result of ontogenetic colour change
188 (Futahashi, Kurita, Mano, & Fukatsu, 2012; Huang & Reinhard, 2012).

189 Ontogenetic colour change is the irreversible change of animal colouration during its
190 development (Booth, 1990). This developmental colour change occurs in vertebrates i.e.,
191 primates, birds, and lizards, and in invertebrates such as Lepidoptera and Odonata (Booth,
192 1990). Natal coat colour change in primates, irreversible plumage colour change in birds,
193 and differently coloured instars in lepidopteran caterpillars are examples of ontogenetic
194 colour change (Grant, 2007; Hawkins, Hill, & Mercadante, 2012; Treves, 1997). During
195 ontogenesis, some animals shift from dull to conspicuous colouration such as in Long-
196 tailed Manakins (*Chiroxiphia linearis*) where males change colour from dull olive-green
197 to bright red and blue (Doucet, McDonald, Foster, & Clay, 2007). This dull juvenile male
198 colouration functions as status signal thereby reducing adult male aggression, whereas
199 bright adult colour increases mating success via female preference (Doucet, Mennill, &
200 Hill, 2007). Contrary to attaining conspicuousness, some animals become dull as they
201 progress to adulthood. For example, the blue tail of *Acanthodactylus beershebensis*
202 lizards and the blue abdominal bands of *Ischnura elegans* and *Ischnura heterosticta*
203 damselflies faded during development (Hawlena, 2009; Huang & Reinhard, 2012;
204 Willink, Duryea, & Svensson, 2019). Conspicuous tails in juvenile *Acanthodactylus*
205 *beershebensis* lizards direct predator attacks to non-vulnerable body parts (tail), thereby
206 increasing survival (Bateman, Fleming, & Rolek, 2014; Hawlena, Boochnik, Abramsky,
207 & Bouskila, 2006). On the other hand, blue abdominal bands in female damselflies reduce

male mating harassment, and are more conspicuous in juveniles, fading in adults (Willink et al., 2019).

Mating systems are thought to select for conspicuousness in animals. In territorial mating systems, where mating success is largely determined by territory defence, conspicuous colouration is predicted to evolve as an honest signal of male fitness and territory defending capability (Suhonen, Rantala, & Honkavaara, 2008). In non-territorial mating systems, males do not defend a territory and mating success is largely determined by how quickly a male can find and reach females (Darwin, 1871; Herberstein, Painting, & Holwell, 2017). Male sensory traits such as larger antennae and eyes and locomotor traits, such as smaller body size and better flight capability, are predicted to evolve in non-territorial mating system rather than conspicuous colouration (Elgar et al., 2018; Herberstein et al., 2017; Jayaweera & Barry, 2017). Conspicuous colouration in males and females however occurs frequently in non-territorial mating systems but their evolutionary significance is poorly understood.

In my thesis I aim to study the evolutionary significance of conspicuous animal colouration in non-territorial mating systems. My focus is on ontogenetic colour changing animals that alter conspicuousness during development. In my thesis, I studied two non-territorial damselfly species *Xanthagrion erythroneurum,* where I investigated the adaptive significance of male conspicuous colouration and *Agriocnemis femina* where I investigated the function of female conspicuous colouration.

Dragonflies and damselflies as a model system

Dragonflies and damselflies (Odonata) are terrestrial insects, however, they depend on freshwater ecosystems for larval development (Corbet, 1999). After mating, female damselflies lay eggs either directly on water, or deposit the eggs on submerged plants (Corbet, 1999; Theischinger & Hawking, 2016). The larval stage can last from days to years depending on temperature and microhabitat (Cardoso-Leite, Vilardi, Guillermo-Ferreira, & Bispo, 2014; Corbet, 1999). In the tropics, the larval stage is shorter and adult stages can emerge as quickly as 30 days whereas at higher latitudes the larval stage can lasts for 3-5 years (Corbet, 1999). The newly emerged odonates are recognisable by their shiny wings and soft body, which harden within a few hours after emergence (Corbet, 1999). It takes a few days after emergence for juvenile males and females to attain sexual maturity (Corbet, 1999; Hinnekint, 1987). In this developmental transition, some species exhibit ontogenetic habitat shifts and colour change which can occur in males, females or both sexes (Corbet, 1999; Shah & Khan, 2019).

Damselfly mating systems can be broadly divided into two categories: territorial and non-territorial (Corbet, 1999). In territorial mating systems, males defend a territory in the breeding area (Suhonen et al., 2008). Territorial males usually perch on vegetation by waterbodies, and patrol surrounding areas (Watanabe & Taguchi, 1990). If territorial males encounter intruder males in their territory, they show aggression, engage in territorial fights and drive intruder males away from the territory (Suhonen et al., 2008; Watanabe & Taguchi, 1990). Once receptive females visit the breeding arena, males will secure a mating and the female lays her eggs in the water (Corbet, 1999; Suhonen et al., 2008). In non-territorial species, on the other hand, males do not defend a territory, rather they search for females and once they spot a female they engage in mating (Corbet, 1999). In this mating system, a large number of males assemble in a breeding arena (Conrad &

254 Pritchard, 1992). The breeding resources, however, are limited and males compete among
255 themselves to access and persist in the breeding area, to acquire suitable spots for
256 perching, mating and ovipositing (Conrad & Pritchard, 1992; Corbet, 1999).

257 Damselflies have been extensively studied to understand the functions of conspicuous
258 male colouration in the context of sexual selection and speciation (Córdoba-Aguilar,
259 2002; Siva-Jothy, 1999; Svensson & Waller, 2013; Watanabe & Taguchi, 1990). These
260 studies were focused on territorial species in which conspicuous male colouration signals
261 male condition and resource holding capacity thereby increasing mating success via
262 female preference and male-male competition for territory defence (Contreras-Garduño,
263 Buzatto, Serrano-Meneses, Nájera-Cordero, & Córdoba-Aguilar, 2008; Drury & Grether,
264 2014; Siva-Jothy, 1999; Watanabe & Taguchi, 1990). Conspicuous male colouration also
265 occurs frequently in many non-territorial damselflies (Shah & Khan, 2019; Theischinger
266 & Hawking, 2016).

267 In mating systems where males do not maintain territories and females are predicted to
268 have limited choice, conspicuous male colouration is not expected to evolve via female
269 mate choice. Non-territorial male damselflies hover around the pond in search of a mate
270 and upon encountering a female the male approaches from behind and grasps the female
271 from above to form a 'tandem'. Therefore, the females cannot see the colour of the
272 approaching males; thereby making intersexual selection an unlikely mechanism to select
273 for conspicuous male colouration. Sherratt and Forbes (2001) proposed that conspicuous
274 male colouration in non-territorial damselflies evolved as antiharassment aposematic
275 signals to reduce unprofitable male-male mating attempts (Sherratt & Forbes, 2001).
276 Fincke (1997), however, argued that males need females' cooperation for mating,
277 therefore female mate choice can select for bright male colouration in non-territorial

damselflies (Fincke, 1997). Furthermore, males compete for limited breeding resources, such as perching and ovipositing sites, without defending an actual territory. Therefore, male-male competition for breeding resource acquisition and utilization can also select for conspicuous male colouration in non-territorial damselflies. Although some evidence exists for the antiharassment aposematic hypothesis (Beatty, Andrés, & Sherratt, 2015), other mechanisms are yet to be tested.

Damselflies are the focus of studies on sex-limited colour polymorphism (Sánchez-Guillén et al., 2018). Female limited colour polymorphism occurs in many damselflies, therefore offers opportunities to study the evolution of sex-limited polymorphism. Evidence suggests that female limited polymorphism increases female fitness and fecundity by reducing male mating harassment through male mimicry and mismatch with the male mate search image (Takahashi, Kagawa, Svensson, & Kawata, 2014). The male mimicry hypothesis suggests that by mimicking the male colour pattern (andromorphs), females receive less mating harassment as males fail to recognise them as potential mates (Sirot, Brockmann, Marnis, & Muschett, 2003; Van Gossum et al., 2011). Males can, however, recognise multiple female morphs as potential mates based on their social experience and prefer the most abundant morph (Miller & Fincke, 1999). Female polymorphism, therefore, can be maintained by negative frequency-dependent selection (Iserbyt, Bots, Van Gossum, & Sherratt, 2013; Takahashi, Yoshimura, Morita, & Watanabe, 2010).

Female limited dichromatism in damselflies can also result from developmental colour change (Huang & Reinhard, 2012). Although genetically determined female limited colour polymorphisms are well studied (Sánchez-Guillén et al., 2018), developmental dichromatism in females is poorly understood. It is thought that juvenile females reduce

male mating harassment by mimicking male colouration or by signalling sexual immaturity through distinct juvenile colouration (Huang & Reinhard, 2012; Takahashi, Morimoto, & Watanabe, 2012). For example, *Ischnura heterosticta* juvenile females resemble males in colour, thereby reducing mating harassment in the pre-reproductive stages. At the adult stage, however, females change colour to the distinct female phenotype (Huang & Reinhard, 2012).

Juvenile female colouration, however, does not always resemble male colouration and are more conspicuous than adult female colouration in many species (Gering, 2013; Vilela, Samuel Ricioli, Del-Claro, & Guillermo-Ferreira, 2017). Conspicuous colouration in females can function as an identification badge for species recognition (Takahashi & Watanabe, 2011). Furthermore, bright colouration can signal females' fitness and fecundity (Takahashi & Watanabe, 2011; Huang & Reinhard, 2012). Consequently, conspicuous female colouration is predicted to evolve via male mate choice or female-female competition to increase female mating success (Amundsen & Forsgren, 2001; Rosvall, 2011). These mechanisms, however, cannot explain the occurrence of conspicuous colouration in juvenile females as they are sexually immature, and selection should reduce rather than increase male mating attention. Further studies are needed to understand the evolution of conspicuous colouration in juvenile females.

Study system: *Xanthagrion erythroneurum* and *Agriocnemis femina*

Xanthagrion erythroneurum (Coenagrionidae: Zygoptera: Odonata) is a medium-sized (30-33 mm body length) damselfly. This species is distributed in Australia, Fiji, and New Caledonia, and is commonly found in stagnant freshwater bodies such as ponds, creeks,

marshes, and dams (Theischinger & Hawking, 2016). The adult males can be distinguished easily from other sympatric damselflies by their red face, red thorax, red abdominal segments one and two (S1 and S2), and by blue bands on abdominal segments eight and nine (S8 and S9). Adult female colouration is similar to male colouration except they lack the blue bands on the abdominal segments S8 and S9 and the red colouration on S1 and S2. In the Sydney region, this species is seen in flight from September to May. *Xanthagrion erythoneurum* males do not defend territories for mating but compete with other males for breeding resources such as perching, mating and oviposition sites.

Agriocnemis femina is a small damselfly (21-23 mm body length) of the Coenagrionidae family (Zygoptera: Odonata). This species is widely distributed in South-east Asia, and frequently occurs on ponds, lakes, marshes, and agricultural lands (Shah & Khan, 2019). Females *Agriocnemis femina* exhibit ontogenetic colour change, where the juvenile females are conspicuous red and become male-like green upon reproductive maturity. Males thoracic colouration is similar to adult females and do not express developmental colour change. Terminal abdominal appendages of males (S9-S10), on the other hand, change from orange to black during ontogenesis. In my study region in the north eastern region of Bangladesh, this species is seen in flight throughout the year although population frequency peaked between April-July (Khan, 2015; Shah & Khan, 2019).

Thesis aims: The aim of my thesis was to determine the functional significance of conspicuous colouration in non-territorial damselflies. This thesis contains five chapters; each chapter is a self-contained manuscript and formatted according to specific journal requirements.

Chapter one: Male ornaments such as conspicuous colour can evolve via female preferences. Alternatively, in species with limited female choice, showy male colouration can evolve to reduce costly male-male mating interactions, which frequently occurs in high male density mating assemblage (Sherratt & Forbes, 2001). We tested these mechanisms to determine the function of the blue abdominal bands that are present on males *Xanthagrion erythroneurum* damselflies, but not on females. We showed that male blue bands do not increase male mating success but function as an antiharassment aposematic signal to reduce costly male-male mating interactions. This chapter is published in Animal Behaviour. Co-author Marie E. Herberstein contributed to the design of the study, data analysis, and critical revision of the manuscript.

Chapter two: Intrasexual colour morphs can persist in a species due to genetically determined polymorphism or developmental constraints (Sánchez-Guillén et al., 2018; Shah & Khan, 2019). We observed red and yellow coloured males in *Xanthagrion erythroneurum* damselflies. We tested if these colour forms are developmental, and whether they are related to sexual maturity. We showed that *Xanthagrion erythroneurum* males exhibit ontogenetic colour change from yellow to red during ontogenesis. With a range of behavioural experiments, we showed that the colour signals sexual maturity in this species. This chapter is published in Ethology. Co-author Marie E. Herberstein contributed to the design of the study and critical revision of the manuscript.

Chapter three: In non-territorial mating systems, selection is predicted to favour traits that facilitate mate location, such as sensory and locomotor organs (Darwin, 1871; Elgar et al., 2018; Herberstein et al., 2017). But as males rarely engage each other in these mating systems, conspicuous male colouration is not predicted to evolve via male-male

competition. Nevertheless, in non-territorial systems, conspicuous male colouration can evolve via female mate choice if it increases mating success. Ornamental male colouration occurs in many non-territorial damselflies (Shah & Khan, 2019; Theischinger & Hawking, 2016). Here, we tested if conspicuous male colouration evolved via female preference and/or male-male competition in the ontogenetic colour changing damselfly, *Xanthagrion erythroneurum.* We showed that male-male competition over access to the breeding territory selects for conspicuous male colouration in this species. Our study provides evidence for the evolution of conspicuous male colouration via male-male competition for resource utilization in non-territorial animals. The paper has been submitted to Behavioural Ecology. Co-author Marie E. Herberstein contributed to the design of the study and critical revision of the manuscript.

Chapter four: The status signalling hypothesis predicts that juvenile male colouration functions as a submissive signal to adults, thereby reducing intrasexual aggression (Hawkins et al., 2012). In *X. erythroneurum* damselflies, we, however, found that juvenile colouration does not reduce male aggression in the breeding territory (chapter three). In chapter four, we aimed to determine whether juvenile males exhibit behavioural adaptations to reduce costly intrasexual interactions. We showed that juvenile males shift habitat away from the primary breeding area (pond) to adjacent woods. We found that by shifting their habitat, juvenile males reduced costly male-male interactions (male aggression and male mating attempts). The manuscript has been submitted to Biology Letters and is co-authored with Marie E. Herberstein, who contributed to the design of the study, and provided critical revision of the manuscript.

Chapter five: Conspicuous female colouration is predicted to evolve via male preference and/or female-female competition. In species where mating does not provide benefits

such as nuptial gifts or parental care, and where mating is guaranteed because of the high male density, ornamental female colouration is unlikely to evolve. I discovered conspicuous female colouration in *Agriocnemis femina* damselflies, where females can be either conspicuous red or inconspicuous green. I showed that red and green females are developmental forms: all juvenile females are red and change colour to green upon sexual maturity. I showed that the conspicuous red colour in juvenile females signals their sexual immaturity and males avoid mating with red females. I concluded that conspicuous female colouration functions as an antiharassment signal for sexually immature females. I initially observed this species in the field and collected ~10% data before starting PhD and collected rest 90% of the data, performed the analysis and wrote the manuscript during my PhD period. I designed, performed experiments, analysed and wrote the manuscript with feedback from Marie E. Herberstein.

Sexually dimorphic blue bands are intra-sexual aposematic signals in non-territorial damselflies

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Abstract

Sexually dimorphic traits in males are thought to evolve via female preference or male-male competition. Alternatively, in species without overt male displays or female mate choice, dimorphic coloration may function as a warning signal to conspecific males thereby avoiding costly harassment. We aimed to determine the function of sexual dimorphic coloration in the damselfly *Xanthagrion erythroneurum* in which males, but not females, have conspicuous blue bands on the tip of the abdomen. We show that the male blue bands and female black abdomen are chromatically and achromatically discriminable against their natural background. Moreover, the male blue bands and their adjacent abdominal segments generate higher internal contrast than female abdominal segments. We conducted two sets of experiments to test alternative hypotheses that the male blue bands are (1) the target of female mate choice, or (2) an intrasexual aposematic signal to avoid male mating harassment. We hid male blue bands by painting them black and measured female preference between the manipulated and the nonmanipulated (control) males. We found no difference in mating success between the control and manipulated males, thereby rejecting the female preference hypothesis. To test whether the blue bands function as a warning signal, we manipulated the females by painting male-like blue bands on their abdomen and measured the male response to those females relative to control females. Females with artificial blue bands on the terminal abdomen

were mated less frequently than control females. However, when we painted blue bands on the anterior abdominal segments, the males did not discriminate between control and painted females. Our study demonstrates that dimorphic coloration advertises the males' unprofitability as mates to conspecifics thereby reducing intrasexual harassment.

Introduction

Conspicuous male coloration can evolve in animals if it improves attractiveness to females, increases success in male-male competition, or both (Darwin, 1871). In sexually dimorphic coloration, also known as ornamental coloration (Taylor & McGraw, 2013), females prefer to mate with males that display more conspicuous ornaments during courtship if the coloration signals male quality such as physiological condition, body mass (Contreras-Garduño et.al., 2008), body size (Serrano-Meneses, Córdoba-Aguilar, Méndez, Layen, & Székely, 2007), immunity (Córdoba-Aguilar, 2002; Weaver, Santos, Tucker, Wilson, & Hill, 2018), sperm quality (Fukuda & Karino, 2014), better territory-defending capabilities (Córdoba-Aguilar, 2002), or higher social status (Bergman, Ho, & Beehner, 2009). Alternatively, in species in which males do not exhibit courtship displays, male-limited dimorphic coloration can still evolve as an intrasexual signal irrespective of female preferences. In this case, male coloration can signal either male competitive ability, thereby avoiding unnecessary fights (Olsson, 1994), or unprofitability as a mate, reducing unwanted mating encounters from other males (Beatty et al., 2015).

Sexually dimorphic blue bands are commonly found in many damselflies of the Coenagrionidae family. In these damselflies, males neither maintain territories nor perform courtship displays (Corbet, 1999). Mate-searching males hover around the breeding ponds looking for a mating partner, and the scenario resembles a scramble competition among males (Herberstein et al., 2017). After encountering a female, the male approaches from behind and grasps the female from above to form a ‘tandem’. The damselflies' visual acuity and sensitivity, however, is maximized for forward and slightly downward vision (Schröder, Walguarnery, & Butler, 2008); therefore, the females cannot

463 see the colour of the approaching males. Thus, females seem to have limited choice over
464 whether they mate or not, or with which partner, making intersexual selection an unlikely
465 mechanism to drive male-limited colour dimorphism. Sherratt and Forbes (2001)
466 proposed that conspicuous male coloration in these damselflies is not a signal to females,
467 but rather a warning signal of unprofitability to other males, reducing costly mating
468 harassment (hereafter, the antiharassment hypothesis) (Sherratt & Forbes, 2001).
469 Erroneous male-male mating is a common occurrence, whenever a large number of males
470 assemble and compete for mates (Corbet, 1999; Miller, 1987). In this circumstance, male-
471 limited conspicuous coloration can evolve to signal unprofitability as a mate to
472 conspecific males.

473 Fincke (1997), however, argued that females can show mating unwillingness and avoid
474 tandem formation by hiding, flying away, feigning death, curling the abdomen and
475 through a wing raise signal (Fincke, 1997). Moreover, even after a tandem formation, the
476 male needs the female's cooperation to bend her abdomen and form a ‘wheel’ to receive
477 the sperm. Females can show resistance at this stage by delaying wheel formation or even
478 by dissociating from the wheel. Under these circumstances, the evolution and
479 maintenance of sexually dimorphic male ornamental coloration could be the result of
480 female preferences (hereafter, the female preference hypothesis), which is yet to be tested
481 in damselflies.

482 In *Xanthagrion erythroneurum* damselflies, males have two blue bands on the dorsum of
483 abdominal segments 8 and 9 (S8 and S9), whereas these segments are black in females
484 (Fig. 1a and b). Animal coloration can function as an effective visual cue only when it is
485 perceived by the intended receiver (Rowland, 1979). Hence, it is necessary to investigate
486 colours in relation to the receivers' visual systems, which differ between taxa (Kelber &

Osorio, 2010; Kemp et al., 2015). In the present study, signal receivers were conspecific damselflies. We therefore quantified the colour and luminance contrast of a damselfly against its background using the damselfly visual system.

Our main aim was to determine the function of sexually dimorphic blue bands in this damselfly by experimentally testing both the female preference hypothesis and the antiharassment hypothesis (Sherratt & Forbes, 2001). If the male-limited blue bands evolved through female preferences, we predicted that mating success of males with blue bands would be higher than that of males without them. On the other hand, if blue bands function as an intrasexual aposematic signal, the presence of the blue bands will repel approaching males. The best way to experimentally validate this hypothesis is to paint the blue bands on females' abdomens and calculate the mating decisions of the approaching males. We predicted that the presence of the blue bands would repel males, and thus females bearing blue bands would be avoided, even though other female cues are still available to the male for identification.

Materials and methods

Study Species

Xanthagrion erythroneurum, commonly known as the red and blue damselfly, is a medium-sized damselfly (19-21 mm) of the Coenagrionidae family (Zygoptera: Odonata). This species is widely distributed across all Australian states and commonly found in ponds, marshes and dams (Theischinger & Hawking, 2016). The adult male can be easily distinguished from the other Coenagrionidae species by the red colour of the face, thorax and first two abdominal segments, and by the blue bands on abdominal

segments 8 (S8) and 9 (S9) (Fig. 1a) (Theischinger & Hawking, 2016). The females are similar to males except the abdomen is dorsally black and does not have the blue bands (Fig. 1b). In the Sydney region, this species can be seen in flight from September to May and their reproductive season lasts throughout this period (personal observation).

We collected adult male and female *X. erythroneurum* damselflies, using an insect sweep net, from a pond on the North Ryde campus of Macquarie University, NSW, Australia. We did not require permission to collect this damselfly species because it is not protected in Australia and we conducted the studies outside national parks or other protected areas.

Reflectance Spectra

We measured the reflectance spectra of abdominal segments S7 and S8 of the males and the females, with a Jazz Ocean Optics spectrophotometer (Ocean Optics, Largo, FL, U.S.A.). We set the spectrophotometer at an integration time of 20 ms with an average of five successive scans. We used a PX-2 pulse xenon light source and took the measurements relative to a white standard WS-1. We immobilized the damselflies by cooling them in a refrigerator at 4^0C for 5 min before taking the spectra. We focused the light source of the spectrophotometer perpendicular to the cuticular surface of the damselflies and measured spectra from a uniform distance of 2 mm. We tried to minimize the environmental light as much as possible and used a black velvet cloth to block the light from any other sources except the probe. To quantify the background spectrum, we measured spectra of the plant leaves from the pond site where the damselflies usually perched. We measured reflectance spectra of the damselflies and background leaves between 300 nm and 700 nm and averaged from three measurements.

533 Colour analysis

534 The question we wanted to address with these spectral measurements was whether
535 conspecifics can discriminate the male- and female-specific abdominal colours against
536 natural back- grounds, and whether the male abdomen possesses higher internal contrast
537 than the female abdomen. Animals use colour (chromatic) cues and luminance
538 (achromatic) cues to discriminate an object from its background. These cues can be
539 calculated using colour discrimination analyses (Kemp et al., 2015). We used a
540 discriminability index (D) to estimate the chromatic discriminability (Ds) of the dorsal
541 coloration of the eighth and ninth abdominal segments of the damselflies (blue in males
542 and black in females) against the natural background. We calculated Ds using the index
543 proposed by Hastad, Victorsson, and Odeen (2005):

544 $$\mathrm{Ds} = \frac{\overline{\Delta S_D} - \overline{\Delta S_B}}{\sqrt{\overline{\Delta S_B}}},$$

545 where $\overline{\Delta S_D}$ is the average of the chromatic distance of each damselfly spectrum to the
546 measured background spectra and $\overline{\Delta S_B}$ is the average chromatic distance between each
547 background leaf sample (Håstad, Victorsson, & Ödeen, 2005). We used a similar rationale
548 for calculating achromatic discriminability (D_L) based on the quantum catches of the
549 green photoreceptor, since bees and other insects use this photoreceptor to detect
550 achromatic contrast (Giurfa, Vorobyev, Kevan, & Menzel, 1996). The discriminability
551 values (chromatic and achromatic) indicate whether the blue bands of the males or black
552 abdomens of the females are detectable against the natural background: a value above
553 zero indicates the signal is visible. To understand whether the males have higher internal
554 contrast than the females, we calculated the chromatic and achromatic contrast between
555 abdominal segments S7 and S8. We used standard daylight D65 as an ambient light

556 spectrum to calculate chromatic and achromatic contrast. Because it is unclear whether
557 this species has a tri- or a tetrachromatic visual system, we calculated the colour contrast
558 and discriminability for both (see supplementary material for details).

559

560 Female Mate Choice Experiments

561 We manipulated the colour of the damselflies using nontoxic colour paint (Tim and Tess
562 poster paint). We used black paint (105 carbon black) to hide the blue bands on the male
563 abdomen. For the control males we applied the black paint on the dorsal side of abdominal
564 segment S7. We kept one manipulated and one control male with a female in an insect
565 mating cage (58 X 32 cm and 34 cm high). We placed the cage close to a natural lake, in
566 the sunlight and observed their sexual interactions from approximately 1 m. We counted
567 the tandems and wheels formed by the control and the manipulated males. Once a tandem
568 was formed, we always ran the trial until the tandem dissociated or formed a wheel. We
569 recorded the tandem duration and termination event (i.e. wheel formation or dissociation).
570 We also recorded wheel duration when it occurred. Each trial ran for a minimum of 30
571 min to provide enough time for the damselflies to overcome the painting stress and to
572 form a tandem. We performed 150 trials; in 108 of these trials, the female formed a
573 tandem with one of the males and in three trials the males formed a tandem with each
574 other. As male - male tandem formation was very rare in the experimental set-up, and our
575 aim was to determine the female choice between the males, we did not use the male -
576 male tandem data for further analysis. Each of the damselflies was used once in a single
577 trial, then released.

578

579 Male mate choice experiments

580 We manipulated female colour using nontoxic colour paint (Tim and Tess poster paint).
581 We used 90 peacock blue and 105 carbon black paint for colouring blue and black,
582 respectively. We painted two blue bands on females matching the colour and brightness
583 of the male blue bands (Supplementary figure. S1). In two separate experiments, we
584 varied the position of the bands: in the first, the blue bands were applied in the same
585 position as on the male (segments S8 and S9) but in the second experiment we painted
586 the blue bands on segment 4 (S4). To control for the paint, we applied black paint over
587 the natural black patches on the control females.

588 We placed four damselflies (two males, one control female and one manipulated female)
589 into an insect mating cage (58 X 32 cm and 34 cm high) at the edge of a pond and observed
590 their interactions. We used four individuals at a time to reflect the naturally high density
591 of males and females on the breeding ground and to ensure tandem formation during the
592 trial. For experiment 1, we placed two males with a control female and an S8 - S9-
593 modified female in the mating cage, and for experiment 2, we placed two males with a
594 control female and an S4-modified female in the mating cage. We terminated a trial when
595 one of the males formed a tandem with a female or if no tandem was formed within 20
596 min. Since we terminated a trial immediately once a tandem was formed, only one of the
597 males could form a tandem, with either the control female or the manipulated female.
598 Each of the damselflies was used only once in a single trial and then released. We
599 conducted the trials between 1000 and 1600 hours which is when mating usually occurs
600 in the field (M. K. Khan, personal observation). For experiment 1 we conducted 53 trials,
601 with 40 resulting in tandem formation. We conducted 48 trials for experiment 2, also
602 resulting in 40 tandem formations. As our aim was to determine male choice between the

603 control and the manipulated female, we only analysed trials in which a male formed a
604 tandem with one of the females.

