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Female mate preferences on high-dimensional shape variation for male species recognition traits

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Abstract

Females in many animal species must discriminate between conspecific and heterospecific males when choosing mates. Such mating preferences that discriminate against heterospecifics may inadvertently also affect the mating success of conspecific males, particularly those with more extreme phenotypes. From this expectation, we hypothesized that female mate choice should cause *Enallagma* females (Odonata: Coenagrionidae) to discriminate against conspecific males with more extreme phenotypes of the claspers males use to grasp females while mating – the main feature of species mate recognition in these species. To test this, we compared cerci sizes and shapes between males that were captured while mating with females to males that were captured at the same time but not mating in three *Enallagma* species. In contrast to our hypothesis, we found only one of forty comparisons of shape variation that was consistent with females discriminating against males with more extreme cerci shapes. Instead, differences in cerci shape between mating and single males suggested that females displayed directional preferences on 1–4 aspects of cerci shape in two of the species in our samples. These results suggest that whereas some directional biases in mating based on cerci shape occur, the intraspecific phenotypic variation in male cerci size and shape is likely not large enough for females to express any significant incidental discrimination among conspecifics with more extreme shapes.

Introduction

Mating decisions generate some of the strongest selection pressures impinging on the phenotypes of species, as these decisions define essential components of fitness for all involved (Kingsolver *et al.*, 2001; Andersson & Simmons, 2006). For males, mating decisions by females can influence which males will produce offspring and which males will not, and of those males that do obtain mates, the number and fitness qualities of female partners will further determine how many offspring they will produce. Likewise for females, mate discrimination may influence the number and quality of their offspring.

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More fundamentally for many species, individuals must also discriminate between conspecifics and heterospecifics to ensure that they produce any offspring at all, and many species have mate recognition systems that facilitate this discrimination (Paterson, 1985; Ryan & Rand, 1993; Boake *et al.*, 1997). In species with specific mate recognition systems, individuals choose among mates based on some phenotype or set of phenotypes, including calls and vocalizations (Otte, 1989; Wells & Henry, 1992; Ryan & Rand, 1993; Gerhardt, 1994; Shaw, 2000; Gerhardt, 2005), colour patterns (Waage, 1979; Price, 1998; Seehausen & van Alphen, 1998; Tynkkynen *et al.*, 2004; van Alphen *et al.*, 2004; Svensson *et al.*, 2007; Price, 2008; Salzburger, 2009; Svensson *et al.*, 2016; Verzijden & Svensson, 2016), pheromones (Lofstedt, 1993; Blows & Allan, 1998; Wyatt, 2003; Bickford *et al.*, 2007; Johansson & Jones, 2007; Higgie & Blows, 2008) and morphological structures (Dufour, 1844; Eberhard, 1985; Arnqvist, 1998;

Hosken & Stockley, 2004; Simmons, 2014). By choosing mates within a restricted range of phenotypes, an individual is maximizing its probability of mating with a conspecific (McPeek & Gavrilets, 2006; Pfennig & Ryan, 2006; Phelps *et al.*, 2006; Svensson *et al.*, 2007). However, in so doing, individuals may also apply selection to these phenotypes in conspecifics of the opposite sex (Pfennig, 1998; Higgie *et al.*, 2000; Hosken & Stockley, 2004; McPeek & Gavrilets, 2006; Pfennig & Ryan, 2006; Higgie & Blows, 2008; Hoskin & Higgie, 2010; Steele *et al.*, 2011). Specifically, such mate choice should continually enforce strong stabilizing selection on conspecific phenotypes because conspecifics with more extreme phenotypes should be mistaken more frequently as heterospecifics and be rejected as mates.

In this study, we test whether females express any discrimination among conspecific males based on the structures that are used in the species mate recognition system in *Enallagma* damselflies (Odonata: Coenagrionidae). Up to eight *Enallagma* species can routinely be found breeding at a lake on a given day, and *Enallagma* males will attempt to mate with females of any *Enallagma* species indiscriminately (Paulson, 1974; Robertson & Paterson, 1982; Miller & Fincke, 1999; Barnard *et al.*, 2017). Females exercise choice about which males are suitable mates, and a substantial component of this choice is based on the morphological features of male secondary structures used to grasp females while mating (Paulson, 1974; Fincke, 1982; Robertson & Paterson, 1982; Tennessen, 1982; Fincke, 1986a, 1997; Miller & Fincke, 1999; Barnard *et al.*, 2017). Adult females spend most of their time away from lakes and only return to oviposit. When a female arrives at a lake, males of all *Enallagma* species in her immediate vicinity will attempt to grasp her thorax with the four secondary appendages on the ends of their abdomens (Fig. 1a). The two male inferior appendages (i.e. the paraprocts) grasp the dorsal and lateral surfaces of her prothorax, and the two male superior appendages (i.e. the cerci) grasp the female's thorax on two mesostigmal plates on the anterodorsal surface of her mesothorax (Fig. 1). Once a male has grasped a female, she decides whether she will mate with this male or reject him – males cannot force copulation (Paulson, 1974; Fincke *et al.*, 2007; Gosden & Svensson, 2007).

