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Tests of hypotheses for morphological and genetic divergence in *Megaloprepus* damselflies across Neotropical forests

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Differences in sexual signalling may initiate speciation by limiting gene flow among diverging populations. The damselfly *Megaloprepus caerulatus* exhibits two, visually obvious 'wing types' across its range. Males from one subspecies have sexually dimorphic, white-banded wings whereas males from the other subspecies lack the sex-specific white wing band. Using mitochondrial (cytochrome *c* subunit I and 16S) and nuclear (H3) markers, and measures of body size, wing ratio and secondary genitalia, we identified distinct genetic and morphological clades from Mexico to Panama; absence of a wing band was ancestral. To determine if relative reflectance properties of male and female wing tips cue sexual and competitor identity, as they do for wing dimorphic males, we noted reactions of males lacking wing bands to conspecifics with manipulated wings. Isolation by distance explained only 18% of the molecular variation among clades. Relative to wing dimorphic demes, wing monomorphic populations showed lower adult density, lower resource defence and fewer male–male interactions, suggesting lower sexual selection on males. However, not all were less sexually dimorphic in body size. Males lacking wing bands reacted to conspecifics with manipulated wings in ways suggesting that signals for potential mates and competitors do not differ across wing types, a conclusion that awaits more data. Wing mono- and dimorphic demes in *Megaloprepus* occur allopatrically over relatively short distances and may be isolated via secondary genitalia or unknown physiological constraints.

ADDITIONAL KEYWORDS: intraspecific competition – isolation by distance – male mate choice – niche conservatism – Odonata – speciation – sexual signalling – wing polymorphism.

INTRODUCTION

Ecological divergence via natural selection has long been viewed as a leading driver of speciation (e.g. Endler & Basolo, 1998; Masta & Maddison, 2002; Rundle & Nosil, 2005; van Doorn *et al.*, 2009; Keller & Seehausen, 2012). Under this hypothesis, ancestral populations that inhabit different environments adapt to local conditions, during which genetic divergence accumulates, as exemplified by speciation via host plant shifts in phytophagous insects (e.g. McMillan *et al.*, 1997; Nosil *et al.*, 2002; Jiggins, 2008), and local adaptation to mimetic patterns in butterflies that results in assortative mating (e.g. Chamberlain *et al.*, 2009; Kronforst & Papa, 2015). Ecological divergence may produce a pattern of isolation-by-environment, where the degree of genetic divergence should be correlated with different environmental states or a continuous environmental cline (e.g. Feder *et al.*, 2003; Paaby *et al.*, 2010).

Alternatively, genetic drift, such as isolation by distance and founder's effects, can lead to non-adaptive divergence (e.g. Oh *et al.*, 2013; Spurgin *et al.*, 2014). Closely related species often share the same ecological niche, which occurs in a limited subset of habitats across an ancestral range. In such cases initial divergence could occur in the absence of niche partitioning (Wiens, 2004). Genetic divergence then occurs randomly and gene flow decreases with geographical distance, giving rise to Wright's (1943) classical isolation-by-distance

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pattern of genetic variation, exemplified by ring species (e.g. Irwin *et al.*, 2005).

A second way that non-adaptive changes can lead to speciation is by sexual selection (reviewed by Panhuis *et al.*, 2001; Ritchie, 2007). Under this hypothesis, divergence in sexual recognition cues can lead to different criteria in mate recognition, effectively curtailing gene flow (Coyne & Orr, 2004). Indeed, divergence in sexual signals coupled with female mate preference is observed in some of the most rapid speciation events, as in Hawaiian *Laupala* crickets (Mendelson & Shaw, 2005), *Heliconius* butterflies (Kronforst *et al.*, 2013), cichlid fish (Seehausen *et al.* 2008) and Darwin's finches (Lamichhaney *et al.*, 2018). Although understudied, the role of male behaviour in reproductive isolation via male–male competition has increasingly been implicated in reinforcement of isolation after speciation (Grether *et al.*, 2009; Becher & Gumm, 2018). Male aggressive behaviour towards conspecifics is thus expected to be greater than towards heterospecifics (e.g. Anderson & Grether, 2010). However, to initiate reproductive isolation via male behaviour would require (1) male, rather than female, mate choice and (2) deme-specific male signals that mediate male–male competition, conditions known in some fish (e.g. Martin & Mendelson, 2016; Moran *et al.*, 2017).

The damselfly *Megaloprepus caerulatus* (Odonata: Coenagrionidae after Dijkstra *et al.*, 2014) is well suited to field tests of effects of isolation via sexual signalling on gene flow and speciation. Visual cues are necessary and sufficient for mate recognition by damselflies in the field (Ribora *et al.*, 2018). Wing patterns of *Megaloprepus* vary across allopatric populations (Fig. 1). Based on wing characteristics, de Selys Longchamps (1886) described three subspecies within the genus: (1) *Megaloprepus*