605

606 Statistical Analyses

607 We applied the Shapiro Wilk test to determine the normality and an F test to compare the
608 variances. We applied the Mann Whitney U test to compare the internal chromatic and
609 achromatic contrast between the males and females. An exact binomial test was used to
610 test whether females preferred males with or without blue bands. We used Cox regression
611 models to analyse effects of colour morphs on tandem duration and wheel duration in the
612 female mate choice experiments. Tandems ending in wheel formation were analysed
613 separately from the tandems ending in dissociation. For the male mate choice experiment,
614 we applied a chi- square test to test whether males preferred mating with control females
615 or manipulated females. We analysed all the data in R version 3.4.1 (R core team, 2017).
616 We used the 'survival' R package for the Cox regression analysis (Therneau & Lumley,
617 2019).

618

619 Results

620 The reflectance spectra of the male blue abdominal band (S8) peaked between 440 nm
621 and 481 nm, while the female abdominal segment S8 did not show any peaks but a gradual
622 increase with increasing wavelengths (Fig. 1c). As in the males, the blue colour we used
623 to create artificial bands on the female abdomen also showed reflectance peaks in this
624 region (471- 472 nm; Supplementary Fig. S1). The background leaf spectra showed a
625 Gaussian peak between 551 nm and 554 nm.

626

627 Colour Analysis

628 Both male abdominal blue bands and female abdominal colouration were chromatically
629 and achromatically discriminable in the trichromatic (Figure 2a-b) and tetrachromatic
630 (Figure 2e-f) damselfly visual system against the natural background. When analysed
631 with a trichromatic visual model, the males had higher internal chromatic contrast (Mann-
632 Whitney U Test: W = 279, N_{male} = 15, N_{female} = 19, $P < 0.001$) and achromatic contrast
633 (Mann-Whitney U Test: W = 285, N_{male} = 15, N_{female} = 19, $P < 0.001$) than the females
634 (Figure 2c-d). Similarly, in tetrachromatic damselfly vision, the males had higher internal
635 chromatic contrast (Mann-Whitney U Test: W = 285, N_{male} = 15, N_{female} = 19, $P < 0.001$)
636 and achromatic contrast (Mann-Whitney U Test: W = 285, N_{male} = 15, N_{female} = 19, P
637 <0.001) than the females (Figure 2g-h).

638

639 Female mate choice

640 The females did not show a preference (Exact binomial test: $P = 0.631$) between the
641 control males (i.e. with blue bands, $N = 51$) or the manipulated males (i.e. without blue
642 bands, $N = 57$) for the tandem formation (Figure 3a). Only 42 out 108 (38%) pairs formed
643 a wheel after tandem formation. The frequency of males that formed wheels after tandem
644 formations did not differ significantly (Exact binomial test: $P = 0.631$) between the
645 control males (45.1%) and the manipulated males (33.3%) (Figures 3b). There was no
646 significant difference in tandem duration of control and manipulated males, either when
647 the tandems dissociated rather than forming wheels (Cox regression analysis, LRR: $\chi 2_1 =$
648 0.672, $P = 0.7$) (Figure 3c), or when wheels were formed (Cox regression analysis, LRR:

649 $\chi 2_1$= 0.403, *P* = 0.4) (Figure 3d). Finally, the wheel duration did not differ significantly
650 between control males and manipulated males (Cox regression analysis, LRR: $\chi 2_1$=
651 0.403, *P* = 0.4) (Figure 3e).

652

653 Male mate choice

654 When females carried blue bands on abdominal segments S8 and S9 (similar to male
655 colouration) males were significantly less likely to form tandems (Chi-square test: $\chi 2_1$ =
656 9.8, *P* = 0.001) compared with control females (Figure 4a). However, when the females
657 carried the blue bands on abdominal segment S4, the males were equally likely to form
658 tandems with the manipulated and control females (Chi-square test: $\chi 2_1$ = 0.8, *P* = 0.371)
659 (Figure 4b).

660

661 Discussion

662 In this study we aimed to determine the function of the male- limited conspicuous blue
663 bands of *X. erythroneurum* damselflies. Such dimorphic male coloration can evolve
664 through female choice, male-male competition or costly male-male mating interactions.
665 Here we tested two of these proposed mechanisms: (1) do females prefer males with blue
666 abdominal bands or (2) do blue abdominal bands deter erroneous mating attempts by
667 conspecific males? We found no effect of the blue bands on male mating success but
668 females with artificial blue bands received fewer mating attempts by conspecific males.
669 Thus, we have shown that dimorphic blue bands in this species function as a warning
670 signal to avoid costly mating harassment from conspecific males.

If the male blue bands are sexually selected ornamental coloration, we predicted that female preference would lead to males with blue bands having greater mating success than males without blue bands. We found, however, no effect of blue bands on male tandem formation or wheel formation. We think that the male's approach behaviour is responsible for the observed lack of female preference for blue bands. As the males approach the females from behind and grab them from above during tandem formation (Corbet, 1999), the females cannot immediately detect the male's coloration and therefore cannot use it to reject an approaching male. One might argue that in a cage experiment a female cannot avoid an approaching male and the outcome observed could be due to restricted movement. While this may be true for the first stage of mating interactions, females can still reject males and dissociate from the tandem or can delay the wheel formation. This is supported by our results where more than half of the tandems dissociated rather than forming a wheel. Nevertheless, the female's cooperation in forming and maintaining the wheel did not differ between the control and manipulated males, suggesting that females did not reject males based on the presence of the sexually dimorphic blue bands.

In contrast, sexually dimorphic conspicuous coloration has been shown to increase male mating success through female preference in many other taxa, including birds (Wells, Safran, & Dale, 2016), lizards (Lisboa, Bajer, Pessoa, Huber, & Costa, 2017) and insects (Rutowski & Rajyaguru, 2013). Similarly, in nonterritorial damselflies, it has been hypothesized that the females might prefer males with conspicuous dimorphic coloration (Fincke, 1997); this hypothesis, however, has not been tested previously. To the best of our knowledge, our study provides the first experimental evidence in damselflies that females do not prefer male colour ornaments. We conclude that male-limited dimorphic

coloration in this, and perhaps in other damselflies, is unlikely to evolve through female preferences.

Rather than function as an intersexual signal, we believe the male blue bands function as an intrasexual signal, as females with blue bands in the same position as males were less attractive than control females. There are two possible mechanistic interpretations for our results: either males failed to recognize manipulated females as potential mates or the blue bands acted as a warning signal to conspecific males. We argue that the males had sufficient cues available to identify females. Odonates use tactile and visual cues for mate recognition (Winfrey & Fincke, 2017) and female abdomen colour is the most important (Gorb, 1998). Additionally, males also use body size (Pezalla, 1979), abdomen shape (Gorb, 1998; Ubukata, 1983), flight pattern (Ubukata, 1983), female display (Gorb, 1992; Utzeri, 1988) thorax coloration and pattern (Miller & Fincke, 1999; Xu, Cerreta, Schultz, & Fincke, 2014) and chemical cues (Frati, Piersanti, Conti, Rebora, & Salerno, 2015) for mate recognition. Gorb (1998) even showed that males can recognize a female based on isolated female body parts (thorax, head). Moreover, the adult females in this species are mono- morphic, and less conspicuous than males, thereby further reducing the probability of recognition error. Hence, males could still identify the manipulated females as potential mates based on their overall coloration and phenotype.

Therefore, the most likely explanation of our results is that the added blue bands on the female's abdomen repelled males and may thus function as a warning or aposematic signal, possibly indicating an unprofitable mating partner. An aposematic colour pattern functions best when the signal generates high contrast against the background and when the pattern possesses high internal contrast (Endler, Krebs, & Davies, 1991; Stevens & Ruxton, 2012). In *X. erythroneurum* males, the two blue bands are separated by black

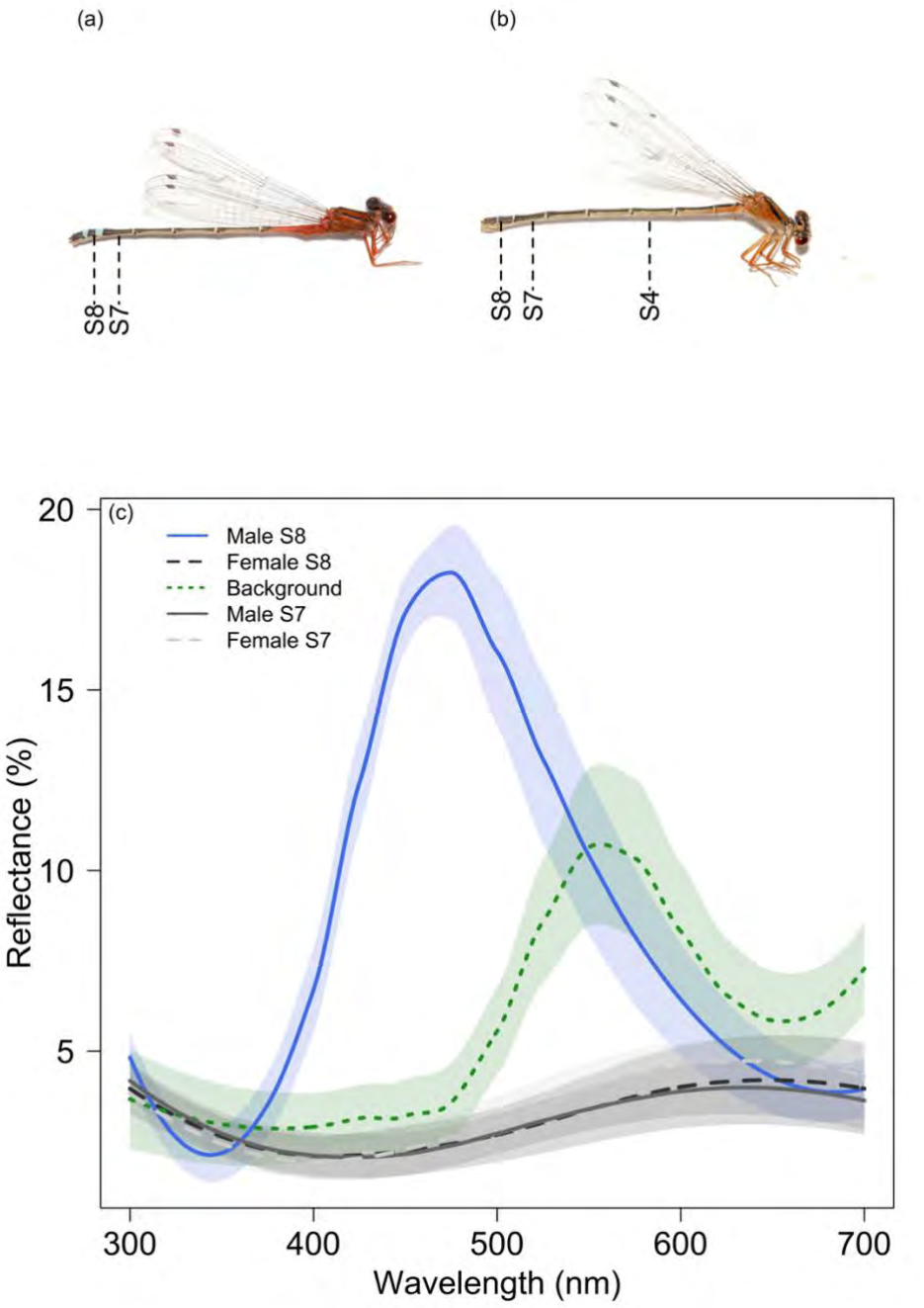
719 abdominal coloration, which is conspicuous against the background and generates high
720 internal contrast (Fig. 2c, d, e, f). The combination of black with a bright colour such as
721 red, yellow or blue is considered a classic aposematic signal (Cott, 1940). Furthermore,
722 aposematic signals function most effectively when presented optimally to the receiver.
723 Therefore, aposematic coloration is often restricted to specific body parts that maximize
724 its presentation. For example, in unpalatable poison dart frogs and unpalatable butterflies,
725 the aposematic coloration is located on the dorsum and the upper wings, respectively,
726 thereby maximizing its presentation to predators (Dreher, Cummings, & Pröhl, 2015;
727 Joshi, Prakash, & Kunte, 2017; Maan & Cummings, 2012; Su, Lim, & Kunte, 2015).
728 Similarly, the painted blue bands on the female's abdomen were effective at repelling
729 males only when present on the dorsal side of the terminal segments. As males approach
730 from behind and grab females from above during tandem formation, the distal end of the
731 dorsal abdomen maximizes advertisement of the aposematic bands to the approaching
732 males. These dorsal blue bands in the terminal segments also function effectively during
733 threat display where a male raises its tail to threaten conspecific males (Utzeri, 1988).

734 The term ‘aposematism’ was originally applied to conspicuous coloration that displays
735 unprofitability (Poulton, 1890) and only later used exclusively to describe predator-prey
736 interaction where bright, vivid coloration of a prey signals their unpalatability to
737 predators. However, aposematic signals are not restricted to interspecific communication,
738 nor are their functions limited to predation avoidance. For example, in *Battus philenor*
739 butterflies, the colour pattern of the larva is an intraspecific aposematic signal that repels
740 conspecific females from ovipositing on the same leaves, thereby reducing intraspecific
741 competition (Papaj & Newsom, 2005). In damselflies, Sherratt and Forbes (2001) applied
742 the concept of aposematism in a sexual context and suggested the term ‘antisexual

743 aposematism' to explain the reduction in sexual harassment through conspicuous
744 coloration. Their hypothesis was later supported in *Nehalennia irene* damselflies, in
745 which abdominal blue coloration on males repels conspecific males (Beatty et al., 2015).
746 Our study further supports this hypothesis as males avoided mating with females bearing
747 the warning signal even when other female-specific cues were present.

748 We conclude that the male-limited blue bands in *X. erythroneurum* damselflies are
749 intrasexual aposematic signals that are likely to reduce costs (time, energy, predation and
750 lost mating opportunities) associated with male-male mating attempts, for both males
751 (Bowcock, Brown, & Shine, 2009; Gering, 2017; Papaj & Newsom, 2005; Rehberg-
752 Besler, Mennill, & Doucet, 2015; Sztatecsny et al., 2012). Even beyond damselflies,
753 conspicuous blue nuptial coloration of male moor frogs, *Rana arvalis*, reduces male-male
754 mating attempts (amplexus formation) during scramble mate search (Sztatecsny et al.,
755 2012). Similarly, males of the Neotropical yellow toad, *Incilius luetkenii*, develop
756 conspicuous yellow colours during the breeding period that reduce the male-male
757 amplexus formation rate (Rehberg-Besler et al., 2015). These studies suggest that
758 conspicuous dimorphic coloration can evolve to reduce intrasexual mating harassment,
759 especially in species in which large numbers of males assemble in a breeding territory to
760 compete for mates.

761



762

763 Figure 1: Photograph of (a) a male and (b) a female *X. erythroneurum;* (c) aggregated
764 reflectance spectra (mean ± SD) of the male S7 (*N*= 15) and S8 (*N* = 35) abdominal
765 segments, female S7 (*N*= 19) and S8 (*N* = 33) abdominal segments, and the background
766 plant leaves (*N* = 25)

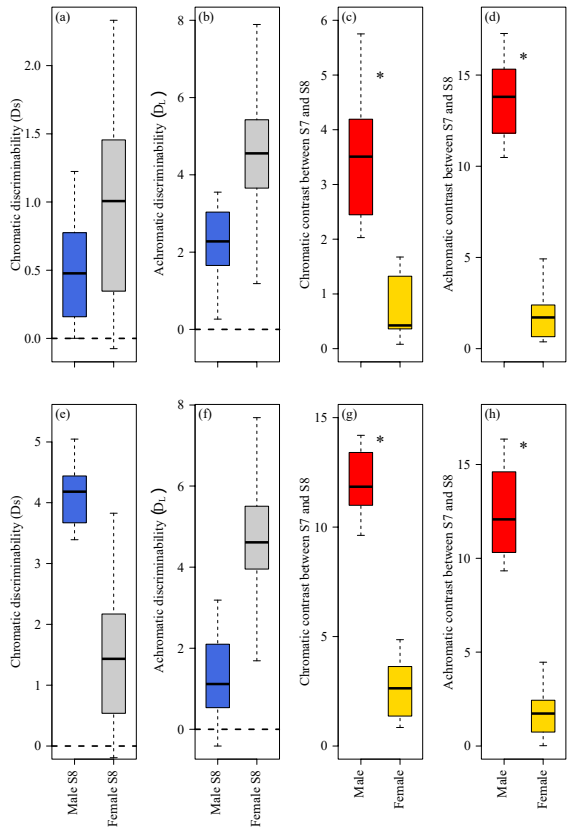
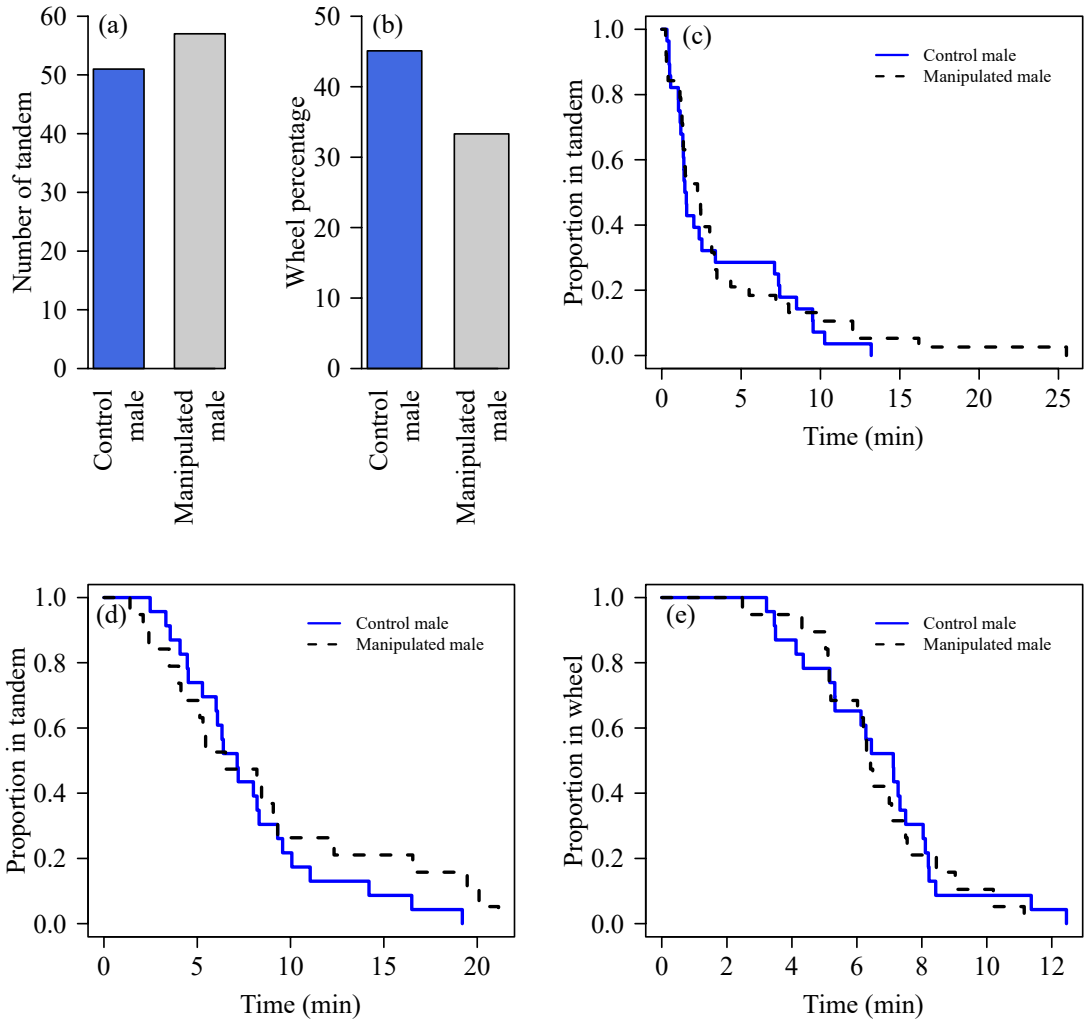
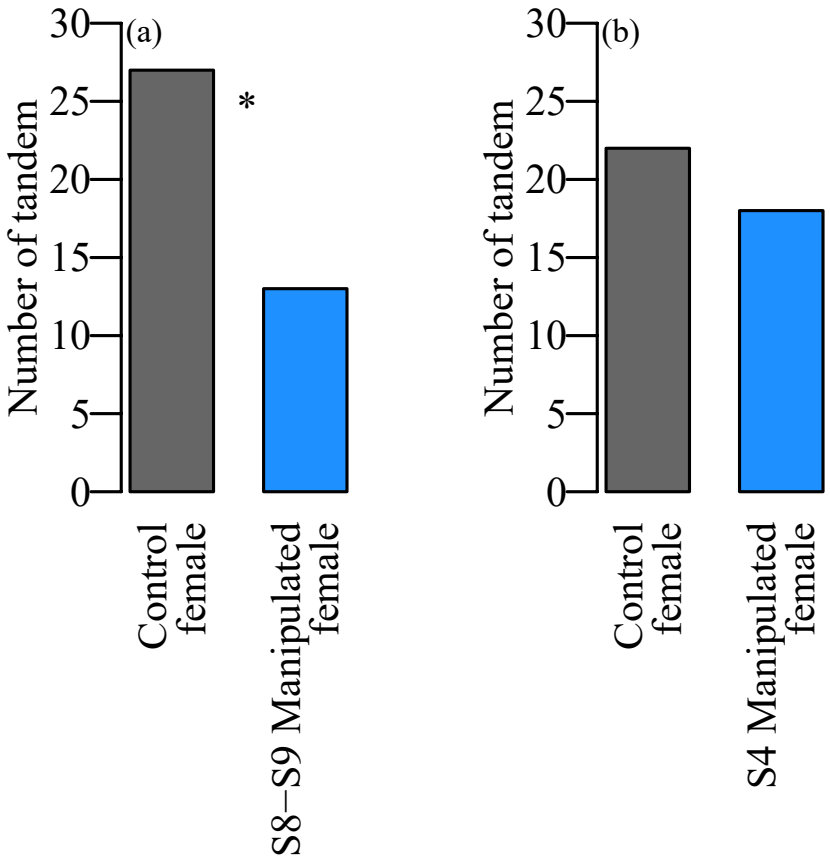


Figure 2: (a) chromatic discriminability (Ds) and (b) achromatic discriminability (D_L) of male and female abdominal (S8) colouration against the natural background in the trichromatic damselfly visual system. (c) chromatic and (d) achromatic contrast between abdominal segment S7 and S8 of male and females in the trichromatic visual system. (e) chromatic discriminability (Ds) and (f) achromatic discriminability (D_L) of male and female S8 segments against the natural background in the tetrachromatic damselfly visual system. (g) chromatic and (h) achromatic contrast between abdominal segments S7 and S8 of males and females in the tetrachromatic visual system. The internal line in the boxes represents the median, and the upper and lower edges of the boxes represent 75th and 25th percentile, respectively. The whiskers extend to the minimum and maximum data points, but exclude outliers which are beyond 1.5 times of the interquartile range. Asterisks (* P <0.001).



780

781 Figure 3: (a) number of tandems formed by the control (with blue at the abdominal
782 segments S8 and S9) ($N = 51$) and the manipulated males (blue bands obscured with black
783 paint) ($N = 58$); (b) percentage of the tandems that formed wheels by control males
784 (45.09%) and manipulated males (33.3%); (c) Proportion of males in tandem that
785 dissociated, over time (control males, $N = 28$; manipulated males, $N = 38$); (d) Proportion
786 of males in tandem that ended in wheel formation, over time (control males, $N = 23$,
787 manipulated males, $N = 19$); (e) Proportion of the control males ($N = 23$) and manipulated
788 males ($N = 19$) in wheels over time.



789

790 Figure 4: (a) the number of control females and S8 and S9 manipulated females (blue
791 bands on segments S8 and S9) ($N = 40$, * $P < 0.05$) and (b) the number of control females
792 and S4 manipulated females (blue bands on segment S4) recorded in mating pairs during
793 the male mate choice experiment ($N = 40$).

794

795 **Supplementary material**

796

797 Supplementary method: Visual modeling

798 We applied the receptor noise model (Vorobyev, Osorio, Bennett, Marshall, & Cuthill,
799 1998; Vorobyev & Osorio, 1998) to analyse how the male and female abdominal
800 coloration will appear to conspecifics. Damselflies can have trichromatic (Huang, Chiou,
801 Marshall, & Reinhard, 2014) or tetrachromatic (Henze, Lind, Kohler, & Kelber, 2013;
802 Outomuro, Söderquist, Johansson, Ödeen, & Nordström, 2017) visual systems. As we do
803 not know the visual system of *X. erythroneurum*, we used both trichromatic and
804 tetrachromatic systems of damselflies from the same family that are the closest related
805 species in the phylogenetic tree. For trichromatic visual modelling we applied the visual
806 system of *Ischnura heterosticta* (Huang et al., 2014) and for the tetrachromatic modelling
807 we used the visual system of *Ischnura elegans* (Henze et al., 2013). We ran the visual
808 modelling in pavo v. 1.3.1 (Maia, Eliason, Bitton, Doucet, & Shawkey, 2013)
809 implemented in R v. 3.4.1 (2017).

810 First, we calculated the quantum catch (Qi) for each photoreceptor i as follows-

811 $$\mathrm{Qi} = \int_{300}^{700} S(\lambda) I(\lambda) Ri\,(\lambda) d\lambda,$$

812 Where λ is the wavelength, $S(\lambda)$ is the reflectance spectra of damselfly integument or
813 each background leaf, $I(\lambda)$ is the light spectrum entering the eye, and $\mathrm{Ri}(\lambda)$ is the
814 spectrum sensitivity of the photoreceptor i. We used a standard daylight spectrum (D65)
815 as $I(\lambda)$ (Wyszecki & Stiles, 1982)

816 Then, we calculated the noise of each class photoreceptor (e_i) as,

817 $$e_i = \frac{\bar{\omega}}{\sqrt{n_i}}.$$

818 Where ω is the Weber fraction assigned to each receptor and n_i is the relative density of the
819 receptor class i. We applied a Weber fraction of 0.12, which was successfully used in damselfly
820 visual modeling (Schultz & Fincke, 2013). The proportion of the photoreceptor of damselflies are
821 not known. So, we applied receptor density of another trichromat, the honey bee (1:0.471:4.412)
822 (Defrize, Théry, & Casas, 2010).