The male cerci and the female mesostigmal plates constitute the species mate recognition system of *Enallagma* (Paulson, 1974; Robertson & Paterson, 1982; Tennessen, 1982; Westfall & May, 2006; Fincke *et al.*, 2007; McPeek *et al.*, 2008, 2009, 2011; Barnard *et al.*, 2017). The female's mesostigmal plates have characteristic shapes that differ among species, and these plates are lined with mechanoreceptors in species-specific patterns (Paulson, 1974; Robertson & Paterson, 1982; Tennessen, 1982; Westfall & May, 2006; McPeek *et al.*, 2009). Likewise, male cerci have characteristic shapes which differ markedly among species (Westfall & May, 2006; McPeek *et al.*, 2008, 2011). A female uses the tactile cues she receives from the contact between the clasping male's cerci and her mesostigmal plates to discriminate whether he is a conspecific or heterospecific. Experimental studies have shown that this is the primary stage of the mating process that maintains reproductive isolation between heterospecifics; females reject heterospecific males, and females refuse to mate with conspecific males whose cerci have been experimentally altered (Paulson, 1974; Robertson & Paterson, 1982; Fincke *et al.*, 2007; Barnard *et al.*, 2017). Also, the distribution of cerci shape variation within each species does not overlap the distributions of any other species with which they have overlapping ranges, and no significant variation in cerci shape is apparent among populations across the ranges of species that span northern North America (McPeek *et al.*, 2011).

Based on these findings, we predicted that conspecific males captured in the process of mating with females would contain males with less extreme phenotypes than males captured at the same time but not in tandem with females. The shapes of female mesostigmal

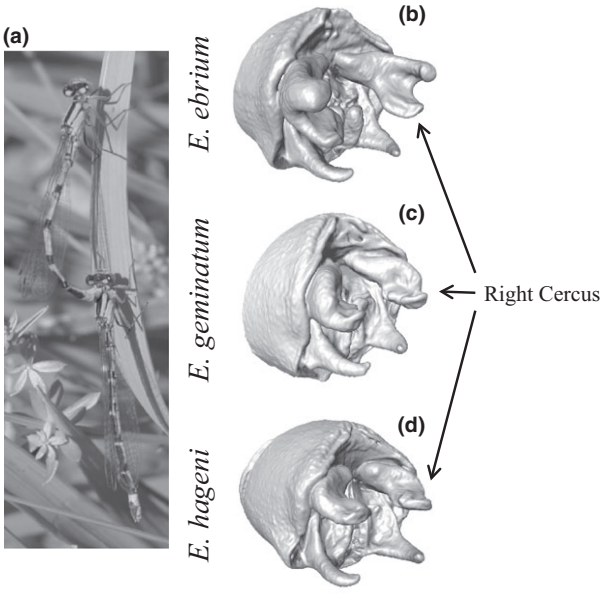


Fig. 1 An illustration of (a) a mating *Enallagma* pair, and representative computer microtomography scans of the entire 10th abdominal segment for males of (b) *Enallagma ebrium,* (c) *Enallagma geminatum* and (d) *Enallagma hageni*. In panel A, a male *E. hageni* (individual above) is grasping a female with his four terminal abdominal appendages on her pro- and mesothorax. Panels b–d show computer tomography reconstructions of the distal end of a male's 10th abdominal segment, illustrating these paired appendages. Arrows point to the right cercus of each male which is the structure analysed in this study. Notice that the two inferior paired structures – the paraprocts – are very similar among the species, whereas the two superior paired structures – the cerci – differ in shape among the species.

plates in a population should favour a particular cerci shape, and males that deviate from that shape should be less likely to be found mating with females. After a female has accepted a male, the male removes the sperm of previous mates and then copulates with her himself (Fincke, 1984): we refer to this period as 'in tandem', which lasts 10–20 min (M. A. McPeek, unpublished data). Once copulation is completed, the male releases the female, and she typically climbs down a plant stem to oviposit below the surface of the water (Fincke, 1986b). Thus, males that are in the process of mating with females are easily identified because they are conspicuously in tandem. Therefore, as in previous studies (e.g. Gosden and Svensson 2008; Steele *et al.*, 2011), we tested our prediction by collecting large numbers of males of three *Enallagma* species that were and were not in tandem with females, quantifying the shapes of their cerci, and using logistic regression to compare differences in cerci shapes between single and tandem males.

Materials and methods

To assess whether female mate preferences that should discriminate against heterospecific males also generate biases in the phenotypes of conspecific males that are found mating, we caught males of three *Enallagma* species that were either in tandem or not in tandem with females on 1 day at the peak of the breeding season. The breeding period for *Enallagma* species, like most coenagrionids, is concentrated in a 1- to 2-week period (Fincke, 1982, 1986a; Cordero, 1995). *Enallagma ebrium* males were sampled on 6 July 2010 at Lower Symes Pond, Ryegate, Vermont, USA (44°14′33.9″N, 72°5′31.2″ W); *Enallagma geminatum* males were sampled on 26 July 2010 at Lower Baker Pond, Wentworth, New Hampshire, USA (43°53′40.3″N, 71°57′38.9″W); and *Enallagma hageni* males were sampled on two dates in consecutive years, on 6 July 2010 and on 28 July 2011, at Martin's Pond, Green Bay, Vermont, USA (44°18′23.8″N, 72°11′ 13.8″W).

Because the shape of the female thoracic plates and male cerci do not change over their adult lifespan, and their shapes are highly consistent across the ranges of species that span much of North America (e.g. McPeek *et al.*, 2011), focusing collections on 1 day should be representative of the entire breeding period. In effect, we are testing the expressed preferences of females based on the phenotypes of their male partners. If our hypothesis was correct, we expected that these female preferences result in the distributions of single males to have more extreme cerci shapes in all three species, because it should not matter when or where mating preferences are being quantified. Thus, intensively sampling on a single day allows us to test our core hypothesis. The comparison between *E. hageni* in 2010 and 2011 at the same lake also served as a very simple check for the consistency of mate selection across successive generations (*Enallagma* are univoltine). On each day, we collected males with aerial nets between 1000 and 1700 h. Males were placed in 2-mL microfuge tubes filled with 70% ethanol and stored in the laboratory until processed.

