1   **Divergence in female damselfly sensory structures is consistent with a species recognition**

2   **function but shows no evidence of reproductive character displacement**

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13   **Abstract**

14   Males and females exchange signals prior to mating that convey information such as sex,

15   species identity, or individual condition. In some animals, tactile signals relayed during physical

16   contact between males and females before and during mating appear to be important for mate

17   choice and reproductive isolation. This is common among odonates, when a male grasps a

18   female's thorax with his terminal appendages prior to copulation, and the female subsequently

19   controls whether copulation occurs by bending her abdomen to complete intromission. It has

20   been hypothesized that mechanosensory sensilla on the female thoracic plates mediate mating

21   decisions, but is has been difficult to test this idea. Here, we use North American damselflies in

22   the genus *Enallagma* (Odonata: Coenagrionidae) to test the hypothesis that variation in female

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23   sensilla traits is important for species recognition. *Enallagma anna* and *E. carunculatum*

24   hybridize in nature, but experience strong reproductive isolation as a consequence of

25   divergence in male terminal appendage morphology. We quantified several mechanosensory

26   sensilla phenotypes on the female thorax among multiple populations of both species and

27   compared divergence in these traits in sympatry versus allopatry. Although these species

28   differed in features of sensilla distribution within the thoracic plates, we found no strong

29   evidence of reproductive character displacement among the sensilla traits we measured in

30   regions of sympatry. Our results suggest that species-specific placement of female

31   mechanoreceptors may be sufficient for species recognition, although other female sensory

32   phenotypes might have diverged in sympatry to reduce interspecific hybridization.

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Introduction

For sexual species, maintenance of species boundaries relies on reproductive isolation (RI) between recently diverged species (Mayr, 1942). Premating reproductive isolating barriers, including behavioral or sexual isolation, often evolve earlier in the speciation process than postmating barriers in a variety of animal taxa (*e.g.*, McMillan et al., 1997; Price and Bouvier, 2002; Mendelson and Wallis, 2003; Dopman et al., 2010; Sánchez-Guillén et al., 2012; Williams and Mendelson, 2014; Castillo et al., 2015; Barnard et al., 2017). Behavioral isolation requires that mate recognition signals and/or preferences diverge between populations, which ultimately results in the ability of individuals to discriminate conspecifics from heterospecifics. Species recognition signals may rely on a variety of sensory modalities such as color (Wiernasz and Kingsolver, 1992; Sætre et al., 1997; Jiggins et al., 2001; Boughman et al., 2005; Kronforst et al., 2006; Williams and Mendelson, 2014), courtship behavior (Stratton and Uetz, 1986), sound/vibration (Ewing and Bennet-Clark, 1968; Wells and Henry, 1998; Shaw, 2000; Gerhardt and Huber, 2002; Arthur et al., 2013), and volatile chemicals (Coyne et al., 1994; Noor and Coyne, 1996; Trabalon et al., 1997; Rafferty and Boughman, 2006). Often, multiple signals act in concert to affect species recognition (*e.g.*, Costanzo and Monteiro, 2007; Girard et al., 2015).

Although much is known about the importance of visual, auditory, and chemical signals and responses in sexual communication and species recognition, we know relatively little about other sensory modalities that may have strong effects on individual mating decisions. Tactile signals have been hypothesized to contribute to mating decisions (Mendelson and Shaw, 2012), but it is unclear whether tactile cues could represent a primary species recognition signal, given that visual, auditory, and chemical cues usually act earlier during the mating sequence.

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56   Research on the prevalence of tactile signals in mating decisions is limited (Coleman, 2008)

57   partly because of the experimental challenge it poses: whereas other sensory modalities

58   present male signals to a focal female from a distance, studying female preference for tactile

59   cues requires contact between males and females, which is not always easily achieved or

60   quantified under controlled conditions.

61   Despite this challenge, understanding the role of tactile signals along the continuum

62   between intraspecific mate choice and interspecific RI is important because it broadens our

63   understanding of the causes and consequences of a common pattern in nature— the rapid

64   divergence of male genital morphology between species. It has been suggested that rapid

65   genital differentiation can cause RI (Dufour 1844), although mechanical incompatibilies

66   between heterospecific male and female genitalia do not appear to be a common cause of RI

67   (Shapiro and Porter, 1989; Masly, 2012; Simmons, 2014). However, observations both within

68   (Eberhard, 1994; Edvardsson and Göran, 2000; Briceño and Eberhard, 2009a; Briceño and

69   Eberhard, 2009b; Frazee and Masly, 2015) and between species (Patterson and Thaeler Jr,

70   1982; Robertson and Paterson, 1982; Eberhard, 1992; Coyne, 1993; Barnard et al., 2017)

71   suggest that male reproductive structures may convey tactile information to females that

72   affects their subsequent behavior and/or physiology. Although female genital structures often

73   appear invariant among closely related species (Shapiro and Porter, 1989), subtle

74   morphological differences (*e.g.*, Kamimura and Mitsumoto, 2011; Yassin and Orgogozo, 2013)

75   could enable females to detect variation among males' morphology. Female variation in

76   detection ability could also occur in signal processing at the level of neurons, neural networks,

77   and/or in the distribution and morphology of sensory structures that receive male tactile

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78   signals. These sensory structures may exist not just in the female genitalia or reproductive

79   tract, but in any region of the female that receives contact from male structures.