823 Finally, we calculated the chromatic contrast (ΔS) between the damselfly spectra and the
824 background using the equation:

825 $$\Delta S = \sqrt{\frac{{e_1}^2(\Delta f_3 - \Delta f_2)^2 + {e_2}^2(\Delta f_3 - \Delta f_1)^2 + {e_2}^2\,(\Delta f_1 - \Delta f_2)^2}{(e_1 e_2)^2 + (e_1 e_3)^2 + (e_2 e_3)^2}}$$

826 Where Δf_i is the log of quantum catches for receptor i between damselfly and the
827 background.

828 Also, to calculate the achromatic contrast we used green photoreceptor like honeybee
829 visual system and the achromatic contrast (ΔL) was calculated as:

830 $$\Delta L = \frac{\Delta f_i}{e_i}$$

831 For the tetrachromatic damselfly visual system we used photoreceptor sensitivities 370
832 nm, 440 nm, 540 nm, 600 nm (Henze et al., 2013) photoreceptor density 2:2.5:2.5:1
833 (Armett-Kibel & Meinertzhagen, 1983) and a Weber fraction 0.12 (Schultz & Fincke,
834 2013). We calculated the chromatic contrast (ΔS) using the equation:

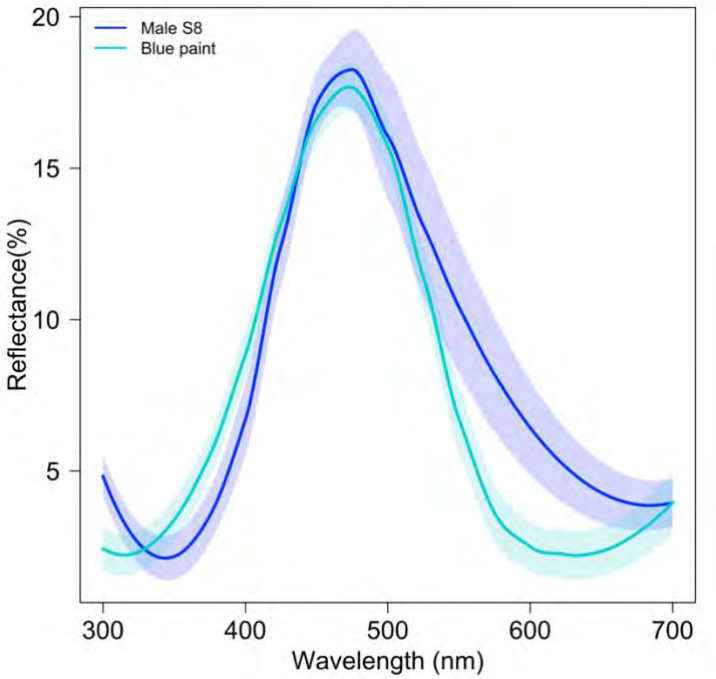
835

836 $$\Delta S = \sqrt{\frac{e_1 e_2{}^2(\Delta f_4 - \Delta f_3)^2 + e_1 e_3{}^2(\Delta f_4 - \Delta f_2)^2 + e_1 e_4{}^2(\Delta f_3 - \Delta f_2)^2 + + e_2 e_3{}^2(\Delta f_4 - \Delta f_1)^2 + e_2 e_4{}^2(\Delta f_3 - \Delta f_1)^2 + e_3 e_4{}^2(\Delta f_2 - \Delta f_1)^2}{(e_1 e_2 e_3)^2 + (e_1 e_2 e_4)^2 + (e_1 e_3 e_4)^2 + (e_2 e_3 e_4)^2}}$$

837 The achromatic contrast for the tetrachromatic visual system was calculated using the
838 same equation for trichromatic visual system.

839

840 Supplementary figure



841

842 Figure S1: Aggregated reflectance spectra of the male blue band at abdominals segment
843 S8 ($N = 35$) and painted blue bands at the female abdominal segments ($N = 8$).

844 Acknowledgments

845 We thank two anonymous referees, Ken Cheng and Jim Mclean for their constructive
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848 the data, and wrote the manuscript. MEH contributed to the design of the study, data
849 analysis and critical revision of the manuscript.

850 Data accessibility

851 The data are deposited in Github repository:
852 https://github.com/KhanKawsar/BlueFunction

Ontogenetic colour change signals sexual maturity in a non-territorial damselfly

Md Kawsar Khan, Marie E. Herberstein

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Abstract

Conspicuous colouration increases male reproductive success through female preferences and/or male-male competition. Despite the advantages of conspicuous colouration, inconspicuous male morphs can exist simultaneously in a population due to genetic diversity, condition dependence, or developmental constraints. We are interested in explaining the male dichromatism in *Xanthagrion erythroneurum* damselflies. We reared these damselflies in outdoor insectaries under natural conditions and showed that this species undergoes ontogenetic colour changes. The younger males are yellow and change colour to red six to seven days after their emergence. We took red and yellow male reflectance spectra and found that red males are brighter than yellow males. Next, we aimed to determine whether ontogenetic colour change signals sexual maturity with field observations and laboratory experiments. Our field observational data showed that red males are in higher abundance in the breeding territory, and they have a higher mating frequency than yellow males. We confirmed these field observations by enclosing a red and a yellow male with two females and found that yellow males do not mate in presence of red males. To determine whether colour change signals sexual maturity, we measured mating success of males before and after colour changes by enclosing a single male at different age (day 3-day 7) and colour (yellow, intermediate and red) with a single female in a mating cage. Males did not mate when yellow but the same male mated after it

876 changed colour to red, suggesting the ontogenetic colour change signals sexual maturity
877 in this species. Our study shows that male dichromatism can be age-dependent and
878 ontogenetic colour change can signal age and sexual readiness in non-territorial insects.
879

Introduction

Across different taxa, females prefer mating with conspicuous males because conspicuousness is associated with better immunity (Faivre, Grégoire, Préault, Cézilly, & Sorci, 2003), higher social status (Bergman et al., 2009), and a larger territory (Vilela, Tosta, Rodrigues, Del-Claro, & Guillermo-Ferreira, 2017), all of which contribute to fitness. Moreover, competitor males avoid fighting with conspicuous males, leaving conspicuous males with larger territories and better access to breeding grounds (Serrano-Meneses et al., 2007). Hence, conspicuousness increases male mating success through female preferences as well as male-male competition. Conspicuous, however, can be costly in terms of colour production and predation. Many animals acquire colour pigments from their diet, therefore they cannot attain conspicuous colouration until they obtain enough resources (Hill, & McGraw, 2006; Taylor, Clark, & McGraw, 2011). Additionally, conspicuous colouration increases visibility to predators, which makes conspicuous males vulnerable to predation (Husak, Macedonia, Fox, & Sauceda, 2006). Conspicuousness also increases visibility of predators to their prey, thereby decreasing foraging success (Grether & Grey, 1996). Many animals mitigate these costs by changing colour. For example, developmental colour change, or ontogenetic colour change, can reduce the costs of sexually selected colouration by expressing dull colouration in the juvenile or pre-reproductive stage and attaining conspicuous colouration upon sexual maturity.

Ontogenetic colour change is an irreversible colour change during the development of an organism. The developmental colour forms may signal age (Nicolaus et al., 2007), fitness (Beeching & Pike, 2010), fecundity (Takahashi & Watanabe, 2011) and/or sexual readiness (Huang & Reinhard, 2012; Wilson, Heinsohn, & Wood, 2006). In cases where

developmental stages signal sexual maturity, juvenile males are often dull coloured and subordinate to conspicuous dominant males. The status signal hypothesis suggests that juvenile males receive less aggression by signalling their subordination, thereby increasing their survival during early life stages (Beauchamp, 2003; Karubian, Sillett, & Webster, 2008). However, dull colouration is not always associated with sexual immaturity. Due to resource constrains, attaining conspicuous colouration can be delayed in a male (Hooper, Tsubaki, & Siva-Jothy, 1999; Ruell et al., 2013). In such cases, inconspicuous but sexually mature males may exist in a population and may deploy alternative strategies to secure mates compared to conspicuous males. Conspicuous males are often territorial whereas the inconspicuous ones are non-territorial (Contreras-Garduño et al., 2008; Watanabe & Taguchi, 1990), remain on the periphery of the territorial males and sneak upon females as they enter the territory (Córdoba-Aguilar & Cordero-Rivera, 2005; Watanabe & Taguchi, 1990). Alternatively, these males may gain access to females if they deceive the conspicuous males, who may misclassify the duller coloured males as either sexually immature or as females (Hawkins et al., 2012).

Intrasexual colour variation is a common phenomenon in odonates either due to genetic variation or because of developmental constraints (Corbet 1999). In odonates, genetic and ontogenetic colour variation can occur in both sexes, or it can be limited to either males or females (Huang & Reinhard, 2012; Sanmartín-Villar, Zhang, & Cordero-Rivera, 2017; Willink, Duryea, Wheat, & Svensson, 2019). Genetically determined female polychromatism occurs in sexually mature and immature in *Ischnura senegalensis, Ischnura elegans*, and *Ischnura genei* damselflies (Sanmartín-Villar & Cordero-Rivera, 2016; Takahashi & Watanabe, 2010; Willink et al., 2019). Likewise, sexually mature male dichromatism (conspicuous orange-winged males and inconspicuous clear-winged

928 males) occurs in *Mnais costalis* damselflies (Tsubaki, 2003). Alternatively, male
929 dichromatism can occur as a result of ontogenetic colour change in territorial and non-
930 territorial damselflies. In territorial odonates, such as *Crocothemis servilia,*
931 *Sympetrum darwinianum* and *Sympetrum frequens*, ontogenetic colour change generates
932 male dichromatism where pre-reproductive juvenile males are inconspicuous yellow and
933 attain conspicuous red colouration upon sexual maturity (Futahashi et al., 2012). Similar
934 developmental colour changes have also been documented in non-territorial species
935 (Henze, Lind, Wilts, & Kelber, 2019; Hinnekint, 1987). Mating frequencies in the field
936 and an indirect correlation between colour change and spermatogenesis suggest that
937 colour change signals sexual maturity in non-territorial damselflies (Hinnekint, 1987).
938 Sexual maturity of colour changing males at different developmental stages could be
939 tested with direct mating experiments, however these tests are yet to be performed.
940 Here, we describe male dichromatism in a non-territorial Australian damselfly,
941 *Xanthagrion erythroneurum*, in which males can be either dull yellow or conspicuous
942 red. We aimed to determine if male dichromatism is age-dependent. We reared freshly
943 emerged damselflies in their natural habitat and found that males changed colour six to
944 seven days after emergence. We measured conspicuousness of yellow and red males by
945 taking reflectance spectra. Finally, with field mating observations and more directly with
946 controlled mating experiments, we tested whether male colour change is a signal of sexual
947 maturity. We predicted that only red males would mate in the field and in the mating
948 experiments.
949
950 Methods and Materials
951 Study species and field site

Xanthagrion erythoneurum (Coenagrionidae: Zygoptera: Odonata) is a non-territorial damselfly. This species is widely distributed in Australia and commonly found in stagnant freshwater bodies such as ponds, creeks, marshes and dams. The adult males can be distinguished easily from other sympatric damselflies by their red face, red thorax, red abdominal segments one and two, and by blue bands on abdominal segments eight and nine (Theischinger & Hawking, 2016). This species is seen in flight from September to April in the Sydney region and its reproductive season lasts throughout the whole of this period (Khan & Herberstein, 2019a).

We carried out field observations at a pond located on the North Ryde campus of Macquarie University, Sydney, Australia (33.772 S, 151.114 E). The shoreline vegetation of the lake serves as a perching and mating spot for the damselflies. The study species *Xanthagrion erythroneurum* coexists with *I. heterosticta, Austroagrion watsoni, Austrolestes annulosus, Diplacodes melanopsis,* and *Orthetrum caledonicum* in this pond (M. K. Khan, personal observation). We surveyed the field and collected data on sunny and partially sunny days from September 2016 to March 2017 and September 2017 to March 2018.

Rearing of *X. erythoneurum* males

We set up a mating cage in the Macquarie University Fauna Park over an artificial pond. We collected mature males and females from the field in early Autumn (March-April) and placed them in the mating cage (58cm × 32cm × 34cm). After mating, the females laid eggs on the water. The following spring and summer, damselflies emerged from the larvae. We collected freshly emerged individuals (N = 40), which were identified by their shiny wings and immature thoracic colouration. We retained the immature males in a

976 rearing cage made of fine mesh (58cm × 32cm × 34cm; 10 males per cage) and reared
977 them from 5 to 25 December 2017 in the insectaries – large outdoor laboratories enclosed
978 by fine fences – situated in the Fauna Park of Macquarie University. To provide substrate
979 where damselflies could perch, we placed small plants inside the cages. We placed the
980 cages over an artificial small pond inside the insectaries so that the damselflies obtained
981 enough moisture. One corner of the cage was dipped in water so that the damselflies could
982 access water for bathing and drinking. We provided cultured *Drosophila* regularly as a
983 food source for the damselflies. The damselflies received the natural light cycle
984 (approximately 14 hours daylight per 24 hours). The diurnal temperature fluctuated from
985 18 - 28^{0}C during the rearing period. We monitored the damselflies every day, inspecting
986 for colour changes. Using a permanent marker, we marked the wings of each damselfly
987 with a unique code, and housed them until colour change occurred.
988
989 Reflective spectrometry

990 We measured the reflective spectra of the damselflies (17 yellow males and 17 red males)
991 using a JAZ EL-200 portable spectrophotometer (boxcar width = 10, integration time =
992 20 ms, scans to average = 5) (Ocean Optics, USA) with a PX-2 pulsed light source. To
993 standardize the measurements, we measured the reflectance relative to a white standard
994 (Ocean Optics, USA). We immobilized the damselflies by placing them in a refrigerator
995 at 4°C for five minutes before taking the spectra. We focused the probe of the
996 spectrophotometer perpendicular to the cuticular surface of the mesothorax from a fixed
997 distance of 2 mm. We used a black velvet cloth to block any other possible light sources
998 apart from the probe. We took three reflectance spectra of each damselfly from 300nm to
999 700nm and subsequently averaged those three measurements. We processed the

reflectance spectra with OceanOptics Spectrasuite software (ver. 1.6.0_11) and binned data to 1 nm wavelength intervals. We calculated the peak wavelength, total brightness, yellow chroma (relative contribution of 550nm-625nm to the total brightness) and red chroma (relative contribution of 605nm-700nm to the total brightness) of the males using the R package pavo v 2.0 (Maia, Gruson, Endler, & White, 2019) implemented in R v 3.5.2 (R core team, 2018).

Occurrence and mating frequencies of the males

We calculated occurrence and mating frequencies of red and yellow males in the field by applying a mark recapture method. We calculated the mating frequency from 10:00-16:00 hours when the abundance of damselflies is high and mating usually occurs in the field (Khan & Herberstein, 2019a). We walked slowly around the shoreline and bushes besides the pond, capturing any damselflies we saw (Khan, 2015). We calculated the frequency of yellow and red males from the numbers of captured males. We marked the wings of captured damselflies to avoid recounting individuals. Mating frequencies were counted by observing occurrences of males and females in a tandem or copulation wheel in the field. For each mating pair, we noted whether a yellow or red male formed the mating pair.

Mating experiment condition

We performed the mating experiments in an insect mating cage (58cm × 32cm × 34cm). We placed the mating cage approximately three meters from the pond, in their natural habitat and measured their sexual interactions from a one-meter distance. We performed the mating trials on sunny or partially sunny days from 10:00 hr to 16:00 hr when mating

usually occurs in the field. We performed each trial for 30 minutes with new individuals. We calculated a successful trial when a male formed a tandem with a female. We performed further analyses based on trials where a male formed a tandem with a female.

Male mating experiments

We conducted two sets of experiments to determine whether yellow males can mate. In the first experiment, we collected red and yellow male damselflies from the field. We placed one red and one yellow male with two females in the mating cage. We conducted 25 trials, each time with new males and females. We recorded the number of tandems formed by the red males and the yellow males.

For the second experiment, we collected the newly emerged males from the field and reared them until they changed colour from yellow to red. We placed different-aged males (from 3-7 days) with a female in the mating cage and observed their reproductive activities. Each male was used twice in this experiment, once on either day 3, day 4, day 5 or day 6 and then again at day 7. We conducted a total of 80 trials; 40 trials with day 3 to day 6 males (10 trials for each day), and 40 trials with the same males at day 7. All the day 3 - day 5 males were yellow, seven day 6 males were intermediate coloured whereas three day 6 males were red, and all day 7 males were red in colour. We counted the number of tandems formed by males of each colour and at each developmental day. We used 80 females for this experiment, each trial with a new female.

Statistical analyses

We applied the F test and Levene test to determine normality and homogeneity of variance of the data. We applied Mann–Whitney U tests when data were not normally distributed, and two sample t-tests when data were normally distributed and variances were equal between the compared groups. We used chi-square tests to compare the

differences between observed and expected mating success of red and yellow males. We conducted all analyses in R v 3.5.2 (R core team, 2018).

Results

Ontogenetic colour change

All of the juvenile males changed colour from pale yellow to bright red (Figure 1a-b). Under the applied experimental rearing conditions, the colour changed six or seven days after emergence. Among the 40 reared individuals, 16 males changed colour after six days, while 24 males changed colour after seven days (Figure 1c).

Reflectance spectra

The reflectance spectra of both yellow and red males showed a peak between 588 nm and 700 nm. There was no significant difference in peak wavelength (Mann Whitney U test, $W = 180.5$, $p = 0.218$) between the red and yellow males (Figure 2a). Total brightness of the red males was significantly higher (Two sample t-test, $t = -7.723$, $df = 32$, $p < 0.001$) than the yellow males (Figure 2b). The chroma of the yellow males was significantly higher (Mann Whitney U test, $W= 279$, $p < 0.001$) than the red males in the 550-625 nm wavelength range (Figure 2c). At the same time, the chroma of the red males was higher (Two sample t-test, $t = -8.812$, $df = 32$, $p < 0.001$) than the yellow males in between 605-700 nm wavelength (Figure 2d). This chromatic difference explains the yellow and red appearance of the males to the human visual system.

Mating frequency in the natural population and in mating experiments

We counted 408 male individuals in the field. Among them, only 9.56 % were yellow, whereas the rest (90.44 %) were red. We observed 102 mating pairs in the field. Under

field conditions, red males (N = 101) had higher mating success ($\chi_2 = 8.78$, df = 1, $p < 0.01$) than yellow males (N = 1). In the cage experiment, when we placed a red male and a yellow male with two females, red males had higher mating success ($\chi_2 = 40$, df = 1, $p < 0.001$) than yellow males. Out of 25 experimental males, 20 red males mated but yellow males did not mate, even once the red male was engaged in a tandem and unable to compete for the second female in the cage. When we placed one male with one female in the cage, day 3 - day 5 yellow males did not mate, even in the absence of a red male (Figure 3). Four out of 10 of the day 6 males mated (2/7 intermediate males and 2/3 red males) (Figure 3). Thirty-four out of 40 day seven red males mated with the females (Figure 3).

Discussion:

In the present study, we described ontogenetic colour changes in *Xanthagrion erythroneurum* damselflies. The younger males are yellow and change colour to red six to seven days after emergence. We showed that red males are brighter than the yellow males. Red males occur more frequently than yellow males in the breeding pond, and have higher mating success than the yellow males. Furthermore, the follow-up mating experiments showed red and intermediate males, but not yellow males, mated with females, thereby suggesting that colour change signals sexual maturity in males.

The higher mating success of red males in the field can occur due to two reasons: 1) the yellow males are sexually mature but they cannot mate because they are unable to access the breeding territory due to the red males' aggression, or 2) the yellow males are sexually immature. In many damselflies, more conspicuous males are in better physical condition

(Contreras-Garduño et al., 2008; Fitzstephens & Getty, 2000), hence in territorial damselflies, conspicuous males maintain territories and secure higher mating success (Watanabe & Taguchi, 1990). Similarly, in non-territorial damselflies, males compete to access breeding territory and breeding resources such as oviposition sites (Corbet, 1999; Herberstein et al., 2017). Access to breeding territory, breeding resources and potential mates largely determines mating success in non-territorial mating systems (Debuse, Addison, & Reynolds, 2003; Shuster & Wade, 2003). In support of that, we found that red males comprised 90% of the total male population in the pond, and they had a higher mating frequency than the yellow males.

To establish whether lower mating frequency of the yellow males is due to their lower occurrence in the breeding pond or because they are sexually immature, we placed a red and a yellow male with two females so that both males had equal mating opportunities. If the yellow males were sexually mature, we predicted they would mate under these conditions. Moreover, if yellow males could mate but were unable to compete with the red males, we predicted that they would sneak a mate after the red male commenced mating. Our results showed that only the red male mated under these conditions, which suggests that the yellow males are sexually immature. However, one might argue that either the yellow males do not mate in the presence of red males, or that females reject yellow males when red males are present. To eliminate these causes, we placed a single yellow male with a single female in a mating cage. Our results showed that none of the yellow males mated, however, the same male mated after it changed colour to red. We therefore concluded that yellow males are not capable of mating and they attain sexual maturity after their colour changes.

Signalling sexual maturity through change in colour has been reported in many taxa including insects, amphibians, reptiles, and birds (Bell & Zamudio, 2012; Chan, Stuart-Fox, & Jessop, 2009; Corbet, 1999; Griggio et al., 2009). *Ischnura heterosticta*, *Ischnura genei, Ischnura elegans* and *Ischnura senegalensis* females change colour during ontogenesis; juvenile females carry fewer eggs and mate less frequently than adult females (Hinnekint, 1987; Huang & Reinhard, 2012; Sanmartín-Villar & Cordero-Rivera, 2016; Takahashi & Watanabe, 2011). In *Ischnura elegans*, the males also change colour from green to blue, which correlates with spermatogenesis (M. J. Henze et al., 2019; Hinnekint, 1987). Outside the odonates, both male and female wood frogs (*Rana sylvatica*) become more conspicuous upon sexual maturity, and remain conspicuous throughout the breeding season (Lambert, Carlson, Smylie, & Swierk, 2017). In king penguins (*Aptenodytus patagonicus*), beak and head colour changes, which signal sexual maturity and also indicate social status (Nicolaus et al., 2007).

The yellow to red shift during ontogenesis that we observed in *X. erythroneurum* is not uncommon in odonates. Similar colour changes are seen in *Crocothemis servilia, Sympetrum darwinianum*, and *Sympetrum frequens* dragonflies (Futahashi et al., 2012). The ommochrome pigments stored in the epidermal chromotophores are the colour producing agent in these dragonflies. When they change colour from yellow to red they reduce the epidermal ommochrome pigments (Futahashi et al., 2012). Xanthommatin and dihydroxanthommatin were detected in the chromatophore in *X. erythroneurum* damselflies (Veron, O'Farrell, & Dixon, 1974). Further studies, however, are needed to understand if the same mechanism underlies colour change in this species.

Xanthagrion erythroneurum males change colour from yellow to red at day six or seven after emergence. This developmental period, which varies in different species and

1142 geographical locations, is required for attaining physical fitness, behavioural
1143 modification, and gonadal development in damselflies (Corbet, 1999). The gonads of the
1144 newly emerged males are undeveloped and often do not contain spermatozoa (Midttun,
1145 1974; Pajunen, 1962). Spermatogenesis occurs gradually during ontogenesis (Jacobs,
1146 1955) and active sperm exists in about a week-old male in *Lestes sponsa* (Uéda, 1989).
1147 In I*schnura elegans,* on the other hand, the newly emerged male contains active sperm,
1148 although the volume increases significantly during ontogenesis (Corbet, 1999). The
1149 initiation of spermatogenesis or the presence of active sperm, however do not always
1150 indicate sexual maturity (Uéda, 1989). In *Lestes sponsa*, active sperm can be found in
1151 dull coloured males, however sexual maturity is not achieved until the sperm volume
1152 reaches a threshold that is attained at colour maturation (Uéda, 1989). The precise
1153 interaction between sperm maturation, sperm volume and colour change in
1154 *X. erythroneurum* is currently unknown, but an obvious next step.
1155 Conspicuous colouration plays a vital role in the reproductive success of a male. Here, we
1156 described ontogenetic colour change in *X. erythroneurum* damselflies, which results in
1157 male dichromatism. Based on our field observations and behavioural experiments, we
1158 showed that ontogenetic colour change signals age and sexual readiness in non-territorial
1159 damselflies. In the immature stage, males are unable to mate, which may be due to a lack
1160 of sperm in their testes, a prediction that still requires confirmation. Our findings raise
1161 the tantalizing questions, why ontogenetic colour change occurs in non-territorial
1162 damselflies. We propose the following mechanisms to explain the existence of male
1163 dichromatism in non-territorial damselflies. After emerging, the males signal their sexual
1164 immaturity with a duller yellow colour. This dull colour is associated with habitat
1165 segregation away from the mating arena (personal observation), which we predict,

1166 reduces aggressive interactions from mature males. After attaining sexual maturity and
1167 bright red colouration, the conspicuous males take up residence in the mating arena, and
1168 compete to access females for mating. Future work is needed to experimentally test the
1169 proposed mechanism of developmental male dichromatism in a non-territorial mating
1170 system.

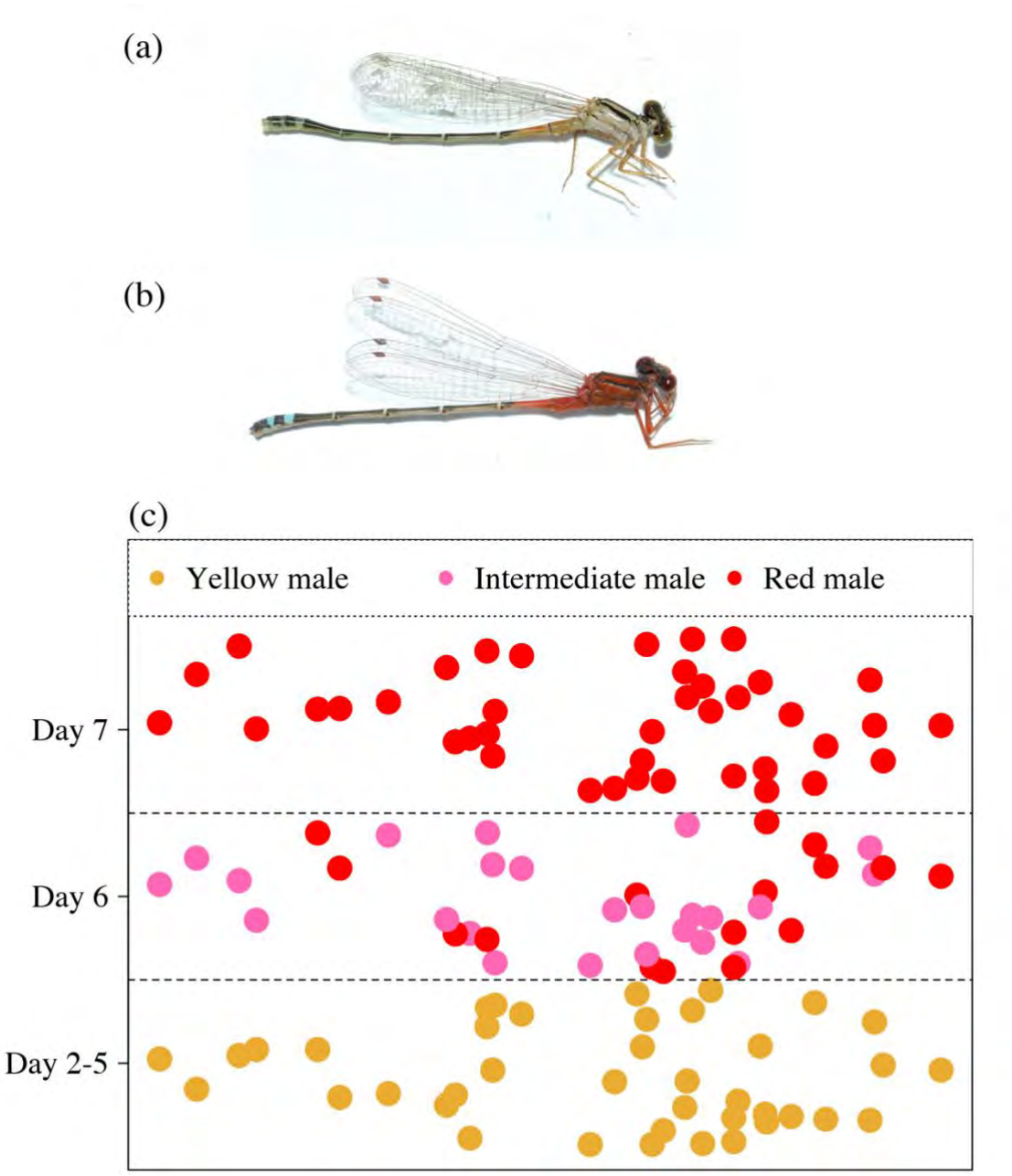

Figure 1: (a) photographs of a yellow and (b) a red male. (c) Each circle represents a male damselfly at a different developmental day. Colour of the circle indicates the colour of a damselfly at a particular developmental day.