The methods used to quantify cerci shape closely followed our previous work using micro-computer tomography scans of these structures (McPeek *et al.*, 2008, 2009, 2011). We refer the reader to Appendix S1 for a detailed description of these methods. All males caught in tandem (hereafter 'tandem males') were scanned, and a random subsample of males caught without a female (hereafter 'single males') and of comparable sample size to the tandem males were scanned from each population. Because of debris clinging to the cerci of some males, a few scanned males in each category were not included in the final data set; final sample sizes are given in Table S1.

We then used the SPHARM software package (McPeek *et al.*, 2008; Shen *et al.*, 2009) to analyse triangular mesh representations of the external surfaces of the cerci using spherical harmonics. (The SPHARM package that implements these spherical harmonics analyses is available at http://enallagma.com/.) The result was a very large set (768) of complex coefficients that quantify the contributions of different spatial frequencies to overall cercus shape. All cerci were standardized to a centroid size of 1.0 before applying spherical harmonics techniques to remove size from the characterization of shape. We included the centroid size before standardization in the analyses as well.

To reduce the dimensionality of the data set, separate principal components (PC) analyses were performed for each species on the covariance matrix among the 768 complex-valued SPHARM coefficients. We chose to perform separate PC analyses on each species so that PCs would quantify only intraspecific variation. We did combine individuals of *E. hageni* from the 2 years for the PC analysis, but analysed differences between tandem and single males each year separately. Because separate PC analyses were carried out on each species, PCs cannot be compared across species. Also, female preferences should not necessarily be based on the greatest axes of cerci shape variation. In this analysis, the PCA is used simply as a transformation that creates a much smaller set of variables that still characterizes a substantial fraction of the total shape variation described by the original 768 complex spherical harmonic coefficients. We chose to maintain ~ 75% of the original variation before we performed the analyses of phenotypic differences between single and tandem males. Therefore, we analysed scores for the first 10 PCs, which explained 68–75% of the total variation in cerci shape within species (Table S1). Each PC describes an axis of shape variation for that species, and each PC axis is centred on the grand mean for the entire data

set (i.e. the origin in the high-dimensional shape space) for that species combination.

To visualize shape variation represented along each PC axis, cerci models were reconstructed at five points along each PC axis: the origin of the axis (i.e. at 0.0), and at ±1 and ±2 standard deviations along the axis. These five points span ~ 95% of the shape variation along that axis. The faces of the models were then coloured according to their shape relative to the model at the PC axis origin (i.e. the reference shape) in the following way. The distances between all corresponding faces on the model at a given position relative to the corresponding face of the model at the PC axis origin were first calculated. If the face on the model in question was closer to its centroid (i.e. the point at the average of all vertices for that shape) than was the corresponding face on the model at the PC origin, the distance for that face was designated as negative, which signifies the face on the model is 'inside' the face of the model at the PC origin. Conversely, if the face on the model in question was farther from its centroid than was the corresponding face on the model at the PC axis origin, the distance is designated as positive, which signifies the face is 'outside' the face of the model at the PC origin. Faces of models were then coloured using a greyscale colour map based on these distances, so that shape change relative to the shape at the origin of the high-dimensional space can be visualized more easily: greater positive distances get lighter shades, greater negative distances get darker shades, and a distance of zero gets the median grey colour.

As an illustration of this visualization method, consider the following simple example. Imagine three elastic spheres that are initially of identical radius. Now, compress one sphere at both poles but keep its volume constant (imagine pressing your hands on either side of a balloon), pull apart the poles of the second sphere while also keeping its volume constant, and leave the third sphere unaltered. Faces near the poles of the compressed sphere will be 'inside' the faces of the uncompressed sphere, but its faces near the equator will be 'outside' those of the uncompressed sphere. Conversely, for the sphere where the poles were pulled apart, its faces near the poles will be outside those of the uncompressed sphere, but its faces near the equator will be inside those of the uncompressed sphere. Using the above scheme to colour the three spheres with the uncompressed sphere as the reference, the uncompressed sphere will be coloured uniformly the median shade of grey, whereas the compressed sphere would be coloured darker at the poles and lighter at the equator, and the sphere with the poles pulled apart would be coloured lighter at the poles and darker at the equator. The images presented do actually show the reconstructed cerci shapes at these points, but differences are subtle enough to require this shading enhancement to visualize the differences.

Statistical analysis

We used logistic regression to quantify the relationships between cerci shape and the probability of being captured in tandem with a female (Janzen & Stern, 1998). The binary response variable (single = 0, tandem = 1) in these analyses was regressed on a model of independent variables containing centroid size, the ten PCs describing cerci shape, and the quadratic terms for each. A logit link function was used to relate the independent variables model to the response variable. The quadratic terms test the hypothesis whether females bias their mating preferences against (negative regression coefficient) or towards (a positive regression coefficient) males with more extreme cerci phenotypes. The linear terms test whether females show any simple directional biases in their preferences. (Obviously, males that are identified as 'single' in these samples may have bred previously, and so these analyses are conservative with respect to identifying cerci shape properties that females may prefer.)

Because the regression coefficients from these logistic regressions are equivalent to measuring selection imposed by the females on male cerci size and shape on these specific days, we present the regression coefficients as standardized selection gradients (Janzen & Stern, 1998). This permits our estimates to be compared to estimates from other studies of such characters. To do this, PCs were first standardized to mean = 0.0 and variance = 1.0, and the resulting logistic regression model was used to predict an absolute fitness value (W_i) for each individual (i.e. predicted values after applying the inverse link function). The odds ratio of the absolute fitness quantity $W_i(1-W_i)$ is then calculated for each individual. The standardized selection gradient is then the regression coefficient from the logistic regression multiplied by the ratio of the mean odds ratio divided by the mean absolute fitness (i.e. standardized selection gradient = slope $\times$ $\overline{(W(1-W)}/\overline{W})$ (Janzen & Stern, 1998). Values for quadratic terms were then doubled (Stinchcombe *et al.*, 2008). Because the PC axes describe independent components of shape variation for each species, each of the tests of statistical significance are likewise independent. All statistical analyses were performed using the GLIMMIX procedure of SAS, version 9.4 (SAS Institute Inc. 2016).

