Mechanical and tactile incompatibilities cause reproductive isolation between two young damselfly species

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Abstract

External male reproductive structures have received considerable attention as an early-acting cause of reproductive isolation (RI), because the morphology of these structures often evolves rapidly between populations. This presents the potential for mechanical incompatibilities with heterospecific female structures during mating and could thus prevent interbreeding between nascent species. Although such mechanical incompatibilities have received little empirical support as a common cause of RI, the potential for mismatch of reproductive structures to cause RI due to incompatible species-specific tactile cues has not been tested. We tested the importance of mechanical and tactile incompatibilities in RI between *Enallagma anna* and *E. carunculatum*, two damselfly species that diverged within the past ~250,000 years and currently hybridize in a sympatric region. We quantified 19 prezygotic and postzygotic RI barriers using both naturally occurring and lab-reared damselflies. We found incomplete mechanical isolation between the two pure species and between hybrid males and pure species females. Interestingly, where mechanical isolation was incomplete, females showed greater resistance and refusal to mate with hybrid or heterospecific males compared to conspecific males, which suggests that tactile incompatibilities involving male reproductive structures can influence female mating decisions and form a strong barrier to gene flow in early stages of speciation.

1

29   **Introduction**

30   Understanding speciation requires identifying how reproductive isolation (RI) is initiated

31   and maintained in the early stages of population divergence (Coyne and Orr 2004; Butlin et al.

32   2012). Over the past century, speciation researchers have used a variety of experimental and

33   comparative approaches to identify which barriers appear most important in causing RI early in

34   the speciation process. These efforts have revealed that sexual isolation and ecological

35   divergence tend to evolve earlier than hybrid sterility and inviability in both plants (*e.g.,* Grant

36   1992; Ramsey et al. 2003; Husband and Sabara 2004; Kay 2006) and in animals (*e.g.,* McMillan

37   et al. 1997; Price and Bouvier 2002; Mendelson and Wallis 2003; Dopman et al. 2010; Sánchez-

38   Guillén et al. 2012; Williams and Mendelson 2014; Castillo et al. 2015). Prezygotic isolation

39   also typically evolves faster in sympatry than in allopatry, and hybrid sterility typically evolves

40   faster than hybrid inviability (Coyne and Orr 1997; Presgraves 2002; Price and Bouvier 2002;

41   Russell 2003). Identifying the traits that diverge to cause RI underlying these broad patterns is a

42   major goal of speciation research.

43   One set of traits that has received much attention because of their rapid rates of

44   evolutionary change is external reproductive structures. In internally fertilizing animals, male

45   intromittent genitalia are among the fastest-evolving external morphological traits, and genital

46   morphological variation can affect reproductive fitness within species (Eberhard 1985; Otronen

47   1998; Danielsson and Askenmo 1999; House and Simmons 2003; Rodriguez et al. 2004; Bertin

48   and Fairbairn 2005; Simmons et al. 2009). Likewise, non-intromittent contact or grasping

49   structures often show similar patterns of rapid, divergent evolution, and divergence in these

50   structures can also affect reproductive success within species (Arnqvist 1989; Bergsten et al.

51   2001; Wojcieszek and Simmons 2012).

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52   Rapid divergence of reproductive structures between populations has been hypothesized

53   to cause RI via two different mechanisms. The first is mechanical incompatibility (Dufour

54   1844), in which structural incompatibilities between male and female genitalia of different

55   species prevent successful copulation and reproduction. Mechanical incompatibilities have been

56   documented in some animal species pairs (Jordan 1896; Standfuss 1896; Federley 1932; Schick

57   1965; Paulson 1974; Sota and Kubota 1998; Tanabe and Sota 2008; Kamimura and Mitsumoto

58   2012; Sánchez-Guillén et al. 2012; Wojcieszek and Simmons 2013; Sánchez-Guillén et al. 2014;

59   Anderson and Langerhans 2015), although this mechanism of RI has not received broad support

60   as a common mechanism of RI between young species (Shapiro and Porter 1989; Masly 2012;

61   Simmons 2014).

62   The second proposed mechanism is tactile incompatibility (de Wilde 1964; Eberhard

63   1992), in which mismatch between male and female genitalia of different species prevents or

64   reduces the success of mating and reproduction because one or both sexes fail to stimulate the

65   other in the proper species-specific manner. The essence of this idea is that female reproductive

66   decisions are based on the pattern of tactile stimuli transmitted by the male, and improper

67   stimulation can result in female refusal to mate, early termination of mating, or lowered

68   postcopulatory fitness, including reduced reproductive fitness in hybrid offspring (Eberhard

69   2010). Tactile isolation likely operates in a similar manner as other sensory modalities involved

70   in mate choice and species recognition such as auditory or chemical signals, in which

71   quantitative variation exists in male traits and female preferences (Ryan and Wilczynski 1991;

72   Shaw 1996; Tregenza and Wedell 1997; Singer 1998; Johansson and Jones 2007). If females

73   discriminate among the mating structures of conspecific mates, female discrimination against

74   heterospecific males can arise as a byproduct of sexual selection within species (reviewed in

75   Panhuis et al. 2001; Turelli et al. 2001; Simmons 2014). Thus, any mismatch between male

76   morphology and female response to stimulation from a particular morphology could result in

77   reduced reproductive success when females mate with a heterospecific male or an interspecific

78   hybrid male. The importance of tactile incompatibilities remains unknown, although there is

79   good reason to expect that these incompatibilities may occur frequently (Simmons 2014), and

80   therefore have the potential to play a significant role in the evolution of RI.

81   Because identifying the effects of tactile incompatibilities requires carefully quantifying

82   mating behavior and physiology, these incompatibilities have often been overlooked in tests of

83   RI involving divergence of reproductive structures (Masly 2012). Nonetheless, some evidence

84   for tactile incompatibility in the absence of mechanical incompatibilities exists in butterflies

85   (Lorkovic 1953, 1958), scarab beetles (Eberhard 1992), *Drosophila* (Coyne 1993; Price et al.

86   2001; Frazee and Masly 2015 – but see LeVasseur-Viens et al. 2015), and sepsid flies (Eberhard

87   2001). Notably, damselflies (Odonata, suborder Zygoptera) are often touted as a prime example

88   of the importance of both mechanical and tactile incompatibilities in RI among closely related

89   species. The potential for either mechanism to cause RI has been particularly well described in

90   the families Lestidae and Coenagrionidae, whose males do not engage in premating courtship or

91   visual displays (Williamson 1906; Krieger and Krieger-Loibl 1958; Loibl 1958; Paulson 1974;

92   Tennessen 1975; Robertson and Paterson 1982; Hilton 1983; Battin 1993; Sánchez-Guillén et al.

93   2012; Sánchez-Guillén et al. 2014). Male damselflies have two sets of paired grasping organs at

94   the end of their abdomen (Fig 1). A male initiates the mating sequence by grasping the female's

95   thorax with these appendages to form the "tandem" position. The species-specific male

96   appendages and female thoracic structures engage such that structural mismatch appears to

97   prevent many heterospecific tandems from forming. Mechanical isolation appears to be a major

98   cause of RI in *Ischnura* (Krieger and Krieger-Loibl 1958; Sánchez-Guillén et al. 2014). For

99   *Ischnura* species pairs with incomplete mechanical isolation, tactile isolation has been suggested

100   to contribute to RI (Sánchez-Guillén et al. 2012; Wellenreuther and Sánchez-Guillén 2016),

101   although this idea has not been tested quantitatively.

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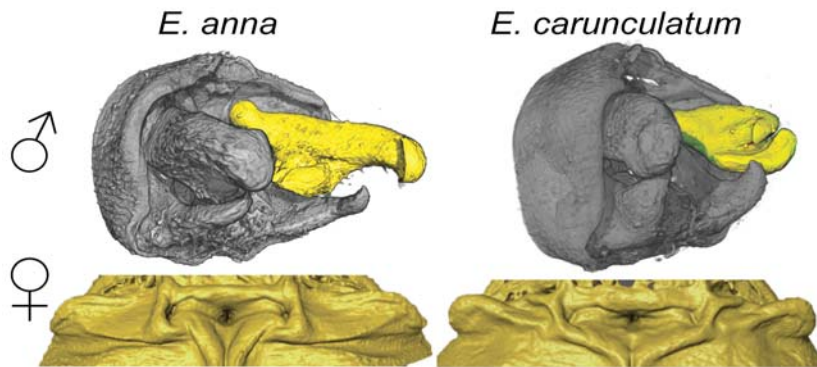
Fig 1. Male grasping appendages and female mesostigmal plate morphology. The right
cercus on each male is shaded yellow.