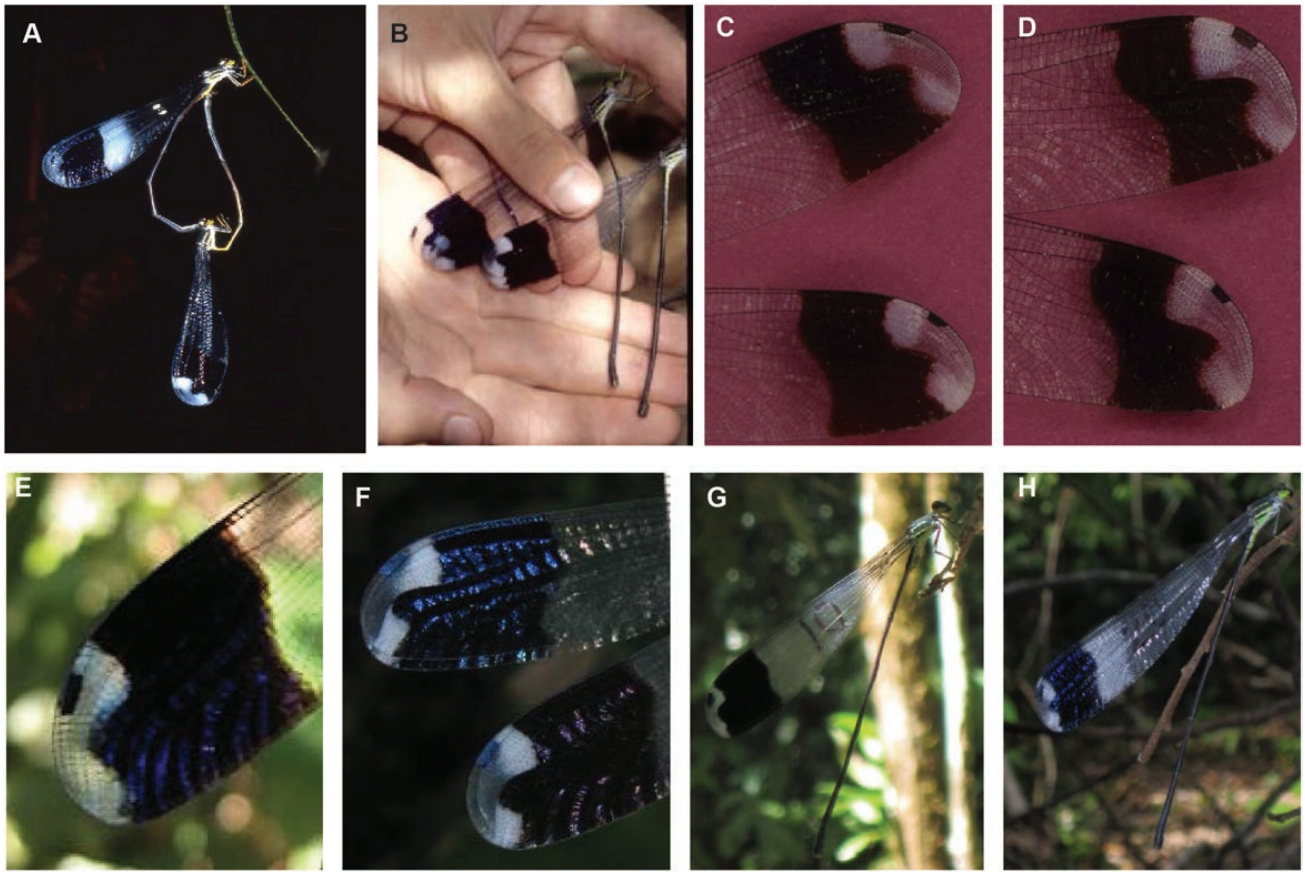

Figure 1. Subspecies of *Megaloprepus caerulatus*. A, *M. c. caerulatus* wing dimorphic male and female (in copula; two small wing dots on are ID marks), BCI, Panama; B, *M. c. latipennis*, wing monomorphic male (left) and female (right), Los Tuxtlas, Mexico; C and D, *M. c. brevistylus*, wing tip of male (C) and female (D), Colombia; E and F, *M. c.* **subsp. nov.**, wingtip of male (E) and female (F), Sirena, Costa Rica; G and H, control male from Sirena (G) and the same male (number 10), doubly manipulated to illustrate white band and enhanced white wing tip (H). Note that in addition to the presence of a male band in A, white on the male's wing tip is nearly absent; in contrast, in B, C–D, and E–G, wing tips of males are more similar to those of females.

caerulatus caerulatus, known from Central America to Colombia, Guyana, Ecuador and Bolivia, (2) *M. caerulatus latipennis*, described from Mexico and Guatemala, and (3) *M. caerulatus brevistigma* found in Colombia east of the Andes, Venezuela and Peru. Of these, only *M. c. caerulatus* exhibits an obvious, sexually dimorphic wing pattern, characterized by the sex-limited, bright, waxy ultraviolet (UV)-reflective white wing bands of males (Fig. 1A), whose pale wing tips contrast with the conspicuous white wing tips of females. Previous wing manipulative experiments indicated that the relative brightness of the wing tips, which best describes the overall difference between the male and female wing tip colour, cues both sexual and competitor recognition to territorial males. Males with normal wing tips but a blackened white wing band still elicited aggressive responses from focal males (Schultz & Fincke, 2009). Evidence to date suggests that *Megalorpepus* females do not discriminate against males based on male phenotype, but rather, gain large males as sires by mating only at defended sites (Fincke, 1992a). The male's UV-reflectance of the white wing bands signals his body size to rivals during territorial fights (Xu & Fincke, 2015). Strong male–male competition favours large territorial males, and size dimorphism is male-biased in this subspecies (Fincke, 1992a, 1998). In contrast to *M. c. caerulatus*, males of the subspecies *M. c. latipennis* (Fig. 1B) and *M. c. brevistigma* lack the white wing band; additionally, these males have relatively more white on wing tips, more similar to their females (Fig. 1B–D). These subspecies differ in wing shape and length of the wing pterostigma (de Selys Longchamps, 1886). Recently, based on only mitochondrial DNA, Feindt *et al.* (2014) found that *M. c. latipennis* from Mexico was genetically distinct from a novel clade on the Osa peninsula in Costa Rica (Fig 1E, F). In none of these subspecies has sexual behaviour or sex recognition cues been described. Nor is it known whether individuals of any *Megaloprepus* subspecies discriminate against another.

