

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

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11

12 **Abstract**

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

## 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).

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).

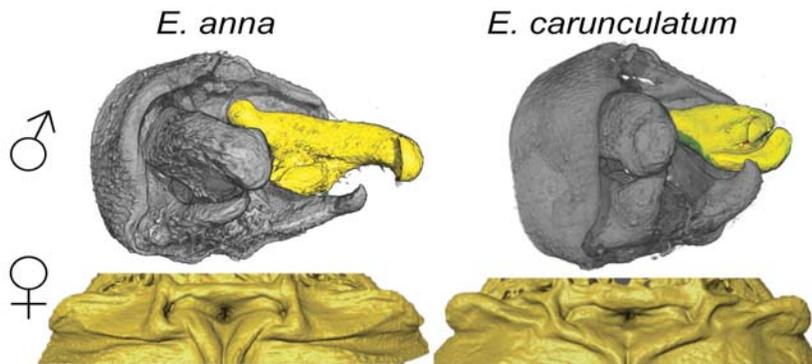
62           The second proposed mechanism is tactile incompatibility (de Wilde 1964; Eberhard  
63 1992), in which mismatch between male and female genitalia of different species prevents or  
64 reduces the success of mating and reproduction because one or both sexes fail to stimulate the  
65 other 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.

102



103

104 **Fig 1. Male grasping appendages and female mesostigmal plate morphology.** The right  
105 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; McPeck 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 (McPeck 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;

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 (Tennesen 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 (Tennesen 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 (McPeck et al. 2008; Callahan and McPeck  
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; McPeck 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 Damsely 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

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  $20.0 \pm 0.19$  °C.  
194 We housed adults in mesh cages (30.5 cm<sup>3</sup>; 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 × hybrid male, 2 *E. carunculatum* female × hybrid male, 6 hybrid female × *E. anna*  
199 male and 4 hybrid female × 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.

204

### 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,

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  $\mu\text{m}$ , and female thoraces at  
215 2.78 or 3.88  $\mu\text{m}$ , 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 McPeck 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

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 Otárola-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).

248

249

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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
<b>Prezygotic</b>	
Visual	$1 - (\text{number heterospecific tandem attempts} / \text{conspecific tandem attempts})$
Precopulatory mechanical	$1 - (\text{number heterospecific tandems} / \text{number heterospecific tandem attempts})$
Tactile I (female resistance)	$1 - (\text{proportion heterospecific tandems without resistance} / \text{proportion conspecific tandems without resistance})$
Tactile II (female refusal)	$1 - (\text{proportion heterospecific matings} / \text{proportion conspecific matings})$
<b>Postzygotic</b>	
Hybrid mechanical I (tandem)	$1 - (\text{number hybrid tandems} / \text{number hybrid tandem attempts})$
Hybrid mechanical II (intromission)	$1 - (\text{number hybrid copulations} / \text{number hybrid intromission attempts})$
Hybrid tactile I (female resistance)	$1 - (\text{proportion hybrid tandems without resistance} / \text{proportion conspecific tandems without resistance})$
Hybrid tactile II (female refusal)	$1 - (\text{proportion hybrid matings} / \text{proportion conspecific matings})$
Hybrid copulation duration	$1 - (\text{mean hybrid copulation duration} / \text{mean conspecific copulation duration})$
Hybrid copulation interruption duration	$1 - (\text{mean conspecific copulation interruption duration} / \text{mean hybrid copulation interruption duration})$
Hybrid oviposition	$1 - (\text{proportion females oviposited, hybrid matings} / \text{proportion females oviposited, conspecific matings})$
Fecundity	$1 - (\text{mean number eggs, hybrid clutch} / \text{mean number eggs, conspecific clutch})$

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

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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*

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

295 *Postzygotic isolation*

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<sub>2</sub>, 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

314 *Strength of RI barriers*

315           We estimated the absolute strength of each individual barrier using the following general  
316 equation (Ramsey et al. 2003; Dopman et al. 2010):