106

107   Mechanical isolation also appears to prevent many heterospecific tandems in *Enallagma,*

108   the most speciose North American genus (Paulson 1974; Miller and Fincke 2004; Fincke et al.

109   2007). Divergence in reproductive structure morphology is associated with a relatively recent

110   *Enallagma* radiation (250,000-15,000 years ago; McPeek et al. 2008). Importantly, the rapid

111   morphological diversification was not accompanied by marked ecological divergence among

112   many *Enallagma* species (Siepielski et al. 2010). Although male cerci (superior terminal

113   appendages) and female thoracic plates show a pattern of correlated evolution within *Enallagma*

114   species (McPeek et al. 2009), species-specific divergence in these structures does not always

115   cause strong mechanical incompatibilities, and interspecific tandems are occasionally observed

116   (Paulson 1974; Tennessen 1975; Bick and Bick 1981; Forbes 1991; Miller and Fincke 2004;

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117   Fincke et al. 2007). After tandem formation, female *Enallagma* control whether or not

118   copulation occurs and they typically refuse to mate with heterospecifics or males whose cercus

119   morphology has been manipulated (Robertson and Paterson 1982). *Enallagma* mesostigmal

120   plates contain mechanoreceptors in species-specific locations that appear to be contacted by the

121   male cerci during tandem, which may allow female assessment of a male's cercus morphology

122   (Robertson and Paterson 1982).

123   Although prezygotic isolating barriers appear to evolve earlier than postzygotic barriers

124   in damselflies (Sánchez-Guillén et al. 2012; Sánchez-Guillén et al. 2014), the relative importance

125   of mechanical and sensory mechanisms of prezygotic RI remains unclear for two reasons. First,

126   it can be difficult to distinguish between mechanical and tactile mechanisms experimentally: if a

127   male-female pair fails to form a tandem, it is often unclear whether the incompatibility is purely

128   mechanical or whether it involves tactile or behavioral cues that cause one sex to reject the other

129   (Tennessen 1975; Robertson and Paterson 1982; Shapiro and Porter 1989). Second, mechanical

130   isolation or male-female "fit" is not always defined in a way that makes quantifying variation in

131   these phenotypes straightforward (Masly 2012). This lack of clarity over what constitutes

132   mechanical incompatibility has led to conflation of mechanical RI (*i.e*., failure of male and

133   female parts to engage) in damselflies with mechanisms that might be better described as tactile

134   (Tennessen 1982).

135   Distinguishing mechanical from tactile mechanisms requires performing detailed mating

136   observations among males and females that possess interspecific variation in reproductive

137   structures and identifying specific features of reproductive morphology that prevent mating or

138   reduce mating success using high-resolution phenotypic data. Here, we take advantage of a large

139   collection of naturally occurring interspecific hybrids and lab-generated hybrids to test the

140   hypothesis that divergence in reproductive structural morphology causes RI at the early stages of

141   speciation in damselflies. We measure 19 potential pre- and postzygotic isolating barriers

142   between *Enallagma anna* and *E. carunculatum*, two species that diverged from a common

143   ancestor sometime in the last ~250,000 generations (McPeek et al. 2008; Callahan and McPeek

144   2016) and co-occur over much of the western United States (Westfall and May 2006). Both

145   species have identical ecologies and overall morphologies (Turgeon et al. 2005; McPeek et al.

146   2009), but display conspicuous differences in the size and shape of the male cerci and female

147   mesostigmal plates (Fig 1). We quantify variation in male and female reproductive structure

148   morphologies, distinguish mechanical and tactile premating incompatibilities, estimate the

149   cumulative strengths of multiple reproductive barriers, and independently test predictions of

150   mechanical and tactile isolation hypotheses (Richards and Robson 1926; Shapiro and Porter

151   1989). If mechanical incompatibilities occur, male *E. anna* × *E. carunculatum* hybrids that

152   possess intermediate cercus morphologies will have less success at forming tandems compared to

153   conspecific males. If tactile incompatibilities occur, males will be able to achieve tandem

154   regardless of their cercus morphology, but females will refuse to mate with males whose

155   morphologies deviate significantly from the conspecific mean phenotype.

156

157   **Materials and Methods**

158   Damselfly cerci and mesostigmal plates are non-intromittent sexual structures that are not

159   directly involved in the transfer of gametes from male to female. However, terminal appendages

160   of male insects and the female structures they contact during mating are often referred to as

161   secondary genital structures. We thus include them as genital traits, consistent with previous

162   definitions (Eberhard 1985; Arnqvist and Rowe 2005; Eberhard 2010; Simmons 2014; Brennan

163   2016) and refer to them generally as "genitalia" in the presentation of our results.

164

165   **Natural population sampling**

166   We studied wild populations of *E. anna* and *E. carunculatum* in July and August 2013 at

167   a site on the Whitefish River (Montana, U.S.A.; 48°22'15"N 114°18'09"W), where putative

168   interspecific hybrids have been reported (Miller and Ivie 1995; Westfall and May 2006). To

169   estimate relative frequencies of each species, we collected solitary males and tandem/copulating

170   male-female pairs during peak activity between 1030-1600 hr. We initially assigned species

171   identity after inspecting cercus and mesostigmal plate morphology with a hand lens or dissecting

172   microscope, respectively. Males and females with morphologies that appeared intermediate

173   were initially designated as hybrids. We reassessed these assignments in the lab after 3-D

174   morphometric analysis (see below). We calculated the proportions of *E. anna*, *E. carunculatum*

175   and hybrid males from all sampling bouts and used these male frequencies to estimate the

176   expected frequencies of each type of male-female pair under random mating.

177   We attempted to cross virgin *E. anna* and *E. carunculatum* to measure postzygotic RI

178   between pure species, but we did not obtain heterospecific copulations in either cross direction.

179   Instead, we established laboratory populations of hybrids and parental species by collecting eggs

180   from mated pairs captured in the field. Mated females oviposited on moist filter paper, which

181   was kept submerged in 2-4 cm of water until larvae hatched. We obtained embryos from 24 *E.*

182   *anna* pure species crosses, 32 *E. carunculatum* pure species crosses, and 8 mixed crosses: 1 *E.*

183   *carunculatum* female × *E. anna* male, 1 *E. anna* female × *E. carunculatum* male, 1 *E.*

184   *carunculatum* female × hybrid male, and 5 hybrid female × *E. anna* male ("hybrid" refers to

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185 damselflies with intermediate cercus or mesostigmal plate morphologies). After sampling, egg

186 collection, and behavioral observation, we stored adult damselflies in 95% ethanol for

187 subsequent morphometric analyses.

188

189 **Laboratory rearing**

190 We transported embryos from the field site to the University of Oklahoma Aquatic

191 Research Facility where the larvae hatched and were reared to adulthood in individual 140 ml

192 cups. The larvae were provided with *Artemia*, *Daphnia*, or *Lumbriculus* as food sources and

193 experienced a natural photoperiod and daily water temperatures that averaged 20.0 ± 0.19 °C.

194 We housed adults in mesh cages (30.5 cm^3; BioQuip), segregated by sex until sexual maturity

195 and provided with adult *Drosophila* as a food source *ad libitum*. We used lab-reared virgin

196 adults to quantify prezygotic barriers, plus additional postzygotic barriers that we could not

197 measure in the field. We mated 24 adult pairs from this first lab generation: 11 *E. anna*, 2 *E.*

198 *anna* female $\times$ hybrid male, 2 *E. carunculatum* female $\times$ hybrid male, 6 hybrid female $\times$ *E. anna*

199 male and 4 hybrid female $\times$ hybrid male. Embryos from the second lab generation contributed

200 fecundity, fertility, and hatch rate data but were not raised to adulthood due to difficulties with

201 rearing them. Mated adults were stored in 95% ethanol after mating (males) or after oviposition

202 (females). Unmated damselflies were maintained to calculate captive lifespan, then preserved in

203 95% ethanol.