The genus exhibits a highly conserved, ecological niche across its geographical range. From Mexico to Bolivia, *Megaloprepus* is restricted to mature wet or moist forests (Hedström & Sahlén, 2001; Fincke & Hedström, 2008; Garrison *et al.*, 2010). Its larvae, which are adapted to a broad range of pH and temperature (Fincke, 1998, 2006), develop only in water-filled tree holes, a limiting reproductive resource, non-randomly distributed across tree species (Fincke, 2006). Unlike most odonates, which take flying insect prey (reviewed by Corbet, 1999), adult *Megaloprepus* are specialized predators of small, orb-weaving spiders, a trait shared across the subfamily (Calvert, 1923; Fincke, 1992b; Clausnitzer & Lindeboom, 2002). These common ecological specializations across *Megaloprepus* subspecies suggest no obvious ecological divergence, a possibility not tested here.

Given the discrete nature of its aquatic larval habitats, dispersal of *Megaloprepus* occurs only by adults, which seldom cross open expanses of land or water (Fincke, 2006; Khazan, 2014). Thus, we expected *Megaloprepus* populations to be increasingly isolated as the distance among disjunct mature forests increases. Such isolation-by-distance serves as a proxy for the null hypothesis of genetic divergence in the absence of other drivers (Holsinger & Weir, 2009).

We used both nuclear and mitochondrial DNA to reconstruct a phylogeny and haplotype network of study populations to test whether genetic divergence is accompanied by non-random divergence in morphology and wing signals, as predicted if cladogenesis is not entirely the result of random drivers. Across populations nested within the two wing types, we assessed the potential for sexual selection via male–male competition by quantifying population density, male agonistic behaviour, resource defence of artificial tree holes, and sexual dimorphism in body size, wing ratio and reflectance. We predicted that relative to wing monomorphic populations, sexual selection and sexual size dimorphism should be greater in wing dimorphic populations. We conducted wing manipulation experiments in a 'wing monomorphic' population whose males lack the white wing band. If the wing type patterns function in mate and competitor recognition, divergence in signal recognition should provide a means to decrease gene flow and promote reproductive isolation among demes. If so, we would expect cues for sex and competitor recognition used by wing monomorphic, un-banded males to differ from those used by white-banded males in a wing dimorphic population determined previously by Schultz & Fincke (2009).

In addition to measuring visual cues that could function in pre-zygotic reproductive isolation, we also measured male secondary genitalia to test whether mechanical cues might function as an isolating mechanism that could change due to drift across geographically isolated demes. In odonates, the secondary genitalia consist of male claspers, which engage with the female mesostigmal plates to form a tandem coupling prior to copula, and the pene on a male's second abdominal segment, which transfers sperm (produced by primary genitalia) to females (Corbet, 1999). Females must raise their abdomens to engage in copula. Male claspers are often species-specific in damselflies (Kennedy, 1922), and are known to be a reproductive isolating mechanism (e.g. Turgeon *et al.*, 2005; Barnard *et al.*, 2017). Changes in clasper morphology make it difficult if not impossible for heterospecific males to form tandems, regardless of visual signals.

Table 1. Abiotic features of our six study sites

Site	Mean temperature (°C)	Mean rainfall (cm)	Duration of dry season (months)	Reference
Los Tuxtlas, MX	26 (16–36)	450	3–4	Soto & Gama (1997) station records
El Jaguar, NI	(8–32)	205	4	Gourdji *et al.* (2015) http://www.jaguarreserve.org/biodiversity.html
Sirena, CR	25.5 (21–32)	550	2	Boinski & Fowler (1989)
Bartola, NI	26 (23–36)	400	3	Wong *et al.* (2009)
La Selva, CR	25.8 (16–37)	400	0	Sanford *et al.* (1994)
BCI, PA	27 (21–30)	262	2-3	Leigh (1999)

All are lowland forests sites except for El Jaguar, which is a tropical cloud forest at 1300 m. Ranges are given in parentheses. Wing dimorphic populations are shown in bold.

MATERIAL AND METHODS

Study populations

Our study populations were all in mature forests, which differed in rainfall patterns and temperature extremes, but did not differ in male wing type with respect to mean rainfall (t = –0.77, P = 0.49) or temperature span (t = –0.42, P = 0.69, Table 1). La Selva was the only site where water-filled tree holes do not dry up seasonally; the others experience prolonged dry seasons. Sampling periods corresponded with times of relatively high adult abundance and activity at each site (i.e. rainy and early dry season). Unless noted otherwise, DNA was taken from adults. The wing monomorphic deme at Los Tuxtlas Biological Station, Veracruz, México (TUX; 18°N, 95°W), was our most northern population, where behavioural observations were made in 1998 and 2000; DNA (20% larval) was collected in 2011 and 2013. Roughly 11 215 km to the south of TUX was our second wing monomorphic population in a tropical cloud forest at 1350 m elevation at El Jaguar reserve and coffee plantation (EJ; 13°14′N, 86°03′W) near Jinoteca, Nicaragua. There, we collected DNA (56% larval) and observed behaviour between April and September, in 2015 and 2016. Our most southern monomorphic population, studied during January 2012, was at Sirena Field Station (SIR; 8°30′N, 83°35′W) in the Parque Nacional Corcovado on the Osa Peninsula of Costa Rica, about 1700 km overland from TUX and 607 km from EJ. Our most northern dimorphic deme, used only for DNA sampling, was in lowland forest in and around Bartola Reserve near the Atlantic coast of Nicaragua (BART; 10°97′N, 84°16′W), about 283 km from EJ. Our second dimorphic population was at La Selva Biological Station, Costa Rica (SELVA; 10°26′N, 83°59′W). There, DNA (22% larval) was sampled in July 1996 and January 2012; behavioural data were taken in June–July 1991. Our most southern wing dimorphic population was at the Smithsonian Field Station on Barro Colorado Island, Panama (BCI; 9°10′N, 79°15′W). Adults were sampled in July 1997 and September–December 2010. We made behavioural observations in September–October 1994, January 1997 and December 2008. BCI is about 528 km overland from SELVA. To better test for divergence by distance, we also included DNA samples collected in 2013 and 2014 from a wing dimorphic population in the lowland forest of Canandé Reserve, Esmeraldas Province, Ecuador (CAN; 00°31′N, 79°14'W), roughly 1178 km overland from BCI, our closest study site.