317

318 
$$RI = 1 - \frac{\text{fitness between species}}{\text{fitness within species}}$$

319  
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,

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

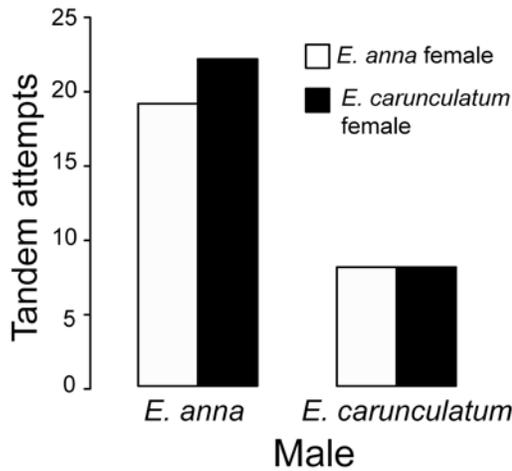
## 349 **Results and Discussion**

### 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;  $\chi_1^2 = 0.010$ ,  $P = 0.76$ ;  
356 Fig 2). *E. carunculatum* males also attempted tandem with females of both species equally  
357 (50.0% (8/16) each;  $\chi_1^2 = 0.00$ ,  $P = 1.0$ ; 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* ( $\chi^2_3 = 65.40, P < 1 \times 10^{-5}$ ). 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.

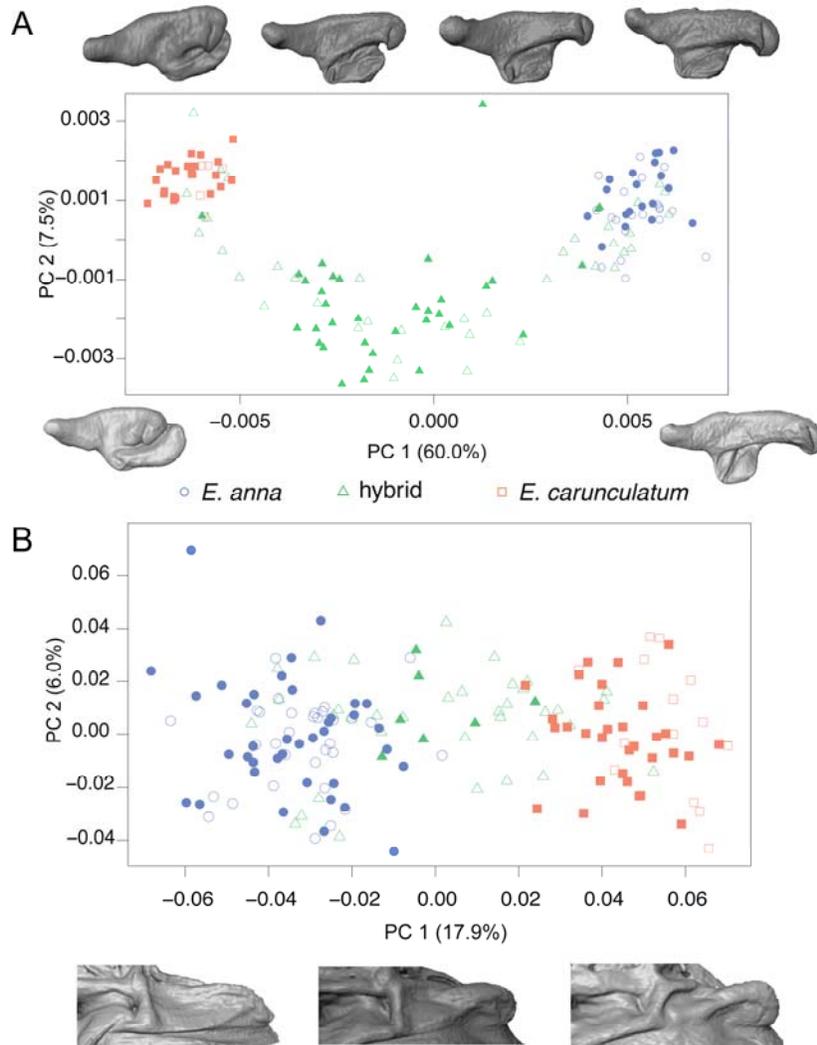
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380 **Hybrids are morphologically distinct from either parental species**