Results

The distributions of cerci sizes and shapes for males captured in tandem with females were different from males captured singly for multiple PCs in two of the three species (Table 1). No term in the logistic regression model was significant for *E. ebrium*, which indicates that the distributions of cerci sizes and shapes were similar for single and tandem males (Table 1).

Table 1 Statistical tests for parameters resulting from logistic regression analyses of males caught singly or in tandem with females in models that include cerci centroid size and the first 10 shape PCs, plus associated quadratic terms of these independent variables as independent variables.

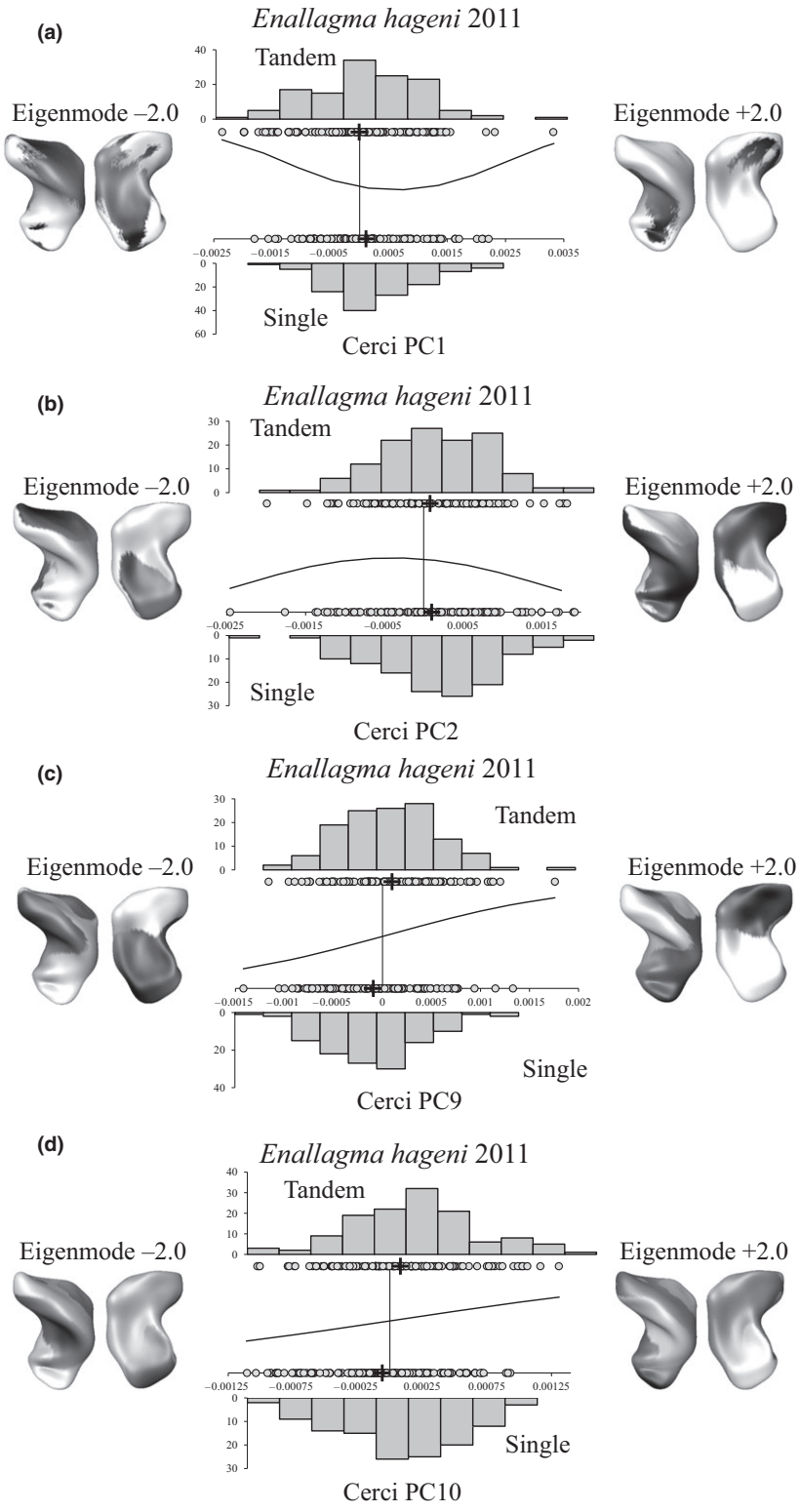
	Enallagma ebrium			*Enallagma geminatum*		
	β	t_{130}	P	β	t_{123}	P
Centroid Size	0.0846	0.73	0.46	0.0403	0.32	0.75
PC1	−0.1157	−0.29	0.77	−0.0263	−0.21	0.83
PC2	0.3012	0.82	0.41	−0.1469	−1.20	0.23
PC3	0.1249	0.90	0.37	0.2335	1.92	0.06
PC4	−0.2275	−1.05	0.29	0.1342	1.09	0.28
PC5	−0.1221	−0.64	0.52	**−0.3076**	**−2.27**	**0.03**
PC6	0.0538	0.45	0.66	**0.3015**	**2.23**	**0.03**
PC7	−0.0127	−0.10	0.92	−0.1240	−0.92	0.36
PC8	0.1593	1.40	0.16	0.2280	1.86	0.07
PC9	0.0213	0.13	0.90	0.0648	0.52	0.60
PC10	−0.0819	−0.71	0.48	**−0.2992**	**−2.15**	**0.03**
Centroid size*Centroid size	−0.0235	−0.14	0.89	−0.1641	−0.75	0.46
PC1*PC1	−0.0310	−0.25	0.81	−0.0999	−0.50	0.62
PC2*PC2	0.2652	1.32	0.19	−0.1598	−0.77	0.44
PC3*PC3	0.0516	0.27	0.79	−0.1028	−0.52	0.60
PC4*PC4	−0.2582	−1.08	0.28	−0.1402	−0.83	0.41
PC5*PC5	0.1463	0.68	0.50	−0.0877	−0.44	0.66
PC6*PC6	0.0744	0.35	0.73	−0.0190	−0.09	0.93
PC7*PC7	−0.2009	−1.12	0.27	−0.2132	−1.02	0.31
PC8*PC8	0.0369	0.21	0.83	0.1179	0.58	0.56
PC9*PC9	0.0934	0.44	0.66	−0.0339	−0.23	0.82
PC10*PC10	0.2483	1.36	0.18	−0.0587	−0.29	0.77