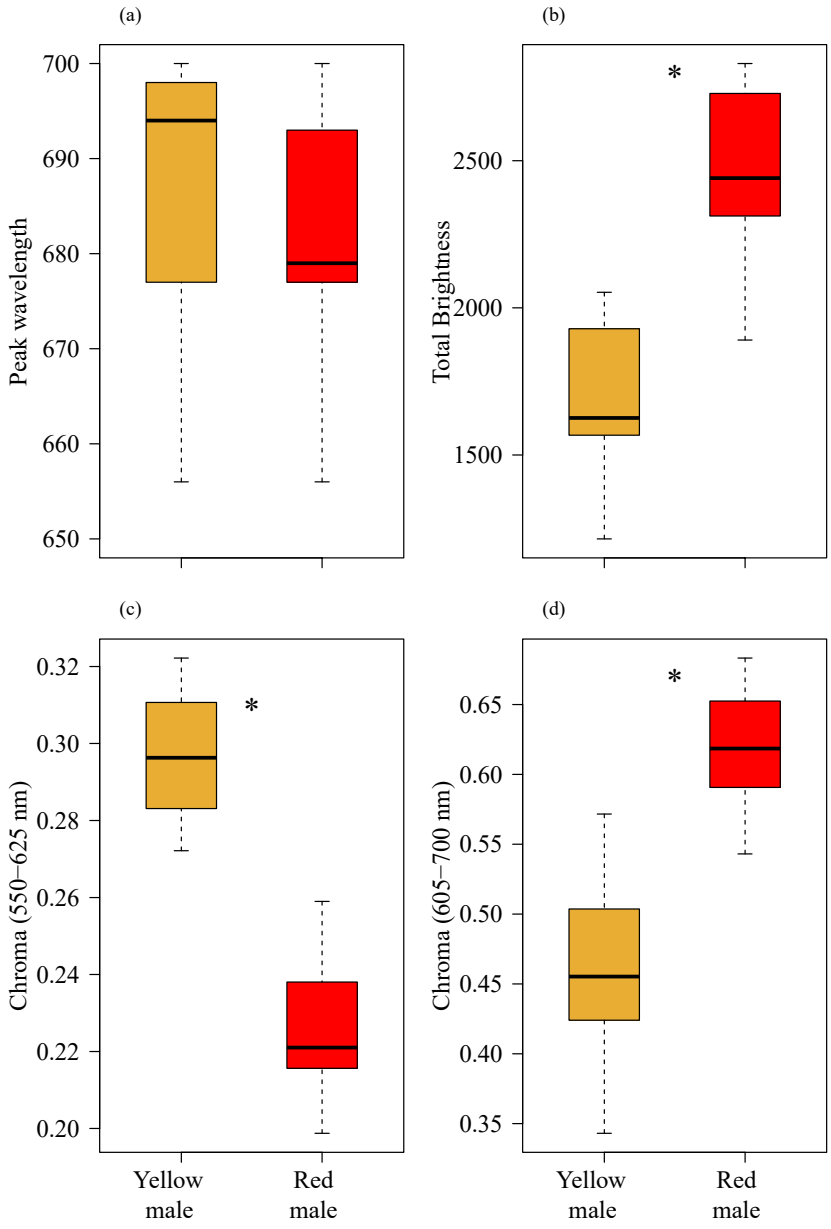

Figure 2: a) The peak wavelength b) total brightness c) yellow chroma (550-625 nm) and d) red chroma (605-700 nm) of the red and yellow males. The internal line in the boxes represents the median, and the upper and lower edges of the boxes represent 75th and 25th percentile, respectively. The whiskers extend to the minimum and maximum data points, but exclude outliers which are beyond 1.5 times of the interquartile range. * denotes significant difference between compared groups.

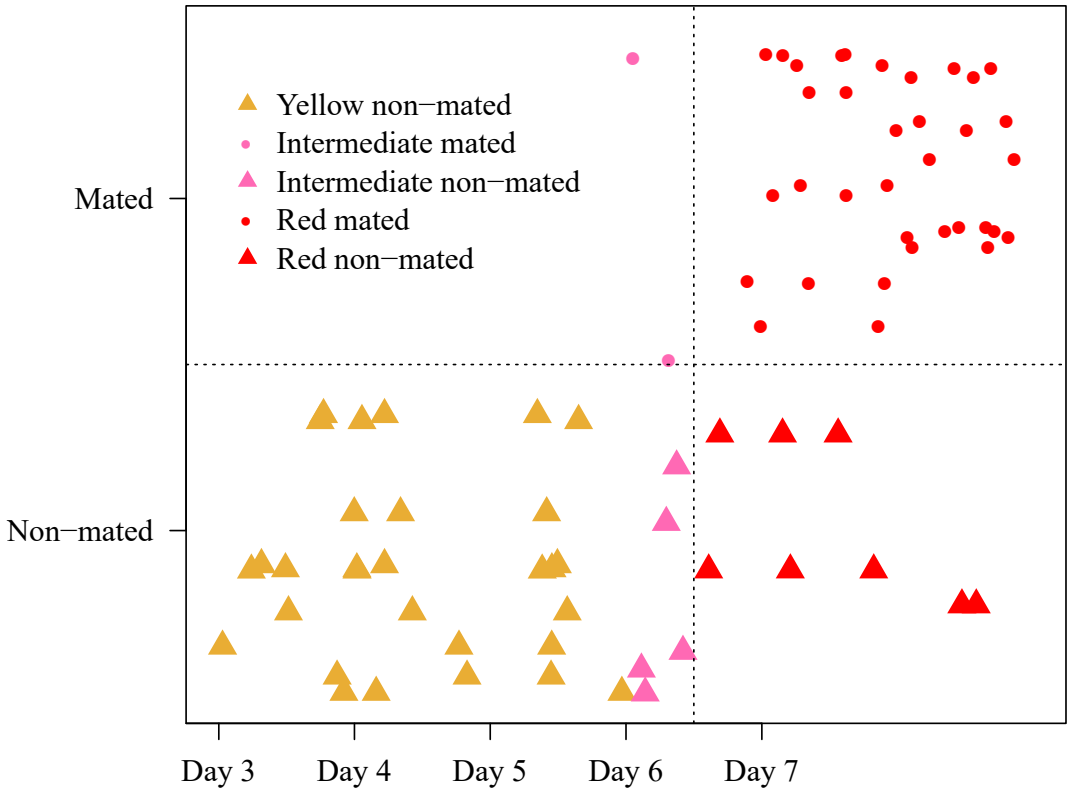

Figure 3: Each circle and triangle represent the mating outcome of a male. The colour of the data points indicates the colour of the experimental males. A triangle indicates a non-mated male, whereas a circle indicates a mated male. Data points above the horizontal lines indicate mated individuals and points below indicate non-mated individuals. Data points on the left of the vertical lines are the males before changing colour whereas data points on the right indicate males after changing colour.

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1197 the data, and wrote the manuscript. MEH contributed to the design of the study, and
1198 critical revision of the manuscript.

1199

Male-male interactions drive the evolution of conspicuous male colouration in a non-territorial mating system

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Abstract

Male ornamentation, such as conspicuous colouration, can evolve through female mate choice or via intrasexual selection that resolves male-male competition. These mechanisms are predicted to select for colour ornaments in territorial males, but it is unclear if they contribute to the evolution conspicuous male coloration in non-territorial mating systems. In non-territorial scramble mating system, selection is predicted to favour mate locating traits instead of conspicuous male colouration, therefore the occurrence of bright male colouration in this mating system remained an enigma. Here, we investigated the drivers of conspicuous male colouration in an ontogenetic colour changing damselfly, *Xanthagrion erythroneurum,* where the juvenile males are yellow and change colour to red upon sexual maturity. We first showed that red males were chromatically and achromatically more conspicuous than the yellow males. Moreover, red males were larger and in better condition than yellow males. We tested female preference in a choice experiment where we artificially manipulated male colour, and found that females did not choose mates based on male colouration. We further tested whether the male colouration affected male-male interactions. We presented red and yellow males in their breeding area, and found that red males received less intra- and interspecific aggression than yellow males. Conspicuous colouration of *X. erythroneurum* male is not a target of female mate choice. Intra- and interspecific male-male interaction

1223 therefore appears to be the driver of conspicuous male colouration in *X. erythroneurum*
1224 and perhaps in non-territorial mating systems.

Introduction

Male armaments and ornaments, such as horns in antelopes, the long tails of peacocks, giant hind legs in beetles, and conspicuous colours in birds, lizards and insects, are not thought to afford survival benefits, but rather evolve through sexual selection (Sherratt & Forbes, 2001). Conspicuous male colouration can evolve via intersexual selection through female preference (Gomez et al., 2009; Kemp, 2007), or via intra-sexual selection through male-male competition for mating and/or to avoid male-male mating attempts (Bajer, Molnár, Török, & Herczeg, 2011; Sherratt & Forbes, 2001). Females prefer conspicuous males if it signals male quality such as physiological condition, body mass, immunity, sperm quality, or higher social status (Bergman et al., 2009; Córdoba-Aguilar, 2002; Córdoba-Aguilar & Cordero-Rivera, 2005). On the other hand, conspicuous colouration can function as an honest signal of male condition, fighting ability and territory defending capacity (Hill, 1991; Ligon & McGraw, 2013; Lim & Li, 2013; Weaver et al., 2017), thereby ensuring larger breeding territories and higher social dominance, which result in higher mating success (Korzan & Fernald, 2007; Setchell & Wickings, 2005).

In non-territorial mating systems, males do not maintain territory or display to females, instead they compete among themselves to access females (Herberstein et al., 2017). Selection is therefore predicted to favour sensory traits such as longer antennae with more olfactory receptors and larger eyes to locate females or locomotory traits such as smaller body, longer legs and better flight capability to reach females promptly (Elgar et al., 2018; Herberstein et al., 2017). Besides locating and moving to females, male-male competition can further influence access to mating area if females assemble in a location for breeding. In these locations, male density can be high with limited mating opportunities.

Conspicuous colouration, if it signals fitness and competitive ability, could allow a male better access to a mating arena by reducing aggression from competitors: a prediction yet to be tested in non-territorial mating systems.

Conspicuous male colouration commonly occurs in many non-territorial damselflies. These systems provide an ideal platform to test the selective agents and benefits of conspicuous male colouration in non-territorial mating systems (Corbet 1999; Bybee et al. 2016). The adult male and female damselflies assemble in waterbodies such as ponds, lake, streams for mating, and ovipositing. Because of the high male density and limited oviposition sites, males compete to access and persist in the mating grounds. In such scramble scenarios, males approach females from behind for mating and females cannot see the colour of males, in the first step of the mating sequence, which is unlikely to be the point of mate selection (Khan & Herberstein, 2019a; Sherratt & Forbes, 2001). Subsequently, however, males require the cooperation of females to lock genitalia (Fincke, 1997; Khan & Herberstein, 2019a) and it has therefore been argued that conspicuous male colour can evolve through female mate choice in non-territorial damselflies, but experimental evidence are limited to support this prediction.

Here we tested the causative agents of the conspicuous male colouration in *Xanthagrion erythroneurum* damselflies. *Xanthagrion erythroneurum* exhibits ontogenetic colour change, where the males change colour from yellow to red, about a week after their emergence (Khan & Herberstein, 2019b). First, we tested if red males are more conspicuous than yellow males for damselfly vision. We then assessed whether the conspicuous colour is an honest signal in this species by testing the prediction that

conspicuous red males will be in better physical condition than yellow males. We experimentally tested both the female preference hypothesis and the male male-competition hypothesis to determine the likely selection mechanism on conspicuous red colour. We predicted the females would prefer red males over yellow males if the conspicuous red colouration evolves through female mate choice. On the other hand, we predicted that red males would receive less aggression from competitor males than the yellow males if male-male competition selects for conspicuous male colouration.

Methods and Materials

Study Species

Xanthagrion erythoneurum is a medium-sized damselfly (21-23mm) belonging to the Coenagrionidae family (Zygoptera: Odonata). This species is widely distributed throughout Australia and commonly found in stagnant freshwater reservoirs such as ponds, marshes, lakes and dams. The adult males can be distinguished from other sympatric damselflies by their red face, red thorax, the red colouration of the first and second abdominal segments and the blue bands on the eighth and ninth abdominal segments (Khan & Herberstein, 2019a; Theischinger & Hawking, 2016). In the Sydney region, this species starts emerging in September and adults are seen in flight until June (personal observation). During this whole period, this species remains reproductively active.

Field site

We collected the damselflies from and carried out experiments at a pond located on the North Ryde campus of Macquarie University, Sydney, Australia (33.772 S, 151.114 E). In this pond, *Xanthagrion erythroneurum* cooccurs with other damselflies including *Ischnura heterosticta, Austroagrion watsoni, Austrolestes annulosus, Diplacodes melanopsis* and *Orthetrum caledonicum.* The sympatric species *Ischnura heterosticta* and *Austroagrion watsoni,* like *Xanthagrion erythroneurum,* are non-territorial species. All three species share the same shoreline vegetations at ponds for perching and mating and submerged vegetations for ovipositing.

Reflective spectrometry

We measured the reflective spectra of the collected males and leaves of the vegetation surrounding the pond to quantify the visual background using a JAZ EL-200 portable spectrophotometer (Ocean Optics, USA) with a PX-2 pulsed light source. We measured all spectra in a dark room by setting the spectrophotometer to a constant boxcar width and integration time settings of 10 and 20 milliseconds respectively, and to average five scans. We measured the reflectance relative to a white standard (Ocean Optics, USA) to standardize the measurements. We first immobilized the damselflies by placing them in a refrigerator at 4^0C for five minutes. Then, we set the probe of the spectrophotometer perpendicular to the cuticular surface of the metathorax from a fixed working distance of two millimetres. We took three reflectance spectra of each male and each background leaf from 300 nm to 700 nm and subsequently averaged those three measurements. We processed the reflectance spectra with OceanOptics Spectrasuite software (ver. 1.6.0_11) and eventually binned to one nm wavelength intervals before minor LOESS smoothing

1317 ($\alpha = 0.35$). We performed the spectral processing using the package 'pavo' v 2.1 (Maia
1318 et al., 2019) in R v 3.5.2 (R core team 2018).

1319 Visual modelling

1320 We calculated the chromatic and achromatic contrast of the red and yellow males against
1321 their background using the receptor noise model (Vorobyev, Brandt, Peitsch, Laughlin,
1322 & Menzel, 2001; Vorobyev & Osorio, 1998). This model calculates the detectability
1323 between two colours in just noticeable difference (JND) units where one JND indicates
1324 that the receiver can distinguish between the colours (Vorobyev et al., 2001). The receptor
1325 noise model has previously been applied in behavioural studies to predict colour
1326 discriminability in various taxa including odonates (Barry, White, Rathnayake, Fabricant,
1327 & Herberstein, 2015; Huang, Chiou, Marshall, & Reinhard, 2014; Khan & Herberstein,
1328 2019a).

1329 We aimed to determine how the colour and luminescence of the red and yellow males are
1330 perceived by the receiver i.e. conspecific and heterospecific damselflies. The visual
1331 system of *X. erythroneurm* is not known. We however, know that the damselflies of the
1332 Coenagrionidae family can have either trichromatic or tetrachromatic visual system. We
1333 therefore applied both systems to calculate the chromatic and achromatic contrast of the
1334 red and yellow males against their backgrounds. We applied photoreceptor sensitivities
1335 of *Ischnura heterosticta*, and *Ischnura elegans* for trichromatic and tetrachromatic
1336 modelling, respectively (Henze, et. al, 2013; Huang et al., 2014; Khan & Herberstein,
1337 2019a). We used the photoreceptor sensitivities of these two species as they are the closest
1338 related species in the phylogenetic tree of our study system whose visual system is known.

We calculated the quantum catches of the photoreceptors by following the methods of Vorobyev and Osorio (1998) (Supplementary method S1). We used standard daylight (D65) as the ambient light spectrum. We then log-transformed the quantum catches according to the Weber-Fencher law (Vorobyev et al., 2001). Finally, we calculated the chromatic and achromatic contrast of the red and yellow males against their background as a function of the log-transformed quantum catches weighted by the noise of each photoreceptor (Vorobyev & Osorio, 1998). We performed the visual modelling in R v 3.5.2 (R core team, 2018) using the package pavo v 2.1 (Maia et al., 2019a).

Male condition

We calculated body length, body mass, lipid and protein content to determine the condition of the males (Castaños, Córdoba-Aguilar, & Munguía-Steyer, 2017). We captured *X. erythroneurum* males from the field using an insect sweep net and brought them back to the Behavioural Ecology Laboratory at Macquarie University for morphometric measurements. We took measurements of the damselflies within two hours after collecting them. We weighed the body mass of the live damselflies on a balance (Mettler toledo, accuracy 0. 01 mg). Next, we immobilized the damselflies by cooling them in a refrigerator at 4^0C for five minutes. We then positioned the damselflies laterally and took digital photographs using a Canon 600D camera mounted with Canon EF 55-250 lens. We measured the total body length of the damselflies from the digital photographs using the ImageJ software (Schneider, Rasband, & Eliceiri, 2012).

Lipid Quantification

We measured the lipid content of the damselflies by the gravimetric method (Barry & Wilder, 2013). First, we euthanised the damselflies by placing them in a -30°C freezer for 10 minutes, then we dried the damselflies at 60^{0}C for 48 hours and then weighed their dried body mass. We then submerged the dried damselflies in chloroform. After 24 hours, we discarded the chloroform, and replaced it with fresh chloroform for another 24 hours. The chloroform was then discarded and the damselflies were air-dried under a fume hood at room temperature for another 24 hours. We further dried the damselflies for another 24 hours at 60^{0}C and later reweighed the dried damselflies. The lipid content of each damselfly was calculated as the difference between the body mass of the damselfly before and after chloroform extraction.

Protein extraction and quantification

We extracted the soluble protein from the damselflies using 0.1 M NaOH (Sigma-Aldrich) as a lysis buffer. First, we finely ground the dried damselflies from above with a polypropylene pestle and added 0.1 N NaOH (100µl per 1mg of insect weight). We then vortexed the lysate, sonicated it for 30 minutes in a water bath, and then heated at 90^{0}C for 15 minutes. Finally, we centrifuged the lysate at 13000 rpm for 10 minutes, discarded the undigested tissues that precipitated, and collected the clarified lysate from the supernatant.

We quantified the protein content in the clarified lysates of the damselflies using Pierce™ BCA protein assay kit (Thermo Fisher Scientific). We used the Bovine Serum Albumin (BSA) supplied with the protein assay kit for preparing the standard solution. We applied

a linear range of standard BSA protein concentrations from 1.35 mg to 0.05 mg for making the standard absorbance curve. We used 0.1 M NaOH (Sigma-Aldrich) as a diluent for the standard solution preparation, for the damselfly lysates preparation and also for the blank control. We took 25 µL of standard BSA for standard solution preparation and 25 µL damselfly lysates for sample protein quantification and then added 0.1 M NaOH (Sigma-Aldrich) to make the final volume 200 µL. We added the standard and sample solution in triplicates in a in 96 well flat-bottomed plate and incubated at 37°C for 30 min. We took the absorbance of the incubated plates at 562 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech). We quantified the relative protein quantity of the lysates using the standard absorbance curve.

Female mate choice experiment

We experimentally tested if females prefer mating with red or yellow males. The yellow males are sexually immature and unable to mate (Khan & Herberstein, 2019). We, therefore, painted red males with yellow colour to determine the effect of male yellow colour on female mate choice. We performed the female mate choice trials by restraining a female in an insect mating cage (58cm × 32cm × 34cm) with a natural red male and a red male that was painted yellow. We manipulated the thorax of the damselflies by painting yellow over red using non-toxic Tim & Tess™ poster paint (Khan & Herberstein, 2019a). We also painted red over the natural red to control for the effect of paintings. We took spectra of the painted damselflies to approximate their natural colour (supplementary figure S1).

We placed the mating cage approximately three meters away from the pond — the natural habitat of the damselflies. We recorded the sexual interactions of the damselflies while

sitting one meter away from the cages. We counted the number of tandems formed by the control red males and the manipulated yellow males. The tandem is the first step of damselfly mating where a male becomes physically connected to a female by his cerci. A tandem event can dissociate if the female does not cooperate, or it can form a wheel if the female cooperates. When a tandem was formed, we continued the trials until the tandem disassociated or formed a wheel. We recorded the duration of tandems when disassociated and when forming a wheel. When a wheel was formed, we recorded the duration of the wheel before disassociation.

We performed the female mate choice trials on sunny days between 10:00 hrs and 16:00 hrs when mating usually occurs in the field (Khan & Herberstein, 2019a). We performed each trial for 30 minutes with two new males and a female; no damselflies were reused. Paint was washed off the damselflies after every trial, and the damselflies were released at the end of the day. The aim of the experiment was to determine female mate choice between the red and yellow males, so in the analysis we included trials where a female choose to form a tandem or wheel with one of the males.

Male-male competition experiment

We conducted three sets of experiments to determine the effect of male colour on male-male interactions by tethering the experimental males. In the first experiment, we tethered a naturally occurring red male and a naturally occurring yellow male and determined the male-male interactions received by the red and yellow males. To determine whether the incurred interactions are the effect of colour or other developmental changes, we painted a red male yellow and tethered it with a red male and determined any male-male interactions. In the third experiment, we altered the colour of yellow males by painting

them red and presented the naturally occurring yellow males with the red-painted yellow males and determined any male-male interactions. If body colour determines the interactions at the pond, we expect the yellow painted red males will receive less aggression than the red males. Similarly, the red-painted yellow males will receive less aggression than yellow males.

We applied a modified damsel-on-a-dowel technique (Fincke, Fargevieille, & Schultz, 2007) to determine the male-male interaction of the red and yellow males in their natural habitat. We glued a live yellow male and a red male 20 cm apart from each other on a dowel using UHU™ glue. The damselflies were glued in perching positions, with their legs attached to the dowel. The dowel was then placed at the edge of the pond. In this pond, *X. erythoneurum* coexists with two heterospecific damselfly species of the Coenagrionidae family: *Ishnura heterosticta and Austroagrion watsoni*, with whom they share breeding area and oviposition sites. We measured the aggressive and non-aggressive responses received by the red and yellow *X. erythoroneurum* males from conspecific males and heterospecific (*Ishnura heterosticta*) males. We observed the responses by sitting approximately one meter away from the dowel, which allowed us to observe the focal damselflies clearly without disturbing regular movements of the approaching damselflies. An approaching damselfly can detect the focal damselfly when it passes within 10 cm of the focal damselfly (Fincke, 2015) and can either show aggression or non-aggression when it passes. When an intruder male passed within 10 cm left or right of the focal male without any physical contact, we counted it as a non-aggressive interaction. On the other hand, when the intruder male bit the focal male, we counted it as an aggressive interaction (Fincke et al., 2007). Finally, if the intruder male tried to form a clasp (grab the focal male and tried to move its cerci to the prothorax of

the focal male) or formed a tandem (intruder male physically connected with the focal male), we counted it as a mating attempt.

We conducted each trial for 10 minutes by placing the tethered damselflies in different locations around the lake. We used the same pair for three consecutive trials unless the focal damselflies were predated by sympatric odonates after one or two trials. We conducted all our experiments on sunny days between 10:00 hrs and 1600 hrs when damselfly density and interactions are high (personal observation). We counted aggressive and non-aggressive responses received by the focal males from approaching conspecific and heterospecific males. We manipulated damselfly colour following the same procedure as described in the female mate choice experiment. After finishing the experiment, we unglued the damselflies by hand, washed off the paint, and released them at the end of the day.

Statistical analyses

We applied Shapiro-Wilk tests to determine normality and F-tests to compare the variance of the data. Two-sample t-tests were applied to compare the chromatic and achromatic contrast of the males against their background. We applied Two-sample t-tests to analyse the total length, and Welch Two Sample t-test to analyse body mass, protein content and lipid content of red males and yellow males – Bonferroni corrections were applied to adjust the p-values.

We applied Generalized linear models (GLMs) to determine whether females are more likely to form tandems and wheels with red males than yellow painted red males. We fitted GLMs with the numbers of tandems and wheels as a response variable and male

colour (red or painted yellow) as covariates. We applied Cox regression models to analyse tandem duration and wheel duration of the red and yellow painted red males. Tandems that transitioned to wheels were analysed separately from tandems that dissociated before forming wheels.

To analyse the aggression received by red and yellow males in male-male competition experiments, we applied generalized linear mixed models (GLMMs) with aggression or non-aggression as a response variable, and the pair identity as a random effect for the experiments involving natural red and yellow males (a) and natural red males with a red male painted yellow (b). We fitted a generalized linear model with a quasi-binomial distribution (to account for the over-dispersion) to analyse the aggression received by the focal males in experiments where we paired a natural yellow male with a yellow male painted red (c). For each analysis, we used the full model by including interactions between the fixed effects and pair identity as random effect (aggression rate ~ focal male + intruder male + focal male* intruder male + 1|id). We, however, tested other possible models: a) focal male, or b) intruder male as covariate, c) focal male and intruder male as covariates without interactions, and d) focal male and intruder male as covariates with interactions and tested the goodness of the models. The model selection criteria are shown in Appendix S1. All the analyses were conducted in R v 3.5.2 (R core team, 2018) using the 'survival' (Therneau & Lumley, 2019), 'lme4' (Bates et al., 2019), and 'MuMIn' (Bartoń, 2019) packages.

Results

Damselfly spectra

The reflectance spectra of the yellow males showed peaks between 588 nm and 700 nm whereas the red males showed peaks between 657 nm and 700 nm (Figure 1a). The reflectance spectra of the background showed a Gaussian peak between 551-554 nm (Figure 1a).

Visual modelling

The chromatic and achromatic contrast between the red and yellow males and the background was more than one JND, suggesting that the males are discriminable against their background by both trichromatic (Figure 1b-1c) and tetrachromatic (Figure 1d-1e) visual systems (Figure 1b-1e). The red males showed both higher chromatic contrast (Mann-Whitney U test: $W = 0$, $p < 0.0001$) and achromatic contrast (Welch two sample t-test: $t = -2.07$, $df = 27.32$, $p < 0.05$) than the yellow males in trichromatic damselfly vision (Figure 1b-1c). Similarly, in tetrachromatic damselfly vision, the red males showed higher chromatic contrast (Two sample t-test: $t = -11.81$, $df = 31$, $p < 0.0001$) and achromatic contrast (Two sample t-test: $t = -2.49$, $df = 30$, $p < 0.05$) than the yellow males (Figure 1d-1e).

Male condition

The red males were longer in total length (Two sample t-test: $t = -5.13$, $df = 75$, $p < 0.0001$) and their body mass was greater (Welch two sample t-test: $t = -16.65$, $df = 70.39$, p

<0.0001) than the yellow males (Figure 2a-2b). Furthermore, the larger red males had higher lipid content (Mann-Whitney U test: $W = 21$, $p < 0.0001$) than yellow males (Figure 2c). Similarly, protein content of red males was significantly higher (Welch two sample t-test: $t = -8.69$, $df = 28.25$, $p < 0.0001$) than in yellow males (Figure 2d).

Female mate choice

In total, 60 tandems were formed during the female mate choice experiment. The number of tandem formations did not differ significantly (GLM: $\chi^2 = 0.46$, $df = 1$, $p = 0.49$) between the red males ($n = 32$) and the yellow painted red males ($n = 28$) (Figure 3a). Over half (51.7%) of the males failed to form a wheel after forming a tandem. The number of males proceeding from tandem to wheel did not differ significantly (GLM: $\chi^2 = 1.63$, $df = 1$, $p = 0.20$) between the red males ($n = 13$) and the yellow painted red males ($n = 16$) (Figure 3b). When the tandems dissociated before forming wheels, the tandem duration was not affected by male colour (Cox regression: $\chi 2 = 1$, $df = 1$, $p = 1$) (Figure 3c). Similarly, the time to wheel formation did not differ significantly (Cox regression: $\chi 2 = 0.62$, $df = 1$, $p = 0.6$) between the red or yellow painted red males (Figure 3d). Finally, the duration of the wheel before disassociation did not differ significantly (Cox regression: $\chi 2 = 0.06$, $df = 1$, $p = 0.06$) between the red males ($n = 13$) and the yellow painted red males ($n = 16$) (Figure 3e).