80   Female sensory structures that reside in body regions that contact species-specific male

81   structures during mating have been documented in several arthropods, including flies

82   (Eberhard, 2001; Ingram et al., 2008) and damselflies (Cordoba-Aguilar, 1999, 2002, 2005;

83   Robertson and Paterson, 1982). Other studies have demonstrated that tactile cues from male

84   grasping organs influence female mating responses, either via experimental manipulation of

85   male structures and desensitization of females (Eberhard, 2002; Briceño et al., 2007; Briceño

86   and Eberhard, 2009a; Eberhard, 2010; Myers et al., 2016), or via comparison of female behavior

87   when females are grasped by males with varying terminal appendage morphologies (Sánchez-

88   Guillén et al., 2012; Sánchez-Guillén et al., 2014; Barnard et al., 2017). Premating tactile

89   isolation may also be important in vision-limited vertebrates. For example, contact cues via the

90   lateral line system may influence female mate choice in a cavefish (Plath et al., 2004; but see

91   Rüschenbaum and Schlupp, 2013).

92   Tactile signals appear to be a significant cause of RI in Zygoptera, the damselfly suborder

93   of Odonata (Krieger and Krieger-Loibl, 1958; Loibl, 1958; Robertson and Paterson, 1982; Corbet,

94   1999). Concentrations of cuticular mechanoreceptors (sensilla) on the female thorax have been

95   described in several coenagrionid damselfly genera. The morphology of these sensilla is

96   consistent with a mechanosensory function and does not indicate that they conduct signals

97   related to olfaction, hygroreception, or temperature reception (McIver, 1975; Robertson and

98   Patterson, 1982). These sensilla reside in areas where males' grasping appendages contact the

99   female thorax before and during mating, which has led to speculation that they allow females

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100   to evaluate male morphologies and discriminate conspecific from heterospecific males (Jurzitza,

101   1974, 1975; Tennessen, 1975; Robertson and Paterson, 1982; Battin, 1993a, 1993b). Each

102   mechanoreceptor is associated with a single sensory neuron (McIver, 1975; Kiel, 1997). The

103   thoracic sensilla thus represent a spatial matrix that can transmit signals to the female central

104   nervous system based on the pattern in which the sensilla are stimulated. Greater numbers of

105   these receptors are expected to enhance a female's sensory resolution by increasing the

106   combinatorial complexity of tactile signals that she can perceive. For example, if a female

107   possesses 25 sensilla, and each sensillum has two response states ("on" if contacted and "off" if

108   not contacted), then the number of unique tactile patterns that the female could distinguish is

109    $2^{25} = 3.4 \times 10^7$. A female that possesses just one additional sensillum would be able to

110   distinguish among roughly twice as many tactile patterns ($2^{26} = 6.7 \times 10^7$). Should individual

111   sensilla respond to quantitative variation in touch (rather than a binary response), this would

112   dramatically increase the number of response states and therefore further enhance tactile

113   acuity (*e.g.*, Gaffin and Brayfield, 2017). Female damselfly thoracic sensilla thus present an

114   external, quantifiable phenotype in which to investigate the mechanistic basis of tactile stimuli

115   and female mating decisions.

116   The North American damselfly genus *Enallagma* includes several recently diverged

117   species that often co-occur in the same habitats (Johnson and Crowley, 1980; McPeek, 1998),

118   and do not engage in premating courtship (Fincke et al., 2007; Barnard et al., 2017) or use

119   chemical cues for mate selection (Rebora et al., 2018). A female's first opportunity to assess a

120   potential mate occurs when the male uses his terminal appendages to grasp the mesostigmal

121   plates on the female thorax to form "tandem", the premating position. The male superior

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122   grasping appendages (cerci) have species-specific morphologies, and differences in the

123   morphology of these structures are the primary cause of RI in this genus (Paulson, 1974;

124   Barnard et al., 2017). Two species, *E. anna* and *E. carunculatum*, occasionally hybridize in

125   nature to produce males and females with reproductive structure morphologies that are

126   intermediate to each of the pure species (Miller and Ivie, 1993; Donnelly, 2008; Johnson, 2009;

127   Barnard et al., 2017). Females of both pure species discriminate strongly against both

128   heterospecific and interspecific hybrid males that take them in tandem, which shows that

129   female *E. anna* and *E. carunculatum* can detect not only large differences in male cercus

130   morphologies, but also more subtle differences such as those that distinguish conspecific and

131   hybrid males (Barnard et al., 2017).

132   Because it appears that mesostigmal sensilla mediate species recognition, they might be

133   expected to show signs of reproductive character displacement (RCD): increased divergence of

134   traits involved in RI in regions of sympatry between *E. anna* and *E. carunculatum* relative to

135   regions of allopatry (Brown and Wilson, 1956; Howard, 1993; Pfennig and Pfennig, 2009). RCD

136   can manifest phenotypically as divergence in either signaling traits or mate preferences, in

137   which sympatric females display stronger discrimination against heterospecific males than do

138   allopatric females of the same species (*e.g.*, Gerhardt, 1994; Gabor and Ryam, 2001; Albert and

139   Schluter, 2004; Wheatcroft and Qvarnstrom, 2017). This strengthening of preference in

140   sympatry may evolve via direct selection on adult prezygotic phenotypes, or via reinforcement,

141   where selection against interspecific hybrids gives rise to selection for enhanced premating

142   isolation between species (Dobzhansky, 1937). *Enallagma anna* and *E. carunculatum* can

143   interbreed, but their hybrids experience significantly reduced fitness (Barnard et al., 2017).

144   Female *Enallagma* experience frequent mating attempts from heterospecific males where both

145   species co-occur (Paulson, 1974; Fincke et al., 2007; Barnard et al., 2017). These findings

146   suggest that in sympatry, females may experience selection for stronger species discrimination

147   ability. Studies of several *Enallagma* species (not including *E. anna* or *E. carunculatum*) have

148   revealed that male cercus shape varies little among populations, even across large geographical

149   regions (McPeek et al., 2011; Siepielski et al., 2018). *Enallagma anna* and *E. carunculatum*

150   appear to show similar patterns, at least in the western part of their distributions (A. Barnard,

151   unpublished data). It is possible, however, that females in sympatry with other species are

152   more sensitive to variation among males than are females of the same species in regions of

153   allopatry, and this variation in sensitivity may be reflected in female sensilla traits.

154   Here, we use sensilla number, density, and location as proxies for female preference, to

155   test the hypothesis that variation in female sensilla phenotypes supports a function in species

156   recognition. We tested this hypothesis by quantifying sensilla on the mesostigmal plates of a

157   large set of *E. anna* and *E. carunculatum* females from multiple populations across the western

158   United States and comparing phenotypes of each pure species from sympatric and allopatric

159   populations to identify patterns consistent with RCD. We predicted that in sympatric

160   populations, females would possess higher sensilla numbers, higher sensilla density, and/or

161   different spatial distributions of sensilla within their mesostigmal plates when compared to

162   females from allopatric populations.