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205 **Morphometric analysis**

206 We photographed ethanol-preserved adults using a Nikon D5100 camera (16.2 MP;

207 Nikon Corporation, Tokyo, Japan) and measured abdomen length (abdominal segments 1-10,

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208 excluding terminal appendages) as a proxy for body size using ImageJ (Abramoff et al. 2004) for

209 175 males and 171 females. To reduce measurement error, we measured each abdomen twice,

210 then used the mean length in subsequent analyses after confirming that repeatability was high for

211 the separate measurements ($r = 0.97$). We obtained 3-D digital reconstructions of male cerci and

212 female mesostigmal plates by scanning 140 male terminal segments and 162 female thoraces in a

213 SkyScan 1172 micro-computed tomography scanner (Bruker microCT, Kontich, Belgium).

214 Male structures were scanned at a voxel resolution of 2.36 or 2.53 um, and female thoraces at

215 2.78 or 3.88 um, and the scan data were converted to image stacks using NRecon version 1.4.4

216 (Bruker microCT).

217   To quantify cercus shape, we digitally segmented the right cercus from each male's

218 image stack and converted it to a solid surface object using Avizo Fire software (FEI Software;

219 Hillsboro, Oregon) as described in McPeek et al. (2008). We measured the volume of each

220 cercus object as a proxy for cercus size, using Avizo's volume measurement tool. To quantify

221 and compare their shapes, each cercus was represented by a mesh of 20,000 triangles with

222 10,002 vertices, each defined by distinct (x, y, z) coordinates (Fig S1). We placed 7 landmarks

223 on common points on each cercus, then used these landmarks to register all digitized cerci in

224 identical orientations within the coordinate plane. To ensure that only shape and not size was

225 compared in the analysis, all objects were standardized to have the same centroid size. Next, we

226 performed spherical harmonic analysis (Shen et al. 2009), which represents the shape of a closed

227 surface in terms of the sum of 3-D sines and cosines on a sphere. We performed the analysis

228 using 18 degrees of spherical harmonic representation, which captures relevant surface detail

229 without introducing excess noise (Shen et al. 2009). The analysis generated 1,083 coefficients to

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230   describe the shape of each cercus, which we reduced into the primary axes of shape

231   differentiation using principal component analysis.

232   Because female mesostigmal plates are relatively flat structures, we represented plate

233   morphology using 3-D geometric morphometrics. For each female plate we assigned 11 fixed

234   landmarks and 248 sliding semi-landmarks to the right anterior thorax of each female (Fig S2)

235   using Landmark software (Wiley et al. 2005). We imported landmark coordinates into R and

236   used the Geomorph package (version 2.1.7; Adams and Otarola-Castillo 2013) to assign 79

237   landmarks as "curve sliders" on the medial thorax and around the plate periphery, and 169

238   "surface sliders" evenly spaced across the plate. We obtained 3-D shape variables for these

239   representations using general Procrustes analysis superimposition (Rohlf 1999), then obtained a

240   smaller set of plate shape variables from the Procrustes-superimposed coordinates using

241   principal component analysis.

242

243   **Measuring pre- and postzygotic reproductive isolating barriers**

244   To measure the strength of RI barriers between *E. anna* and *E. carunculatum*, we

245   quantified 19 potential pre- and postzygotic isolating mechanisms that act from the beginning of

246   the mating sequence through an individual's life history. Table 1 summarizes these RI measures

247   and describes the equations used to estimate the absolute strength of each (Dopman et al. 2010).

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253 **Table 1.** Formulas for the absolute strength of each reproductive isolating barrier measured,
254 listed in the order in which they act during the mating sequence and subsequent life history of an
255 individual. In the postzygotic barrier formulas, "heterospecific" includes male-female pairs
256 composed of both pure species and any male-female pair involving at least one hybrid partner.

Barrier	RI formula
Prezygotic	
Visual	1 – (number heterospecific tandem attempts / conspecific tandem attempts)
Precopulatory mechanical	1 – (number heterospecific tandems / number heterospecific tandem attempts)
Tactile I (female resistance)	1 – (proportion heterospecific tandems without resistance / proportion conspecific tandems without resistance)
Tactile II (female refusal)	1 – (proportion heterospecific matings / proportion conspecific matings)
Postzygotic	
Hybrid mechanical I (tandem)	1 – (number hybrid tandems / number hybrid tandem attempts)
Hybrid mechanical II (intromission)	1 – (number hybrid copulations / number hybrid intromission attempts)
Hybrid tactile I (female resistance)	1 – (proportion hybrid tandems without resistance / proportion conspecific tandems without resistance)
Hybrid tactile II (female refusal)	1 – (proportion hybrid matings / proportion conspecific matings)
Hybrid copulation duration	1 – (mean hybrid copulation duration / mean conspecific copulation duration)
Hybrid copulation interruption duration	1 – (mean conspecific copulation interruption duration / mean hybrid copulation interruption duration)
Hybrid oviposition	1 – (proportion females oviposited, hybrid matings / proportion females oviposited, conspecific matings)
Fecundity	1 – (mean number eggs, hybrid clutch / mean number eggs, conspecific clutch)

Fertility	1 – (mean fertilized eggs, hybrid clutch / mean fertilized eggs, conspecific clutch)
Egg hatching	1 – (proportion hatched eggs, heterospecific clutch / proportion hatched eggs, conspecific clutch)
Embryo development	1 – (mean days from oviposition to hybrid egg hatching, hybrids/ mean days from oviposition to pure species egg hatching)
Larval maturation time	1 – (mean days from pure species hatch to adult emergence / mean days from hybrid hatch to adult emergence)
Larval survivorship	1 – (proportion hybrid larvae that reach adulthood / (proportion pure species larvae that reach adulthood)
Adult sex ratio	1 – (hybrid sex ratio / pure species sex ratio)
Adult lifespan	1 – (hybrid lifespan / pure species lifespan)

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259 *Mate discrimination*

260   We measured males' visual discrimination of potential mates by restraining individual *E.*

261 *anna* and *E. carunculatum* females on wooden dowels near the water and measuring the

262 frequencies of each type of male that attempted tandem with them. We attached live females of

263 each species by their legs to wooden dowels using Duco cement (ITW Devcon, Glenview, IL,

264 USA; Miller and Fincke 1999) and placed individual dowels level with surrounding vegetation

265 within 5 m of the water's edge at the field site. Over 20-minute intervals, we captured each male

266 that either attempted or achieved tandem with a restrained female and assigned them to species

267 by examining the cerci with a hand lens. Males were held in paper envelopes until the end of the

268 observation period to prevent the possibility of a second encounter with the restrained female and

269 were then released.

270

271 *Mating assays*

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272   We measured several premating RI barriers using no-choice mating experiments in which

273   females were placed in mesh cages with either heterospecific, hybrid, or conspecific males. We

274   used both field-caught and lab-reared damselflies, and used only virgin females in each mating

275   assay. To obtain virgins in the field, we captured newly emerged females, identified by their

276   pale teneral coloration. We assigned species identity as described above, then housed virgin

277   females in cages until they reached sexual maturity (~10 days post-emergence). We placed 2-5

278   individuals of each sex in a cage under partial shade in the grass and observed behaviors between

279   1000-1600 hr.

280   We quantified precopulatory mechanical RI by measuring the frequency of tandem

281   attempts in which the male was unable to securely grasp a female for longer than five seconds.

282   A secure hold was confirmed by observing the male flying while engaged with the female, or

283   attempting to fly without losing contact while the female remained perched. We measured

284   copulatory mechanical RI as the proportion of copulation attempts in which the male and female

285   failed to achieve genital coupling. This estimates mechanical incompatibility between male

286   grasping appendages and female thoracic plates and excludes the possibility of male loss of

287   interest, because males were often observed repeatedly attempting tandem on the same female

288   despite being unable to grasp her.

289   We quantified two types of precopulatory tactile incompatibilities using pairs that formed

290   tandems. First, we recorded whether each female showed resistance behaviors during tandem

291   (*e.g.*, head shaking, wing flapping, dorsal abdominal extension, or body repositioning)

292   (Tennessen 1975; Xu and Fincke 2011). Second, we recorded whether females in tandem

293   cooperated in copulation or refused to mate.

294

Postzygotic isolation

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296   We quantified several postmating RI barriers using the progeny from interspecific

297   crosses, beginning at copulation (Table 1). We measured oviposition success as the proportion

298   of females from each cross type that oviposited. When females failed to oviposit within three

299   days post-mating, we checked their male partners for motile sperm by anesthetizing them with

300   CO_2, immediately dissecting out the seminal vesicle, gently squashing it under a coverslip, and

301   examining the contents under a Zeiss Axio Imager 2 stereomicroscope (100× total

302   magnification). We dissected females that failed to oviposit to check the oviduct for mature eggs

303   and the bursa copulatrix for sperm.