Male-male competition

The yellow males received significantly higher aggressive responses than red males from conspecific males (estimate: 4.48 ± 0.38, $z = 11.74$, $p < 0.001$) in the male-male competition experiment when natural red and yellow males were presented to intruder

males (Figure 4a). Also, there was a significant interaction between focal males and intruder males (estimate: -1.59 ± 0.67, z = -2.37, $p < 0.05$) showing that the probability of receiving aggression from heterospecific males was higher in yellow males than red males (Figure 4b; supplementary Table 2). Similarly, when yellow painted red males were presented with natural red males, yellow painted red males received significantly higher aggression than natural red males from conspecific and heterospecific males (Figure 4c-d; supplementary Table 4-5). Furthermore, when yellow males were painted red, and presented with the natural yellow males, the natural yellow males received higher aggression from conspecific and heterospecific males than the red painted yellow males (Figure 4e-f; supplementary Table 7-8).

Discussion

Conspicuous male colouration can evolve through female mate choice, male-male competition for mating, to reduce male-male mating attempts or through a combination of all three (Clutton-Brock, 2007; Sherratt & Forbes, 2001). Although the colour production mechanism of *X. erythroneurum* is unknown, conspicuous red colour is generally considered costly to produce and maintain (Hill, 1996; Johnson & Candolin, 2017). We therefore predicted that the red colouration is an honest signal of good male condition in *X. erythroneurum* damselflies. Accordingly, we found that red males were in better nutritional and physiological condition than yellow males. We further predicted that conspicuous red colouration would offer males higher mating success via female preference. Instead, we found that the females did not prefer red males over yellow males when given a choice. However, we showed that the yellow males received more aggression than the red males from conspecific as well as from heterospecific males in

1570 their breeding grounds. This indicates that the conspicuous red colouration favours males'
1571 access to breeding area and to females through intra- and interspecific male-male
1572 competition.

1573

1574 The female mate choice experiments showed that the number of tandems did not differ
1575 between red and yellow males. In the cage experiment, females cannot avoid tandem as
1576 they cannot fly away from the approaching males. A female, however, can reject a mate
1577 by dissociating from the tandem (Khan & Herberstein, 2019a). In support of that, we
1578 found that the females rejected 51.7% of mating attempts in our trials. The rejection rate,
1579 however, was not different between the red and the yellow males. A female can further
1580 express refusal by delaying the wheel formation, or by dissociating from the wheel
1581 quickly before sperm transfer (Khan & Herberstein, 2019a). If females preferred red
1582 males, we would have expected 1) tandem durations before dissociation are longer for
1583 red males, 2) red males attain wheel formation more quickly, and 3) the wheel durations
1584 are longer for red males. We, however, did not find significant differences between the
1585 red and the yellow males in any of these choice indicators. Females are unlikely to detect
1586 the coloration of the males as they approach from behind to form a tandem. The females
1587 probably use tactile cues and clasping strength rather than colour to estimate male quality
1588 (Barnard, Fincke, McPeek, & Masly, 2017; Barnard & Masly, 2018). Taken together, our
1589 results strongly suggest that female preferences are not selective agents of male
1590 colouration. Cryptic female mate choice, however, might select for the male conspicuous
1591 colouration, but further studies are required for affirm that prediction.

1592

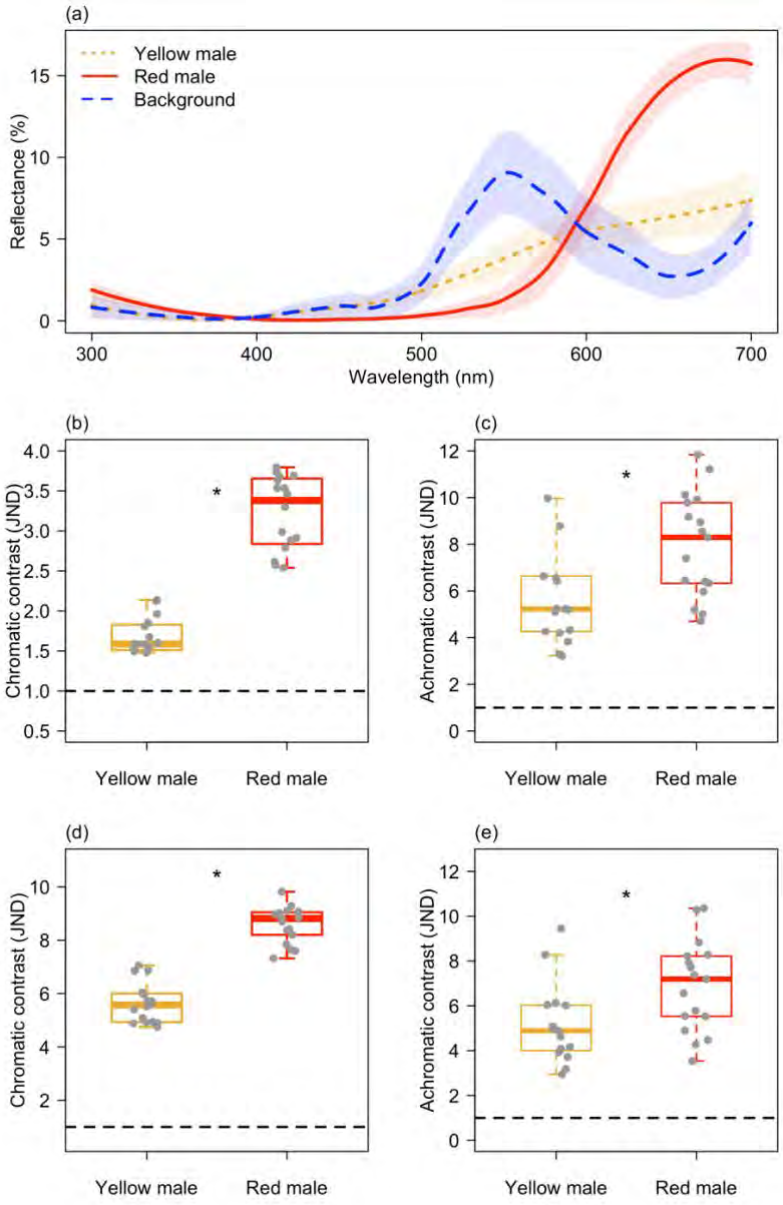
Conspicuous male colouration can evolve by intrasexual selection if conspicuousness increases male mating success by reducing conspecific aggression in the breeding area. Thus, we predicted that red males would receive less aggression than yellow males. Our results confirmed this prediction: yellow males received more overall aggression than red males. We also found yellow painted red males received more aggression than red males, and red painted yellow males received less aggression than yellow males, thereby suggesting males' body colour reduces conspecific aggression. The red males received less aggression probably because of the red colour signals male quality and competitive ability, thereby serving as a status badge to resolve costly disputes without direct physical contact. Our findings are consistent with previous findings suggesting that red colouration is a signal of male condition and dominance, and functions to intimidate rivals in lizards (Healey, Uller, & Olsson, 2007; Whiting et al., 2006), fishes (Dijkstra, Seehausen, & Groothuis, 2005), birds (Pryke & Griffith, 2006) and primates (Setchell & Wickings, 2005). Furthermore, the reduced aggression towards red painted yellow males compared to yellow males support the hypothesis that red is inherently intimidating to rivals (Baird, Baird, & Shine, 2013; Barlow, 1983; Pryke, 2009; Rowland, Bolyard, & Halpern, 1995) even when additional phenotypic information (e.g. size) was available.

Interspecific interactions can be a significant evolutionary force to shape traits in sympatric species (Tynkkynen, Rantala, & Suhonen, 2004; Tynkkynen, Kotiaho, Luojumäki, & Suhonen, 2005). Here, we showed that the natural and painted red males received less heterospecific aggression than the natural and painted yellow males. The heterospecific aggression can occur due to an interspecific recognition error where males are phenotypically similar or because of male competition for common resources. Our study species, *Xanthagrion erythroneurum* with a red thorax and *Ischnura heterosticta*

with a blue thorax are phenotypically dissimilar, therefore recognition error is probably an unlikely mechanism for the observed interspecific aggression. On the other hand, both species assemble at the pond for breeding, share the same perching sites for mating, foraging, and resting in between mate searching, and also share the same oviposition sites suggesting interspecific competition for breeding resources are a possible mechanism. Our findings support the idea that conspicuous colouration can reduce interspecific aggression (Drury & Grether, 2014) to acquire shared breeding resources (Lipshutz, 2018; Peiman & Robinson, 2010). This suggests that, in *X. erythroneurum*, and probably also in other non-territorial scramble competition mating damselflies, the conspicuous colouration has evolved via intra- and interspecific male-male competition.

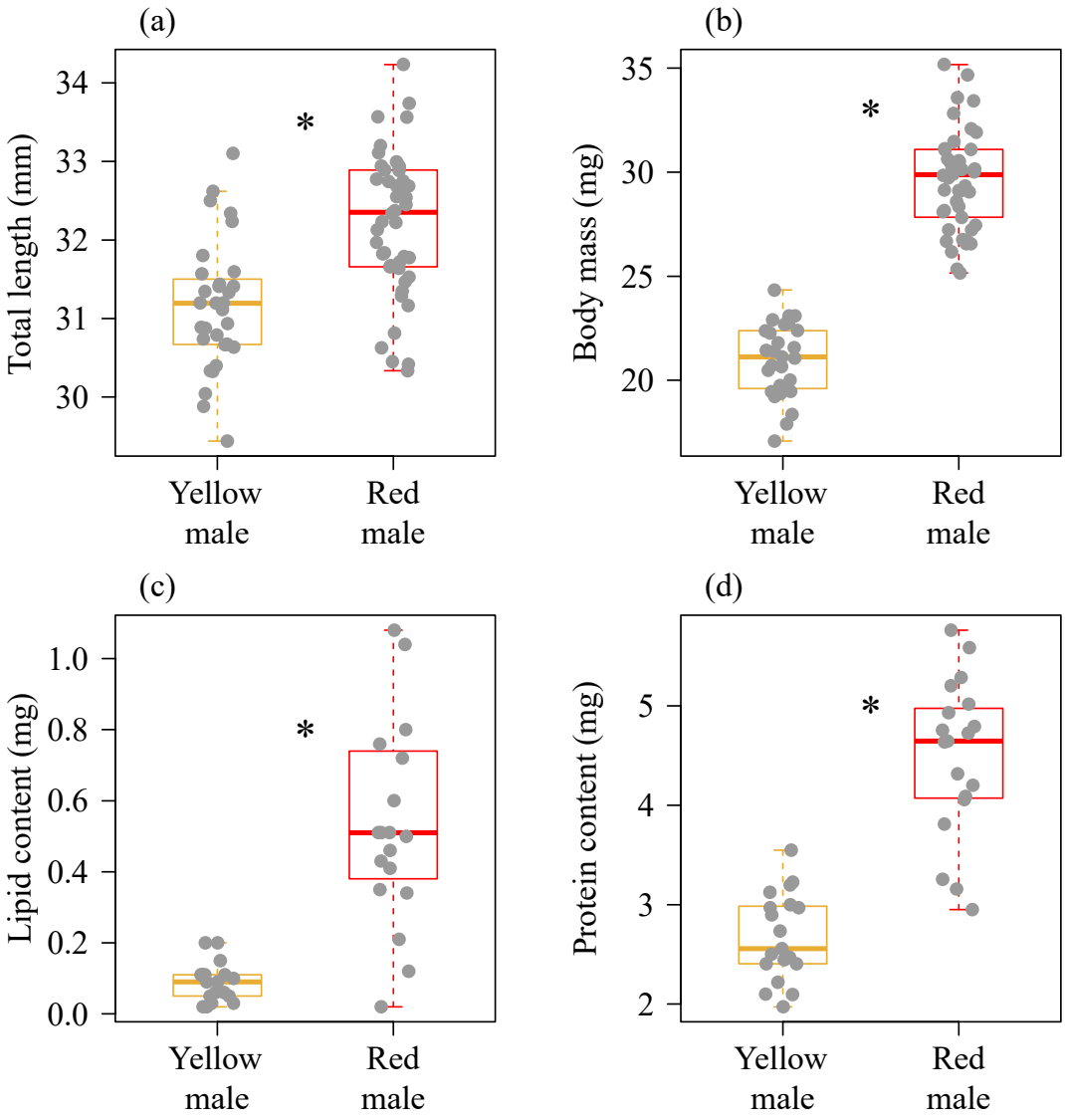
Conspicuous male colouration can also evolve through intrasexual selection to avoid costly male-male mating attempts (Sherratt & Forbes, 2001). This anti-harassment aposematic hypothesis has been supported in moor frogs showing that the males attain conspicuous colouration upon reproductive maturity to avoid male-male amplexus formation (Sztatecsny et al., 2012). Similarly, sexually dimorphic abdominal blue bands in non-territorial damselflies reduce intraspecific male-male tandem formation (Beatty et al., 2015; Khan & Herberstein, 2019a). In our male-male competition experiment, while presenting the males, we found that conspecific males formed tandems with 11 out of 20 experimental yellow males, but never with the red males (data not shown). This preliminary result suggests that the conspicuous red colouration might also function to avoid male-male tandem formation in this species. Further behavioural choice experiments to test conspecific male mate choice between the red and yellow males are needed to better understand the anti-harassment signal of the red colour.

Our study combined colour vision modelling with laboratory and behavioural experiments to explain the function of the conspicuous colouration in a non-territorial mating system. We showed that adult red males have better physiological and nutritional condition than the pre-reproductive yellow males. We demonstrated that the conspicuous colouration is not a target of female mate choice but reduces inter- and intraspecific male aggression in the breeding ground. Our findings suggest that conspicuous male colouration can evolve through male-male competition in non-territorial mating species. Because our study presents clear intra-sexual advantage of red males, the question of why yellow males persist in the population remains elusive. Further studies are needed to explain if being yellow is a resource limited constrain or it is an adaptation to reduce the foraging and predation risks associated with a red colour.



1652

1653 Figure 1: a) Reflectance spectra (mean ± standard deviation) of the red males (n = 17),
1654 yellow males (n = 17) and background leaves (n = 30); b) chromatic and c) achromatic
1655 contrast of the red and yellow males against their background in trichromatic damselfly
1656 vision; d) chromatic and e) achromatic contrast of the red and yellow males in
1657 tetrachromatic damselfly vision. Boxplots depict the median, the 25th and 75th percentile
1658 with the whiskers extended to the minimum and maximum data points. Dots are data
1659 points; outliers that were > 1.5 times the interquartile range. (* p <0.05).



1660

1661 Figure 2: a) Total length of the yellow males (n = 31); b) and red males (n = 46); b) body

1662 mass of the yellow males (n = 27) and red males (n = 46); c) lipid content of the yellow

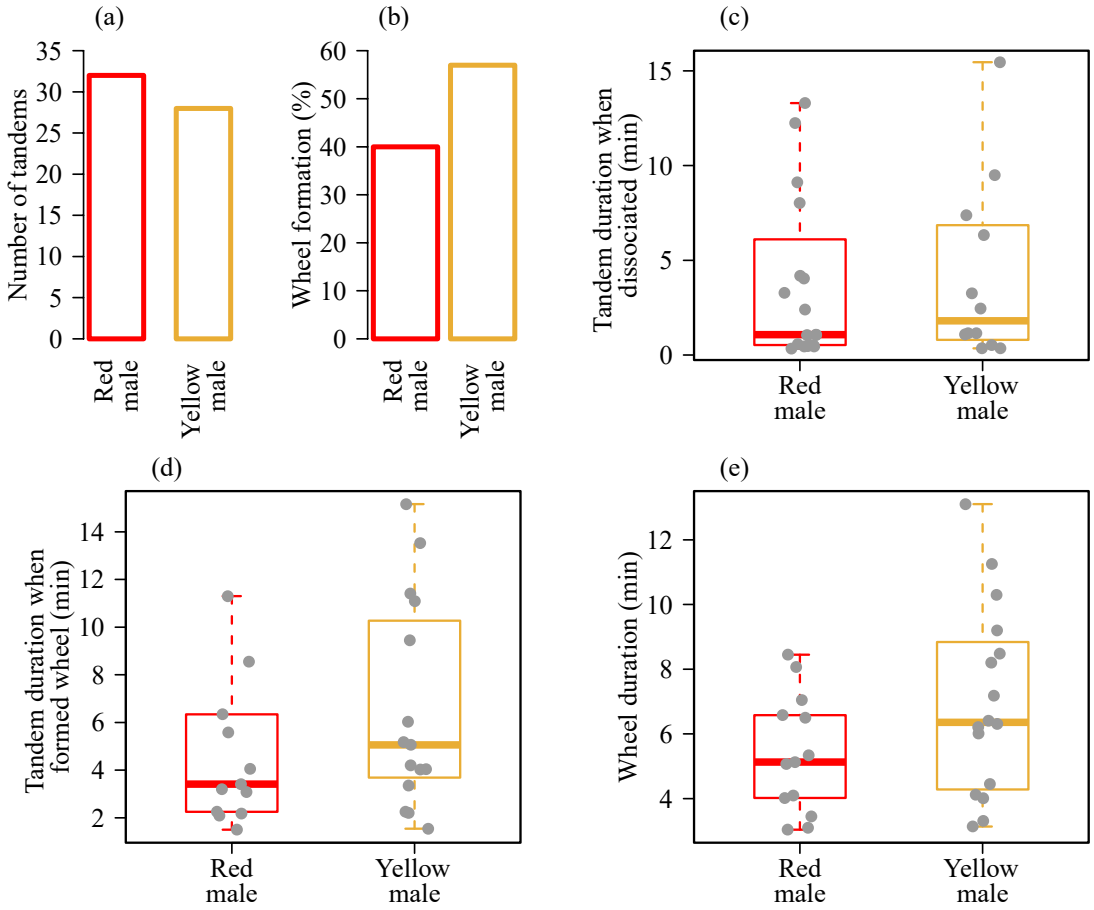
1663 males (n = 19), and red males (n = 19), and d) protein content of the red males (n = 19)

1664 and yellow males (n = 19). Boxplots depict the median, the 25th and 75th percentile with

1665 the whiskers extended to the minimum and maximum data points. Dots are data points;

1666 outliers that were > 1.5 times the interquartile range were excluded. (* p <0.0001).

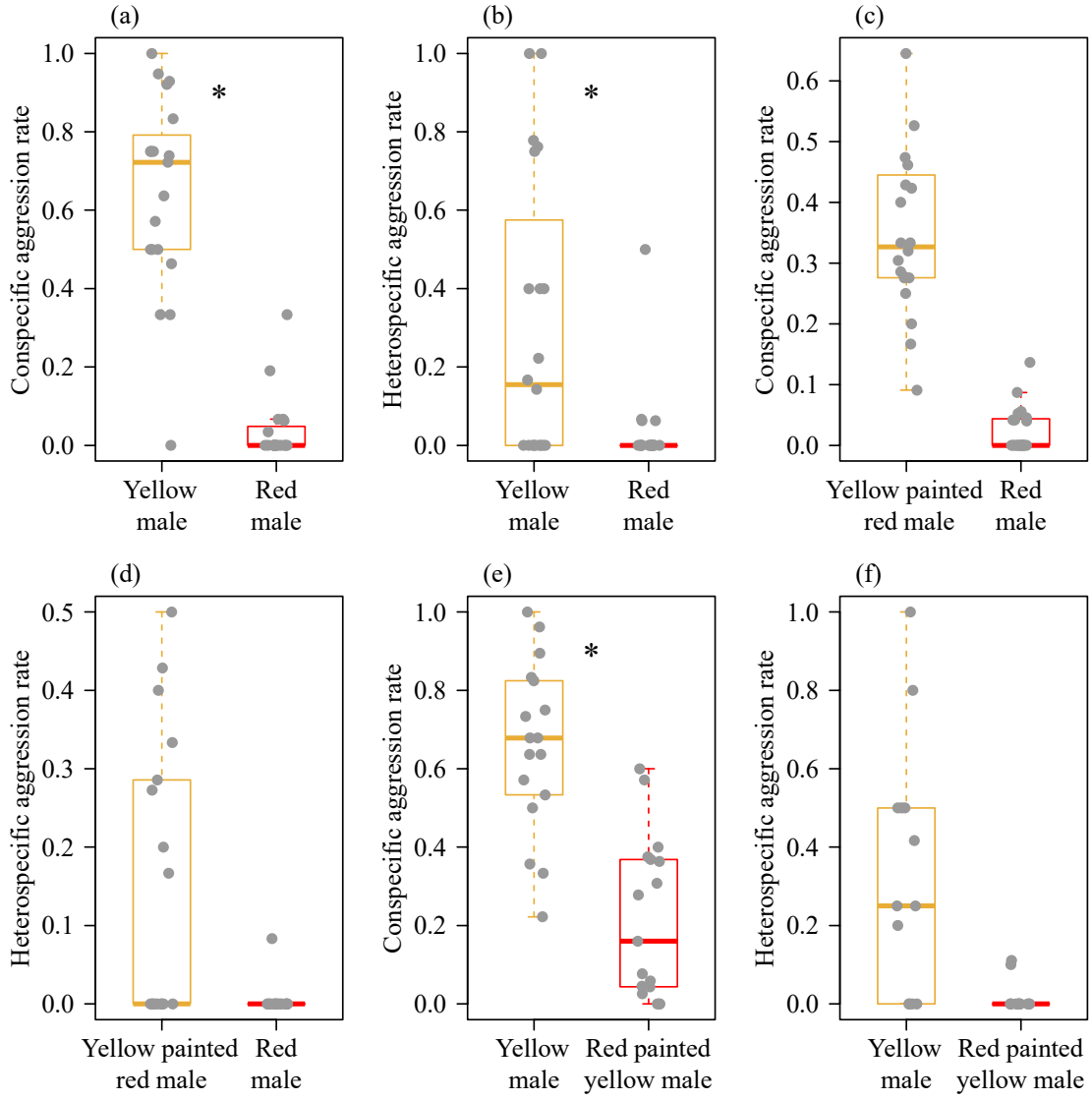
1667



1668

1669 Figure 3: a) Number of tandems formed by red males (n = 32), and yellow males (n =
1670 28); b) percentage of the tandems involving red males (n = 13) and yellow males (n = 16)
1671 that ended in wheel formation; c) duration of red and yellow male tandems that ended in
1672 dissociation rather than wheel formation; d) duration of red and yellow male tandems
1673 that ended in wheel formation, and e) wheel duration of red and yellow males. Boxplots
1674 depict the median, the 25th and 75th percentile with the whiskers extended to the
1675 minimum and maximum data points. Dots are data points; outliers that were > 1.5 times
1676 the interquartile range were excluded.

1677



1678

1679 Figure 4: Aggressions (number of attacks/number of approaches) received by natural red
1680 and natural yellow males (n = 20) from a) conspecific males and b) heterospecific males;
1681 c) aggressions received by natural red males, and yellow painted red males (n = 20) from
1682 conspecific males and d) heterospecific males; e) aggressions received by natural yellow
1683 males and red painted yellow males (n = 18) from conspecific males, and f) heterospecific
1684 males. Boxplots depict the median, the 25th and 75th percentile with the whiskers
1685 extended to the minimum and maximum data points. Dots are data points; outliers that
1686 were > 1.5 times the interquartile range were excluded.

Supporting material

Supplementary method S1:

We applied the receptor noise model to analyse how the red and yellow males will appear to conspecific and heterospecific damselflies. Damselflies can have either trichromatic (Huang et al., 2014) or tetrachromatic visual systems (Henze et al., 2013; Outomuro et al., 2017). The photoreceptor sensitivities of the *X. erythroneurum* damselfly is unknown, therefore we used both trichromatic and tetrachromatic system (Khan & Herberstein, 2019a). We ran the visual modelling in pavo v. 2.0 (Maia et al., 2019) implemented in R v. 3.5.2.

We first calculated the quantum catch (Qi) for each photoreceptor i as follows-

$$\mathrm{Qi} = \int_{300}^{700} S(\lambda) I(\lambda) Ri\,(\lambda) d\lambda,$$

Where λ is the wavelength, $S(\lambda)$ is the reflectance spectra of damselfly integument or each background leaf, $I(\lambda)$ is the light spectrum entering the eye, and $\mathrm{Ri}(\lambda)$ is the spectrum sensitivity of the photoreceptor i. The standard daylight spectrum (D65) was used as $I(\lambda)$ (Wyszecki & Stiles, 1982)

We then calculated the noise of each class photoreceptor (e_i) as,

$$e_i = \frac{\bar{\omega}}{\sqrt{n_i}}.$$

Where ω is the Weber fraction assigned to each receptor and n_i is the relative density of the receptor class *i*. A weber fraction of 0.12 was applied, which was also used for damselfly visual

modelling in previous studies (Khan & Herberstein, 2019a). We applied receptor density of honey bee (1:0.471:4.412) for trichromatic modelling (Defrize et al., 2010).

Finally, we calculated the chromatic contrast (ΔS) between the damselfly spectra and the background using the equation:

$$\Delta S = \sqrt{\frac{e_1{}^2(\Delta f_3 - \Delta f_2)^2 + e_2{}^2(\Delta f_3 - \Delta f_1)^2 + e_2{}^2\,(\Delta f_1 - \Delta f_2)^2}{(e_1e_2)^2 + (e_1e_3)^2 + (e_2e_3)^2}}$$

Where Δf_i is the log of quantum catches for receptor *i* between damselfly and the background.

We used green photoreceptor to calculate the achromatic contrast (ΔL); which was calculated as:

$$\Delta L = \frac{\Delta f i}{e_i}$$

We used photoreceptor sensitivities of *Ischnura elegans* (370 nm, 440 nm, 540 nm, and 600 nm) (Henze et al., 2013), photoreceptor density 2:2.5:2.5:1 (Armett-Kibel & Meinertzhagen, 1983), and a Weber fraction 0.12 (Khan & Herberstein, 2019a) for the tetrachromatic damselfly modelling. We calculated the chromatic contrast (ΔS) using the equation:

$$\Delta S = \sqrt{\frac{\begin{matrix} e_1e_2{}^2(\Delta f_4 - \Delta f_3)^2 + e_1e_3{}^2(\Delta f_4 - \Delta f_2)^2 + e_1e_4{}^2(\Delta f_3 - \Delta f_2)^2 + \\ + e_2e_3{}^2(\Delta f_4 - \Delta f_1)^2 + e_2e_4{}^2(\Delta f_3 - \Delta f_1)^2 + e_3e_4{}^2(\Delta f_2 - \Delta f_1)^2 \end{matrix}}{(e_1e_2e_3)^2 + (e_1e_2e_4)^2 + (e_1e_3e_4)^2 + (e_2e_3e_4)^2}}$$

We used the same equation to calculate the achromatic contrast for both tri- and tetrachromnatic visual system.

Supplementary tables:

Table 1. Best-fit generalized linear mixed model explaining variation in the aggression received by the focal males from the intruder males (male-male competition: experiment a). We analysed the aggression received by the focal males (red and yellow males) from intruder males (conspecific and heterospecific males) using a generalized linear mixed model. Explanatory variables in the best-fit model included the interaction between focal males and intruder males as a fixed effect. Intercept gives the estimate of the number of aggressions received by the red males from conspecific males ($n = 20$). Significant values ($Pr(>|z|) < 0.05$) are in bold. Variance of random effects (σ^2): 0.358

Aggression received by the red and yellow males from intruder males				
Fixed effects	**Estimate**	**Standard error**	**Z value**	**Pr(>|z|)**
Intercept	-3.465	0.379	-9.148	**<0.001**
Focal male	4.481	0.382	11.738	**<0.001**
Intruder male	0.039	0.630	0.062	0.951
Focal male*intruder male	-1.586	0.671	-2.365	**0.018**

Table 2: Probability of aggression received by the focal males (natural red males and yellow males) from the intruder males

Focal male	Intruder male	Aggression probability (%)
Natural red male	Conspecific male	3.03
Natural yellow male	Conspecific male	73.4
Natural red male	Heterospecific male	3.14
Natural yellow male	Heterospecific male	37.02

Table 3. Best model selection among from the possible models. Estimates of the log-likelihood (LL), adjusted Akaike's information criterion (AICc), change in AICc relative to the leading model (ΔAICc), and relative weights (*w*) are provided for each model. Bold indicates the most informative model.