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164   **Materials and methods**

165   **Population sampling**

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166   We measured the sensilla traits of 29 *E. anna* females across 13 populations, and 74 *E.*

167   *carunculatum* females across 19 populations (Fig. 1, Table 1). We classified each population as

168   allopatric, locally allopatric, or sympatric. Sympatric populations are those where *E. anna* and

169   *E. carunculatum* co-occur temporally as well as spatially. Because *E. anna*'s geographic range

170   falls completely within *E. carunculatum*'s range, only *E. carunculatum* has completely allopatric

171   populations. We designated populations as locally allopatric at sites within the area of range

172   overlap, but where only one species is known to occur based on occurrence data from

173   OdonataCentral (Abbott, 2016). Although some specimens were collected as early as 1945, the

174   majority of samples (82 of 103) we studied were collected between 2012 and 2016.

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176   **Trait imaging and quantification**

177   We photographed each damselfly using a Nikon D5100 camera (16.2 MP; Nikon

178   Corporation, Tokyo, Japan). We dissected the ventral thoracic cuticle from each female using

179   forceps and imaged the mesostigmal plates using scanning electron microscopy. Specimens

180   were mounted on aluminum stubs with carbon tape, sputter-coated with gold-palladium, and

181   imaged at, 200X magnification and 3kV using a Zeiss NEON scanning electron microscope.

182   To avoid any potential bias during measurements, we blind-coded image files before

183   measuring all traits. We measured abdomen length (abdominal segments 1-10, excluding

184   terminal appendages) on the full-body photos as an estimate for body size using the segmented

185   line tool in ImageJ (Abramoff et al., 2004). We quantified sensilla traits on the right

186   mesostigmal plate of each female damselfly unless the right plate was dirty or damaged, in

187   which case we quantified the left plate (n = 20). Sensilla counts on a subset of 57 females

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188   showed that left plate and right plate sensilla counts were highly correlated within individual

189   females (r = 0.85). In cases where we quantified the left plate, we flipped the image

190   horizontally, so it was in the same orientation as a right plate. We standardized the position of

191   the mesostigmal plate in each image by cropping and rotating the image so that the lower

192   medial corner of the plate was in line with the lower left corner of each image. We counted

193   sensilla and obtained their x and y coordinates in ImageJ using the multi-point selection tool.

194   We traced an outline around the plate image, excluding the lateral carina (Fig. S1), using a

195   Wacom Cintiq 12WX tablet and stylus (Wacom, Saitama, Japan) and the freehand selection tool

196   in ImageJ. This procedure produced x and y coordinates that described the plate outline. We

197   performed all measurements twice for each specimen. Measurements across the two technical

198   replicates were highly correlated ($r_{abdomen}$ = 0.95, n = 78; r_{count} = 0.95, n = 103; $r_{plate\ area}$ = 0.99, n

199   = 86), so we used the mean trait values of the two replicates in subsequent analyses.

200   Seventeen samples were imaged at angles that allowed counting of the sensilla, but distorted

201   the plate shape or distances between the sensilla. Those samples are included in analyses of

202   sensilla number, but were not included in the analyses of sensilla density or distribution.

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204   **Sensilla trait analyses**

205   We conducted all morphometric and statistical analyses using R v. 3.4.1 (R Core Team,

206   2015). We used the mesostigmal plate outline coordinates to calculate each plate's two-

207   dimensional area. To calculate the area of the sensilla-covered region of each plate, we

208   generated a polygon connecting the coordinates of the outermost sensilla and calculated the

209   area within this outline. We determined the proportion of each plate that was covered by

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210   sensilla by dividing the sensilla area by total plate area. We calculated sensilla density in two

211   ways. First, we divided sensilla number by the area of the sensilla-covered region. This

212   measures the number of sensilla that occur in a particular area, but does not capture the

213   relative arrangement of sensilla within that area. Second, we computed the nearest neighbor

214   distances among all sensilla within each plate based on their *x* and *y* coordinates and then

215   calculated the mean and median nearest neighbor distances between the sensilla for each

216   female. Nearest neighbor mean and median distances were highly correlated ($r_{E.\ carunculatum}$ =

217   0.83; $r_{E.\ anna}$ = 0.81), so we used the mean values for these measures in our analyses.

218   To determine whether larger females possess more sensilla, we regressed sensilla

219   number against abdomen length. We found no significant relationship between these traits in

220   either species (*E. anna*: R^2_{adj} = -0.007, $F_{1,25}$ = 0.82, P = 0.737; *E. carunculatum*: R^2_{adj} = 0.01, $F_{1,48}$ =

221   0.52, P = 0.47). We thus present the results that compare sensilla counts without correcting for

222   differences in body size.

223

224   **Sensilla spatial analyses**

225   To quantify sensilla distributions within each plate, we generated kernel density

226   estimates (KDEs) for populations with at least four sampled individuals (two *E. anna* and six *E.*

227   *carunculatum* populations) using the R package ks (Duong, 2016). First, we randomly selected

228   one of the two replicate sets of sensilla and plate outline coordinates for each female. To

229   prepare the coordinate data for KDE analyses, we concatenated the sensilla and plate

230   coordinates for each female and adjusted all plate outlines to have an area of one. This

231   standardized each set of sensilla coordinates for size, while maintaining their relative positions

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232   within each plate. Next, we translated each set of coordinates to place the origin of the

233   coordinate system at the plate outline's centroid. We concatenated sensilla coordinates for all

234   females sampled within each population to compute a representative KDE for each population.

235   Within each species, we conducted pairwise tests to compare each population's KDE

236   against every other population using the function kde.test with the default settings. This test

237   returns a *P*-value that reflects the probability of generating the two respective KDEs from the

238   same distribution of points. Because we performed multiple pairwise tests among *E.*

239   *carunculatum* populations, we adjusted the resulting *P*-values using the false discovery rate

240   (Benjamini & Hochberg, 1995).