	Enallagma hageni 2010			*Enallagma hageni* 2011		
	β	t_{126}	P	β	t_{230}	P
Centroid size	**−0.2880**	**−2.18**	**0.03**	**0.1761**	**2.85**	**0.005**
PC1	0.0372	0.23	0.82	−0.0820	−0.83	0.405
PC2	−0.0130	−0.13	0.89	−0.1010	−1.59	0.11
PC3	−0.3487	−0.76	0.45	−0.0150	−0.13	0.90
PC4	−0.1409	−0.97	0.33	−0.0350	−0.37	0.71
PC5	0.0035	0.03	0.97	−0.0770	−1.15	0.25
PC6	0.1468	1.53	0.13	−0.0077	−0.12	0.90
PC7	0.3031	1.68	0.10	−0.1072	−1.28	0.20
PC8	−0.1004	−0.67	0.50	0.0532	0.7	0.49
PC9	−0.0107	−0.11	0.91	**0.1874**	**2.95**	**0.004**
PC10	**−0.3171**	**−3.17**	**0.002**	**0.1710**	**2.78**	**0.006**
Centroid size*Centroid size	−0.1027	−0.44	0.66	0.0099	0.11	0.91
PC1*PC1	−0.2873	−1.57	0.12	**0.2139**	**2.31**	**0.02**
PC2*PC2	−0.2180	−1.36	0.18	**−0.1880**	**−2.17**	**0.03**
PC3*PC3	−0.0590	−0.15	0.88	0.0358	0.39	0.70
PC4*PC4	−0.1575	−0.83	0.41	−0.1327	−1.38	0.17
PC5*PC5	0.1977	1.13	0.26	0.1998	1.91	0.06
PC6*PC6	−0.0871	−0.49	0.63	0.0378	0.38	0.71
PC7*PC7	0.1140	0.44	0.66	0.0792	0.76	0.45
PC8*PC8	−0.0069	−0.04	0.97	−0.0374	−0.47	0.64
PC9*PC9	0.1091	0.94	0.35	0.0824	0.92	0.36
PC10*PC10	−0.1910	−1.26	0.21	−0.0016	−0.02	0.99

Each column presents the results for each species, and separate analyses are presented for *E. hageni* in 2010 and 2011. The *t*-test with associated *P* value is presented for tests that each regression parameter in the model is different from zero, and error degrees of freedom for these tests are given in the subscript. Those that are significantly different from zero at the $P < 0.05$ level are identified in bold. The column labelled β presents the model parameters as standardized selection gradients according to the methods of Janzen & Stern (1998), and the quadratic estimates have been doubled (Stinchcombe *et al.*, 2008).

In contrast, the distributions of cerci size and/or shape of tandem and single males were significantly different in multiple features for *E. geminatum* and *E. hageni*. However, only *E. hageni* in 2011 showed any significant quadratic terms which indicated a female mating bias for or against males with more extreme phenotypes (Table 1). In fact, these two significant PCs for *E. hageni* in 2011 showed contrasting results. The quadratic term for PC2 was significant with a negative coefficient, indicating that males with more extreme phenotypes on PC2 had a lower probability of being found in tandem with a female (Fig. 2b). This PC quantified variation mainly in the dorsoventral thickness near the middle of the cercus, suggesting that females showed some level of discrimination against males with too thick or too thin cerci (Fig. 2b). In contrast, PC1 for *E. hageni* in 2011 was a positive coefficient and therefore indicated a higher probability of being in tandem for males with more extreme phenotypes on this shape axis. One obvious feature for PC1 was that males at both extremes of this axis had distal ends of the cerci that were broader and longer than the average cercus (Fig. 2a). However, these results for all species provided no compelling support overall for our major hypothesis that females would discriminate against conspecific males with more extreme phenotypes.