304   We calculated fecundity by counting all eggs laid by each mated female within three days

305   of mating. The date of egg hatch was recorded as the first day that larvae were observed. We

306   calculated the proportion of eggs that hatched from each clutch by counting the number of

307   unhatched eggs that remained in the filter paper seven days after first hatch. We calculated

308   fertility of lab-reared matings by counting the number of fertilized eggs, as indicated by a dark

309   spot that develops on the apical end of the egg (Corbet 1999). We calculated embryo

310   development timing as days from oviposition to egg hatch, larval maturation as days from egg

311   hatch to adult emergence, larval survivorship as the proportion of hatched larvae that emerged as

312   to adults, plus adult sex ratio and total adult lifespan.

313

Strength of RI barriers

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315   We estimated the absolute strength of each individual barrier using the following general

316   equation (Ramsey et al. 2003; Dopman et al. 2010):

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$$RI = 1 - \frac{fitness\ between\ species}{fitness\ within\ species}$$

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320   This equation yields a value between -1 and 1 in which 0 indicates no barrier to gene flow, 1

321   indicates full RI, and negative values indicate a hybrid fitness advantage. We estimated each RI

322   barrier's sequential strength (*SS*) based on its absolute strength (*AS*) and the absolute strengths of

323   all preceding barriers, as described in (Dopman et al. 2010):

324

$$SS_n = AS_n \left(1 - \sum_{i=1}^{n-1} SS_i \right)$$

325

326   We calculated total RI (*T*) between *E. anna* and *E. carunculatum* as the sum of the sequential

327   strengths of all barriers, then calculated each barrier's relative contribution to total RI (SS_n / T)

328   (Dopman et al. 2010; Table S1).

329

330   **Statistical analyses**

331   We compared males' sexual approaches toward con- and heterospecific females,

332   observed vs. expected frequency of heterospecific pairs, and adult sex ratios using binomial tests.

333   We compared presence or absence of female resistance behaviors, female mating refusal or

334   cooperation, frequency of copulation interruptions, and oviposition success among parental

335   species and hybrid pairs using Fisher Exact tests. We examined the relationship between male

336   abdomen length and cercus size using linear regression. We compared copulation and copulation

337   interruption durations between conspecific and non-conspecific matings using *t*-tests. We

338   compared abdomen length, fecundity, fertility, proportion eggs hatched, developmental timing,

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339   and adult lifespan among *E. anna*, *E. carunculatum*, and hybrids using analysis of variance

340   (ANOVA), after arcsin-transformation of proportion data. When an ANOVA indicated a

341   significant difference existed among the three groups for any measure, we conducted Tukey

342   post-hoc tests to identify the differences among groups. For both forms of premating tactile

343   isolation data, we omitted all cross types with sample size < 6 from statistical analyses. When

344   possible, we combined data (field and lab, or lab generations 1 and 2) to increase statistical

345   power, after confirming with ANOVA that measurements not differ significantly between the

346   two groups. All analyses were conducted in R version 3.1.1 (R Core Team 2015). Means are

347   reported as $\pm$ 1 SEM.

348

349   **Results and Discussion**

350   **Males mate indiscriminately and hybridization occurs at low frequency in nature**

351   At the Whitefish River site, *E. anna* males outnumbered *E. carunculatum* males by a

352   factor of ~1.5. This was observed for both solitary males (*E. anna*: n = 165, *E. carunculatum*: n

353   = 108, over 8 sampling days) and male-female pairs (*E. anna*: n = 44, *E. carunculatum*, n = 28,

354   over 9 sampling days). *E. anna* males attempted tandem with *E. carunculatum* females (46.3%;

355   19 of 41) as frequently as they did with *E. anna* females (53.7%; 22 of 41; $\chi^2_1 = 0.010$, $P = 0.76$;

356   Fig 2). *E. carunculatum* males also attempted tandem with females of both species equally

357   (50.0% (8/16) each; $\chi^2_1 = 0.00$, $P = 1.0$; Fig 2). These results show that premating interactions

358   between *E. anna* and *E. carunculatum* are random, similar to observations from other *Enallagma*

359   (Paulson 1974; Fincke et al. 2007; Xu and Fincke 2011) and *Ischnura* species (Sánchez-Guillén

360   et al. 2012; Sánchez-Guillén et al. 2014).

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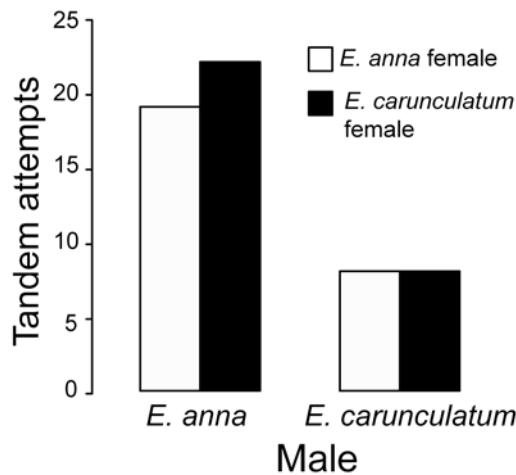
363

364   **Fig 2. Male visual isolation.** Number of male tandem attempts on conspecific and

365   heterospecific females at the Whitefish River site.

366

367   Despite this lack of habitat and visual isolation in sympatry, heterospecific pairs were

368   rarely captured in the field. In more than one month at the field site, we captured only two

369   heterospecific male-female pairs, one in each cross direction. Based on the relative frequencies

370   of each pure species, heterospecific pairs occur significantly less often than expected under

371   random mating between *E. anna* and *E. carunculatum* ($\mathcal{X}^2_3 = 65.40$, $P < 1 \times 10^{-5}$). This suggests

372   that although males may frequently initiate tandems with heterospecific females, such pairs

373   likely remain in tandem only briefly. However, even the rare occurrence of both types of

374   heterospecific tandems suggests that pure species may interbreed at low frequencies in the wild,

375   and our collection of field-caught individuals supports this notion: 41 of 630 males and 7 of 547

376   females we collected possessed intermediate reproductive structure morphologies that were

377   visibly different from either pure species.

378

379

Hybrids are morphologically distinct from either parental species

381   Among males, the first 5 principal component (PC) scores explained >77% of the cercus

382   shape variance. PC1 (60.05%) distinguished pure species and represented differences in overall

383   cercus length, from short (*E. carunculatum*) to long (*E. anna*), with hybrids showing a range of

384   intermediate scores (Fig 3A). PC2 (7.50%) represented a difference in the relative angles of the

385   upper and lower projections of the cercus, with many hybrids occupying a different space along

386   this axis than parental species. Most field-caught hybrids had distinctly intermediate cercus

387   morphologies, whereas the lab-reared males from heterospecific or backcross pairs possessed

388   morphologies that spanned the entire range of variation between *E. anna* and *E. carunculatum*

389   males (Fig 3A).