241   We generated an average plate outline for each population on which to visualize the

242   KDEs. The total number of coordinates that describe each plate outline varied among females,

243   ranging from 647-1078 for *E. anna* and 688-1028 for *E. carunculatum*. We standardized the

244   number of coordinates representing each plate by retaining the points for each of the upper

245   and lower medial corners and randomly sampled 198 points in between. We then treated each

246   of these, 200 points as landmarks (the corners represented fixed landmarks and the remaining

247   points were designated as sliding semilandmarks) and used the R package geomorph (Adams

248   and Otarola-Castillo, 2013) to perform general Procrustes analysis (Rohlf, 1999) and obtain an

249   average two-dimensional plate shape for each population.

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251   **Statistical analyses**

252   Some populations were well-sampled whereas others were represented by a single

253   female (Table 1). To avoid psedoreplication, for each population with N > 1, our analyses of

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254   sensilla number, density, and area of each mesostigmal plate covered by sensilla used the

255   population mean of each trait value, so that each population was represented by a single

256   measurement. We arcsin transformed proportion data prior to analysis. To compare traits

257   between *E. anna* and *E. carunculatum*, we used Welch's *t*-tests. We compared traits among

258   sympatric, locally allopatric, and fully allopatric *E. carunculatum* populations using Kruskal-

259   Wallis tests, and between sympatric and locally allopatric *E. anna* populations using Welch's t-

260   tests. To understand the relationships between sensilla number, sensilla density, and the area

261   of the plate occupied by sensilla, we performed linear regressions between each pair of traits.

262   Due to the limitation of single samples from some populations, we analyzed *E. carunculatum*

263   populations in two ways: we first included all samples, then conducted a separate analysis that

264   excluded populations with N < 4. Both analyses yield similar results; we report the results for

265   the analysis using all samples in the main text and provide results from the subset of samples

266   with N ≥ 4 in the supplemental material (Table S1).

267

268   **Results**

269   ***Enallagma anna* and *E. carunculatum* females possess distinct sensilla traits**

270   *Enallagma anna* females possessed significantly more sensilla per plate ($\bar{x}$ = 49 $\pm$ 2) than

271   *E. carunculatum* females ($\bar{x}$ = 28 $\pm$ 1, $t_{35.1}$ = 11.13, P = 4.6 × 10^{-13}; Fig. 2A). *Enallagma anna*

272   females also possessed sensilla distributed over a larger proportion of each plate ($t_{39.7}$ = 11.1, P

273   = 8.6 × 10^{-14}; Fig. 2B), and larger mean distances between sensilla (t_{54} = 6.7, P = 1.3 × 10^{-8}; Fig.

274   2C). This ultimately results in a lower density of sensilla per unit area in *E. anna* compared to *E.*

275   *carunculatum* ($t_{99.6}$ = -12.96, P = 2.2 × 10^{-16}; Fig. 2D). The sensilla also occurred in different

276   locations on the mesostigmal plates of each species: they were more medially located in *E.*

277   *anna* and more laterally located in *E. carunculatum* (Figs. 3, 4).

278   Both species showed a strong positive relationship between sensilla number and the

279   absolute area of the plate occupied by sensilla (*E. anna*: R^2_{adj} = 0.33, $F_{1,27}$ = 14.71, *P* = 0.0007; *E.*

280   *carunculatum*: R^2_{adj} = 0.33, $F_{1,72}$ = 37.68, *P* = 4.1 × 10^{-8}). Consistent with this result, linear

281   regressions also revealed that females with more sensilla also had a larger proportion of the

282   plate occupied by sensilla (*E. anna*: R^2_{adj} = 0.26, $F_{1, 27}$ = 10.65, *P* = 0.003; *E. carunculatum:* R^2_{adj} =

283   0.20, $F_{1, 65}$ = 18.93, *P* = 4.4 × 10^{-5}). Females with more sensilla had smaller mean distances

284   between neighboring sensilla (*E. anna*: R^2_{adj} = 0.11, $F_{1, 27}$ = 4.34, *P* = 0.046; *E. carunculatum*: R^2_{adj}

285   = 0.09, $F_{1,72}$ = 3.80, *P* = 0.01). Overall, these results show that a greater number of sensilla was

286   more strongly associated with a sensilla distribution that covers a larger area of the

287   mesostigmal plate rather than a greater concentration sensilla within in a smaller area.

288

289   **E. carunculatum sensilla traits do not show a strong pattern of reproductive character**

290   **displacement**

291   We made several non-mutually exclusive predictions expected under RCD for the

292   sensilla traits we measured in sympatric populations relative to allopatric populations. In

293   particular, we predicted to observe at least one of the following phenotypic differences in

294   sympatric females relative to allopatric females: (1) more numerous sensilla, (2) denser sensilla,

295   (3) sensilla concentrated in different regions of the mesostigmal plates. We did not find

296   significant differences in any of these traits between sympatric and locally allopatric *E. anna*

297   females (Table 2). However, because our *E. anna* samples included only four females from

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298   three locally allopatric populations, we could not perform a robust comparison of *E. anna*

299   sensilla traits between populations that do, or do not encounter *E. carunculatum*. We thus

300   focus our analysis on comparisons between sympatric and allopatric *E. carunculatum*

301   populations, for which we had larger sample sizes.

302   Sympatric, locally allopatric, and fully allopatric *E. carunculatum* populations did not

303   differ significantly from one another in sensilla number (Kruskal-Wallis χ^2_2 = 0.69, *P* = 0.71),

304   proportion of the mesostigmal plate covered by sensilla (Kruskal-Wallis χ^2_2 = 2.16, *P* = 0.34), or

305   sensilla density (overall density: Kruskal-Wallis χ^2_2 = 0.12, *P* = 0.94; mean distance between

306   sensilla: Kruskal-Wallis χ^2_2 = 3.53, *P* = 0.17). In addition to divergence of mean trait values, RCD

307   can also result in reduced trait variance in sympatry without affecting the mean (Pfennig and

308   Pfennig 2009). Sympatric *E. carunculatum* populations displayed less interpopulation variance

309   than allopatric populations in both mean sensilla number (Figure 2A) and mean proportion of

310   the plate covered by sensilla (Figure 2B). However, these trends were not statistically

311   significant (sensilla number: Bartlett's K^2_1 = 0.83, *P* = 0.36; proportion of plate covered by

312   sensilla: Bartlett's K^2_1 = 1.86 *P* = 0.17).