Females did express directional biases for male cerci shapes in both *E. hageni* and *E. geminatum* and cerci size in *E. hageni*. On the sampling day in 2010, the predicted probability of being in tandem increased with decreasing cerci size for *E. hageni*, but the opposite was true in 2011 (Table 1). Shape differences between single and tandem *E. hageni* males at Martin's Pond on the day in 2011 did not match those in 2010. *Enallagma hageni* 2011 PC9 and PC10 both capture similar shape features. Both of these PCs indicated that tandem males on average had raised mediodorsal surfaces and distal



tips that were lower on the dorsoventral axis of the cercus (i.e. dark dorsal and light ventral surfaces of the distal tip in Fig. 2c, d). In contrast, in 2010 only *E. hageni* PC10 was significant, but the selection gradient was reversed to favour males with lower PC10 scores (Fig. 3). Thus, like selection on cerci size, the linear

Fig. 2 Relationships among mating status and PCs that were statistically significant ($P < 0.05$) in the logistic regression analysis for *Enallagma hageni* at Martin's Pond, VT, on 28 July 2011: (a) PC1, (b) PC2, (c) PC9 and (d) PC10. The centre panel in each group shows the predicted relationship between the cercus PC and mating status, with data for each above (tandem males) and below (single males). The mean phenotype for each mating category is identified with +. Histograms above and below show the frequency distributions of males in each category. The images on either side illustrate the differences in cercus shape at either end of the PC axis, with the cercus representation to the left showing the shape at −2.0 standard deviations from the origin along this axis, and to the right showing the shape at +2.0 standard deviations from the origin. The left image in each pair shows the dorsal surface and the right image shows the ventral surface. The cercus is oriented so that where it is attached to the abdomen is at the top, and the distal tip is down. The cerci are coloured so that areas that are farther from the centroid relative to the cercus at the origin are lighter, and areas that are closer to the centroid relative to the cercus at the origin are darker.

selection gradients were in different directions on PC10 on the 2 days sampled in the 2 years for *E. hageni*.

Although the PCs capture orthogonal axes of variation, PC5 and PC6 for *E. geminatum* identified the same general shape difference between single and tandem males on the anterior surfaces: much of the dorsal surface was raised (light area) and the main distal contact surface (i.e. medial and mediodorsal surface) protruded less (dark area) on average in tandem males (Fig. 2a, b). *Enallagma geminatum* PC7 also identified the raised dorsal surface but with a longer and wider (in the dorsoventral direction) distal cercus tip on average in tandem males (Fig. 2c). Finally, *E. geminatum* PC10 indicated that tandem males had cerci that were overall broader in the dorsoventral direction as compared to single males (Fig. 2d).

Discussion

We found little to no support for our hypothesis that *Enallagma* males with more extreme cerci shapes would be found less frequently in tandem with females as a side effect of females discriminating conspecifics from heterospecifics. Only one in forty tests (i.e. the quadratic terms for cerci shape PCs in the four logistic regression models) in these species was consistent with our hypothesis (PC2 for *E. hageni* in 2011; Table 1, Fig. 2b). In fact, we found one other PC for this same comparison that was consistent with females preferring males with more extreme phenotypes (Fig. 2a). Thus, we can offer no unequivocal example of female mate preferences consistent with stabilizing selection operating on cerci shape of conspecific males in the species mate recognition systems of these three species.

Why would females not discriminate against more extreme males? The most likely explanation is that the distribution of male cerci shape within a population is too small now to see any significant effect. That is, females do discriminate against such males; however, it is simply not detectable against the range of variation currently present in the population (Schluter 1988). In this case, almost all conspecific males would fall within the acceptable range for females. Consistent with this, we note that the only two results identifying mating biases for or against more extreme males were for PC1 and PC2 (i.e. the two PCs with the largest variances) in *E. hageni* in 2011. Also consistent with this idea, previous analyses have shown that populations of six *Enallagma* species, including *E. geminatum* and *E. hageni*, display no detectable interpopulation variation in cerci shape across their entire ranges, even for species whose ranges extend from British Columbia and California to New Hampshire and Maine, and the distributions of male cerci for *Enallagma* species all found breeding at

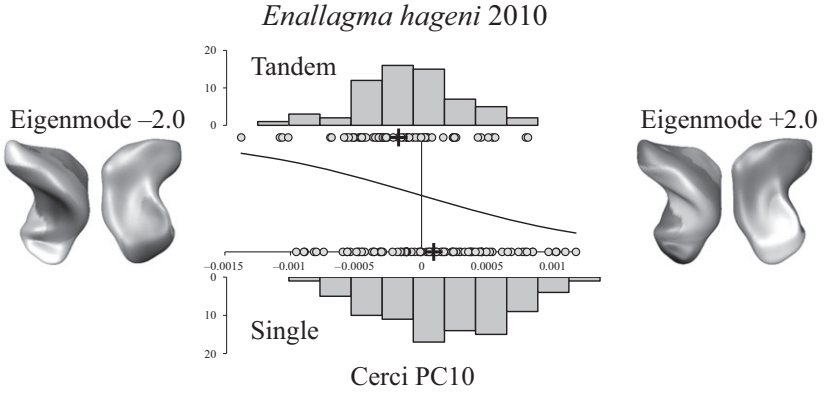


Fig. 3 Relationships among mating status and PCs that were statistically significant in the logistic regression analysis for *Enallagma hageni* at Martin's Pond, VT, on 6 July 2010 for PC10. The figure panels are as described in Fig. 2.