390
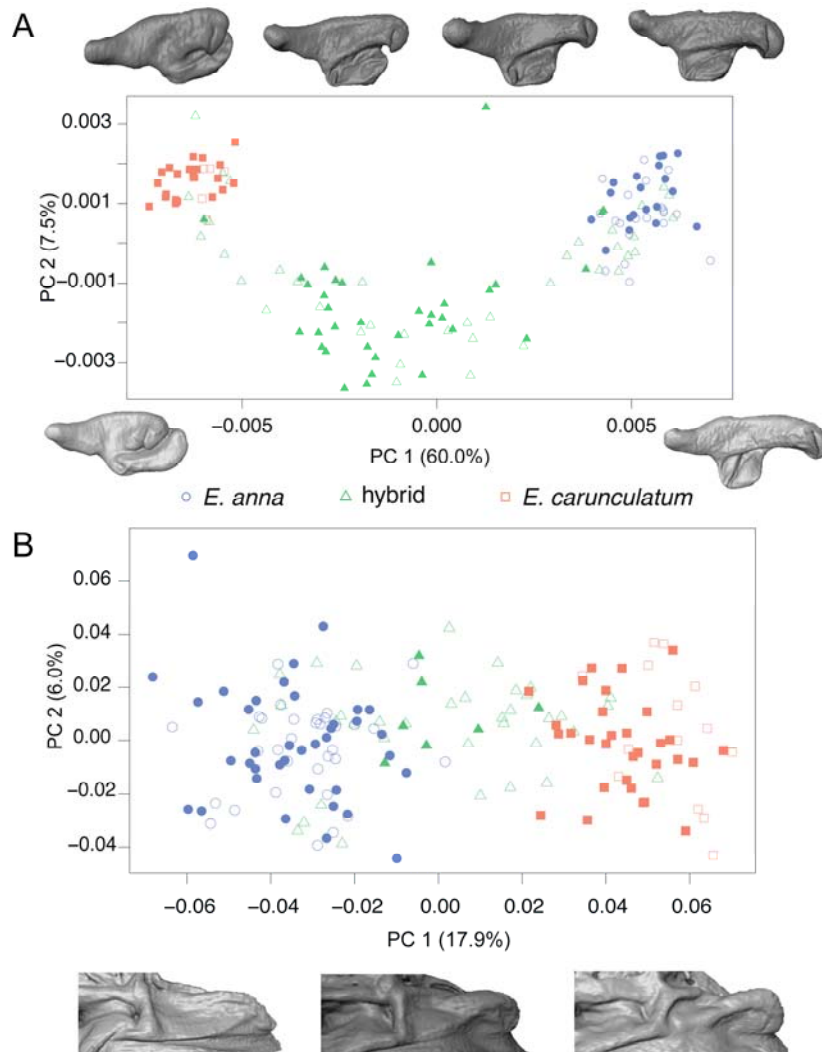
Fig 3. Variation in *E. anna*, *E. carunculatum*, and hybrid male and female reproductive structure morphologies. **(A)** Distribution of the first two principal components (PC) that represent variation in male cercus shape. Cercus representations above the plot show the range of hybrid male variation across the PC1 axis and representations below the plot show parental species morphologies. **(B)** Distribution of the first two principal components (PC) that represent variation in female mesostigmal plate shape. Examples of representative parental species and hybrid morphologies are shown below the plot (left = *E. anna*, middle = hybrid, right = *E. carunculatum*). The percentage of variation explained by each PC axis is shown in parentheses. Open symbols represent lab-reared individuals, filled symbols represent field-caught individuals.

401

402   Among females, the first 6 principal components scores accounted for >42% of the

403   variance in mesostigmal plate shape. *E. anna* and *E. carunculatum* specimens formed separate

404   clusters on PC1 (17.9%), but there was considerable overlap between hybrids and *E. anna* on

405   PC1 (Fig 3B). This overlap might reflect limitations of the resolving power of our morphometric

406   approach to distinguish intraspecific variation from intermediate hybrid morphology of these

407   complex female structures. Additional PC axes indicated that parental species and hybrid plate

408   shapes showed similar levels of variation in several features, including the angle of the plate's

409   anterior edge relative to the thorax (PC2; 6.0%), curvatures of the plate's lateral edge (PC3;

410   5.5%) and plate surface (PC5; 4.6%), and dimensions of the space between the bilateral plates

411   (PC4; 5.3%). Because slight variation in the manual placement of the fixed landmarks on each

412   female has the potential to contribute to this apparent overlap between *E. anna* and the hybrid

413   females, we repeated the entire analysis beginning with placement of landmarks on a subset of

414   157 plates selected at random. Repeatability was high among landmark coordinates in both sets

415   ($r > 0.99$) and both replicate analyses produced similar results (Fig S3).

416   Our behavioral, rearing, and morphometric data confirm that individuals with

417   intermediate reproductive structure morphologies are hybrids between *E. anna* and *E.

418   carunculatum* and not a separate species as originally suggested (Miller and Ivie 1995).

419   Interestingly, the collection of lab-reared hybrids (both F_1 and backcross) included cercus and

420   plate phenotypes not observed in the field-caught samples (Fig 3). Some lab-reared hybrid

421   morphologies were even indistinguishable from those of the parental species, which could be the

422   result of collecting eggs in the field from mated females that may have been storing sperm from

423   previous conspecific matings. Alternatively, some field-caught adult damselflies that we

21

424   designated as pure species may in fact have been hybrids that maintained "phenotypic integrity"

425   with one parental species despite having highly admixed genomes (Poelstra et al. 2014). Despite

426   this possibility of occasional misidentification, the majority of field-caught individuals we

427   identified as hybrid possess morphologies that fall well outside of the distributions of either pure

428   species. This is particularly true for cercus shape, which has pronounced differences between *E.*

429   *anna* and *E. carunculatum*.

430   The distributions of the field-caught versus lab-reared hybrids also show that hybrid

431   genital morphology appears to be under selection in the wild. In particular, the distribution of

432   male morphologies shows that the field-caught hybrids cluster equally distant from the pure

433   species. This result suggests that although interspecific mating occurs in the field, F_1 hybrids

434   either rarely backcross with parental species or backcross hybrids rarely survive to reproductive

435   age. Our lab-rearing data show that hybrids can in fact backcross with parental species and

436   advanced backcross individuals are viable and fertile (see below). However, future genomic

437   studies will be needed to reveal the direction and genomic extent of introgression and the

438   frequency of F_1 versus advanced-generation hybrids in the wild.

439

440   **Mechanical incompatibilities cause substantial, asymmetric reproductive isolation**

441   Between pure species, precopulatory mechanical RI was incomplete in both directions of

442   interspecific cross, and RI appears asymmetric: 25% (7/28) of *E. anna* males achieved tandems

443   with *E. carunculatum* females, whereas 66.7% (6/9) of *E. carunculatum* males achieved tandems

444   with *E. anna* females (Fig 4A). These data show that mechanical isolation is relatively weak

445   between *E. carunculatum* males and *E. anna* females, which presents the opportunity for

446   interspecific matings. Mechanical isolation due to males' inability to grasp heterospecific

22

447   females is frequently evoked as the major contributor to RI in coenagrionid damselflies (Paulson

448   1974; Robertson and Paterson 1982; Fincke et al. 2007; Bourret et al. 2012; Wellenreuther and

449   Sánchez-Guillén 2016), although several exceptions exist (Paulson 1974; Tennessen 1975; Bick

450   and Bick 1981; Forbes 1991; Miller and Fincke 2004). Our results suggest that mechanical

451   incompatibilities are not sufficiently strong enough to completely exclude the possibility of

452   hybridization in *Enallagma*. Additionally, it has been suggested that species with longer cerci

453   are better at grasping females of other species (Paulson 1974), but our data show that *E. anna*

454   males, whose cerci are roughly twice as long as *E. carunculatum* cerci, were less capable of

455   grasping heterospecific females compared to *E. carunculatum* males.

456   The existence of incomplete precopulatory mechanical incompatibilities between *E. anna*

457   and *E. carunculatum* suggests that the intermediate cercus morphology of hybrid males might

458   reduce their ability to form tandems with pure species females. Eighty-six percent (30/35) of

459   hybrid males we tested achieved tandem with *E. anna* females, and 63.6% (7/11) achieved

460   tandem with *E. carunculatum* females (Fig 4A). Thus, male hybrids achieved tandem with both

461   pure species more frequently than males of either pure species achieved tandem with

462   heterospecific females. These results show that although hybrid males were less successful at

463   forming tandems with females than conspecific males, they were more successful than

464   heterospecific males.

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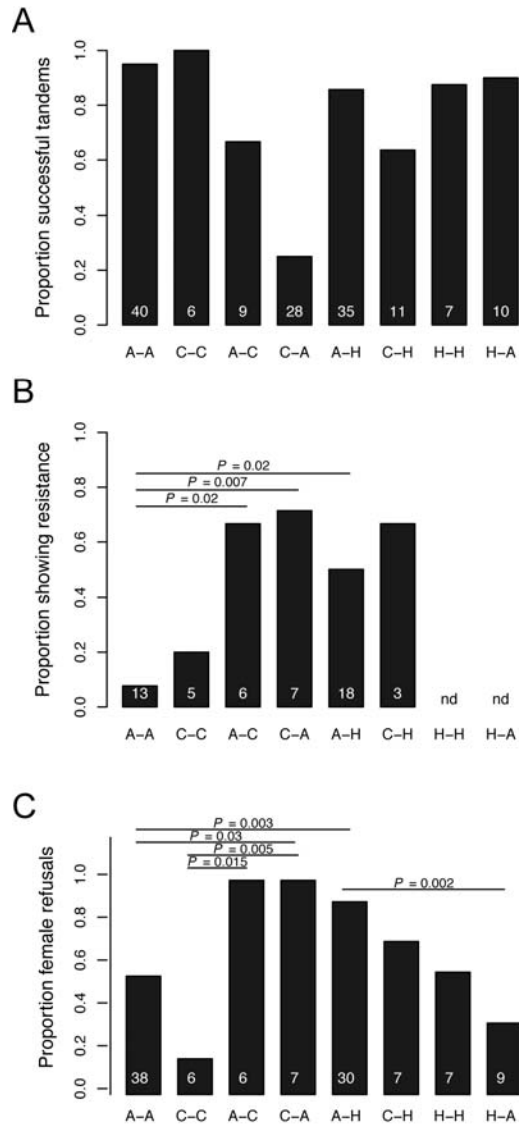
Fig 4. Sequentially-acting mechanisms of prezygotic reproductive isolation. (A) Mechanical isolation. **(B)** Proportion of tandems in which females displayed resistance behaviors (field-caught only). **(C)** Proportion of tandems in which females refused to copulate (field-caught and lab-reared data). Crosses shown on the x-axis list female first. A = *E. anna*, C = *E. carunculatum*, H = hybrid. Numbers at the base of the bars in panels A-C show the numbers of male-female pairs that were measured. "nd" refers to cross types for which no data were collected.