Best model selection					
Model	**d.f.**	**LL**	**AICc**	**ΔAICc**	***w***
~focal male*intruder male + (1|id)	**5**	**-160.274**	**330.8**	**0.00**	**0.787**
~focal male + intruder male + (1|id)	4	-162.630	333.5	2.61	0.213
~ focal male	3	-177.196	360.5	29.66	0.000
~ intruder male	3	-339.887	685.9	355.05	0.000

Table 4. Best-fit generalized linear mixed model explaining variation in aggression received by the focal males from intruder males (male-male competition: experiment b). We analysed the aggression received by the focal male (yellow painted red males and natural red male) from conspecific and heterospecific intruder males using

a generalized linear mixed model. Explanatory variables in the best-fit model included the interaction between focal male and intruder male as a fixed effect. Intercept gives the estimate of the number of aggressions received by the red males from the conspecific males ($n = 20$). Significant values ($Pr(>|z|) < 0.05$) are in bold. Variance of random effects (σ^2): 0.035

Aggression received by red and yellow painted red males from intruder male				
Fixed effects	Estimate	Standard error	Z value	Pr(>|z|)
Intercept	-3.355	0.290	-11.565	**<0.001**
Focal male	2.742	0.300	9.132	**<0.001**
Intruder male	-0.676	0.285	-2.375	**0.018**

Table 5: Probability of aggression received by focal males (red males and yellow painted red males) from intruder males

Focal male	**Intruder male**	**Aggression probability (%)**
Red male	Conspecific male	3.37
Yellow painted red male	Conspecific male	35.13
Red male	Heterospecific male	1.74
Yellow painted red male	Heterospecific male	21.59

Table 6. Best model selection among from the possible models. Estimates of the log-likelihood (LL), adjusted Akaike's information criterion (AICc), change in AICc relative to the leading model (ΔAICc), and relative weights (w) are provided for each model. Bold indicates the most informative model.

Best model selection

Model	**d.f.**	**LL**	**AICc**	**ΔAICc**	**w**
~focal male + intruder male + (1|id)	**4**	**-146.828**	**301.8**	**0.00**	**0.658**
~focal male * intruder male + (1|id)	5	-146.720	303.7	1.88	0.257
~ focal male	3	-149.911	305.9	4.09	0.085
~ intruder male	3	-220.448	447.0	145.16	0.000

Table 7. Best-fit generalized linear mixed model explaining the aggression received by the focal males from intruder males (male-male competition: experiment c). We analysed the aggression received by the focal male (red painted yellow males and natural yellow males) from conspecific and heterospecific intruder males using a generalized linear model with a quasibinomial distribution. Explanatory variables in the best-fit model included focal males and intruder males as a covariate. Intercept gives the estimate of the number of aggressions received by the red males from the conspecific males. (N = 18). Significant values (Pr(>|z|) < 0.05) are in bold.

Aggression received by red and yellow painted red males from intruder male

Fixed effects	Estimate	Standard error	Z value	Pr(>|z|)
Intercept	-1.528	0.198	-7.731	**<0.001**
Focal male	2.359	0.245	9.616	**<0.001**
Intruder male	-1.647	0.345	-4.768	**<0.001**

Table 8: Probability of aggression received by focal males (yellow males and red painted yellow males) from intruder males

Focal male	Intruder male	Aggression probability (%)
Yellow male	Conspecific male	69.65
Red painted yellow male	Conspecific male	17.83
Yellow male	Heterospecific male	30.67
Red painted yellow male	Heterospecific male	4.01

Table 9. Best model selection among from the possible models. Estimates of the log-likelihood (LL), adjusted Akaike's information criterion (AICc), change in AICc relative to the leading model (ΔAICc), and relative weights (w) are provided for each model. Bold indicates the most informative model.

Best model selection

Model	d.f.	LL	AICc	ΔAICc	w
~focal male + intruder male + (1|id)	**3**	**-166.391**	**209.9**	**0.00**	**0.702**
~focal male * intruder male + (1|id)	4	-166.066	211.6	1.77	0.298
~ focal male	2	-186.634	232.4	22.45	0.000
~ intruder male	2	-251.219	310.7	100.74	0.000

Supplementary Figure:

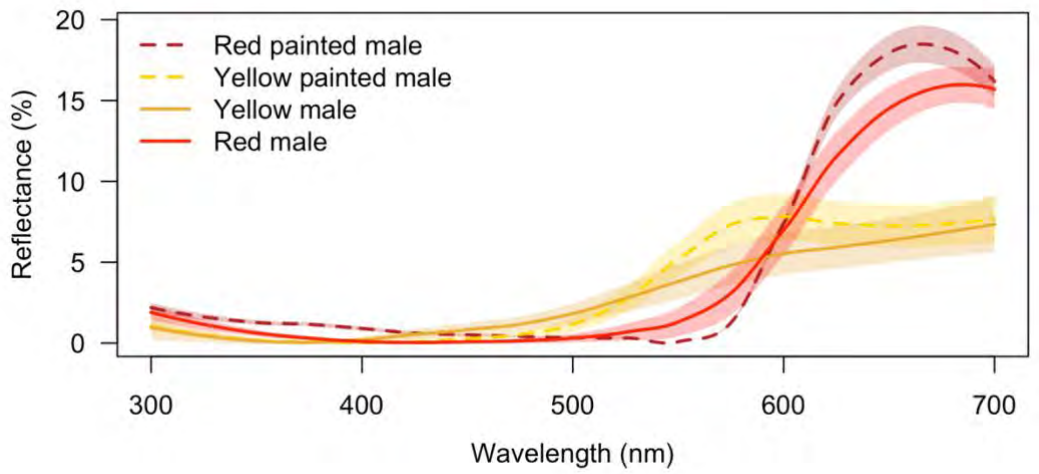


Figure S1: Reflectance spectra (mean ± standard deviation) of the red males (n = 17), yellow males (n = 17), painted red males (n = 10) and painted yellow males (n = 8).

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Ontogenetic habitat shifts reduce costly male-male interactions

Md Kawsar Khan, Marie E. Herberstein

Abstract

Ontogenetic habitat shifts are predicted to increase the fitness and survival of individuals by allowing effective utilization of spatially distributed resources. Evidence supports nutritional requirements and predation pressure as drivers of habitat shifts. Likewise, intraspecific interactions are thought to lead to ontogenetic habitat shifts, however, empirical evidence is lacking. Here, we test if intraspecific male-male interactions are responsible for ontogenetic habitat shifts in *Xanthagrion erythroneurum*, a damselfly that undergoes developmental colour change. The juvenile males are yellow and change colour to red with sexual maturity. Field observations showed that the density of juvenile males is higher in adjacent woods than in primary mating arenas by ponds. We measured male-male interactions by the pond and in the woods, predicting the habitat switch would reduce male antagonistic interactions such as male aggression and male-male mating attempts. We showed that juvenile males receive less aggression in woods than in the pond mating arena. We conclude that lower population density and lower male encounter rates in the woods reduce the cost of male aggression for juvenile males. Our study provides evidence that stage-dependent habitat choice resulting from intrasexual antagonistic interactions may drive ontogenetic habitat shifts.

Introduction

Different developmental stages often require specific habitats (Moran, 1994), and habitat shifts at different developmental stages can generate stage-structured populations (Shine, Shine, & Shine, 2003). Habitat shifts are thought to increase fitness and survival at different life stages by optimizing the utilization of spatially distributed resources (Miller & Rudolf, 2011). Some evidence exists supporting physiological limitations, differential nutritional requirements, and predator avoidance as selective agents of ontogenetic habitat shifts (Grof-Tisza, Holyoak, Antell, & Karban, 2015; Moran, 1994). Antagonistic interactions within and between sympatric species are predicted to be potential drivers of ontogenetic habitat shifts (Delaney & Warner, 2017; Martin, Hoover, & Richardson, 2013; Morris, 2003), but are poorly supported by experimental evidence.

Antagonistic interactions between juveniles and adults are common in many vertebrate and invertebrate taxa, including damselflies (Corbet, 1999; Delaney & Warner, 2017; Martin et al., 2013). Damselflies shift from aquatic to terrestrial habitats after emerging from their larval stage (Corbet, 1999). The adults, however, return to aquatic habitats for breeding as the females oviposit in the water or on submerged plants (Corbet, 1999). Adult males assemble in mating arenas (lakes, ponds, or streams) and engage in scramble competition where they aggressively attack conspecifics to defend or access breeding ground (Corbet, 1999). In high-density assemblages, males often attempt to mate with conspecific males (Beatty et al., 2015; Miller, 1987). Adult male aggression and male mating attempts are costly for juvenile males (Gering, 2017). We predict that these costly interactions can be mitigated if juveniles relocate away from the aquatic breeding ground, thereby causing ontogenetic habitat shifts.

In this study, we test this prediction on *Xanthagrion erythroneurum* damselflies, which exhibit ontogenetic colour change whereby the juvenile males change colour from dull yellow to conspicuous red upon sexual maturity (Khan & Herberstein, 2019b). Juvenile dull colouration signals subordinance in animals, and can reduce adult aggression (Hawkins et al., 2012). In our species, dull juvenile colouration, however, does not reduce adult aggression within breeding area (Khan & Herberstein, unpublished data). We calculated the proportion of juvenile and adult males active in the pond breeding ground and in the adjacent woods, away from the breeding area, to quantify the developmental habitat shift. According to our prediction, juvenile males are expected to occupy woods over pond habitat if it reduces adult male aggression. We performed a behavioural experiment to determine male aggression and mating attempts with juvenile males in the pond and wood habitats. In accordance with our prediction, we found that juvenile males prefer wood habitats where they receive less aggression than in pond habitats.

Methods and materials

Study species and field sites

Xanthagrion erythroneurum is an Australian damselfly commonly found in ponds, lakes, and marshes. *Xanthagrion erythroneurum* exhibits ontogenetic colour changes: the juvenile males are yellow and attain a conspicuous red colour 6-7 days after emergence (Khan & Herberstein, 2019b). This yellow to red colour shift signals sexual maturity in males as only red males mate. Yellow males never mate, even in the absence of competitors (Khan & Herberstein, 2019b). Adult females, like adult males, are red in

colour, whereas juvenile (age 1- 7 days) and young adult females (7- 14 days) exhibit yellow body colour like juvenile males (Khan and Herberstein, unpublished data).

We conducted field studies at a pond situated on the North Ryde campus of Macquarie University, Sydney, Australia (33.772 S, 151.114 E). In the Sydney region, *Xanthagrion erythroneurum* start emerging in September. The juveniles are seen in flight until December, whereas the adults remain active until June (Khan & Herberstein, 2019a). We did not require permits for this study as it was conducted outside national parks and protected areas, and *Xanthagrion erythroneurum* is not protected in Australia.

Habitat selection

To determine whether red and yellow males prefer different habitats, we calculated damselfly frequencies by a pond (vegetation surrounding the pond, <5 meters from the pond edge) and within nearby woods (>10 meters from the pond edge, in bushy patches under tree cover). Selecting sunny days only, we slowly walked along the edge of the pond and woods and captured damselflies using an insect sweep net. During a single sample event, we collected males for 25-35 minutes in either habitat and then counted the number of red, yellow, and newly emerged yellow males captured in the pond and woods. We identified newly emerged males by their shiny wings. We released red and yellow males after counting them, however we kept newly emerged males for 2- 4 hours to allow their wings to harden before release. We marked the wings of damselflies before releasing them to avoid counting the same individuals more than once. We calculated damselfly frequencies in 2017 (n = 7 days), 2018 (n = 6 days), and 2019 (n = 3 days) between September - October when juveniles and adults co-occurred.

Male-male interaction

Xanthagrion erythroneurum females are non-aggressive to conspecifics, therefore, we only considered male-male interactions when assessing the effects of ontogenetic habitat shifts. Our interest is in understanding the habitat selection of yellow males, thus we experimentally investigated if yellow males received less male-male interactions (male approaches, male aggression, male-male copulation attempts) in the woods than by the pond using a modified damsel-on-a-dowel technique (Fincke et al., 2007). We glued the legs of live yellow males to dowels using UHU™ glue. We placed the dowels at the edge of the pond and in the woods, and measured the interactions from conspecific males. We observed the responses by sitting approximately one meter away from the dowel, which allowed us to observe focal damselflies clearly without disturbing regular movements of approaching damselflies. An approaching male can detect the focal male when it passes within 10 cm of the focal male (Fincke, 2015). We counted numbers of approaches when intruder males passed within 10 cm of focal males on the right or left. An approach can result in an aggressive response, a non-aggressive response or a mating attempt. If approaching males passed focal males without any physical contact, we recorded it as a non-aggressive response. When approaching males bit the focal males, we counted it as aggressive response. Finally, when approaching males tried to clasp the focal male (moving their cerci to the focal male's prothorax), or formed a tandem (physically connected to the focal male with their cerci) we counted it as a mating attempt. We measured interactions for a focal male for 10 minutes (one trial). We performed all trials (n = 41) on sunny days and used each male only once. At the end of the experiment, males were unglued, body length was measured and their wings were marked before they were released. As our hypothesis did not pertain to the habitat selection of red males, we did not include them in this set up.

1916 Statistical analyses

1917 We calculated the proportions of red, yellow, and newly emerged males collected from
1918 the pond and woods. The proportions of a male colour type can vary from 0 to 1 in their
1919 habitats. These type data such as proportions and rates are best fitted by beta regression
1920 model (Cribari-Neto & Zeileis, 2010). Therefore, we fitted a beta regression model to
1921 determine whether males prefer a certain habitat type. Beta regression models cannot deal
1922 with extreme values of 1 and 0, so we transformed male proportions by using the equation
1923 ($y * (n - 1) + 0.5) / n$, where y is the calculated proportion and n is the sample size
1924 (Smithson & Verkuilen, 2006). We fitted beta regression models using proportions of the
1925 three male categories as response variables, and habitat (pond or woods) as a covariate.

1926 We estimated population density in the pond and woods as the number of damselflies
1927 collected per minute (Iserbyt et al., 2013). We applied a linear mixed effect model (LMM)
1928 using male density as the response variable and habitat (pond or woods) as a fixed factor.
1929 We used study days as a random factor to account for abiotic factors (temperature, cloud
1930 cover, wind speed, and humidity) that might affect damselfly assemblage and density.
1931 We used the r.squaredGLMM function of the R package 'MuMIn' to determine the effect
1932 size of the model (Johnson, 2014).

1933 We fitted a generalized linear model (GLM) using overdispersed Poisson distribution
1934 (quasipoisson) to determine effects of habitat on the number of approaches received by
1935 focal males. We tested possible models with habitat and total body length as covariates
1936 and selected the best model using quasi Akaike's information criterion (QAIC). To
1937 account for zero inflation and overdispersion, we fitted zero-inflated generalized linear
1938 mixed models (ZIGLMM) to determine aggression and mating attempts received by focal

males in the pond and woods. We tested a number of models with habitat (pond and woods) and total body length of the focal damselfly as fixed effects, and experimental days as a random effect. We choose the best-fitting models using Akaike's information criterion corrected for small sample sizes (AICc) (see supplementary material for model selection). All models were fitted in R v 3.5.2 using the packages 'betareg' (Cribari-Neto & Zeileis, 2010), 'lme4' (Bates et al., 2019), and 'glmmADMB' (Fournier et al., 2012).

Results

Male proportions and population density in pond and woods

Proportions of newly emerged males were greater in the pond than the woods (beta regression: estimate = 2.01 ± 0.04, $z = 48.68$, $p < 0.001$, pseudo $R^2 = 0.98$; figure 1a). By contrast, proportions of juvenile yellow males were higher in the woods than the pond (beta regression: estimate = 1.88 ± 0.04, $z = 39.40$, $p < 0.001$, pseudo $R^2 = 0.97$; figure 1b). The proportion of adult red males, on the other hand, was higher in the pond habitat than the woods (beta regression: estimate = 1.04 ± 0.23, $z = 5.04$, $p < 0.001$, pseudo $R^2 = 0.59$; figure 1c). Population density of damselflies was higher in the pond than the woods (GLM: estimate = 1.10 ± 0.18, $t = 5.85$, $p < 0.001$, $R^2 = 0.46$; figure 1d).

Male-male interactions

The juvenile focal males received more approaches from the intruder males in the pond than in the woods (GLM: estimate = 2.33 ± 0.32, $t = 7.22$, $p < 0.001$, $R^2 = 0.78$; figure 2a). Similarly, the focal juvenile males in the pond received more aggression than those

in the woods (ZIGLMM: estimate = 2.88 ± 0.48, $z = 5.99$, $p < 0.001$; figure 2b). The focal males also received fewer mating attempts in the woods than the pond (ZIGLMM: estimate = 3.24 ± 1.06, $z = 3.05$, $p < 0.01$). Body size of the focal male, however, did not have a significant effect on received aggression (ZIGLMM: estimate = 0.18 ± 0.12, $z = 1.50$, $p = 0.13$) or mating attempts (ZIGLMM: estimate = 0.14 ± 0.36, $z = 0.39$, $p = 0.69$) and was removed from final models.

Discussion

Ontogenetic habitat shifts can occur due to nutritional requirements, to avoid predation, and to reduce intra- and interspecific antagonistic interactions. We showed that the proportion of juvenile males was higher in the woods, whereas adult males occurred in higher proportions in the pond. We further measured adult male aggression towards, and mating attempts with juvenile males and showed that juvenile males received less aggression and fewer mating attempts in the woods than the pond. We conclude that male antagonistic interactions (aggression and mating attempts) in the pond are significant contributors to the observed ontogenetic habitat shifts in *X. erythroneurum* damselflies.

Sexually mature *X. erythroneurm* males assemble in a mating arena (pond) and engage in scramble competition where they aggressively attack conspecific males (personal observation). These aggressive male-male interactions reduce male fitness and longevity (Gering, 2017). The juvenile males, on the other hand, do not benefit from occupying the pond as they are not sexually mature and do not mate. Thus, by moving to a different habitat, they can reduce adult aggression. Accordingly, our results showed that juvenile males can effectively evade male aggression by shifting to the woods. The mechanisms

for this are two-fold: firstly, fewer aggressive red males occur in the woods and secondly, overall population density, and therefore male interactions, are lower in the woods than the pond. Other possible mechanisms that we did not test here include a lower detection probability of yellow males due to greater background matching in the woods.

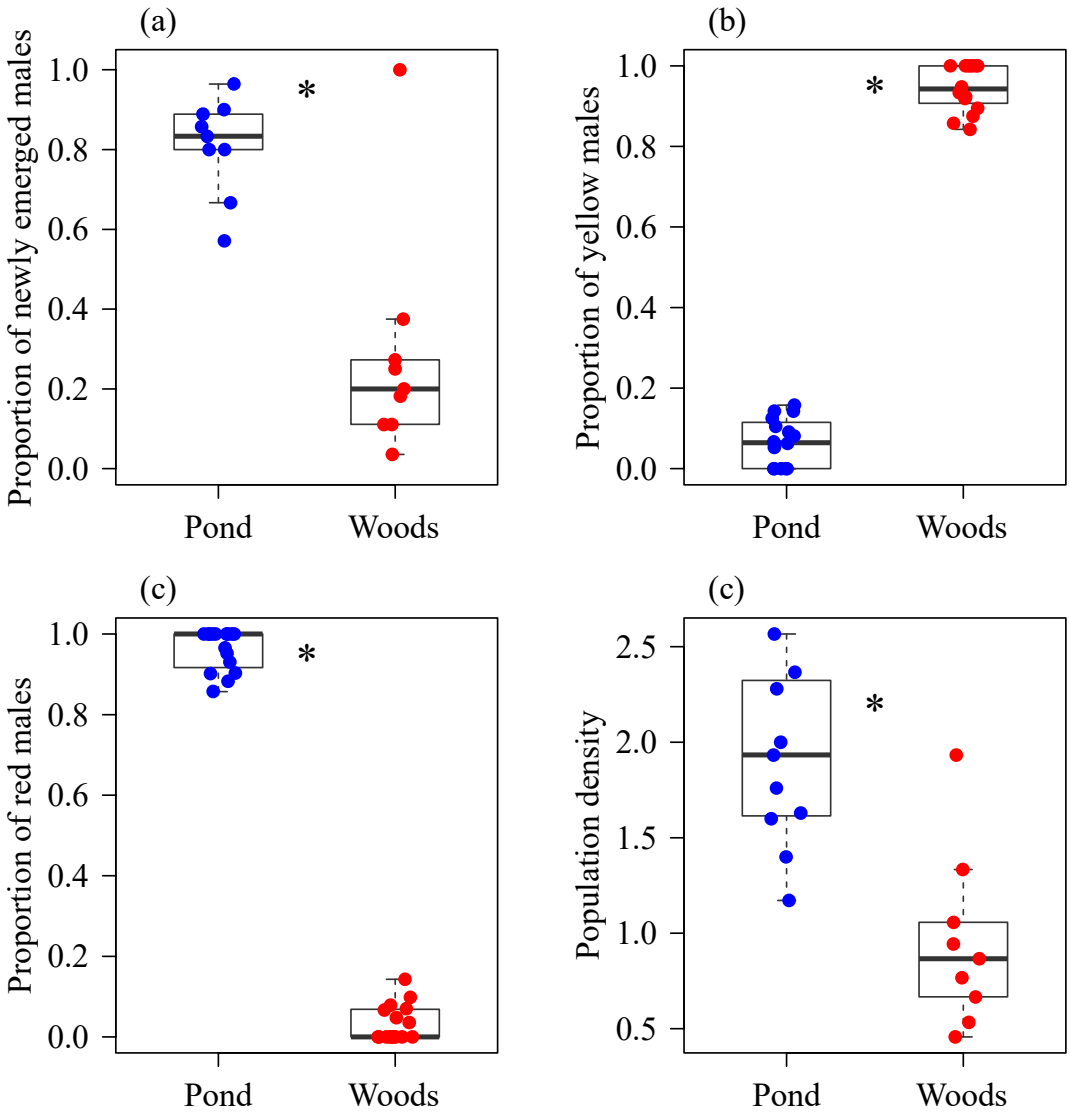
In addition to aggressive interactions, male-male mating attempts frequently occur in damselflies, especially in high male density assemblages (Miller, 1987). These male-male mating attempts are costly in terms of time, energy and unsuccessful mating attempts (Gering, 2017). Male morphological traits, such as blue abdominal bands, conspicuous body colouration, and behaviour such as refusal display, can reduce male-male mating interactions (Khan & Herberstein, 2019a; Sherratt & Forbes, 2001). Our experiment showed that juvenile males that reside in the pond habitat are likely to incur male-male mating attempts. The adult males probably misidentified juvenile males as females because of the body colour similarities between juvenile males and juvenile females. Since adult males and females are similarly red in colour, misidentification among adult males is also possible and reduced due to the male-specific abdominal blue bands that function as antiharassment aposematic signals and reduce male-male mating interactions (Beatty et al., 2015; Khan & Herberstein, 2019a). While juvenile males also have these blue bands, they are much paler (personal observation) and likely to be less effective than habitat shifts.

While our study makes a convincing case that juvenile males enjoy a selective benefit from habitat shifts based on reduced aggression and male-male mating attempts, we

2006 cannot, however, exclude the possibility that the woods additionally provide selective
2007 benefits such as food resources or protection from predators.

2008

2009



2010

2011 Figure 1: Male proportions and population density in pond and woods. a) Proportions of
2012 newly emerged males (n = 8), b) proportions of yellow males (n = 16), c) proportions of
2013 red males in the pond and the woods (n = 16), d) population density (number of captured
2014 individuals/minute) in the pond (n = 11) and the woods (n = 9). Bold lines indicate
2015 medians. Boxes enclose 25th to 75th percentiles. Error bars enclose the data range,
2016 excluding outliers. Dots are data points of each study day; dots that are vertically outside
2017 the error bars are outliers, > 1.5 times the interquartile range.

2018

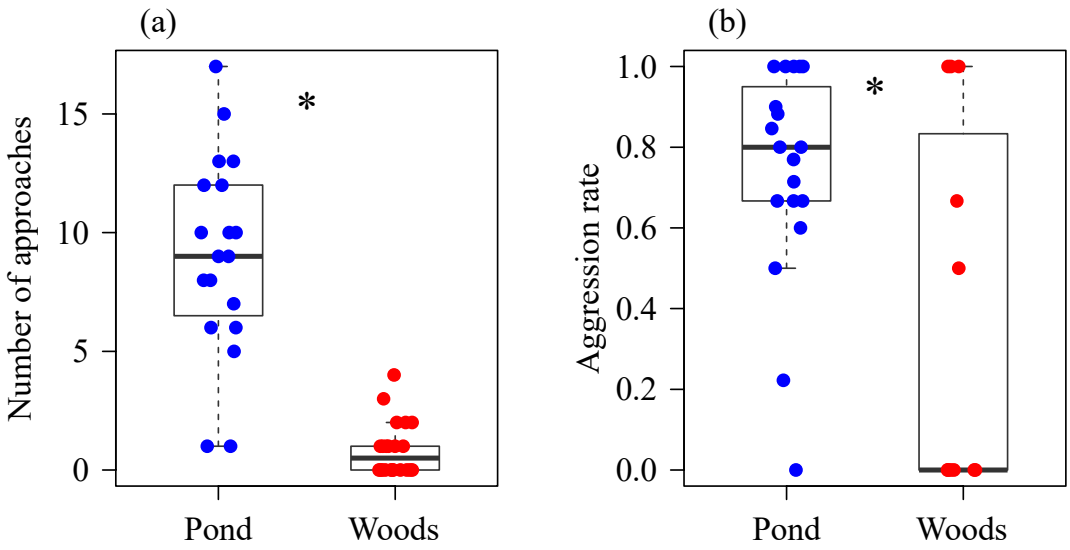


Figure 2: Male-male interactions in the pond and the woods. a) Number of approaches and b) aggression rate (number of attacks/number of approaches) received by the focal males in the pond (n = 19) and the woods (n = 22). Bold lines indicate medians. Boxes enclose 25th to 75th percentiles. Error bars enclose the data range, excluding outliers. Dots are data points; dots that are vertically outside the error bars are outliers, > 1.5 times the interquartile range.

Data accessibility: All raw data are available via github https://github.com/KhanKawsar/Habitat-shift

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Female red colouration is an anti-harassment signal in damselflies

Md Kawsar Khan

Abstract

Conspicuous female colouration can evolve through male mate choice or via female-female competition thereby increasing female mating success. However, when mating is not beneficial such as in the pre-reproductive females, selection should favour cryptic rather than conspicuous colouration to avoid male detection and the associated harassment. Yet, conspicuous female colouration occurs in many juvenile animals, however its evolution remains an enigma. Here, I studied conspicuous female colouration in *Agriocnemis femina* damselflies, in which the conspicuous red colour of the juvenile females changes to a less conspicuous green approximately a week after their emergence. I measured female fecundity and found that red females are lower in fecundity. Finally, I calculated the occurrence frequency and mating frequency of red and green females in several populations over a three-year period. The results demonstrate that red females mated less frequently than green females even when red females were abundant in the populations. I concluded that conspicuous female colouration functions as a warning signal of sexual unprofitability, thereby reducing sexual harassment for females and unprofitable mating for males.

Introduction

Female ornamentation such as conspicuous colouration can evolve as a by-product of genetic correlation with males (Amundsen, 2000; Darwin, 1871). This non-adaptive hypothesis of female colouration has been supported by both theoretical and empirical evidence (Kraaijeveld, 2014; Poissant, Wilson, & Coltman, 2010). Recent phylogenetic comparative studies, however, demonstrated that female conspicuous colouration has often evolved independently of male colouration (Burns, 1998; Dale, Dey, Delhey, Kempenaers, & Valcu, 2015). Alternatively, conspicuous female colouration may be selected for if it provides adaptive benefits such as increased survival (Wallace, 1877; West-Eberhard, 1983) or mating advantages (Clutton-Brock, 2009). There is evidence for male mate preference for conspicuous female colouration (Amundsen & Forsgren, 2001; Griggio et al., 2009), and selective advantage of conspicuousness during female-female competition for mating (Berglund & Rosenqvist, 2009; Rosvall, 2011). Conspicuous female colouration can therefore evolve in animals in which females compete over mates because mating provides either direct or indirect benefits to females (Amundsen, 2000; Rosvall, 2011) or because males are limited (Kokko & Mappes, 2005).