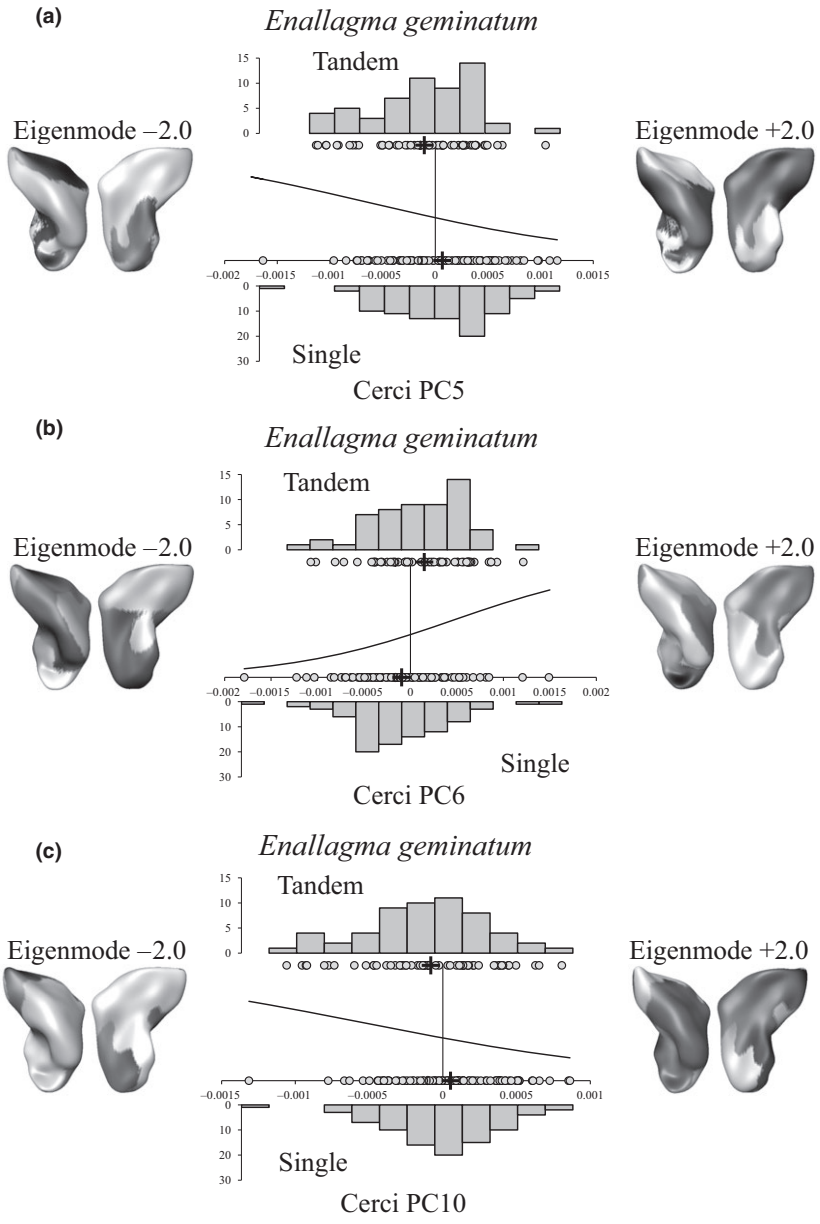


Fig. 4 Relationships among mating status and PCs that were statistically significant in the logistic regression analysis for *Enallagma geminatum* Lower Baker Pond, NH, on 26 July 2010: (a) PC5, (b) PC6 and (c) PC10. The figure panels are as described in Fig. 2.

the same lake do not overlap in multivariate shape space (McPeek *et al.*, 2008, 2011). Thus, past selection in these species mate recognition systems may have moved species far enough apart that females now have little chance of mistaking a conspecific male for a heterospecific male (McPeek & Gavrilets, 2006), even though *Enallagma* males will try to mate with females of any species indiscriminately.

Instead, we detected directional mating biases for cerci shape variation in two species and within the same species in consecutive years. If these directional biases in mating preferences persisted throughout the entire breeding period and then across generations, we would expect the resulting sexual selection to alter the phenotypic distribution of male cerci over time. However, lack of significant interpopulation variation across the ranges of entire species indicates that this does not occur (McPeek *et al.*, 2011). Alternatively, these linear mating biases may be transient or reverse over the course of the breeding season so that the cumulative effects of varying directional selection sum to a net effect of stabilizing selection overall (Arnold & Wade,

1984b, a; Travis, 1989; Wade & Kalisz, 1990; McGlothlin *et al.*, 2010). Consistent with this, the direction of mating bias on cerci size and PC 10 was reversed on the sample days in 2010 and 2011 for *E. hageni*. We obtained similar results for female mating biases for overall male body size fluctuating in direction across successive days over the breeding season for *Enallagma aspersum* (Steele *et al.*, 2011). To explain why the specific features of cerci shape identified in this study are favoured or disfavoured by females, we would have to understand the contact surface relationships and the mechanical fit between these male and female parts during mating: an understanding that we currently lack but are trying to develop.

There has been too little work done on selection for these types of structures in other species to allow us to evaluate whether these results for *Enallagma* are generally indicative of selection on such secondary sexual structures. A few studies have measured phenotypic selection on male claspers in other insects. As in the present study, Wojcieszek & Simmons (2011) identified significant quadratic selection gradients consistent with both stabilizing and disruptive selection acting on various shape metrics of male genital morphology in *Antichiropus variabilis* millipedes. Dougherty & Shuker (2016) identified selection on clasper length in only one of two *Lygaeus* species. In contrast, Koshio *et al.* (2007) found no selection on clasper length in the moth, *Elcysma westwoodii*, but did identify selection on fluctuating asymmetry of clasper lengths. Kelly (2014) explicitly examined selection from mate choice on male clasper shape in the stick insect, *Micrarchus hystriculeus*, but found no selection on either clasper size or shape.

Other damselfly groups also have mate recognition systems that minimize heterospecific matings. For example, sympatric *Calopteryx* species differ in wing spot coloration, and these differences are used by males and females to discriminate among conspecific and heterospecific mates (Waage, 1979; Tynkkynen *et al.*, 2004, 2006; Svensson *et al.*, 2007, 2010, 2016). Consistent with our original hypothesis about the form of mate selection on mate recognition traits, one experimental study found that females imposed stabilizing selection but no directional biases on wing spot size for *Calopteryx splendens* males (Svensson *et al.*, 2016).