474

475   Mechanical incompatibility involving the primary genitalia (intromittent organs) may

476   also cause RI. No heterospecific matings occurred during our behavioral observations, so we

477   could not directly measure copulatory mechanical RI between *E. anna* and *E. carunculatum.*

478   However, among the tandem pairs involving hybrids in which the female initiated copulation (2

479   *E. anna*, 2 *E. carunculatum*, and 3 hybrid females), all 7 pairs achieved genital coupling.

480   Although this sample size is modest, this result suggests that no copulatory mechanical

481   incompatibility exists between hybrids and parental species. This is not unexpected, as *E. anna*

482   and *E. carunculatum* penes have similar morphologies (Kennedy 1919). Taken together, the

483   results from these mating assays show that as the morphological mismatch between interacting

484   male and female mating structures increases, the possibility of forming tandem and mating

485   decreases.

486

487   **Tactile incompatibilities cause substantial RI when mechanical isolation is incomplete**

488   A significantly greater proportion of lab-reared *E. anna* females (12/22) engaged in

489   resistance behaviors during conspecific tandems than did field-caught *E. anna* females (1/13,

490   Fisher exact test, $P = 0.01$). For this reason, we analyzed presence/absence of female resistance

491   during tandem separately for field-caught and lab-reared populations. In the field, *E. anna*

492   females were significantly more likely to resist during tandems with heterospecific males (67%;

493   4 of 6) or hybrid males (50%; 9 of 18) than with conspecific males (7.7%; 1 of 13; Fisher exact

494   tests, $P_{heterospecific} = 0.02$, $P_{hybrid} = 0.02$; Fig 4B). Additionally, 71.4% (5/7) of *E. carunculatum*

495   females displayed resistance behaviors during tandem with *E. anna* males in the field, which was

496   significantly greater than 7.7% of *E. anna* females ($P = 0.007$; Fig 4B).

25

497   Surprisingly, lab-reared *E. anna* females resisted during tandems with conspecific males

498   as frequently as they resisted during tandems with hybrid males (54.5%, 12 of 22 vs. 81.8%, 9 of

499   11, respectively; *P* = 0.25; Fig S4). *E. anna* and hybrid females also showed similar levels of

500   resistance during tandem with *E. anna* males (14.3%, 1 of 7 of hybrid females resisted; *P* = 0.09;

501   Fig S4). A comparison of the two reciprocal *E. anna* × hybrid crosses, however, showed that *E.*

502   *anna* females were significantly more likely to resist during tandem with hybrid males (81.8%)

503   than were hybrid females (14.3%; *P* = 0.01; Fig S4). Female resistance during tandem with a

504   conspecific male is not unusual (Tennessen 1975; Fincke 2015), but because the field-caught and

505   lab-reared *E. anna* populations behaved so differently, and the field data reflects behavior in a

506   natural setting, we used the field-caught female data to calculate this form of tactile isolation

507   (Table S1).

508   Field-caught and lab-reared females showed similar copulatory refusal rates: 94.7%

509   (18/19) field-caught and 81.8% (9/11) lab-reared *E. anna* females refused hybrid males (Fisher

510   exact test, *P* = 0.54), and 69.2% (9/13) field-caught and 51.9% (12/25) lab-reared *E. anna*

511   females refused conspecific males (*P* = 0.31). We therefore pooled field-caught and lab-reared

512   data to analyze female copulation refusal or acceptance. Ninety percent (27/30) of *E. anna*

513   females taken in tandem by hybrid males refused to copulate, which was significantly greater

514   than the 55.3% (21/38) of *E. anna* females that refused conspecific males (*P* = 0.003; Fig 4C).

515   All six *E. anna* females observed in tandem with *E. carunculatum* males refused to copulate,

516   although this level of refusal was not statistically different from the conspecific refusal rate (*P* =

517   0.07; Fig 4C). This is likely due to the low number of heterospecific pairs we could observe. *E.*

518   *carunculatum* females, in contrast, were significantly more likely to refuse an *E. anna* male

519   (100%, 7 of 7) than a conspecific male (16.7%, 1 of 6; *P* = 0.005; Fig 4C). *E. anna* females also

26

520   refused to mate with *E. carunculatum* males more frequently than did *E. carunculatum* females

521   (*P* = 0.015). We obtained a similar result for the reciprocal cross, where more female *E.*

522   *carunculatum* females refused *E. anna* males than did *E. anna* females (*P* = 0.03; Fig 4C).

523   Females' behavioral responses to different types of males reveal strong assortative

524   mating between *E. anna* and *E. carunculatum* when premating mechanical isolation fails.

525   Tactile isolation also predicts that pure species females should refuse to mate with hybrid males

526   because intermediate cerci fail to relay the proper tactile species recognition signal to the female.

527   Our behavioral data support this prediction for *E. anna* females, which mated with hybrid males

528   less frequently than with conspecific males. The finding that some *E. anna* females mated with

529   hybrid males, but none mated with *E. carunculatum* males suggests that females display some

530   latitude in their preferences and are more likely to refuse males whose cercus morphology

531   greatly deviates from a conspecific phenotype. Although incomplete mechanical isolation has

532   been documented in several *Enallagma* species pairs, few cases of hybridization are known,

533   based on morphological or genetic evidence (Catling 2001; Turgeon et al. 2005; Donnelly 2008).

534   This suggests that even with incomplete mechanical isolation, tactile isolation might prevent

535   interbreeding among most *Enallagma* species. A full understanding of tactile isolation will

536   require quantitative study of the mechanoreceptors on female plates to understand how patterns

537   of phenotypic variation might contribute to RI.

538   The relative sizes of male and female reproductive structures may influence both

539   mechanical and tactile mechanisms of RI. Larger males tended to have larger cerci, as indicated

540   by regressing cercus volume on abdomen length (*E. anna*: $F_{1, 26}$ = 18.80, R^2 = 0.397, *P* = 0.0002;

541   *E. carunculatum*: $F_{1, 17}$ = 7.744, R^2 = 0.273, *P* = 0.013). Hybrids, however, showed a weaker

542   relationship between body size and cercus size, because hybrids display more variation in cercus

27

543   morphology than either parental species ($F_{1,55} = 6.70$, $R^2 = 0.092$, $P = 0.01$). A size mismatch in

544   male and female structures either within or between species may contribute to mechanical

545   incompatibilities, although our current data do not allow us to examine that relationship robustly.

546

547   **Postmating barriers contribute little to reproductive isolation**

548   Compared to the strong premating RI caused by mechanical and tactile incompatibilities

549   of male and female reproductive structures, we found relatively weak RI from postmating

550   barriers. Copulation duration was similar among conspecific mating pairs and pairs including at

551   least one hybrid partner (t_{25} = -0.028, P = 0.98; Fig 5A). Sixty percent (6/10) of conspecific

552   matings experienced interruptions, which was not significantly different from the hybrid matings

553   (61.5%, 8 of 13; Fisher exact test, P = 1.0). The total duration of these interruptions was also not

554   significantly different between conspecific or hybrid pairs ($t_{13.26}$= -1.51, P = 0.15; Fig 5B).