313   Interestingly, although mean trait values did not differ significantly between sympatric

314   and allopatric populations, sensilla traits displayed considerable variation within the

315   populations we sampled. For example, within a single population, a particular female might

316   have twice as many sensilla than another female (Fig. 3). This pattern was also observed in the

317   *E. anna* populations we studied.

318   KDE comparisons did not reveal significant differences in sensilla distributions between

319   sympatric and allopatric *E. carunculatum* populations (Table 3). However, the analysis revealed

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320   significant differences in sensilla distributions between several pairs of allopatric *E.*

321   *carunculatum* populations (Fig. 4E), which indicates that populations isolated from *E. anna* vary

322   more among themselves than do populations sympatric with *E. anna*, which share similar

323   sensilla patterns.   This result is consistent with those described above that indicated higher

324   variance in sensilla traits among allopatric populations compared to sympatric populations.

325

326   **Discussion**

327   *Enallagma anna* and *E. carunculatum* females possess different numbers of sensilla in

328   species-specific distributions on their mesostigmal plates. This result supports the idea that

329   receptors that receive male stimuli will occur in patterns that correspond to the male organs

330   during contact (Eberhard, 2010). An association between male morphology and female sensilla

331   has been described for African *Enallagma* species (Robertson and Paterson, 1982), and our

332   results show a similar pattern for two North American species. *Enallagma anna* male cerci are

333   considerably larger than *E. carunculatum* cerci, and the observation that *E. anna* females had a

334   larger number of sensilla compared to *E. carunculatum* females is consistent with the likelihood

335   that *E. anna* male cerci make greater spatial contact with the mesostigmal plates.

336   When species make secondary contact after initial divergence in allopatry, the possible

337   outcomes are increased species divergence (*e.g.*, Sætre et al., 1997; Noor, 2000; Naisbit et al.,

338   2001; Yukilevich, 2012; Dyer et al., 2014), decreased species divergence (*e.g.*, Ritchie et al.,

339   1989; Shurtliff et al., 2013; Yang et al., 2016), local extinction of one species due to

340   reproductive exclusion (Hochkirch et al., 2007, Groning and Hochkirch, 2008), or no change in

341   either direction (Abbott et al., 2013). Because *E. anna* and *E. carunculatum* produce

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342   reproductively disadvantaged hybrids (Barnard et al., 2017), selection is expected to favor

343   increased premating isolation when the species are sympatric. Within each species, we

344   predicted that female sensilla traits in sympatric populations would diverge from those of

345   allopatric populations indicative of a shift in female preferences to avoid mating with

346   heterospecifics. Contrary to this prediction, sympatric and allopatric *E. carunculatum*

347   populations were not significantly different in mean sensilla trait values (Fig. 2) or sensilla

348   density distributions (Fig. 4E).

349   Although we observed a trend toward more sensilla in sympatric *E. anna* populations

350   relative to allopatric populations (Figs. 2A, 3A), it is difficult to conduct a robust comparison for

351   this species because *E. anna*'s entire geographic range overlaps with *E. carunculatum*'s range

352   and *E. anna* are often relatively rare (Acorn, 2004; A. Barnard, personal obs.). It was therefore

353   difficult to collect sufficient *E. anna* samples from populations that do not co-occur with *E.*

354   *carunculatum*. We might, however, expect a stronger pattern of RCD in sympatric *E. anna*

355   females because *E. carunculatum* males can take them in tandem relatively easily, whereas *E.*

356   *anna* males are typically unsuccessful at taking *E. carunculatum* females in tandem (Barnard et

357   al., 2017). This means that *E. anna* females may have more opportunities for mating mistakes

358   than *E. carunculatum* females, which can result in stronger asymmetric RCD (Lemmon, 2009;

359   Pfennig and Pfennig, 2009).

360   There are at least three potential explanations for the absence of RCD in the form of

361   significant differences in the sensilla traits we measured between sympatric and allopatric

362   populations of *E. carunculatum*. First, species-specific sensilla distributions may be sufficiently

363   different to allow females to recognize when they are taken in tandem by heterospecific or

364   conspecific males. If this is true, small degrees of variation within the overall species pattern

365   among females might not affect females' species-recognition abilities. Indeed, a recent study

366   found that intraspecific variation in male cercus morphology appears too minor for *Enallagma*

367   females to show strong discrimination among conspecific males that grasp them (Siepielski et

368   al., 2018). Although RCD is most easily facilitated when the trait under selection already differs

369   between species (Pfennig and Pfennig, 2009), these sensilla traits may have already diverged

370   sufficiently enough to preclude strong selection on further divergence.

371   Second, it is possible that the external sensilla phenotypes we measured are not

372   representative of proximate female sensory traits, and the variation that directs mating

373   decisions occurs within the female nervous system. For example, individual sensilla might differ

374   in response rate or ability to distinguish different levels of pressure applied by the cerci and

375   grasping pressure might differ between males of each species. The direction of mechanosensor

376   deflection is also important for stimulus detection (Keil, 1997), and different species' cercus

377   morphologies may contact sensilla from different angles. Female mate preferences may also be

378   influenced by the relative frequencies with which females encounter heterospecific and

379   conspecific males and female sexual experience (*e.g.*, Svensson et al., 2014).

380   Finally, although we did not detect a statistically significant difference between group

381   means, the small differences we observed may still have biological relevance. If gaining just

382   one additional mechanosensor can (at least) double a female's tactile discriminatory power

383   (Gaffin and Brayfield, 2017), then females in a population with a seemingly minor upward shift

384   in sensilla number could gain a substantial increase in their ability to detect and avoid mating

385   with heterospecifics. Similarly, it is difficult to determine the features of sensilla density

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386   distributions that may influence female preference solely by conducting statistical tests

387   between KDEs. Small spatial differences within largely similar patterns may not contribute a

388   signal large enough to be captured in a statistical test, but still reflect salient variation in the

389   way females receive tactile stimuli. This might include three dimensional spatial differences

390   that we were unable to measure here.