For comparison with a previous phylogeny that used ND1 and 16S rRNA (Feindt *et al.*, 2014), in our genetic analysis we included all of their 111 molecular sequences of 16S, along with our own sequences of 16S (we did not find much signal in ND1, so did not sequence it or include it here). Their samples were from Los Tuxtlas (TUX, Mexico), BCI (BCI, Panamá), La Selva (SELVA, Costa Rica) and Corcovado National Park, Costa Rica, four of the six populations that we sampled, based on latitude and longitude. Due to some discrepancies, we refer to their genetic data from Corcovado as 'CNP' to distinguish it from our data (SIR), taken on the same population.

Genetic structure of study populations

To characterize genetic variation within and across study populations, we used the nuclear protein coding histone 3 (H3) fragment, and the mitochondrial protein coding cytochrome *c* oxidase subunit I (CO1) and ribosomal (16S) fragment. The regions selected were amplified by PCR using standard protocols (e.g. Ware *et al.*, 2007; 2014). The thermal cycler programme was 94 °C for 150 s, then 35 cycles of 94 °C for 30 s, 46–56 °C for 60 s and 72 °C for 60 s, and concluded with 10 min at 72 °C. PCR products were visualized in 1.5–2% agarose gels stained with ethidium bromide. Sequencing PCR in both

directions was done with the ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Carlsbad, CA, USA), and sequences were then purified by using a DyeEx 96 Kit from Qiagen (Valencia, CA, USA), dried and re-eluted with formamide, and run on an ABI Prism 3730xl DNA analyser. We aligned ribosomal fragments with reference to secondary structure. All other fragments were aligned using Clustalx 2.0 (Larkin *et al.*, 2007), followed by manual alignment in Mesquite (v.2.75; Maddison & Maddison, 2008). All sequences have been deposited at GenBank (see Supporting Information, Table S1 for accession numbers).

The phylogenetic tree of our study populations was based on the reconstruction of our concatenated data set, and single gene fragment data sets using maximum likelihood (i.e. 250 sequences, 2197 nucleotides, with 446 parsimony-informative characters). We used *Microstigma*, *Pseudostigma* and *Mecistogaster* species as outgroups. For the phylogenetic analyses of each data set (CO1, H3, 16S, CO1+H3+16S), we used maximum likelihood inference via IQTREE (Nguyen *et al.*, 2015; Trifinopoulos *et al.*, 2016). For the concatenated data set, we partitioned the data set based on gene fragment (H3, CO1 and 16S each considered as separate gene fragments). Substitution models for each of the data sets were determined in IQTREE prior to tree reconstruction [Akaike's information criterion suggests the following models for combined: GTR+I+G4; Kalyaanamoorthy *et al.* (2017); H3: HKY+F+I+G4, CO1: TIM+F+I+G4, 16S: TIM+F+G4]. Bootstrap values for reconstructed trees from the two data sets were obtained using the standard bootstrap approach in IQTREE, with 1000 replications (Minh *et al.*, 2013). To evaluate differences in haplotypes among gene fragments, we ran haplotype analyses of each data set in POPART (http://popart.otago.ac.nz), using the minimum spanning algorithm. DNAsp (Librado and Rozas, 2009) and Genalex (Peakall and Smouse, 2012) were used to estimate population genetic structure (ΦPT: FST) via AMOVA, and to calculate haplotype diversity and Tajima's *D*. To test for isolation by distance, we used a Mantel test on a matrix of genetic distances and the decimal longitudinal and latitudinal geographical locations of study populations in DNAsp.

We estimated the ancestral state of the two male wing types with mesquite, using the maximum likelihood Mk1 model, Markov k-state 1 parameter model and parsimony. Briefly, the wing monomorphic state was coded as '0' and dimorphic state as '1'; these were non-directional.

Population density, male–male competition and sexual dimorphism

Typically, on sunny days when the damselflies were most active (0900–1600 h), we caught sexually mature individual *M. caerulatus* with insect nets, and marked each with a unique number on the hindwing using an indelible black marker. We removed a middle leg and stored it in 95% ethanol for DNA analyses. We measured population density as the number of sightings of unique individuals per man-hour of search effort. Unmarked individuals that were not at territories and avoided capture were considered unique. An unmarked male seen at a territory over consecutive days, most likely the resident male, was conservatively considered to be the same individual until marked. To measure the rate of new territory acquisition by males, we placed artificial tree holes (2–9-litre plastic basins) in light gaps caused by recent tree falls, sites readily used by egg-laying females and defended by males (Fincke, 1992a). We identified residents by a male's characteristic perch above the hole, and his periodic checks for females there. We quantified the number of males and females at defended territories, fights between unique males, and the duration of our observations.