555   Although it has been suggested that Lepidoptera (Lorkovic 1958) and *Ischnura* (Córdoba-

556   Aguilar and Cordero-Rivera 2008) use copulatory morphology or stimulation to identify

557   conspecifics, our results indicate that this type of tactile discrimination during copula does not

558   occur in *Enallagma*.
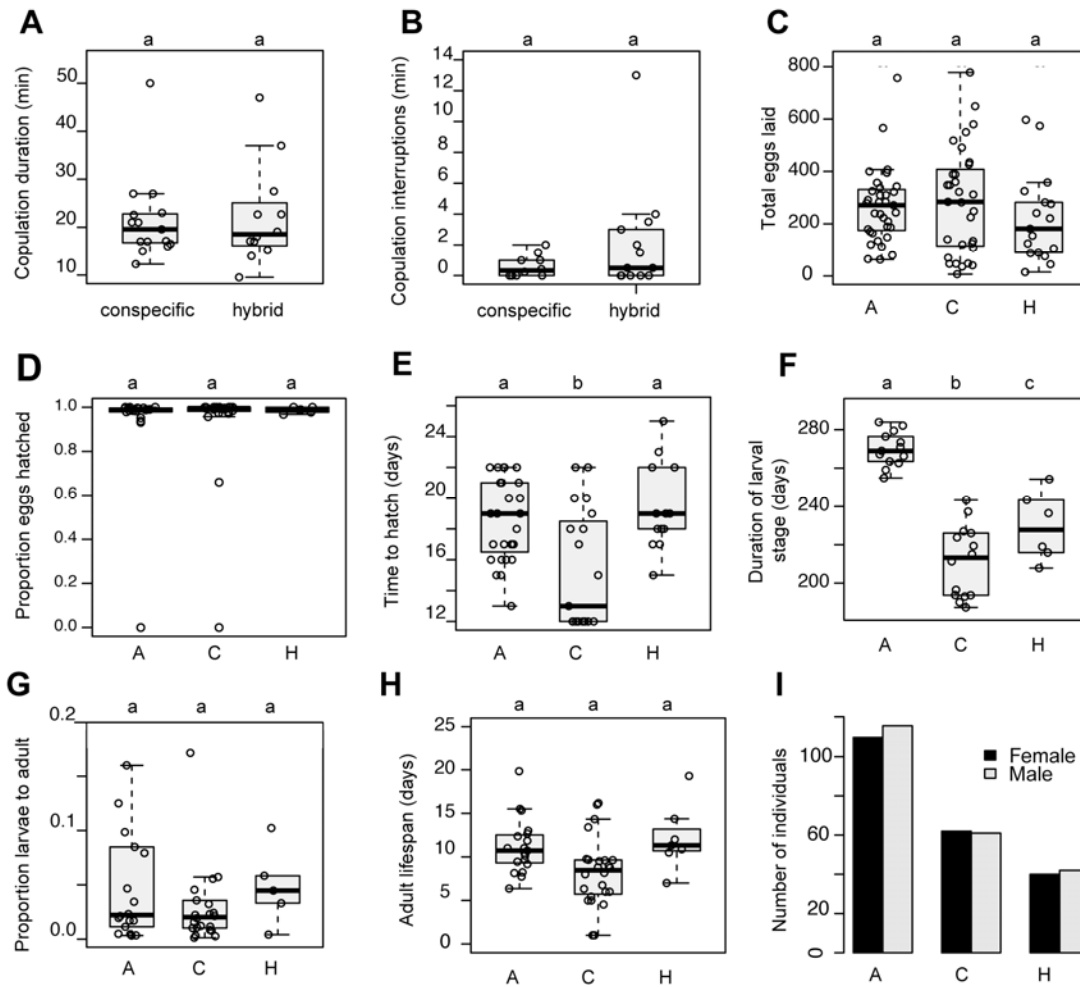
Fig 5. Sequentially-acting mechanisms of postmating reproductive isolation. (A) Copulation duration. **(B)** Length of copulation interruptions. **(C)** Fecundity (field-caught and lab-reared data). **(D)** Proportion hatched eggs per clutch. **(E)** Embryonic development timing. **(F)** Larval maturation timing. **(G)** Larval survivorship. **(H)** Adult lifespan. **(I)** Adult sex ratios. In panels A and B, each point represents one male-female pair. In panels C-G, each point represents one clutch. Within each panel, letters indicate homogeneous groups assigned at the statistical cutoff at $\alpha = 0.05$. Boxplots show the interquartile range. The line within the box shows the median and whiskers extend to the most extreme observation within ±1.5 times the interquartile range. Each open circle represents one mating pair (A, B), one clutch (C-E, G), or the clutch mean (F,H).

570

571   Similar proportions of *E. anna* (81.8%; %, 9 of 11) and hybrid females (83.3%, 5 of 6)

572   oviposited after mating with *E. anna* males (Fisher exact test, $P = 1.0$). Two *E. anna* females

573   mated with hybrid males, but neither laid any eggs. In contrast, two *E. carunculatum* females

574   mated with hybrid males and both oviposited. Two of the three hybrid females that mated with

575   hybrid males also oviposited. Dissections of females that failed to oviposit confirmed that they

576   had been inseminated and possessed mature eggs, and dissections of hybrid males in these

577   matings confirmed that hybrid males produce motile sperm. *E. anna, E. carunculatum*, and

578   hybrid parings also produced comparable numbers of eggs ($F_{2,80} = 0.79$, $P = 0.46$; Fig 5C).

579   Although there appears to be a trend towards smaller clutches or complete failure to oviposit in

580   females mated to hybrids, small samples prevent us from drawing strong conclusions about

581   whether tactile incompatibilities might contribute to postcopulatory isolating mechanisms.

582   The second generation of lab-reared damselflies consisted solely of *E. anna* and

583   advanced generation hybrid clutches, because in generation 1, *E. carunculatum* adults emerged

584   earliest and few were available for crosses with *E. anna* or hybrids. In generation 2, *E. anna* and

585   hybrid clutches had similar fertilization rates ($F_{1,17} = 0.51$, $P = 0.49$). In generation 1, *E. anna*,

586   *E. carunculatum*, and hybrid clutches had similar proportions of hatched eggs (Kruskal-Wallis

587    $\mathcal{X}^2_2 = 1.3385$, $P = 0.51$; Fig 5C). In generation 2, *E. anna*, and hybrid clutches had similar

588   proportions of hatched eggs ($t_{17.97} = 0.49404$, $P = 0.63$, Fig S6). Oviposition date had a

589   significant effect on hatch timing in generation 1 ($F_{1,41} = 49.1$, $P = 1.6 \times 10^{-8}$), but not in

590   generation 2 ($F_{1,41} = 2.96$, $P = 0.11$). We therefore analyzed hatch timing separately for each

591   generation. In generation 1, *E. carunculatum* larvae hatched earlier (15.4 ± 0.9 days, n =19

592   families) than *E. anna* (19.2 ± 0.7 days, n =17 families) and hybrid larvae (20.0 ± 1.3 days, n =7

30

593 families; ANCOVA with oviposition date as covariate, $F_{2, 39} = 10.8$, $P = 2 \times 10^{-4}$). In generation

594 2, *E. anna* and hybrid hatch rates did not differ significantly ($t_{11.92}= -1.22$, $P = 0.25$; Fig 5D). If

595 *E. carunculatum* larvae develop at a faster rate in the wild as they did in the lab, this could

596 contribute to RI via seasonal temporal isolation, in which early-emerging *E. carunculatum* adults

597 are less likely to encounter, and thus potentially interbreed with, *E. anna* adults. Detecting and

598 measuring this potential temporal barrier will require regular sampling throughout the breeding

599 season.

600   An anomalous water quality problem at the Aquatic Research Facility where larvae were

601 housed caused substantial larval mortality of generation 2, so we analyzed larval development

602 timing for generation 1 only (Fig 5F). An ANCOVA with oviposition date as a covariate and

603 Tukey post-hoc tests indicated that hybrids and parental species spent significantly different

604 lengths of time in the larval stage ($F_{2, 29} = 97.3$; $P < 1.4 \times 10^{-13}$). *E. carunculatum* (n =13

605 families) larvae reached adulthood an average of 58.6 ± 2.5 days earlier than *E. anna* (n =14

606 families; $P < 1 \times 10^{-5}$) and 18.2 ± 7.3 days earlier than hybrids (n =6 families; $P = 0.056$).

607 Hybrid larvae also developed significantly faster than *E. anna* ($P = 3 \times 10^{-5}$). Although *E.*

608 *carunculatum* larvae developed faster than *E. anna* and hybrid larvae in the lab, mean adult

609 abdomen length was similar among all three groups for both males ($F_{2, 19} = 0.334$; $P = 0.72$) and

610 females ($F_{2, 21} = 3.30$; $P = 0.57$; Fig S5). These results suggest that hybrid development was not

611 affected by intrinsic genetic incompatibilities.