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



391

392 **Fig 3. Variation in *E. anna*, *E. carunculatum*, and hybrid male and female reproductive**

393 **structure morphologies. (A)** Distribution of the first two principal components (PC) that

394 represent variation in male cercus shape. Cercus representations above the plot show the range

395 of hybrid male variation across the PC1 axis and representations below the plot show parental

396 species morphologies. **(B)** Distribution of the first two principal components (PC) that represent

397 variation in female mesostigmal plate shape. Examples of representative parental species and

398 hybrid morphologies are shown below the plot (left = *E. anna*, middle = hybrid, right = *E.*

399 *carunculatum*). The percentage of variation explained by each PC axis is shown in parentheses.

400 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<sub>1</sub> 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

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<sub>1</sub> 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<sub>1</sub> versus advanced-generation hybrids in the wild.

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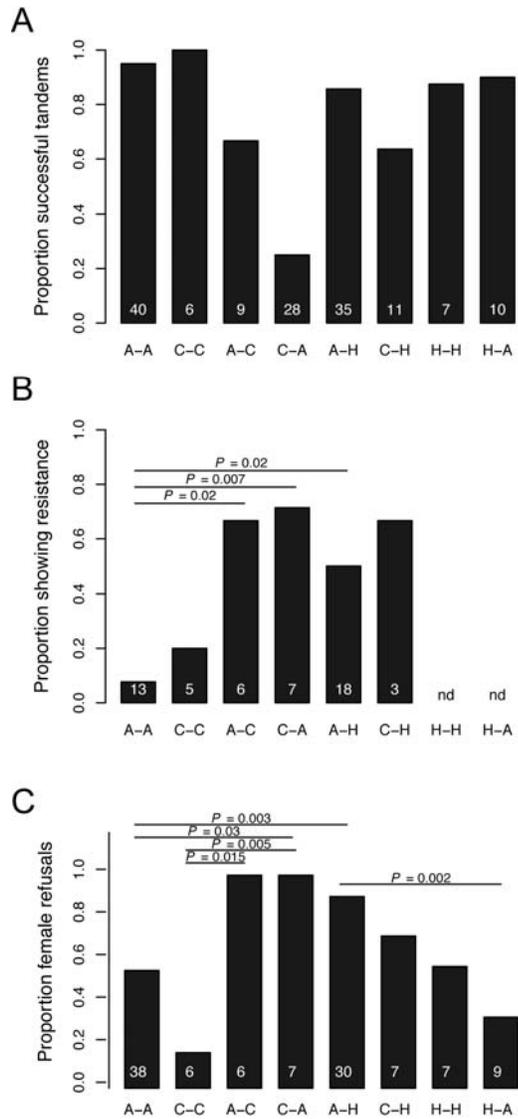
#### 440 **Mechanical incompatibilities cause substantial, asymmetric reproductive isolation**

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

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.

465



466

467 **Fig 4. Sequentially-acting mechanisms of prezygotic reproductive isolation. (A)**

468 Mechanical isolation. **(B)** Proportion of tandems in which females displayed resistance

469 behaviors (field-caught only). **(C)** Proportion of tandems in which females refused to copulate

470 (field-caught and lab-reared data). Crosses shown on the x-axis list female first. A = *E. anna*, C

471 = *E. carunculatum*, H = hybrid. Numbers at the base of the bars in panels A-C show the

472 numbers of male-female pairs that were measured. “nd” refers to cross types for which no data

473 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).

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 (Tennesen 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

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.