We measured wing and abdomen length of most captured individuals with electronic calipers, noting sex and relative age based on wing wear. To minimize handling of individuals, which were critical for field observations and experiments, we measured 'wing ratio' as wing width/wing length, our proxy for wing shape. We took photographs of fore- and hind wings with a ruler in the same plane (Fig. 1F), and used ImageJ (National Institutes of Health, Bethesda, MD, USA) to measure wing length and width across the longest and widest part of the wing.

To compare the relative reflectance of wing tips across wing types, for three of our study populations we measured spectra of wing tips using an OceanOptics SD2000 spectrometer with a PS-2 xenon light source. We analysed the mean spectrum of four spectra for each wing tip (i.e. upper and the lower tip on dorsal and ventral left hindwing). We interpolated the raw spectra between 300 and 700 nm and calculated mean brightness ($\overline{R}_{300-700}$) in Avicol v.6 (Gomez, 2006), the relevant variable for wing dimorphic males on BCI (Schultz & Fincke, 2009, hereafter 'the BCI study').

Wing signal manipulations in a wing monomorphic population

We used the wing monomorphic population of Sirena to test: (1) if cues to sex differ from those in the wing dimorphic BCI study, and (2) if resident males fail to recognize males with novel wing bands as competitors. To counter the paucity of available adults, we used each of six resident males as his own control across wing manipulation treatments. We presented six unique males and five unique females to focal males;

logistics prevented testing all treatments across all focal males (see Table S2).

To test whether the white wing tips of a female cue sexual identity to potential mates, we presented a focal male with females in the following order: (1) natural control female, (2) the same female modified with her white tips blackened and (3) the same female with her wing tips painted white (to see if that reversed the male's behaviour). To determine whether males reacted sexually towards a male with brighter, more female-like wing tips, we presented a focal male with: (1) a natural control male and (2) the same male modified with white wing tips. If sexual cues are similar across wing types, we predicted that females, and males with whiter wing tips would elicit more sexual reactions than control males or females with blackened tips. To test whether wing bands also influenced competitor recognition, we presented a focal male with: (1) a control male without wing bands and (2) the same male with white-painted wing bands. If the presence of novel wing bands decreases competitor recognition, we expected this treatment would elicit fewer aggressive reactions relative to control males. For comparison with reactions of doubly treated males in the BCI study, in three cases we first painted a male's wing tips white and subsequently added white bands (Fig. 1H). In three cases, we reversed the above order. In our binary analyses, we used only responses elicited by singly manipulated males.

For the sex recognition experiment, we painted the wing tips of males with a thin layer of UV-reflective zinc white gouache paint (Winsor & Newton, Piscataway, NJ, USA), whose spectral characteristics are similar to the natural colour (Xu & Fincke, 2015). We blackened the white patch of the female tip with lampblack paint. To test effects of the novel white band on male aggressive behaviour, treatment males were painted with a white band in the same wing position as occurs on wing dimorphic males (see Schultz & Fincke, 2009).

We tethered presented individuals with # 8 fly-fishing line (0.076 mm diameter, Black Knight Industries, Inc., Oil City, PA, USA) around the groove between the head and the thorax, which did not interfere with tandem formation, and tied the other end to a stick (0.5 m long) held by the presenter. We began by allowing the individual to flutter roughly 4 m away and directly in front of a perched, resident male. We noted the distance and time to his response (i.e. leaving the perch), and scored the most extreme reaction as: neutral (return to perch, hover), sexual (grab, tandem attempt, tandem) or aggressive (chase, hit). A presentation ended if the male reacted sexually or aggressively; otherwise, we used the most extreme reaction of three trials for analysis. Tethered individuals were then released and flew away; we re-sighted some individuals days later.

SEM IMAGING AND ILLUSTRATION OF MALE SEXUAL MORPHOLOGY

Specimens for illustration were loaned from the Philadelphia Academy of Natural Sciences. Specimens were identified as male *M. caerulatus* from regions within Central America (see Table S3 for dates and specimen localities). Reference images used for illustrations of secondary genitalia were taken after removing the dorsal side of the second and third abdominal segments. We mounted secondary genitalia on scanning electron microscopy (SEM) stubs, sputter coated them and imaged them using a Hitachi S4700 FE-SEM microscope (American Museum of Natural History) at 30–50× magnification. We traced structural morphology of primary and secondary genitalia from SEM reference images of 16 males using IPAD drawing software (IDRAW) using ImageJ software to measure cerci length, cerci width, paraproct length and angle of paraproct hook (see Fig. S1). We used character mapping in MESQUITE to reconstruct the ancestral states of male genitalia using parsimony and likelihood. Wing type was coded as '0' or '1'; continuous characters associated with secondary genitalia were mapped as relatively short (0), intermediate (1) or long (2).

STATISICAL ANALYSES OF MORPHOLOGY AND BEHAVIOUR

We compared population density, forewing and abdomen length, and wing ratio between sexes among study populations using general linear models (GLMs) in SAS v.9.1 (SAS Institute Inc., Cary, NC, USA). Bonferroni post-hoc tests and least square (LS) means, where appropriate, were used for comparisons. Additionally, we used a mixed model with wing type as a fixed effect and population as a random variable to confirm any conclusion that wing type alone explained differences in the above variables. The number of man-hours of search was a covariate in the analysis of population density across sites, and minutes observed was a covariate in the calculation of fight frequency. After testing for all interactions, we dropped insignificant ones from the models to gain statistical power.