391   These possible explanations highlight the interesting avenues that female damselfly

392   sensilla provide for investigating the mechanisms underlying how females evaluate male tactile

393   signals to make mating decisions. The ability to quantify the number and locations of female

394   mechanoreceptors in a region contacted by male reproductive structures complements our

395   understanding of patterns of variation in male morphologies (McPeek et al., 2008; McPeek et

396   al., 2009; McPeek et al., 2011; Barnard et al., 2017). Females of both species display substantial

397   intrapopulation variation in sensilla traits (Fig. 3) and this variation may play a role in sexual

398   selection and female preferences within species. Behavioral studies will be crucial to link

399   mechanoreceptor phenotypes to female mating decisions and clarify how sensilla traits

400   influence both species recognition and sexual selection. For example, do females with more

401   sensilla make fewer mating mistakes than females with fewer sensilla (Lemmon, 2009)?

402   Another outstanding question of this system is how the cerci stimulate individual sensilla during

403   tandem. This might be determined by flash-freezing male-female tandem pairs and using

404   micro-CT scanning to understand how the male and female structures interact, similar to a

405   recent approach used in seed beetles (Dougherty and Simmons, 2017). Once we understand

406   how cerci contact the sensilla, functional tests of sensilla electrophysiology could reveal how

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407   individual sensilla respond to stimulation and indicate whether certain sensilla make greater

408   contributions to reproductive decision-making than others.

409   Female preference can drive sexual selection, promote trait divergence, and cause RI

410   between species (Ritchie, 1996). A longstanding presumption in the literature on genital

411   evolution and speciation has been that female reproductive morphologies are less variant or

412   species-specific than male genitalia (reviewed in Shapiro and Porter, 1989). However, recent

413   studies of variation in female reproductive structures suggest that variation does exist among

414   individuals and species (Ah-King et al., 2014), and our data highlight the importance of looking

415   beyond the easily-quantified external morphologies. When male reproductive structure

416   morphologies are obviously divergent, but female morphologies are not, females may possess

417   important variation at neurophysiological levels that affects how they evaluate male tactile

418   signals, similar to the way females evaluate signals in other sensory modalities.

419

420   **Acknowledgements**

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664   **Figure legends**

665   **Figure 1**. **Sampling sites and species ranges.** *Enallagma anna*'s geographic range (red) occurs

666   within *E. carunculatum*'s geographic range (orange). Names of sites associated with each

667   number are described in Table 1. Symbol color indicates the species sampled and symbol shape

668   indicates the population type. (Species ranges are adapted from Johnson, 2009; Paulson, 2009,,

669   2011).

670

671   **Figure 2. *Enallagma anna* and *E. carunculatum* sensilla traits by population type. (A)** The

672   number of sensilla on one mesostigmal plate. **(B)** Proportion of the plate that contains sensilla.

673   **(C)** Mean nearest neighbor distances between sensilla. **(D)** Sensilla density in the region of the

674   plate that contains sensilla. Within each panel, each open circle represents the mean of one

675   population. Boxplots show the interquartile range. The line within the box shows the median

676   and whiskers extend to the most extreme observation within 1.5 times the interquartile range.

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678   **Figure 3. Individual trait values for sensilla number, sensilla density, and proportion of plate**

679   **containing sensilla.** Each symbol represents a single female, separated by population along the

680   *y*-axis. Horizontal lines indicate the mean value for each population type (completely allopatric,

681   locally allopatric, or sympatric), calculated from population means. Populations are described

682   in Table 1.

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684   **Figure 4. Sensilla locations. (A)** White box indicates the location of right mesostigmal plate on

685   the thorax. **(B)** Ultrastructural details of individual sensilla. Scale bar represents 10 μm. **(C, D)**

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686   Scanning electron micrographs show the locations of sensilla (yellow) on the mesostigmal

687   plates of *E. anna* (C) and *E. carunculatum* (D). Scale bars represent 100 μm. **(E, F)** Population

688   kernel density estimates for *E. carunculatum* (E) and *E. anna* (F) sensilla. The shading indicates

689   different regions of sensilla density: red represents the 75-99$^{\text{th}}$ percentile of sensilla density,

690   orange represents the 50-74$^{\text{th}}$ percentile, and yellow represents the 25$^{\text{th}}$-49$^{\text{th}}$ percentile. Each

691   outline represents the average mesostigmal plate shape for the population. Asterisks indicate

692   *E. carunculatum* populations whose KDEs are significantly different (* $P < 0.05$, *** $P < 0.001$).

693 **Table 1**. Sampling sites for *E. anna* and *E. carunculatum* populations.

Type	Site (site number*)	Species	Latitude	Longitude	Year collected	N†	Source‡
Sympatric	Big Spring, UT (1)	Ea	40.7407	-112.6472	2016	10	AB
		Ec				4	
	Big Sandy Creek, MT (2)	Ec	48.4519	-109.9199	2015	1	AB
	Creston, MT (3)	Ea	48.2437	-114.1406	1972	1	BM
	Dry Sheep Creek, NE (4)	Ea	41.9999	-103.9706	2012	1	BM
	Fish Springs Run, CA (5)	Ea	37.0794	-118.2539	1998	2	BM
	Grace Coolidge Creek, SD (6)	Ea	43.8072	-103.4502	1969	1	BM
	Horseshoe Springs, UT (7)	Ea	40.6203	-112.7099	2016	1	AB
		Ec				1	
	Long Valley Creek, CA (8)	Ea	39.7315	-120.0434	1973	5	DP
	Murray Creek, NV (9)	Ea	39.2669	-114.8687	2001	1	
	Malad River, UT (10)	Ec	41.8652	-112.1692	1983	2	BM
	Niwot Ditch, CO (11)	Ea	40.1632	-105.1544	2015	2	AB
		Ec				1	