In male-biased mating systems, however, securing a mate is not thought to be a challenge for females; rather females experience repeated mating attempts from males, which is defined as male mating harassment (Clutton-brock & Parker, 1995). Male mating harassment can reduce female fitness (Helinski & Harrington, 2012), fecundity (Gosden, Svensson, Andrade, & Whitlock, 2009; Rossi, Nonacs, & Pitts-Singer, 2010), and longevity (Mühlhäuser & Blanckenhorn, 2002). Selection has favoured female phenotypic traits that reduce male mating harassment such as female-limited polymorphism (Takahashi et al., 2014), male mimicry (Huang & Reinhard, 2012; Iserbyt

et al., 2013; Takahashi & Watanabe, 2011), cryptic female colouration (Fincke, 2015), and behaviours such as death feigning (Khelifa, 2017), and mating refusal displays (Chan et al., 2009; Ide, 2011). Female limited colour polymorphism reduces mating harassment by not conforming to the male mate search image. Males search for the common morph for mating in a frequency dependent manner, thereby reducing mating harassment of the rare morph (Gosden et al., 2009; Van Gossum, Stoks, & De Bruyn, 2001). Alternatively, male mimicry and cryptic female colouration reduces male mating harassment by reducing detection by searching males (Fincke, 2015). After male detection, females can exhibit death feigning and mating refusal displays to avoid unprofitable mating interactions.

Mating can be unprofitable for pre-reproductive females irrespective of the mating systems. As pre-reproductive females do not have mature eggs, they are unlikely to produce viable offspring if mated unless sperm is stored for long periods. Rather, mating would incur costs for both males and females, such as increase predation risk (Almbro & Kullberg, 2008; Kemp, 2012), reduce foraging time (Arnqvist & Nilsson, 2000), that would consequently reduce fitness and fecundity upon adulthood (Kreiter & Wise, 2001; Taborsky, 2006; Zajitschek, Hunt, Jennions, Hall, & Brooks, 2009). Juvenile females can avoid mating harassment by staying away from the breeding area (Corbet, 1999; Hinnekint, 1987), displaying refusal behaviour (Chan et al., 2009), signalling unwillingness via pheromones (Ferrero et al., 2013), mimicking male colouration (Hammers, Sánchez-Guillén, & Van Gossum, 2009; Huang & Reinhard, 2012; Willink et al., 2019), or avoiding male detection by inconspicuous colouration (Baldauf, Bakker, Kullmann, & Thünken, 2011; Fincke, 2015; Vilela et al., 2017). Dull colouration further reduces predator and prey detection, which in turn increases survival and foraging

(McQueen et al., 2019; Outomuro et al., 2017). Dull juvenile female colouration therefore has evolved via natural and sexual selection and is predominant in nature (Corbet, 1999; McQueen et al., 2019). Yet surprisingly, conspicuous female colouration occurs in juvenile females, especially in damselflies (Corbet, 1999; Hammers et al., 2009), however, why it does, remains an evolutionary enigma.

Here, I aim to determine the selective benefits of the conspicuous female colouration using *Agriocnemis femina* damselflies as a model system. Thoracic colouration of *A. femina* can be either conspicuous red or cryptic green in females, but always green in males. I reared these female damselflies under field conditions, and found that female colour forms are developmental; juvenile females are red, and they change colour to green upon reaching sexual maturity. Then, I tested if the red females are more conspicuous than the green females using a model of damselfly vision. I also performed morphological analysis to determine if the colour signals fitness and fecundity. I examined the occurrence frequency and mating frequency of red and green females in several populations to determine the function of the conspicuous juvenile female colouration. I predicted: 1) conspicuous red females would be subject to more mating attempts than green females, if female red colour attracts males, 2) red females would mate in proportion to their occurrence frequency if males neither prefer nor avoid the red females, and 3) red females would mate less than their occurrence frequency if males avoid mating with the red females.

Materials and Methods

Study species

Agriocnemis femaina is a small damselfly (~22mm body length) of the Coenagrionidae family (Zygoptera: Odonata). This species is widely distributed in South-east Asia, and frequently occurs on ponds, lakes, marshes, and agricultural lands (Shah & Khan, 2019). *Agriocnemis femina* females exhibit ontogenetic colour change where the females' thoracic colouration change from red to green but males' thoracic colouration remain unchanged (Figure 1a-b). Ontogenetic habitat shift is common in damselflies (Corbet, 1999). This species, however, does not show such ontogenetic dispersion; instead, the red and green females cohabit with the males throughout the lifecycle (personal observation).

Study sites

Agriocnemis femina is a common species in the north-eastern region of Bangladesh (Khan, 2015, 2018). I conducted the field studies between April and July on three different populations (one study site in 2014 and three study sites in 2015 and 2017) in the north-eastern region of Bangladesh. No permission was required to conduct the field work or to collect the specimens because this species is not endangered and the field sites were not part of national parks or protected areas.

Damselfly rearing

I collected damselflies with shiny wings, as shiny wings indicate that they are newly emerged. I reared the damselflies in insect rearing cages (30×30×60 cm). The cages were placed in the field from 25th May to 10th June 2017 in the damselflies' natural habitat, where they received natural light (approximately 13.30 hours per day) and precipitation. Inside the cage, I kept small submerged plants in a small water tank so that the damselflies

could drink water from the tank and perch on the plants. The damselflies were provided field caught insects every morning as food supply. I also sprayed the cage with water twice a day (at 0800 and 1500) to keep the damselflies hydrated. I monitored the damselflies for colour change at regular intervals.

Reflectance spectrometry

I measured the reflective spectra of *A. femina* males and females using a JAZ EL-200 spectrophotometer (Ocean Optics, USA) with a PX-2 pulsed light source. I also measured spectra of the leaf vegetations around the marshes to quantify the visual background. I set the spectrophotometer to a constant boxcar width of 10 and integration time of 50 milliseconds. I took all spectra in a dark room and measured the reflectance relative to a white standard (Ocean Optics, USA) to standardize the measurements. I set the spectrophotometer probe perpendicular to the damselflies or leaves. I recorded three reflectance spectra from the thorax and abdomen of each damselfly and each background leaf, then averaged the three measurements to quantify the spectra of the damselfly or the plant. I processed the reflectance spectra with OceanOptics Spectrasuite software (ver. 1.6.0_11) and binned the spectra to 1 nm wavelength intervals before minor LOESS smoothing ($\alpha = 0.35$).

Visual modelling

I applied the receptor noise model to determine the colour (chromatic) and luminescence (achromatic) contrast of the green and red females against their background (Vorobyev et al., 2001; Vorobyev & Osorio, 1998). This model calculates the detectability between

two colours for a specific observer in just noticeable difference (JND) units. A JND value greater than 1 suggests that the observer can distinguish between the compared colours (Vorobyev et al., 2001). The receptor noise model has previously been applied in behavioural studies to predict colour discriminability in various taxa including odonates (Barry et al., 2015; Chan et al., 2009; Huang & Reinhard, 2012).

In this study, I modelled the colour differences in a damselfly visual system to determine: 1) if the conspecific males can distinguish the red and green females against their background 2) if the red females are more conspicuous than the green females, and 3) whether the males can distinguish between red and green females. The photoreceptor sensitivities of *Agriocnemis femina* are unknown. The known visual system of Coenagrionidae damselflies (the same family as *A. femina*) showed evidence for trichromatic and tertachromatic visual systems (Henze et al., 2013; Huang et al., 2014). Hence, I used both trichromatic and tetrachromatic models to calculate the chromatic and achromatic contrast of the red and green females against their backgrounds (See supplementary method for details). I applied the photoreceptor sensitivities of *Ischnura heterosticta* and *Ischnura elegans* for trichromatic and tetrachromatic visual modelling respectively (Henze et al., 2013; Huang et al., 2014; Khan & Herberstein, 2019a).

Female fecundity estimation

I captured female damselflies from the field using an insect sweep net and immediately transferred them into 95% ethanol. I transported the damselflies back to the laboratory for fecundity estimation. Then, I took morphometric measurements such as total body length, body mass, and measured volume of the fourth abdominal segment as an indirect

indicator of fecundity (Iserbyt et al., 2011). I also measured female fecundity directly by examining whether females possess eggs and by counting egg numbers when they were present. First, I placed the damselflies on an absorbent paper for exactly two minutes to evaporate the ethanol and then weighted the damselfly on a balance (Mettler toledo, accuracy 0.01 mg). Next, I positioned the damselflies laterally and took digital photographs using a Canon 600D camera with Canon EF 55-250 lens. I measured the total body length, and the length and width of the fourth abdominal segment of the damselflies from the digital photographs using the ImageJ software (Schneider et al., 2012). Later, I calculated the volume of the fourth abdominal segment (S4) using equation (S4 volume = 1/2 × width S4 × π × length S4) (Iserbyt et al., 2011). Finally, I dissected the females under a stereomicroscope and counted the total number of eggs.

Occurrence frequency and mating frequency

I calculated the occurrence frequency and mating frequency of females in the field to test whether mating is frequency dependent in this species. I captured damselflies using an insect sweep net while walking through the marshes. I sweep the net in a 'figures of eight' manner, which captured all damselflies in the vegetation without any bias (Van Gossum, Beirinckx, Forbes, & Sherratt, 2007). I marked the wings of the damselflies before releasing them to avoid repeated counting of the same damselflies. First, I counted the number of non-mating females to determine the occurrence frequency of the red and green females. Then, to determine the mating frequency of the females, I counted the numbers of the mating females over the next three days so that the relative abundance frequency remains approximately the same. I conducted all field studies on sunny days.

2218

2219 Statistical analyses

2220 I calculated the chromatic and achromatic contrast of a damselfly against all background
2221 leaves and then averaged them to determine the chromatic and achromatic contrast of a
2222 damselfly against its background. I calculated the effect size (Cohen's d) using the
2223 equation:

2224 $$\text{Cohen's } d = \frac{M2 - M1}{\sqrt{\frac{SS1 + SS2}{N1 + N2}}},$$

2225 where M2 and M1 are mean, SS1 and SS2 are sum of squares, and N1 and N2 is the
2226 sample number of the compared groups, respectively. I calculated 95% confidence
2227 interval of the estimated effect size using a bootstrapping method with 10000 replicates.
2228 I fitted linear mixed effects models to analyse the fitness (total body length and body
2229 mass) of the females using female colour as covariate and field sites as a random effect.
2230 I used r.squaredGLMM function of the R package 'MuMIn' to determine the effect size
2231 of the models (Johnson, 2014). I applied a beta regression model to analyse the mating
2232 rate of the females using the mating proportions as response variable, and female colour
2233 and female abundance frequency as covariates. I choose the best-fitting beta regression
2234 model using Akaike's information criterion (AIC) (see supplementary table 1 for model
2235 selection).

2236 To determine if males have a baseline preference for or avoidance of one of the female
2237 colours or if matings are frequency dependent, I measured male mate choice (Manly $\hat{\beta}$)
2238 for the red and green females in each study population using the equation:

$$\hat{\beta} = \frac{\frac{e_1}{A_1}}{\frac{e_1}{A_1} + \frac{e_2}{A_2}},$$

where e_1 and e_2 are the numbers of mated red and green females observed in the field, and A_1 and A_2 are the total number of red and green females observed during the survey. The Manly Beta, $\widehat{\beta}$, can range between 0 and 1, with a value of 0.5 indicating no preference or avoidance. I applied a one sample t-test to determine if $\hat{\beta}$ differed from 0.5, a significantly lower $\hat{\beta}$ indicates baseline avoidance of that female form. I calculated the effect size Cohen's d for this one sample t-test as: Cohen's d = (0.5 - mean ($\hat{\beta}$))/ standard deviation ($\hat{\beta}$), and calculated the confidence interval by bootstrapping method with 10000 replicates. I analysed all the data in R version 3.5.2 (R Development Core Team, 2018) using packages 'lme4' (Bates et al., 2019), 'pavo' (Maia, et. al., 2019), and 'bootES' (Kirby & Gerlanc, 2013), 'MuMIn' (Bartoń, 2019), 'betareg' (Cribari-Neto & Zeileis, 2010).

Results

Colour change

In the rearing condition, all newly emerged *Agriocnemis femina* males and females attained a green and red colour respectively a few hours after emergence. All the captive-reared red females (n = 18) then changed colour to green six to seven days after their emergence. The males, however did not exhibit further colour change and stayed green. After changing colour to green, the females did not reverse colour change to red again after another three days of observation.

Reflectance spectra

The reflectance spectra of the male and green female thorax showed a similar Gaussian peak between 526 – 585 nm and 526 – 560 nm respectively whereas the red female thorax spectra peaked between 668 – 694 nm (Figure 1b), which was distinctly different from the thorax of males and green females. The abdominal spectra of males and green females peaked between 526 – 530 nm (Figure 1c). The red female abdomens showed a peak between 667 – 698nm, which was distinctly different from green males and green females (Figure 1c). The background leaves showed a Gaussian peak between 548 – 553nm, which was very similar to males and the green females (Figure 1b-1c)

Visual modelling

In tetrachromatic damselfly vision, the chromatic and achromatic contrast between the background and the thorax (Figure 2a-2b) and abdomen (Figure 2d-2e) of green and red females was greater than one JND. Therefore, a male would be able to discern the females against their background using either the colour or luminescence channel. Moreover, the chromatic and achromatic contrast between red and green females was greater than five JND, suggesting that the males could discriminate between the red and green females (Figure 2c, 2f). The achromatic contrast of the red female thorax against the background was significantly greater than that of the green female thorax (Mann Whitney U test: $W = 0$, $p < 0.001$; Cohen's $d = 6.84$, 95% CI = [4.28, 9.28]). Similarly, the chromatic contrast of the red female thorax against the background was significantly greater than that of the green female thorax (Mann Whitney U test: $W = 60$, $p < 0.001$; Cohen's $d = 1.56$, 95% CI = [0.88, 2.26]). Furthermore, the abdomen of red females showed significantly greater

achromatic contrast than that of green females (Welch Two Sample t-test: t = -17.01, df = 19.91, p <0.001; Cohen's d = 5.25, 95% CI = [3.89, 6.94]) but the chromatic contrast was not significantly different (Two Sample t-test: t = 0.98, df = 36, p = 0.33; Cohen's d = 0.32, 95% CI = [-0.35, 0.97]). The interpretation did not change when we used the trichromatic visual system (Supplementary material).

Female fecundity

Red females had shorter total body length than the green females (LMM: estimate = -0.82 ± 0.15, t = -5.55, p < 0.001, R_2 = 0.22) (Figure 3a). Additionally, the body mass of the red females was significantly lower (LMM: estimate = -2.32 ± 0.30, t = -7.84, p < 0.001, R_2 = 0.22) than that of the green females (Figure 3b). The volume of the fourth abdominal segment of red females were smaller than that of the green females (Two Sample t-test: t = -3.93, df = 46, p < 0.001, Cohen's d = 1.14, 95% CI = [0.49 - 1.69]) (Fig 3c). The egg analysis of the females showed that the red females (9 out of 10) did not have eggs (Exact binomial test: p <0.001) (Figure 3d), while green females carried on average 28.4 ± 10.09 eggs.

Frequency independent mating

The beta regression model showed that female occurrence frequency did not have a significant effect on mating (beta regression: estimate: 0.53 ± 1.02, z = 0.52, p = 0.60, R_2 = 0.95) (Figure 4a). Female colour, however, did have a significant effect on mating, with red females mating significantly less frequently than green females (beta regression:

2306 estimate: -4.78 ± 0.26, z = -18.46, $p < 0.001$, $R_2 = 0.95$). The $\hat{\beta}$ for the red females was
2307 less than 0.5 in all studied populations. A baseline mating avoidance was observed for
2308 the red females (One Sample t-test: t = 22.26, df = 6, $p < 0.001$, Cohen's d = 8.41; 95%
2309 CI = [7.25, 8.85]) (Figure 4b).

2310

2311 Discussion

2312 Conspicuous female colouration can evolve through male mate choice or through mate
2313 avoidance. Here, I tested if these mechanisms can explain the conspicuous female
2314 colouration in *Agriocnemis femina* damselflies, where the females can have either red or
2315 green colouration. I measured the conspicuousness of the females against their
2316 background using damselfly vision, and found that the red females are more conspicuous
2317 than the green females. I also showed that the red females are sexually immature with no
2318 developed eggs. Finally, I found that red females mated less frequently than the green
2319 females, irrespective of their abundance.

2320 If conspicuous female colouration evolved through male mate preference for brighter
2321 female colouration, we would expect greater mating frequency in the red females than
2322 the green females. Contrary to these expectations, we found that red females mated less
2323 frequently than green females, thereby rejecting the male preference for conspicuous
2324 female colouration hypothesis. The lower mating rates of red female could be because:
2325 1) males failed to recognise the red females as potential mates (recognition error), or 2)
2326 males had no preference for female colour, hence the lower mating for the red females
2327 results from their lower frequency in the populations (frequency dependent mating), or
2328 3) the red colour repelled males that avoided mating with the red females (anti-harassment

aposematism). Damselflies use visual cues (thorax colour and pattern, abdomen colour), and morphological cues (body size, abdomen size and shape) for mate recognition (Gorb, 1998; Miller & Fincke, 1999; Pezalla, 1979; Winfrey & Fincke, 2017; Xu et al., 2014). Therefore, one might argue that males failed to recognise the red females as mates because of their colour and size differences to green females. However, in addition to colour and morphology, male damselflies also uses behavioural (flight pattern, display), tactile and chemical cues to identify mates (Frati et al., 2015; S. Gorb, 1992; Ubukata, 1983; Utzeri, 1988; Winfrey & Fincke, 2017). Therefore, the males could potentially recognise differently coloured females based on these other cues. In support of this, it has been shown in several female limited colour polymorphic damselflies that males recognise and mate with two or more differently coloured females (Iserbyt et al., 2013; Sánchez-Guillén et al., 2017; Takahashi et al., 2014, 2010). In fact, male mate choice is plastic in damselflies and based on social experience, males learn to change their preference from one female colour morphs to another (Miller & Fincke, 1999; Sánchez-Guillén et al., 2013; Van Gossum, De Bruyn, & Stoks, 2005). Furthermore, males can even learn to accept novel coloured females as potential mates (Xu et al., 2014), so recognition error is unlikely to be the mechanism behind the lower mating of the red females.

Male mate choice can be frequency dependent, where males mate with the most common female morph without exhibiting preference for a particular female colour. In *Ischnura elegans*, *Nehalennia irene,* and *Ischnura senegalensis* damselflies, males do not show a preference for any female colour, rather male mate choice varies among populations relative to the most abundant female morphs (Gosden et al., 2009; Iserbyt et al., 2013; Takahashi et al., 2010; Van Gossum et al., 2001). Here, however, red females were mated

less frequently irrespective of their frequency in the population, suggesting that males have a frequency independent avoidance of red females. Overall, the results indicate that the conspicuous female red colour repelled males thereby reducing their mating frequency.

The fecundity analysis showed that red females were sexually immature without any developed egg. Therefore, mating would be costly in terms of time and energy for the pre-reproductive females (Takahashi & Watanabe, 2010). Similarly, it would be unprofitable for the males to mate with the red females as time, energy and resources (sperm) would be wasted on red females that cannot produce viable offspring (Perry & Tse, 2013; Scharf, Peter, & Martin, 2013). Therefore, selection should favour traits to reduce these unprofitable matings. Considering these data, it is likely that sexually immature females advertise their unprofitability as mates with their conspicuous red colour.

Conspicuous colouration when signalling unprofitability is known as aposematic colouration (Ruxton, Allen, Sherratt, & Speed, 2018). Aposematic colouration can act as an interspecific signal, such as the conspicuous colours of poison frogs and tiger wood moth advertise their unprofitability and toxicity to predators, thereby reducing predation (Lindstedt et al., 2011; Saporito, Zuercher, Roberts, Gerow, & Donnelly, 2007). Aposematic colouration, however, can function as an intraspecific signal to warn off conspecifics, for example, the bright colouration of the swallowtail caterpillar that repels conspecific females from laying eggs on the same plant (Papaj & Newsom, 2005). The concept of aposematic colouration has also been applied in sexual contexts, where the conspicuous male colour repels conspecific males thereby reducing male-male mating. This has been referred to as anti-harassment aposematism (Beatty et al., 2015; Khan &

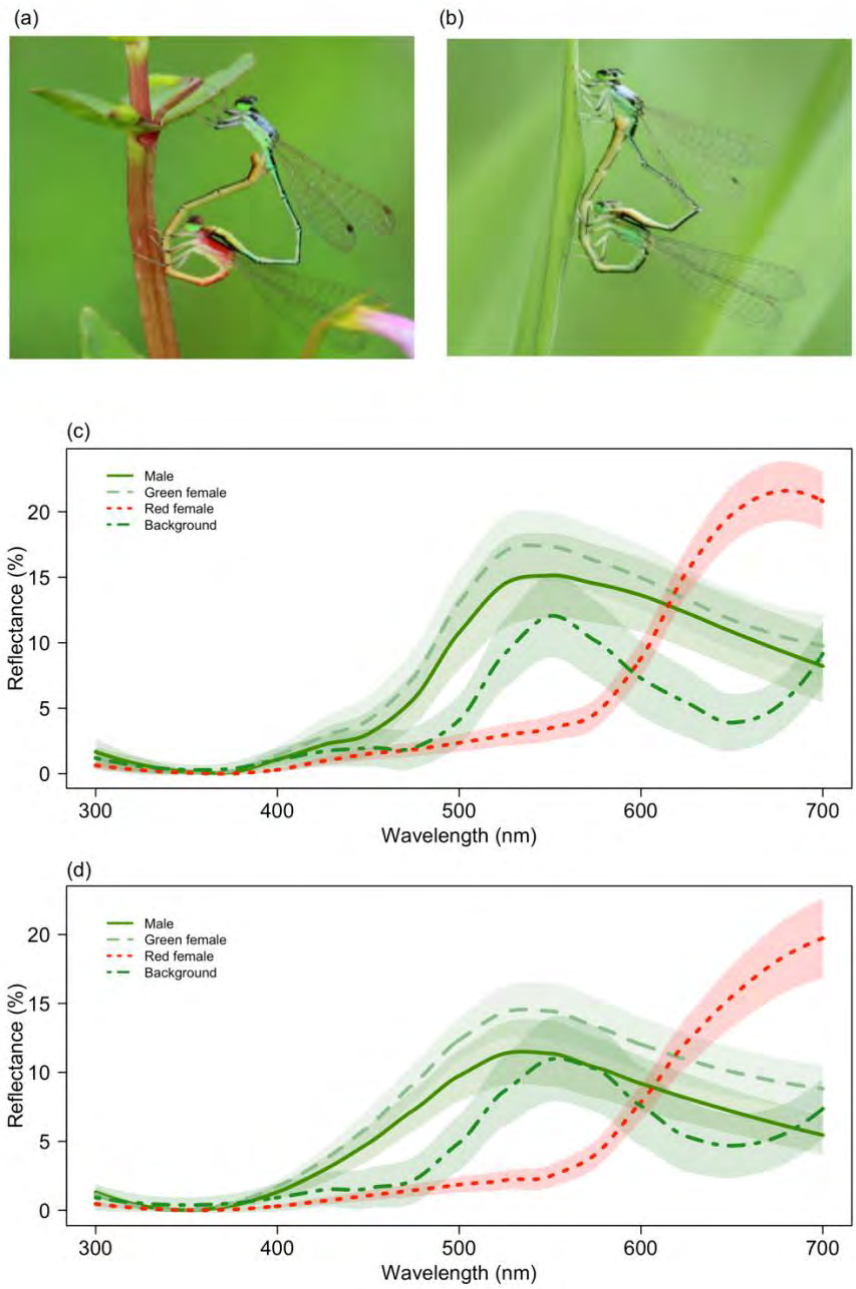
Herberstein, 2019a; Sherratt & Forbes, 2001). Here, for the first time, I showed that conspicuous female colouration functions as an anti-harassment aposematic signal to reduce unwanted mating harassment from conspecific males.

The damselfly visual modelling suggests that, the aposematic red colour of the female thorax and abdomen is highly conspicuous to the receiver male. Aposematic signals are often conspicuous, as this facilitates receiver learning, reduces mistakes and retains the memory of the conspicuous signals for longer (Gamberale-Stille, 2000; Skelhorn, Halpin, & Rowe, 2016). Moreover, red is considered as classic aposematic colour (Cott, 1940), as it generates high contrast against green backgrounds, and therefore maximizes the detection of the aposematic signal. Dorsal red colour in poison frogs, and red color in the upper wings of butterflies maximizes the display of the aposematic signal to their predator birds, thereby increasing the efficacy of the aposematic signal to warn off the predators (Dreher et al., 2015; Kang, Cho, Lee, & Jablonski, 2016; Maan & Cummings, 2012; Su et al., 2015). Similarly, thoracic and abdominal red colouration of *A. femina* females would maximize the presentation of the aposematic signal to the males that typically approaches from behind when intending to mate.

Is the aposematic colouration of female *A. femina* an exception or might we expect similar functions for conspicuous female colour in other animals or odonates? The conspicuous rufous colour of the common cuckoo, *Cuculus canorus*, reduce male mating harassment (Lee, Kim, Yoo, & Yoo, 2019). A preliminary study on the congeneric *Agriocnemis pygmaea*, where the juvenile females are red and change colour to green upon adulthood, suggests a similar function for red immature females (supplementary material). Moreover, female red colouration is known from at least 20 out of 44 known species of the *Agriocnemis* genus (Khan, unpublished data), suggesting that red colouration has

evolved in this genus to reduce juvenile mating harassment. Furthermore, conspicuous colouration in juvenile females is known to occur in other genera, e.g., *Ischnura capreolus*, *Argiocnemis rubescens* and *Mortonagrion aborense* (Vilela et al., 2017); however, the adaptive significance of their conspicuous colouration has not yet been tested experimentally.

While mating during the pre-reproductive period can be detrimental for female fitness, and fecundity, juvenile mating harassment is common in species where juvenile and adult females cohabit with mature males (Hammers et al., 2009; Sirot & Brockmann, 2001). Selection is expected to favour juvenile female traits that reduces male mating harassment during pre-reproductive stages. In *Ischnura heterosticta* and *Ischnura elegans* damselflies, the juvenile females reduce mating harassment by mimicking the male colouration (Huang & Reinhard, 2012; Willink et al., 2019). In this study, I present a novel antiharassment strategy in *Agriocnemis femina* damselflies: the conspicuous red colouration of the juvenile females functions as an anti-harassment aposematic signal, thereby reducing male mating harassment. This finding raises the tantalising questions why the red female colouration is not maintained into adulthood, as it has been shown previously that multiple female colour morphs reduce harassment and increase fecundity (Takahashi et al., 2014). Further studies are needed to determine predation and foraging consequences of the conspicuous juvenile female colouration.



2421

2422 Figure 1: Reflectance spectra of the damselflies. a) photograph of a mating wheel of a
2423 male and a red female and b) mating wheel of a male and a green female. c) aggregated
2424 reflectance spectra (mean ± SD) of the thorax of males (n = 22), green females (n = 20),
2425 red females (n = 21) and background plant leaves (n = 21). d) aggregated reflectance
2426 spectra (mean ± SD) of the abdomen of males (n = 11), green females (n = 18), red
2427 females (n = 20) and background plant leaves (n = 21).