We compared the relative brightness of wing tips among three sites and between the two sexes with ANOVA. Relative brightness, measured as values between 0 and 1, was logit-transformed (i.e. $\ln(x/(1-x))$). We used Tukey's honest significant difference test for post-hoc comparisons. All statistical tests for spectral analyses were conducted in R v.3.3.0 (R Core Team, 2012).

We used the Wilcoxon signed rank test on binary data (sexual vs. non-sexual; aggressive vs. non-aggressive) to test for treatment differences in the subset of focal males that were presented with both controls and

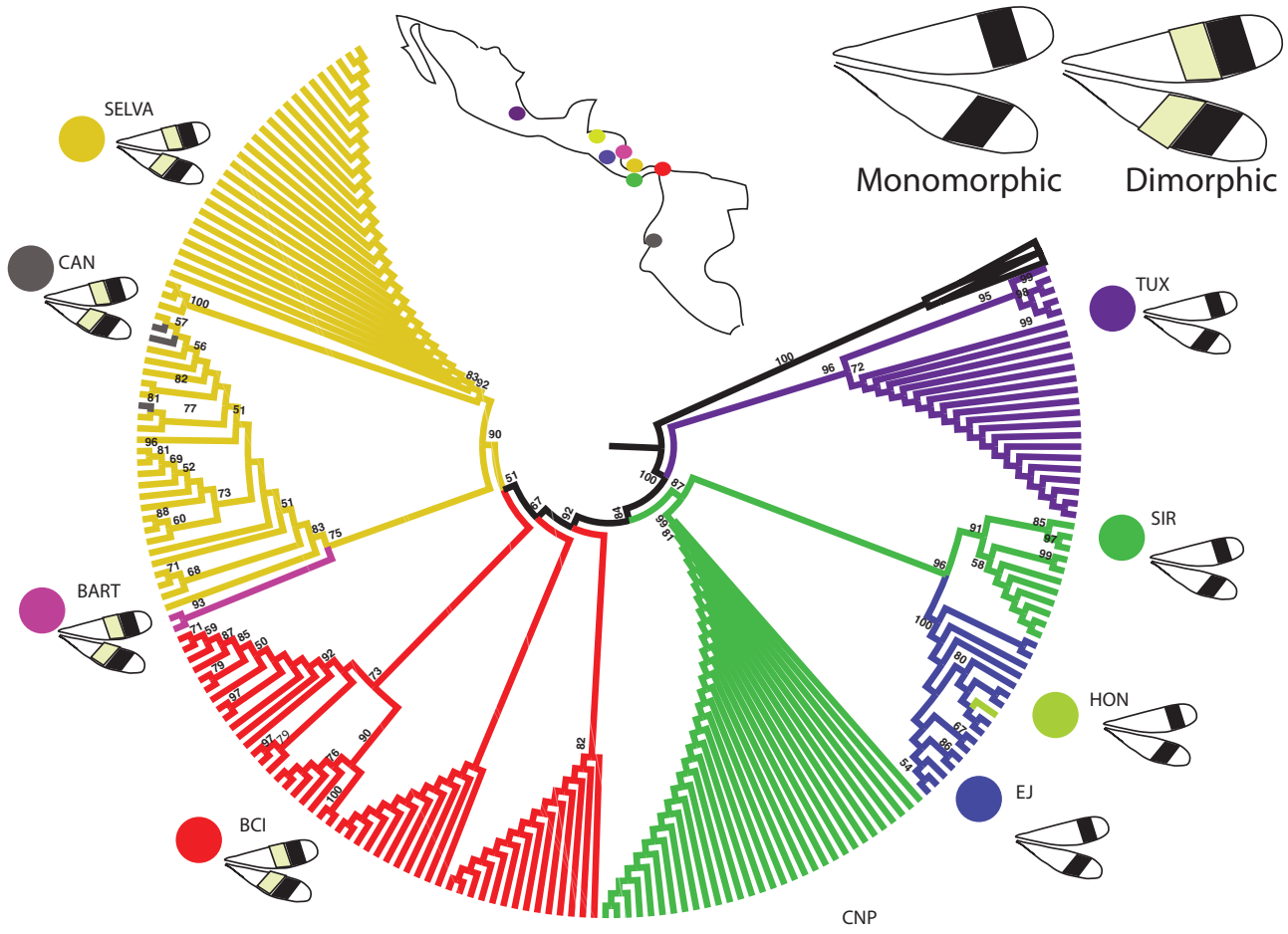

Figure 2. Phylogeny of study populations (majority rule consensus tree from maximum likelihood IQTREE); bootstrap support values are shown at branches. 'CNP' is the same population as 'SIR' (see Discussion); data from the former were presented by Feindt *et al.* (2014).

individuals with the relevant wing manipulation. To evaluate trends in male behaviour, we used Fisher's exact test on binomial data of responses from all six focal males. Throughout, means are presented ±SE.

RESULTS

Ancestral wing type and genetic structure of populations

Wing monomorphic populations all had more basal nodes in the phylogeny than wing dimorphic populations, with strong bootstrap support (Fig. 2). Ancestral state reconstruction in MESQUITE suggested that the monomorphic wing type was the ancestral male condition in *M. caerulatus* with total congruence between parsimony and maximum likelihood analyses. Pooling populations across monomorphic and dimorphic wing types revealed 68% of the genetic variation was within, and 32% was among, the two wing types, which exhibited distinct genetic differences ($\Phi PT = 0.323$, $P < 0.001$).