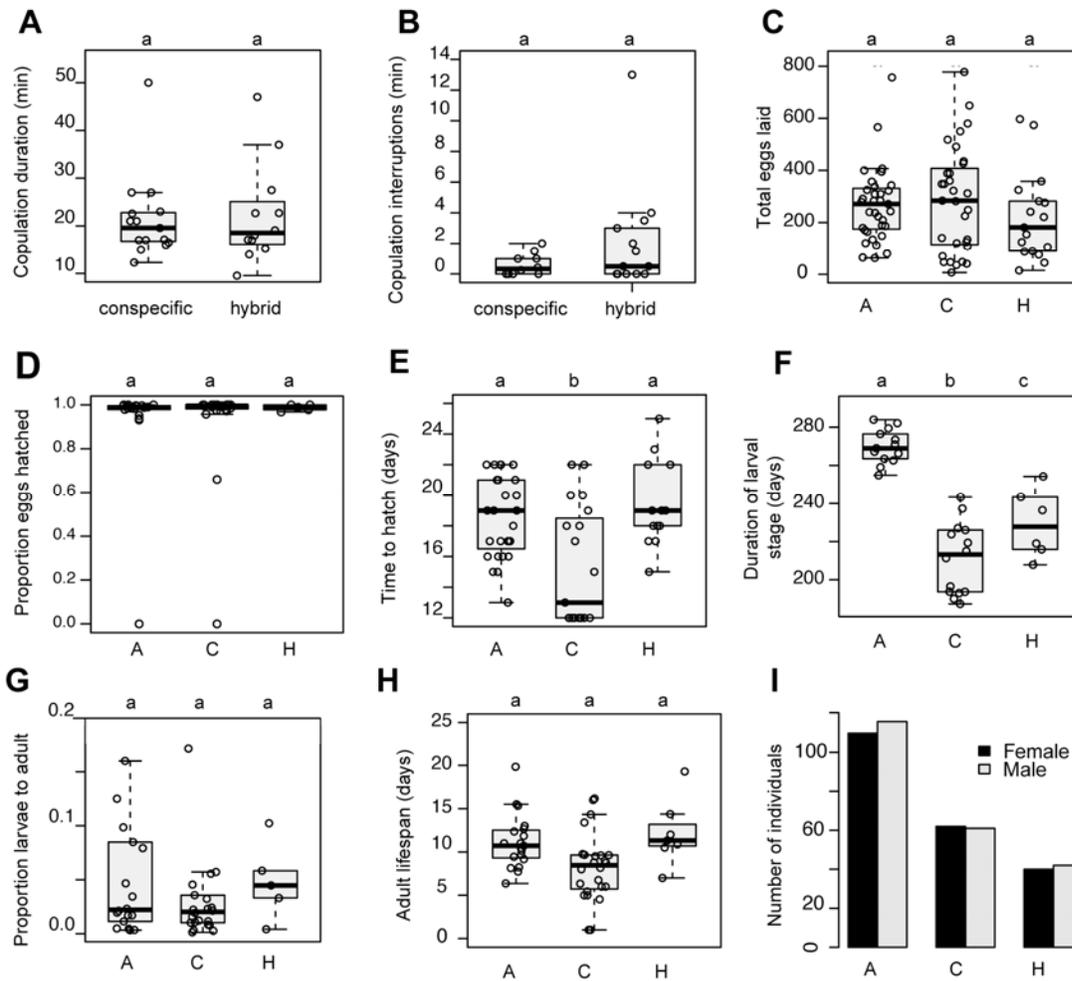
538 The relative sizes of male and female reproductive structures may influence both  
539 mechanical and tactile mechanisms of RI. Larger males tended to have larger cerci, as indicated  
540 by regressing cercus volume on abdomen length (*E. anna*:  $F_{1,26} = 18.80$ ,  $R^2 = 0.397$ ,  $P = 0.0002$ ;  
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

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*.