More generally, mate recognition systems in animals use a variety of different phenotypic traits to signal species identity, including coloration (Waage, 1979; Price, 1998; Seehausen & van Alphen, 1998; Tynkkynen *et al.*, 2004; van Alphen *et al.*, 2004; Svensson *et al.*, 2007; Price, 2008; Salzburger, 2009; Svensson *et al.*, 2016; Verzijden & Svensson, 2016), pheromones (Lofstedt, 1993; Blows & Allan, 1998; Wyatt, 2003; Bickford *et al.*, 2007; Johansson & Jones, 2007; Higgie & Blows, 2008), calls and vocalizations (Otte, 1989; Wells & Henry, 1992; Ryan & Rand, 1993; Gerhardt, 1994; Shaw, 2000; Gerhardt, 2005; Seddon & Tobias, 2010), and even electric signals (Feulner *et al.*, 2009). In all these systems, mate choice against heterospecifics has the potential to impart selection on the phenotypic distribution of conspecific mates (Ryan & Rand, 1993; Boake *et al.*, 1997; Pfennig, 1998; Pfennig & Ryan, 2006; Phelps *et al.*, 2006; Mendelson & Shaw, 2012). However, with the exception of those studies involving insects, many noted above, we are not aware of studies that have directly shown female mate choice generating incidental sexual selection on such species recognition traits. Future studies in this broader set of mate recognition systems should focus on how discrimination against heterospecifics may impart selection on the traits of conspecifics.

One mate recognition system that has been extensively studied for selection on conspecifics is the cuticular hydrocarbons of Australian *Drosophila serrata* and *Drosophila birchii* (Blows & Allan, 1998; Higgie *et al.*, 2000; Higgie & Blows, 2008) and North American *Drosophila subquinaria* and *Drosophila recens* (Dyer *et al.*, 2014; Rundle & Dyer, 2015). In these and related *Drosophila* species, mate choice is influenced by a contact pheromone system of hydrocarbons that decorate the cuticles of males and females, and individuals of both sexes exercise choice among individuals of the other sex. As with interspecific differences in *Enallagma* cerci shape, the hydrocarbon profiles of *Drosophila* males and females do not overlap the profiles of other *Drosophila* species with which it is sympatric (Blows & Allan, 1998; Dyer *et al.*, 2014). Also, *Drosophila* hydrocarbon profiles differ between populations that are sympatric and allopatric with respect to congeners, and these population differences can be attributed to selection causing differentiation in sympatry (Higgie & Blows, 2008; Dyer *et al.*, 2014; Rundle & Dyer, 2015). Studies of intraspecific sexual mate choice and selection also consistently identify significant linear mating biases acting on both sexes within populations, but these linear biases differ among populations (Chenoweth & Blows, 2003, 2005; Rundle *et al.*, 2005, 2008; Dyer *et al.*, 2014; Gershman *et al.*, 2014; Gosden *et al.*, 2014), and much of this interpopulation variability shows no patterning associated with differentiation from other *Drosophila* species (Rundle *et al.*, 2008). In addition, significant quadratic selection gradients that are consistent with both stabilizing and disruptive selection have been routinely measured on *D. serrata* male and female hydrocarbon profiles, and these too showed no consistency between the sexes or across populations (Chenoweth & Blows, 2005; Rundle *et al.*, 2005, 2008; Rundle & Chenoweth, 2011). And yet, for all this phenotypic selection, *Drosophila* populations show little interpopulation variability in cuticular hydrocarbon profiles when compared among analogous regions of their ranges (i.e. comparing populations that are either sympatric or allopatric with respect to a given congener; Chenoweth *et al.*, 2010; Dyer *et al.*, 2014). Thus, our results on *Enallagma* cerci from studies at various scales are largely

consistent with what has been found for *Drosophila* cuticular hydrocarbons.

In fact, taken together, our studies of mate choice on cerci shape (this study) and cerci shape evolution (McPeek *et al*., 2008, 2009, 2011) over multiple timescales and those of similar structures such as cuticular hydrocarbons in *Drosophila* offer all the same paradoxes identified in more comprehensive analyses of evolutionary character change. Many different explanations may explain why, despite strong directional selection and ample genetic variation being so frequently identified in nature, so little intergenerational phenotypic change actually occurs in all types of traits and species (Merilä *et al*., 2001; Ozgul *et al*., 2009; Marrot *et al*., 2018). Certainly, numerous examples of rapid evolutionary responses to selection have been documented (e.g. Hendry & Kinnison, 1999; Reznick & Ghalambor, 2001; Grant & Grant, 2002; Travis *et al*., 2014). However, longer term patterns of character evolution show that only small excursions of character change occur in the short term, and major trait diversification happened in the deeper histories of clades (Estes & Arnold, 2007; Uyeda *et al*., 2011; Arnold, 2014). The puzzle remains about why selection that should change the phenotypes of species over space and time often results in little appreciable evolution in the short term. This is particularly puzzling for traits that are involved in species recognition during mating. We suspect this is simply because past selection has erased much phenotypic variation. However, if females across taxa generally lack mating biases against extreme mate phenotypes, but consistently express directional mating biases (as in *Enallagma* and *Drosophila*), then the overall process that maintains the integrities of species identities across their ranges and through time remains elusive.

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Author contributions

AMS and MAM designed the study, collected the damselflies, analysed the data and wrote the manuscript; and SJM collected the data and wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Supplemental methods description.

Table S1 Summary of principal components analyses of right cercus shape for the four species/year combinations and final sample sizes used in the analyses.

Data deposited at Dryad: https://doi.org/10.5061/dryad.368n7d9

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