POPART minimum spanning networks suggested haplotype differences among populations (Fig. 3). Mitochondrial gene fragments recovered multiple haplotypes; CO1 differences indicated three haplotypes, which could suggest three specific sub- or full species. The more slowly evolving nuclear fragment recovered only one main haplotype that was shared by the majority of samples, with a small number of individuals from La Selva having some additional nucleotide differences.

The phylogeny of *Megaloprepus* was strongly supported as a monophyletic genus in combined and single gene trees. The Los Tuxtlas clade was the most basal node, sister to the remaining clades. The next

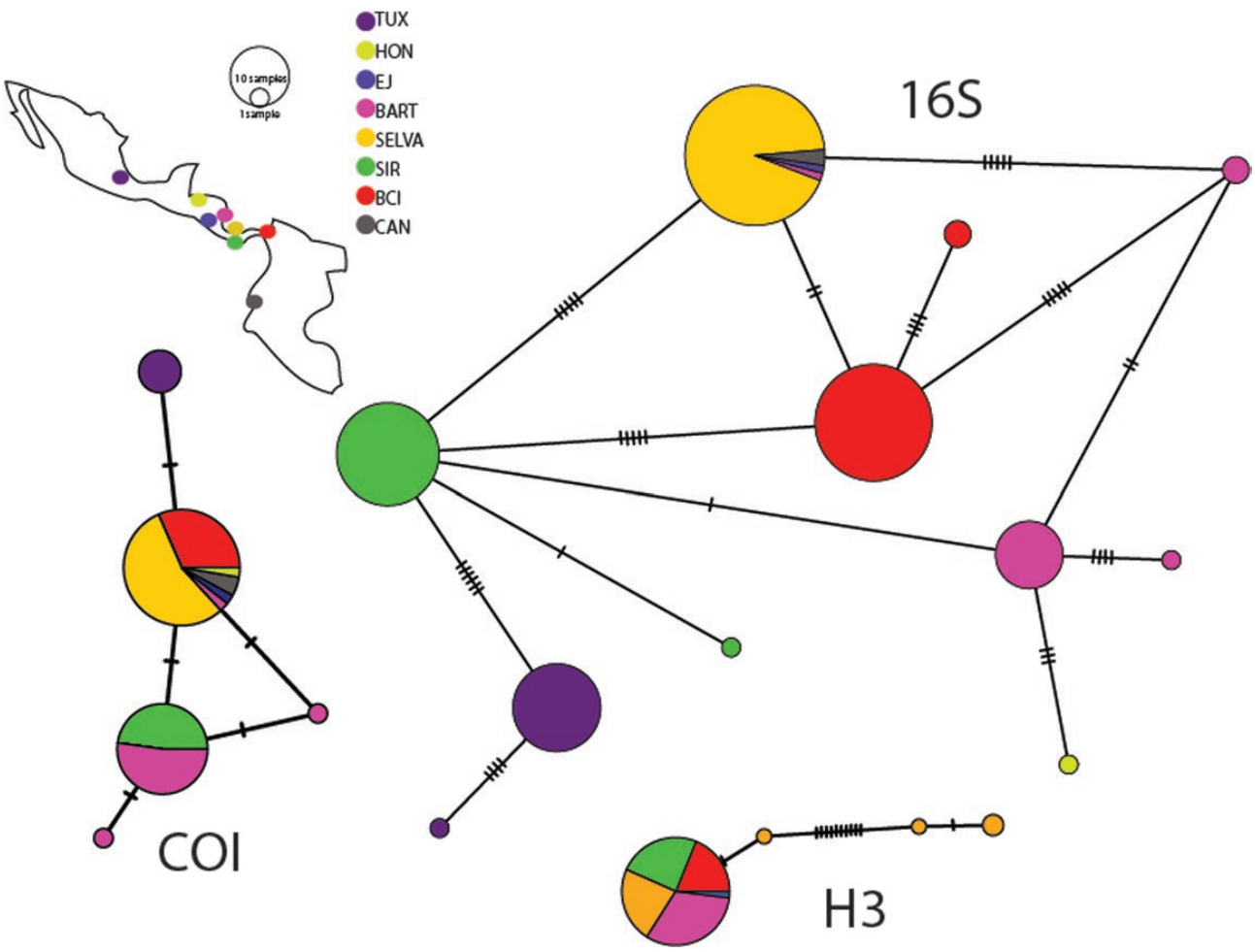

Figure 3. Distribution of genetic variation within and among *Megaloprepus* clades (POPART haplotype minimum spanning networks) based on results with CO1 gene fragment, H3 gene fragment and 16S gene fragment

node comprised a Corcovado (Costa Rica), Honduras, and El Jaguar (Nicaragua) clade. Within this grouping, a Sirena sub-clade included the monomorphic EJ Nicaraguan samples; the single monomorphic sample from Cusoco National Park, Honduras, was recovered within this sub-clade. The SIR+EJ sub-clade was sister to the Feindt *et al.* (2014) CNP samples. The wing dimorphic La Selva population, which included the wing dimorphic Ecuadorian samples, was strongly supported as monophyletic. In contrast, the wing dimorphic BCI population was paraphyletic, and split among three clades (82% to < 50% bootstrap support).