559

560 **Fig 5. Sequentially-acting mechanisms of postmating reproductive isolation. (A)** Copulation

561 duration. **(B)** Length of copulation interruptions. **(C)** Fecundity (field-caught and lab-reared

562 data). **(D)** Proportion hatched eggs per clutch. **(E)** Embryonic development timing. **(F)** Larval

563 maturation timing. **(G)** Larval survivorship. **(H)** Adult lifespan. **(I)** Adult sex ratios. In panels

564 A and B, each point represents one male-female pair. In panels C-G, each point represents one

565 clutch. Within each panel, letters indicate homogeneous groups assigned at the statistical cutoff

566 at  $\alpha = 0.05$ . Boxplots show the interquartile range. The line within the box shows the median

567 and whiskers extend to the most extreme observation within  $\pm 1.5$  times the interquartile range.

568 Each open circle represents one mating pair (A, B), one clutch (C-E, G), or the clutch mean

569 (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 pairings 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  $\chi^2_2 = 1.3385$ ,  $P = 0.51$ ; 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 ( $15.4 \pm 0.9$  days,  $n = 19$   
592 families) than *E. anna* ( $19.2 \pm 0.7$  days,  $n = 17$  families) and hybrid larvae ( $20.0 \pm 1.3$  days,  $n = 7$

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  $58.6 \pm 2.5$  days earlier than *E. anna* (n=14  
606 families;  $P < 1 \times 10^{-5}$ ) and  $18.2 \pm 7.3$  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  $\chi^2_{2} = 4.4$ ,  $P = 0.1$ ; 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,

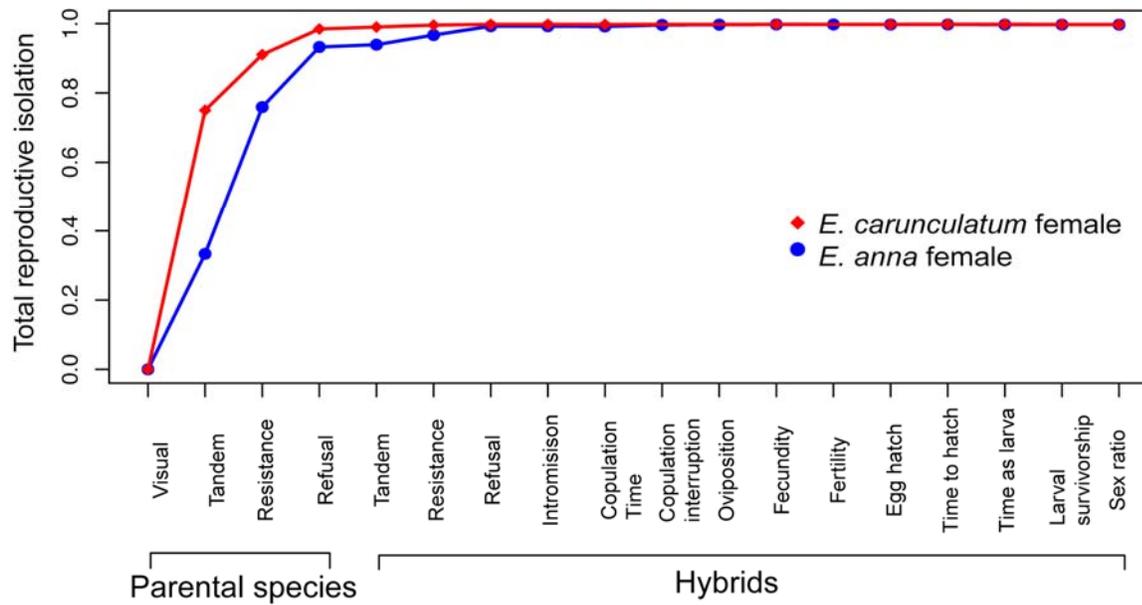
616 adult lifespans under laboratory conditions did not differ significantly (ANCOVA with  
617 emergence date as covariate,  $F_{2,48} = 1.35$ ,  $P = 0.29$ ; Fig 5H). Finally, adult sex ratios were not  
618 significantly different from the expected 1:1 ratio for any group (Fig 5I), which shows that  
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

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

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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