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	Pondera Coulee, MT (12)	Ea	48.1892	-111.3268	2015	1	AB
		Ec				1	
Locally allopatric	Beaver Creek, WY (13)	Ea	42.6417	-108.3475	2015	1	AB
	Indian Road Camp, MT (14)	Ec	46.3336	-111.5254	2015	4	AB
	Jackson, WY (15)	Ea	43.5363	-110.7629	1971	2	BM
	Muddy Creek, MT (16)	Ea	47.9796	-112.1565	2015	1	AB
	Strawberry River, UT (17)	Ec	40.1692	-110.4229	2016	1	AB
	West Greenbelt, CO (18)	Ec	39.7742	-105.1350	2014	9	AB
Allopatric	Bull Lake, MT (19)	Ec	48.2262	-115.8404	2015	1	AB
	Crab Creek, WA (20)	Ec	46.8317	-119.8431	2016	20	DP
	Clear Lake, IN (21)	Ec	41.7360	-84.8397	1945	1	BM
	Columbia River, WA (22)	Ec	45.83	-122.77	1952	2	BM
	Douglas Lake, MI (23)	Ec	45.5606	-84.6741	2016	17	OF
	Flathead River, MT (24)	Ec	47.3678	-114.5776	2015	4	AB
	Home Lake, CO (25)	Ec	37.5756	-106.0937	2015	1	AB

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Little Lake, CA (26)	Ec	35.9490	-117.9023	1967	1	DP
Drumond Island, MI (27)	Ec	46.00	-83.66	2002	1	BM
Snake River, ID (28)	Ec	43.7231	-112.0865	1983	2	BM

694   *Site number corresponds to the locations numbered in Figure 1

695   † N: number of females that were imaged and measured for this study.

696   ‡ Sources: A. Barnard (AB), Ola Fincke (OF), Bill Mauffray (BM), and Dennis Paulson (DP).

697 **Table 2.** Statistical comparison of sensilla traits in locally allopatric and sympatric *E. anna*

698 populations.

Trait	Mean ± s.e.m.		t	d.f.	P
	Local allopatry (N* = 3)	Sympatry (N = 10)			
Sensilla number	39.8 ± 3.8	48.5 ± 2.3	-1.93	3.6	0.13
Proportion plate containing sensilla	0.67 ± 0.27	0.69 ± 0.15	-0.25	2.8	0.82
Sensilla density (sensilla mm^{-1})	1.5 ± 0.2	1.6 ± 0.1	-0.43	2.9	0.70
Mean distance (μm) between sensilla pairs	, 20.0 ± 1.0	, 19.3 ± 0.5	0.62	3.13	0.58

699 * N: number of populations analyzed.

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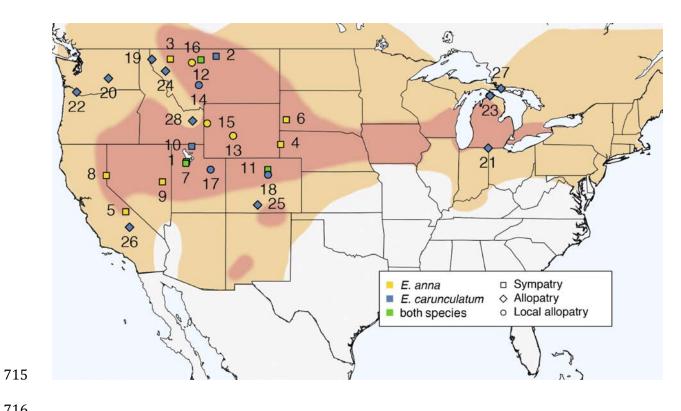709 **Table 3**. Results of pairwise comparisons of sensilla kernel density estimates for *E.*

710 *carunculatum* populations. False discovery rate-adjusted *P*-values are reported[†].

Population	Big Springs, UT	Crab Creek, WA	Douglas Lake, MI	Flathead River, MT	West Green-belt, CO	N*	Population type
Big Springs, UT	1					4	sympatric
Crab Creek, WA		1				20	allopatric
Douglas Lake, MI	0.263	**2.53e^{-10}**	1			17	allopatric
Flathead River, MT	1	**0.0103**	0.263	1		4	allopatric
West Greenbelt, CO	1	0.0625	0.3835	0.502	1	4	Locally allopatric
Indian River, MT	1	1	**0.0103**	0.0625	0.3115	4	Locally allopatric

711 * N: number of females whose sensilla coordinates were used to calculate KDEs.

712 † Bold values indicate *P* < 0.05.

713 **Figure 1**.

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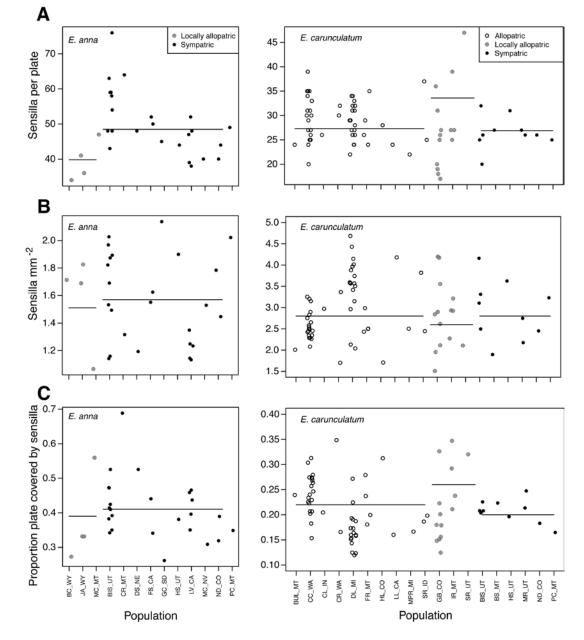
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718   **Figure 2**.