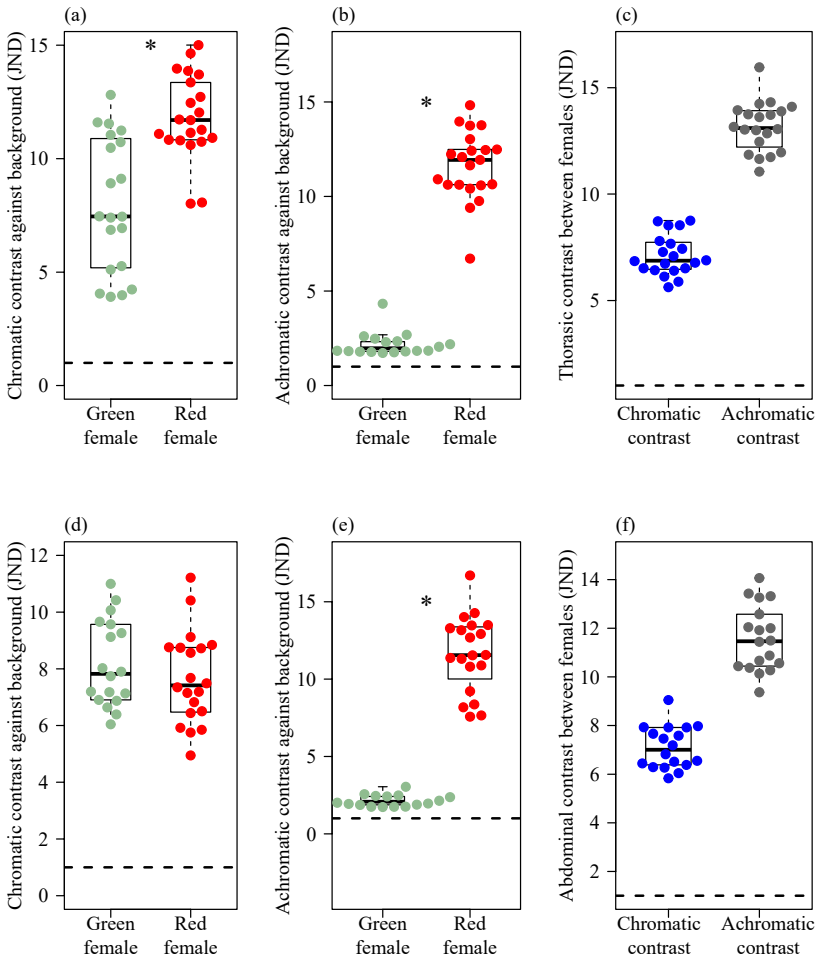


Figure 2: Discriminability of damselflies in the tetrachromatic damselfly vision (a) chromatic contrast and (b) achromatic contrast of thoraxes of red (n = 21) and green females (n = 20) against the natural background; (c) chromatic and achromatic contrast between the thorax of red (n = 20) and green females (n = 20); (d) chromatic contrast and (e) achromatic contrast of the abdomen of red (n =20) and green females (n = 18) against the natural background; (f) chromatic and achromatic contrast between the abdomen of red (n = 18) and green females (n = 18). The box plots represent the median, and the 75th and 25th percentiles. The whiskers extend to the minimum and maximum, but exclude outliers that are beyond 1.5 times the interquartile range. Circles indicate individual values. * $p < 0.001$.

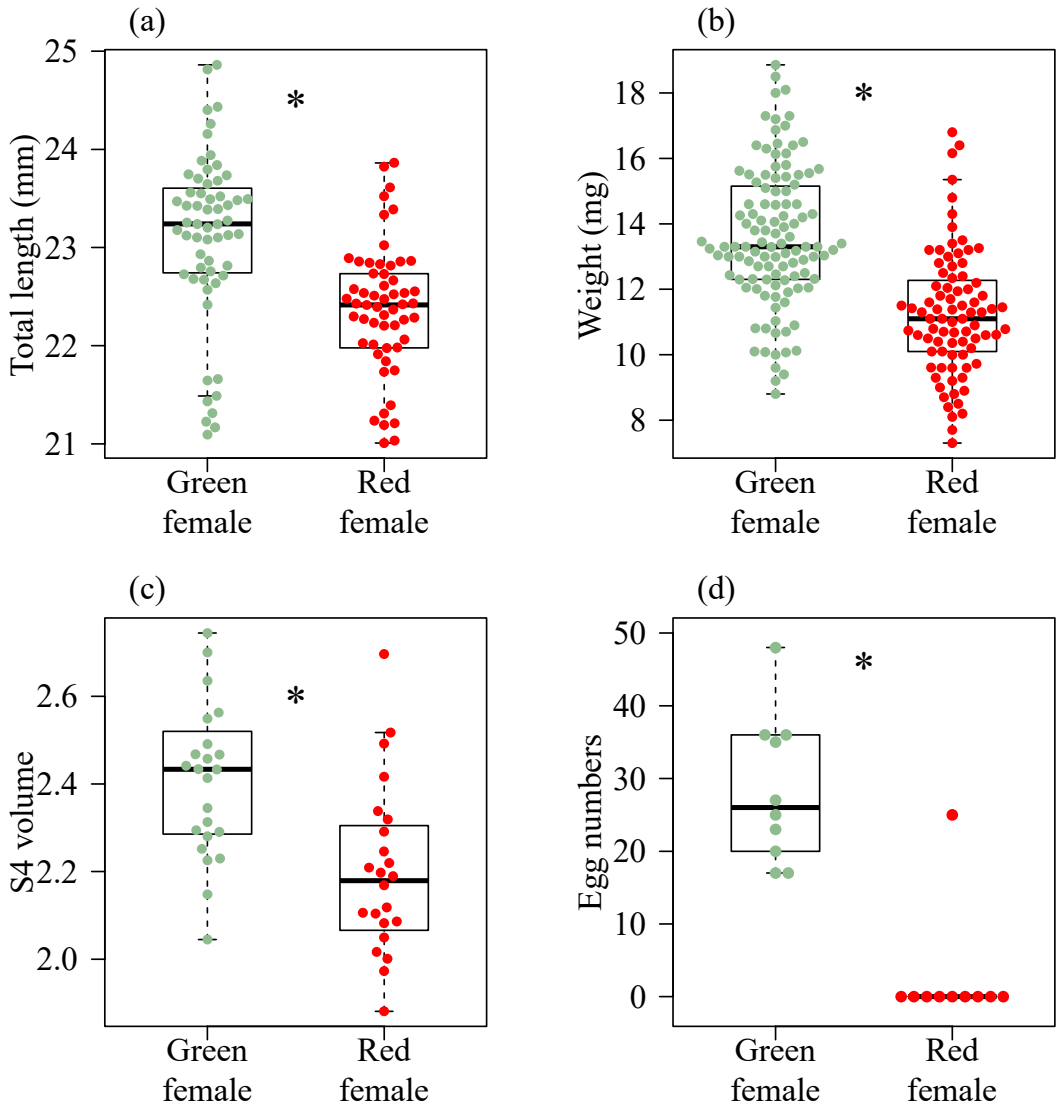


Figure 3: Fitness and fecundity of the female damselflies a) total body length of red females (n = 59); b) and green females (n = 59); b) body mass of red females (n = 83) and green females (n = 112); c) volume of fourth abdominal segment of red females (n = 19) and green females (n = 24); d) egg counts of red females (n = 10) and green females (n = 10). Boxplots depict the median, the 25th and 75th percentile. The whiskers extend to the minimum and maximum, but exclude outliers that are beyond 1.5 times the interquartile range. Circles indicate individual values * p <0.0001.

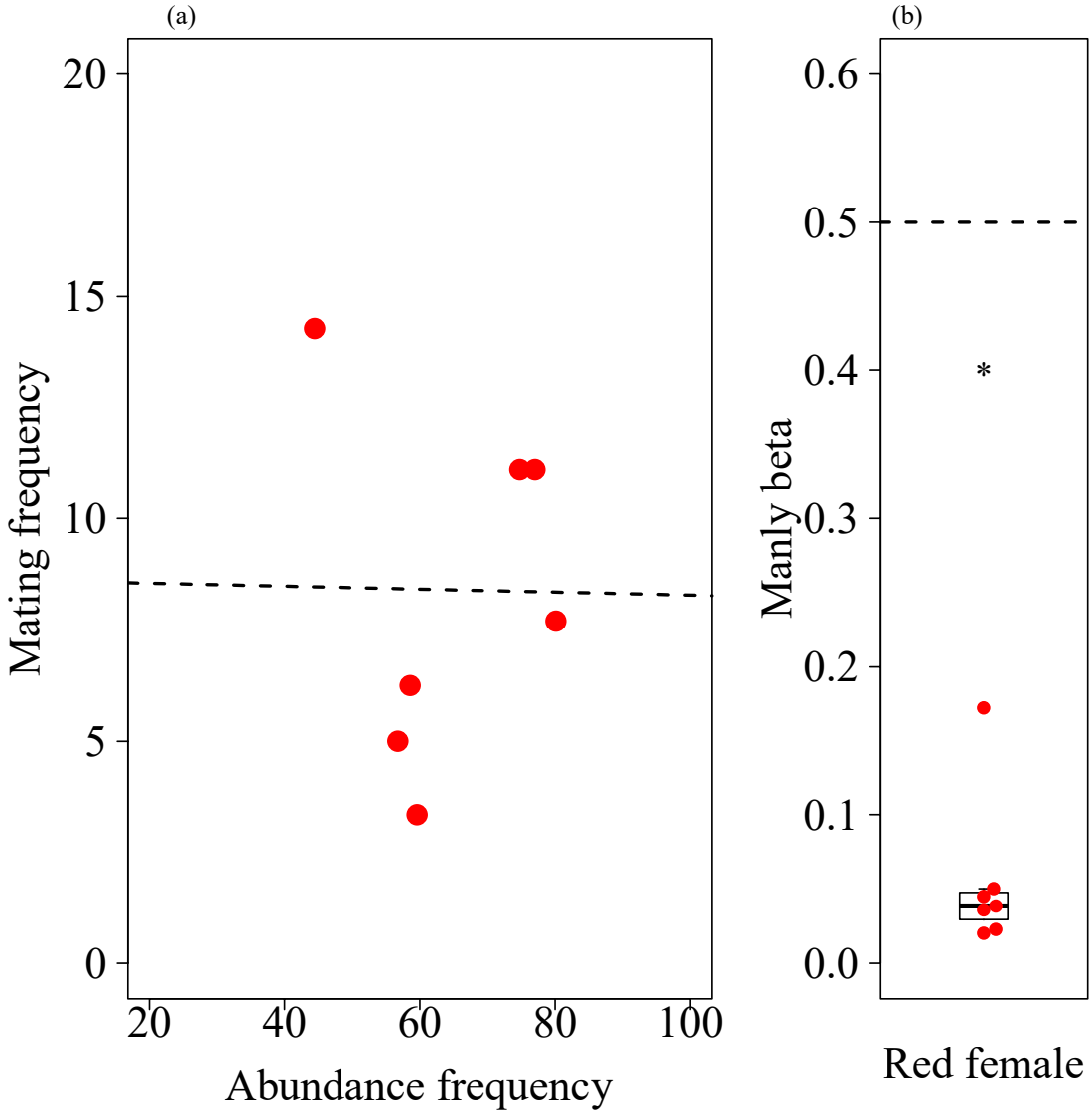


Figure 4: The relation between occurrence frequency and mating frequency. a) abundance frequency and mating frequency of red females in the studied populations (n = 7). horizontal line indicates the linear regression line. b) Manly beta ($\widehat{\beta}$) of red females in seven studied populations. The horizontal dashed line specifies $\widehat{\beta}$ 0.5; values less than 0.5 indicate baseline avoidance of red females. Boxplots depict the median, the 25th and 75th percentile. The whiskers extend to the minimum and maximum, but exclude outliers that are beyond 1.5 times the interquartile range. Each circle represents $\widehat{\beta}$ of a population* p <0.001.

Supplementary material

Supplementary method:

Visual modelling:

First, I calculated the quantum catch (Qi) for each photoreceptor i as follows-

$$\mathrm{Qi} = \int_{300}^{700} S(\lambda) I(\lambda) Ri\,(\lambda) d\lambda,$$

Where λ is the wavelength, $S(\lambda)$ is the reflectance spectra of damselfly integument or each background leaf, $I(\lambda)$ is the light spectrum entering the eye, and $\mathrm{Ri}(\lambda)$ is the spectrum sensitivity of the photoreceptor i. I used a standard daylight spectrum (D65) as $I(\lambda)$ (Wyszecki & Stiles, 1982).

Then, I calculated the noise of each class of photoreceptor (e_i) as,

$$e_i = \frac{\bar{\omega}}{\sqrt{n_i}}.$$

where ω is the Weber fraction assigned to each receptor and n_i is the relative density of the receptor class *i*. I applied a Weber fraction of 0.12, and photoreceptor proportions 1:0.471:4.412, which have been applied to damselfly visual modelling previously (Khan & Herberstein, 2019a; Schultz & Fincke, 2013).

Finally, the chromatic contrast (ΔS) between a damselfly spectrum and the background was calculated using the equation:

$$\Delta S = \sqrt{\frac{{e_1}^2(\Delta f_3 - \Delta f_2)^2 + {e_2}^2(\Delta f_3 - \Delta f_1)^2 + {e_2}^2\,(\Delta f_1 - \Delta f_2)^2}{(e_1 e_2)^2 + (e_1 e_3)^2 + (e_2 e_3)^2}}$$

where Δf_i is the log of quantum catches for receptor *i* between the damselfly and the background.

The achromatic contrast (ΔL) was calculated as green receptor contrast with the equation:

$$\Delta L = \frac{\Delta f_i}{e_i}$$

For the tetrachromatic damselfly modelling, I used photoreceptor sensitivities 370 nm, 440 nm, 540 nm, 600 nm (Henze et al., 2013) with a photoreceptor density 2:2.5:2.5:1 (Armett-Kibel & Meinertzhagen, 1983), and a Weber fraction 0.12 (Schultz & Fincke, 2013). The chromatic contrast (ΔS) was calculated as the equation:

$$\Delta S = \sqrt{\frac{(e_1e_2)^2(\Delta f_4 - \Delta f_3)^2 + (e_1e_3)^2(\Delta f_4 - \Delta f_2)^2 + (e_1e_4)^2(\Delta f_3 - \Delta f_2)^2 + \\ + (e_2e_3)^2(\Delta f_4 - \Delta f_1)^2 + (e_2e_4)^2(\Delta f_3 - \Delta f_1)^2 + (e_3e_4)^2(\Delta f_2 - \Delta f_1)^2}{(e_1e_2e_3)^2 + (e_1e_2e_4)^2 + (e_1e_3e_4)^2 + (e_2e_3e_4)^2}}$$

The achromatic contrast for the tetrachromatic visual system was calculated using the same equation as for the trichromatic visual system. I preformed the visual modelling in R v 3.5.2 (R core team, 2018) using the package pavo v 2.1 (Maia et al., 2019).

Occurrence frequency and mating frequency of *Agriocnemis pygmaea*

I calculated occurrence frequency and mating frequency of the *Agriocnemis pygmaea* females by following the same method described in the main text.

I transformed the mating frequency by using the equation (y * (n − 1) + 0.5)/n to account the extreme values 1 and 0, where n is the sample size (Smithson & Verkuilen, 2006). I

applied a beta regression model to analyse the mating rate of the females using the transformed percentage of mating as response variable, and female colour and female abundance frequency as covariates.

Supplementary result:

Trichromatic visual modelling:

The chromatic and achromatic contrast between the background and the thorax and abdomen of green and red females was greater than one JND in trichromatic damselfly vision. Therefore, a male with a trichromatic vision would be able to discern the females against their background using either the colour or luminescence channel. Moreover, the chromatic and achromatic contrast between red and green females was greater than one JND, suggesting that the males could discriminate between the red and green females.

In trichromatic vision, the achromatic contrast of the red females thoraxes against the background was significantly greater than that of the green females (Mann Whitney U test: W = 0, $p < 0.001$; Cohen's d = 7.24, 95% CI = [4.56, 9.81]), but the chromatic contrast was not significantly different (Two Sample t-test: t = 1.18, df = 39, $p = 0.25$; Cohen's d = 0.37, 95% CI =[-0.27, 1.01). Similarly, the abdomen of red females showed significantly greater achromatic contrast than green females (Welch Two Sample t-test: t = -23.67, df = 24.08, $p <0.001$; Cohen's d = 5.40, 95% CI = [4.05, 7.08]), but chromatic contrast was not significantly different in trichromatic (Welch Two Sample t-test: t = 1.17, df = 33.03, $p = 0.251$; Cohen's d = 3.9, 95% CI = [2.92, 4.72]).

2518 Occurrence frequency and mating frequency of *Agriocnemis pygmaea*

2519 Similar to *A. femina* females, *A. pygmaea* red females mated significantly less frequently
2520 than green females (beta regression: estimate: - -6.06 ± 0.14, z = -41.89, $p < 0.001$, R^2 =
2521 0.99). The $\hat{\beta}$ for the red females was less than 0.5 in all seven surveyed populations. A
2522 baseline mating avoidance was observed for the red females (One Sample t-test: t = -
2523 131.08, df = 6, $p < 0.001$, Cohen's d = 49.54; 95% CI = [48.61, 50.07]).

2524

Supplementary Figure:

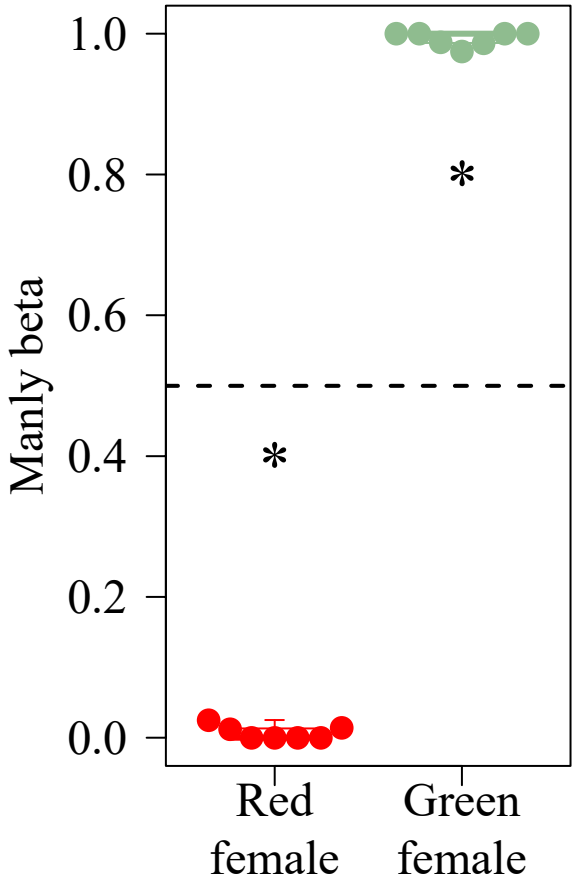


Figure S1: Manly beta ($\widehat{\beta}$) of red and green females in seven studied populations. The horizontal dashed line specifies $\widehat{\beta}$ 0.5; values less than 0.5 indicate baseline avoidance of red females. Boxplots depict the median, the 25th and 75th percentile. Each circle represents $\widehat{\beta}$ of a population* p <0.001

Supplementary table:

Table S1. Best model selection among from the possible models. Estimates of the Akaike's information criterion (AIC) is provided for each model. Bold indicates the most informative model.

Best model selection	
Model	**AICc**
~female colour * female frequency	-46.26
~ female colour + female frequency	-44.26
~ female colour	**-48.05**
~ female frequency	-7.36

Data accessibility: All raw data are available via github (https://github.com/KhanKawsar/AntiharassmentAposematism)

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Conclusions

Inter- and intrasexual colour variation such as colour polymorphism, polyphenism and polychromatism frequently occurs due to sexual selection, and ontogenetic colour change (Booth, 1990; White & Kemp, 2016). The causes and consequences of conspicuous colouration (sexual dimorphism and ontogenetic colour changes) at different life stages are well studied in territorial systems, (Córdoba-Aguilar, 2002; Siva-Jothy, 1999; Watanabe & Taguchi, 1990) but poorly understood in non-territorial mating systems. Here, I aim to study the adaptive significance of conspicuous colouration in ontogenetic colour changing damselflies. I tested existing hypotheses to determine the function of conspicuous colouration of males and females at different developmental stages.

Selection is not predicted to act on ornamental male colouration in non-territorial mating systems (Clutton-Brock, 2007; Darwin, 1871). Male conspicuous colouration, however, evolved in many non-territorial species and their function remains an evolutionary puzzle. In non-territorial damselflies, conspicuous male colouration is predicted to evolved via three non-mutually exclusive hypotheses; 1. intersexual selection through female preference (Corbet, 1999; Córdoba-Aguilar & Cordero-Rivera, 2008; Fincke, 1997), 2. intrasexual selection via male-male competition for matings (Conrad & Pritchard, 1992), and 3. intrasexual selection to avoid male-male mating attempts (Sherratt & Forbes, 2001). I tested these hypotheses to determine the function of conspicuous male colouration (blue abdominal bands and red thoracic colouration) in *Xanthagrion erythroneurum.*

In non-territorial damselflies, males do not perform courtship display and females do not seem to actively choose their mate (Corbet, 1999). Males, however, need the cooperation of females to form a mating wheel for sperm transfer. Consequently, male ornamental colour could evolve via female mate choice. *Xanthagrion erythroneurum* females, however, did not prefer males with blue bands over males without blue bands (Chapter 1) and red males over yellow males (Chapter 3). The absence of female preference for any male colouration is probably because of the specific damselfly mating system where males approach and grab females from behind to form a tandem (first step of mating where males attached with females with their cerci). As a consequence of that females possibly cannot see the approaching males properly.

My research found support for hypothesis 2 that male conspicuous colouration evolved through intrasexual selection via male-male competition for mating. The conspicuous red *Xanthagrion erythroneurum* males received less aggression than yellow males from conspecific males as well as from heterospecific males. In this mating system, males assemble in the breeding area where they search for potential mates. Although males do not defend a breeding ground like territorial damselflies, male-male competition in this mating system can arise when more than one male tries to mate with the same female, or tries to acquire the same mating, perching and oviposition sites. Furthermore, males share these breeding resources with sympatric species resulting in interspecific male-male competition as well. My research suggests that conspicuous red colouration of male function as honest signal of identity thereby reducing conspecific and heterospecific aggressions. The observed lower aggression towards red males in the breeding area is likely to assist red males with accessing and maintaining access to the breeding resources, which ultimately will increase mating frequency.

According to the antiharassment aposematic hypothesis (hypothesis 3), conspicuous colouration of non-territorial damselflies has been predicted to evolve to reduce male-male mating attempts (Sherratt & Forbes, 2001). Male-male mating attempts frequently occur in damselfly and other for example, moor frogs (Sztatecsny et al., 2012) mating system when a large number of males assemble for mating (Corbet, 1999; Miller, 1987). These male-male mating attempts are costly for males in terms of energy, time, lost mating opportunity, and they reduce male fitness and survival (Gering, 2017). My research showed that conspicuous blue abdominal bands and red thoracic colour of males reduce male-male mating attempts (Chapter 1 and 3). Taken together, my findings provide evidence that conspicuous colouration in non-territorial damselflies has a dual function - to reduce male-male mating attempts and to increase mating success. Conspicuous colouration of males, however is likely to increase visibility to predators, therefore could increase predation risks, a prediction I could not experimentally test during my PhD.

Ontogenetic colour change can signal sexual maturity in animals (Nicolaus et al., 2007). I tested how developmental colour change is associated with sexual maturity in *X. erythroneurum* and *Agriocnemis femina* damselflies. I showed that the red to green colour shift in *A. femina* females and yellow to red shift in *X. erythroneurum* males signal sexual maturity (Chapter 2 and Chapter 5). The status signalling hypothesis suggests that dull juvenile colouration reduces aggressions from adult males (Hawkins et al., 2012). Juvenile yellow colour of *Xanthagrion erythroneurum*, however, did not experience reduced aggressions in the breeding area, instead yellow males received more aggression than red males (Chapter 3). Juvenile males, however, can alter their behaviour to reduce antagonistic interactions (Keren-Rotem, Bouskila, & Geffen, 2006; Martin et al., 2013).

Accordingly, my findings showed that yellow males shifted habitats from ponds (their emerging and primary mating arena) to woods. In the woods, yellow males received less antagonistic interactions (aggressions and male-male mating attempts) than in ponds (Chapter 4). This finding supports the idea that ontogenetic habitat shifts can evolve to avoid intraspecific agnostic interactions. However, my results raise the question why juvenile males are not red or attain red colouration more quickly to reduce the adult aggressions. It is likely that the juvenile males cannot change colour to red because of resource constrains or to avoid predation during pre-reproductive period.

Different selection pressure can act on males and females resulting in sexual dimorphism, sex limited-colour change or sex-limited ontogenetic colour change (Booth, 1990; Svensson, 2017). I found the opposite pattern of colour change in males and females in the two studied species; *Xanthagrion erythroneurum* males become more conspicuous during ontogenesis whereas in *Agriocnemis femina* females become less conspicuous during ontogenesis (Chapter 2 and Chapter 5). Conspicuous juvenile colouration is unlikely to evolve via male mate choice as mating with pre-reproductive females will not produce viable offspring unless females can store sperms for a sufficient duration. Even so, the pervasive last male sperm precedence in damselflies is unlikely to select for mating with young and immature females (Córdoba-Aguilar & Cordero-Rivera, 2008).

Conspicuous colouration, however, can function as anti-harassment aposematic signal to reduce unprofitable mating encounters (Chapter 1; Beatty et al., 2015; Sztatecsny et al., 2012). We therefore, predicted that the conspicuous juvenile colouration might act as signal to reduce male mating harassment during pre-reproductive season. I studied population frequency and mating frequency in multiple populations and found that red females were less likely to mate, irrespective of their frequency in the population. I

conclude that red colouration of the damselflies functions as anti-harassment aposematic signals in damselflies to reduce mating harassment during pre-reproductive stages. These results raise further intriguing questions such as why the females change from red to green in this species. It is possible that the colour change is required to secure matings in adult females or to reduce predation risk posed by the red females because of their increased conspicuousness to predators, ideas that are still to be tested.

Future directions

My thesis identified the selective benefits of conspicuous colouration at different ontogenetic stages in male and female damselflies, however, there are additional unanswered questions required to understand the significance of ontogenetic colour change. For example, is ontogenetic colour change an adaptive strategy or a non-adaptive consequences of resource constraints? I showed that there is clear advantage of being red, why, then, are juvenile males yellow? There are two possible explanations: first, the production of red colour pigment is costly (such as carotenoid based red colour pigment (Hill, 2000)) and thus yellow males are not able to produce red because of resource constraints (Hill, 1991). Second, the red colour is not costly (such as pterin based red colours) but the animals remain dull yellow because of the associated selective benefits such as predator avoidance and prey detection (Fabricant, Kemp, Krajíček, Bosáková, & Herberstein, 2013). Future studies, should explore the mechanism of colour production to understand the cost of colour production. Moreover, predator and prey detection of the red and yellow males against their natural habitats will reveal if dull juvenile colouration provides concealment and camouflage relative to red males.

In *Agriocnemis femina* damselflies juvenile red colouration reduced mating harassment. Harassment by males reduces females fitness and fecundity and is a common phenomenon in scramble competition mating systems (Takahashi et al., 2014). Our study raises the question why the females change from red to green despite the selective benefits of red colouration. We believe, although red colour lowers mating harassment, it also increases predator and prey detection as red females are more conspicuous than green females against their natural green backgrounds. On the other hand, the green colour probably increases mating harassment but reduces predator and prey detection. It is likely that mating harassment is more deleterious for juveniles than adults therefore the juveniles trade off sexual harassment with higher predation. However, observations on predator-prey interactions of the red and green females in their natural habitats are needed to understand the trade-offs and to decipher the causes of ontogenetic colour change in these damselflies.

In conclusion, in this thesis, I aimed to understand the functional significance of conspicuous colouration in ontogenetic colour changing damselflies. I showed that conspicuous male colouration in non-territorial damselflies evolve through male-male competition for mating and to reduce unprofitable male-male mating interactions but not via female mate choice. I showed juvenile males exhibit ontogenetic habitat shift that reduces intraspecific antagonistic interactions. On the other hand, conspicuous female colouration functions to lower unprofitable mating encounters in the pre-reproductive stages.

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