612   Larval survivorship in the lab was similar for both parental species' and hybrid clutches

613 (Kruskal-Wallis $\mathcal{X}_2^2 == 4.4$, $P = 0.1$; Fig 5G). Of those individuals that reached adulthood, adult

614 lifespans under laboratory conditions did not differ significantly (ANCOVA with emergence

615 date as covariate, $F_{2, 48} = 1.35$, $P = 0.29$; Fig 5H). Of those individuals that reached adulthood,

616   adult lifespans under laboratory conditions did not differ significantly (ANCOVA with

617   emergence date as covariate, $F_{2, 48} = 1.35$, $P = 0.29$; Fig 5H). Finally, adult sex ratios were not

618   significantly different from the expected 1:1 ratio for any group (Fig 5I), which shows that

619   among pure species and hybrids, both sexes had similar viability. The combination of our

620   postmating isolation results demonstrate that neither strong intrinsic nor extrinsic (*e.g.*,

621   ecological selection against hybrids in the field) postzygotic barriers exist between *E. anna* and

622   *E. carunculatum*.

623

624   **Divergent reproductive structures cause reproductive isolation early during speciation**

625   Fig 6 shows the cumulative strength of RI barriers measured for each reciprocal cross.

626   Premating mechanical and tactile incompatibilities form the most substantial barriers to gene

627   flow between *E. anna* and *E. carunculatum*, whereas later-acting barriers contribute little to total

628   RI. Our results thus unequivocally demonstrate the potential of divergent mating structures to

629   cause RI in the early stages of speciation via mechanical and tactile mechanisms. These

630   incompatibilities also appear to provide particularly strong barriers to gene flow, as they act as

631   both a premating barrier between pure species and also as a postzygotic barrier that reduces

632   hybrid male mating success. Such incompatibilities represent a potent barrier to gene flow and

633   may be a common characteristic of traits that are under sexual selection within species (Stratton

634   and Uetz 1986; Naisbit et al. 2001; Höbel et al. 2003; Svedin et al. 2008; Van Der Sluijs et al.

635   2008).

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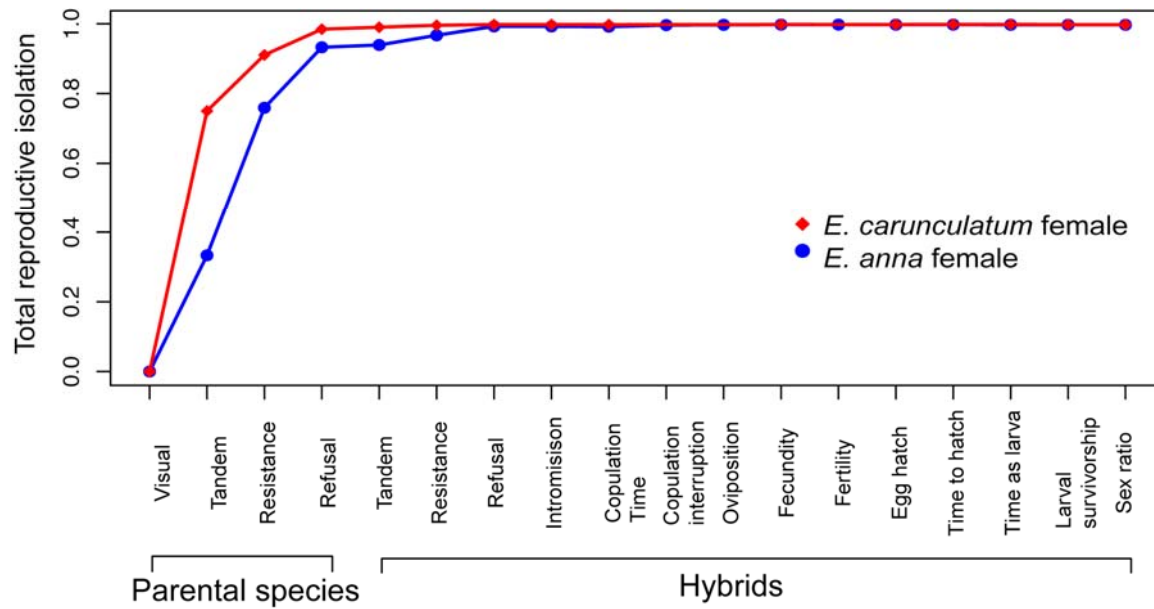
Fig 6. Sequential strength of reproductive isolating barriers, beginning with male-female encounter and proceeding through the reproductive sequence and life history. Estimates of the strength of the first four barriers were obtained from conspecific and heterospecific crosses only, and estimates of the remaining barriers also include crosses involving hybrid individuals. Estimates for the values of the strength of three barriers from the *E. carunculatum* female × hybrid male cross (copulation interruption duration, oviposition, and fertility) are represented by the best-fit line at these barriers.

Our results also show that premating barriers appear to have evolved first in *Enallagma*. Because *E. anna* × *E. carunculatum* hybrids appear to survive as well as parental species and suffer no intrinsic fertility deficits, the primary factor likely to affect their fitness is with whom they can mate. We observed that *E. anna* females often refuse to mate with conspecific males, indicating strong intraspecific discrimination. If the male cerci are under sexual selection similar to non-intromittent mating structures in other taxa (reviewed in Simmons 2014), and if females rely on the same tactile cues for both intraspecific mate choice and species discrimination, then

33

653   female discrimination among conspecific males could extend to discrimination of

654   heterospecifics. Prezygotic RI has been shown to evolve rapidly under laboratory settings due to

655   assortative mating, independent of local adaptation (Castillo et al. 2015), which supports the

656   plausibility of rapid evolution of RI driven by sexual selection in the wild. Female

657   discrimination against males with intermediate cerci also provides an opportunity for

658   reinforcement to strengthen premating isolation between *E. anna* and *E. carunculatum*— a

659   potential example of sexual selection rather than natural selection driving reinforcement (Naisbit

660   et al. 2001). Reinforcement could result in shifting or narrowing of female preferences (Ritchie

661   1996) or an increase in female discrimination in regions of sympatry (Noor 1999), two ideas that

662   deserve further study in these species. Alternatively, evolution of cercus morphology may be

663   driven by sexual conflict over mating rate, in which selection favors females that are less easily

664   grasped by males (Fincke et al. 2007).

665

666   Many researchers have dismissed genital mechanical incompatibilities as having an

667   important role in RI and speciation (reviewed in Shapiro and Porter 1989; Eberhard 2010),

668   primarily because of the small number of convincing cases that show strict support for it. We

669   might be better equipped to investigate the reproductive consequences of the widespread pattern

670   of rapid, divergent evolution of male genitalia if we broaden our scope to include explicitly

671   tactile mechanisms. This may require dropping the genital "lock-and-key" imagery – which

672   often evokes an "all or nothing" scenario in causing RI – in favor of a framework that allows for

673   more variation, similar to our understanding of auditory, visual, and chemical communication

674   signals. Indeed, our data show that mechanical isolation can be strong yet incomplete, and that

675   tactile isolation can form a strong subsequent mating barrier. A full understanding of the

34

676   contribution of mechanical incompatibilities in RI will require detailed morphological study to

677   understand how male and female structures interact (Willkommen et al. 2015) and which

678   features cause morphological mismatch. A deeper understanding of tactile RI mechanisms will

679   require detailed studies of sensory mechanisms and the neurobiological basis of female

680   reproductive decisions, all of which are admittedly challenging to investigate. Where females

681   discriminate against heterospecific reproductive structures (*e.g*., Bath et al. 2012), the female

682   nervous system poses a potentially more complex spectrum of incompatibilities compared to

683   genitalia. Taxa such as damselflies or stick insects (Myers et al. 2016) provide ideal systems to

684   begin to tease apart mechanical and tactile contributions to RI. Neural circuits that integrate

685   olfactory and auditory cues with internal physiological processes to influence female mating

686   decisions are being mapped in *Drosophila* (Bussell et al. 2014; Feng et al. 2014; Zhou et al.

687   2014), paving the way for similar mechanistic understanding of sensory modalities in emerging

688   model systems. Although odonates have a unique mode of mating that presents multiple

689   opportunities for both mechanical and tactile mismatch, our results highlight the potential

690   contribution of tactile signals involving the genitalia to RI among internally fertilizing animals.

691

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