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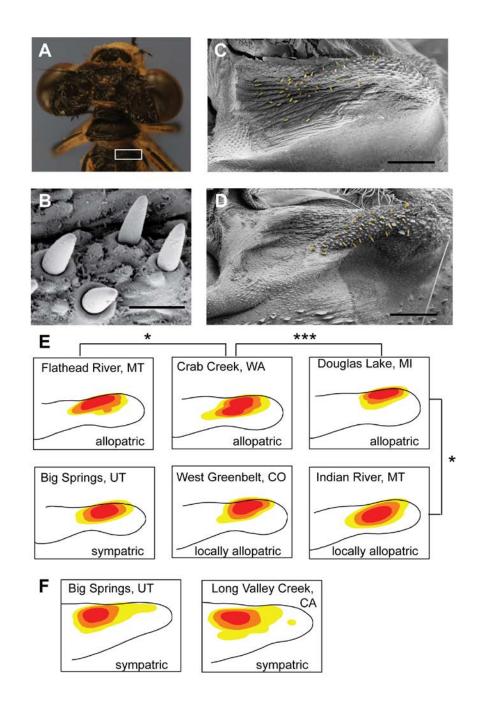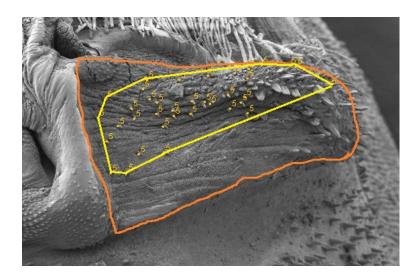721   **Figure 3**.
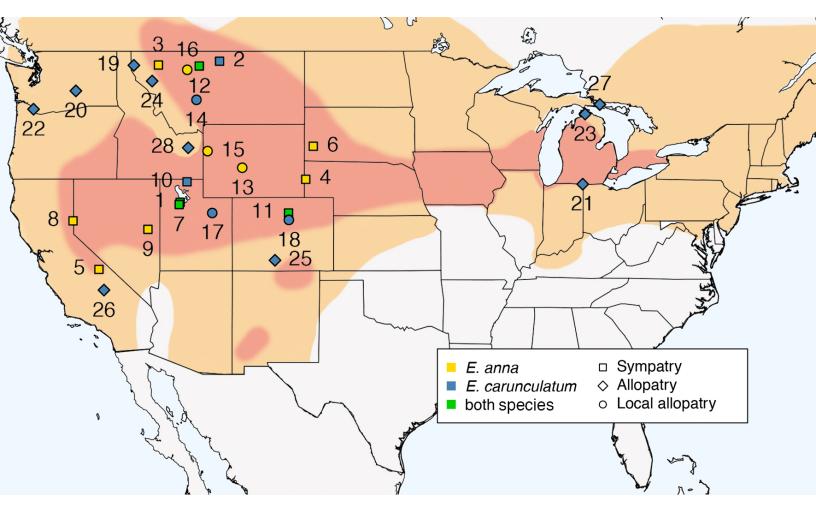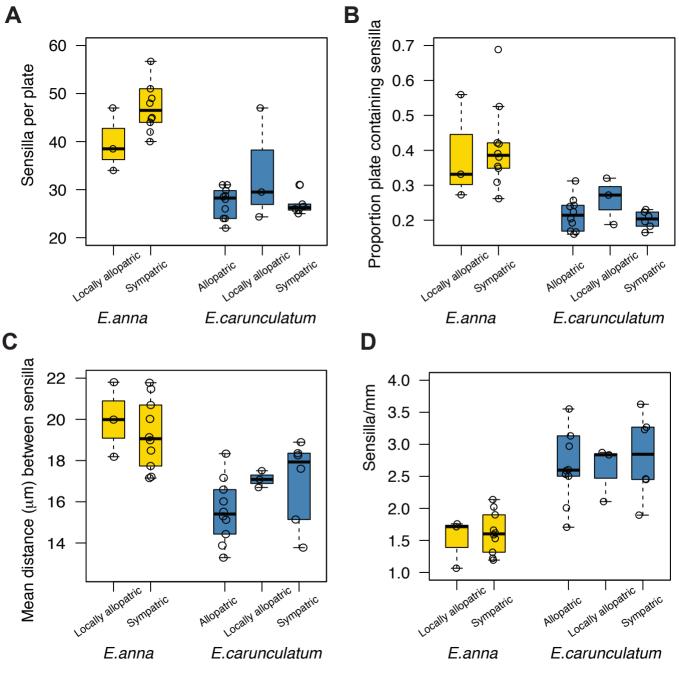
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725 **Figure 4**.

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Figure S1. Method to obtain (*x*, *y*) coordinates of mesostigmal plate outline and individual

sensilla from scanning electron microscope images. The orange line shows the outline that

represents the boundaries of the mesostigmal plate. Yellow dots indicate individual sensilla.

The yellow line around the sensilla shows the polygon generated by connecting the outermost

sensilla.

736 **Table S1.** Statistical comparison of sensilla traits among sympatric, locally allopatric, and fully

737 allopatric *E. carunculatum* populations.

Trait	Mean ± s.e.m.			$\chi^2_2{}^\dagger$	*P*
	Sympatry S (N = 1)	Local allopatry (N = 2)	Allopatry (N = 3)		
Sensilla number	25.8	26.9 ± 2.6	29.0 ± 0.4	2.79	0.25
Proportion plate containing sensilla	0.21	0.23 ± 0.30	0.21 ± 0.23	0.04	0.98
Sensilla density (sensilla mm^{-2})	15.1	17.3 ± 0.2	15.9 ± 0.8	2.14	0.34
Mean distance (μm) between sensilla pairs	3.27	2.85 ± 0.02	2.91 ± 0.32	0.86	0.65

738 * N refers to the number of populations analyzed.

739 $\dagger \chi^2_2$ refers to the Kruskal-Wallis chi-squared value with 2 degrees of freedom.

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Legend:
- *E. anna* — yellow square
- *E. carunculatum* — blue square
- both species — green square
- Sympatry — open square
- Allopatry — open diamond
- Local allopatry — open circle

A

E. anna — Locally allopatric ⊕, Sympatric ●

E. carunculatum — Allopatric ○, Locally allopatric ⊕, Sympatric ●

Sensilla per plate

B

E. anna

E. carunculatum

Sensilla mm^{-2}

C

E. anna

E. carunculatum

Proportion plate covered by sensilla

Population

BC_WY, JA_WY, MC_MT, BIS_UT, CR_MT, DS_NE, FS_CA, GC_SD, HS_UT, LV_CA, MC_NV, ND_CO, PC_MT

BUL_MT, CC_WA, CL_IN, CR_WA, DL_MI, FR_MT, HL_CO, LL_CA, MPR_MI, SR_ID, GB_CO, IR_MT, SR_UT, BIS_UT, BS_MT, HS_UT, MR_UT, ND_CO, PC_MT