Tests of genetic structure suggested low gene flow among the above clades. Using an AMOVA based on countries for which we had 16S data, our most complete data set (TUX, HON, EJ, BART, SELVA, CNP/SIR, BCI, CAN), we estimated a ΦPT value of ~0.8 (0.88, DNAsp; 0.82, GenAlEx, $P < 0.001$). Because ΦPT values range from 0 to 1, with 1 indicating strong genetic structure and 0 indicating panmixia, a value of 0.8 suggests rather strong genetic structure (i.e. 18% within-population differences and 82% among-population differences). Furthermore, haplotype diversity, H, among countries was high (0.784, with a variance of 0.00023 and a standard deviation of 0.015). A positive Tajima's D statistic (0.0869, $P = 0.10$, 1 d.f.) suggested a non-significant trend toward a small population size or balancing selection. Mantel tests on the genetic distance matrix and geographical locations indicated that isolation by distance explained only 18% of the variation among our study populations ($R^2 = 0.180$, $P = 0.01$), indicating that much of the genetic structure results from a factor(s) other than geographical isolation among populations.

Male–male competition across populations and wing types

Accounting for observation time, population density of *Megaloprepus* adults differed among populations

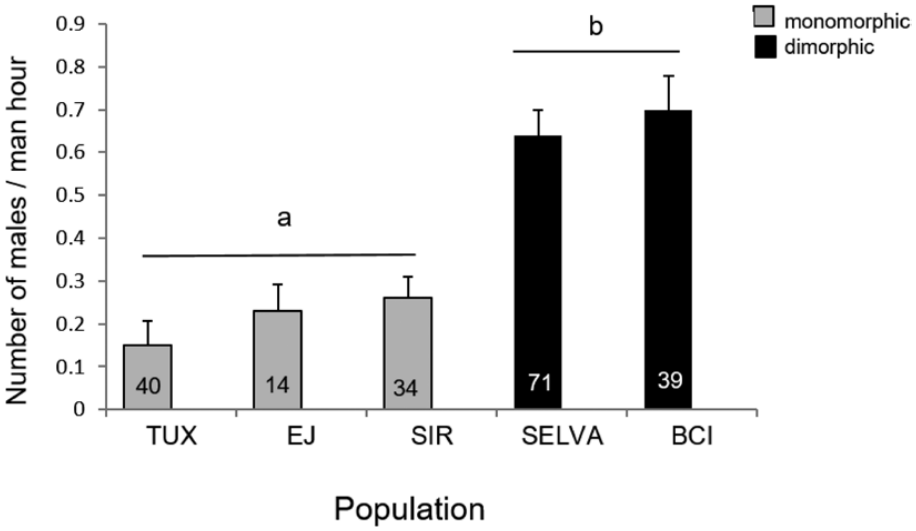

Figure 4. Population density of adult *Megaloprepus* (sexes pooled) measured as unique individuals seen per day, accounting for man-hours of observation per day, at four study sites. Bars connect populations with no difference in density. Numbers indicate total observation days.

Table 2. Frequency of artificial tree holes by site at which one or malemale was seen, total fights with unique males, mean (± SE) number of days that sites were checked and duration that each was observed

Site	Wing type	Holes (*N*)	Defended (*N*)	Total fights	Mean days checked	Mean duration observed (min)
Los Tuxtlas	Monomorphic	5	3	1	9.0 ± 0.6	275.8 ± 34.9
El Jaguar	Monomorphic	20	12	2	5.1 ± 0.3	91.6 ± 11.4
Sirena	Monomorphic	7	2	3	14.1 ± 2.6	505.0 ± 199.9
La Selva	Dimorphic	8	7	8	6.2 ± 0.7	69.0 ± 11.8
BCI	Dimorphic	21	20	13	7.1 ± 0.8	108.9 ± 16.3

($F_{4,192}$ = 18.46, P < 0.0001, Fig. 4) and, as predicted, was greater in wing dimorphic populations than in wing monomorphic populations (mixed model GLM, $F_{1,2}$ = 45.54, P = 0.02). Newly available artificial tree holes in wing dimorphic populations were more likely to be defended by males than holes in monomorphic populations (Fisher's exact test, P = 0.0005).

In the wing monomorphic demes of Los Tuxtlas, Sirena and El Jaguar, the same resident male was seen repeatedly at a given territory over a period of days; agonistic behaviour with rivals was characterized by chases and face-offs, similar to territorial behaviour of wing dimorphic males at La Selva and BCI (Fincke, 1998; Xu & Fincke, 2015, respectively). Nevertheless, at artificial holes defended by at least one male, using observation time as a covariate, fights at defended sites were less frequent in wing monomorphic demes (LS mean = 0.12 ± 0.07) than in wing dimorphic demes (LS mean = 0.70 ± 0.18; $F_{1,59}$ = 15.12, P = 0.0003, Table 2). Even at defended natural tree fall gaps with multiple tree holes, over the course of our study we saw only one agonistic interaction between two rival males in Sirena (during 69 min), and at a large fallen tree in Los Tuxtlas, only two interactions (of the same pair of males during a 3.6-h session). In contrast, on multiple occasions at natural sites at La Selva and BCI, as many as three or four different males interacted over similar time spans on multiple days.

Population variation in sexual dimorphism

Forewing length differed by sex ($F_{1,924}$ = 38.37, P < 0.0001) and population ($F_{3,924}$ = 26.90, P < 0.0001) with a significant interaction effect between sex and population ($F_{3,924}$ = 3.55, P = 0.014; Fig. 5A). Specifically, males had longer wings than females in both wing dimorphic La Selva and BCI populations, but also in the wing monomorphic Los Tuxtlas population. In the wing monomorphic Sirena population, there was no difference between the sexes in wing length (t_{22} = –0.42, P = 0.68). Similarly,

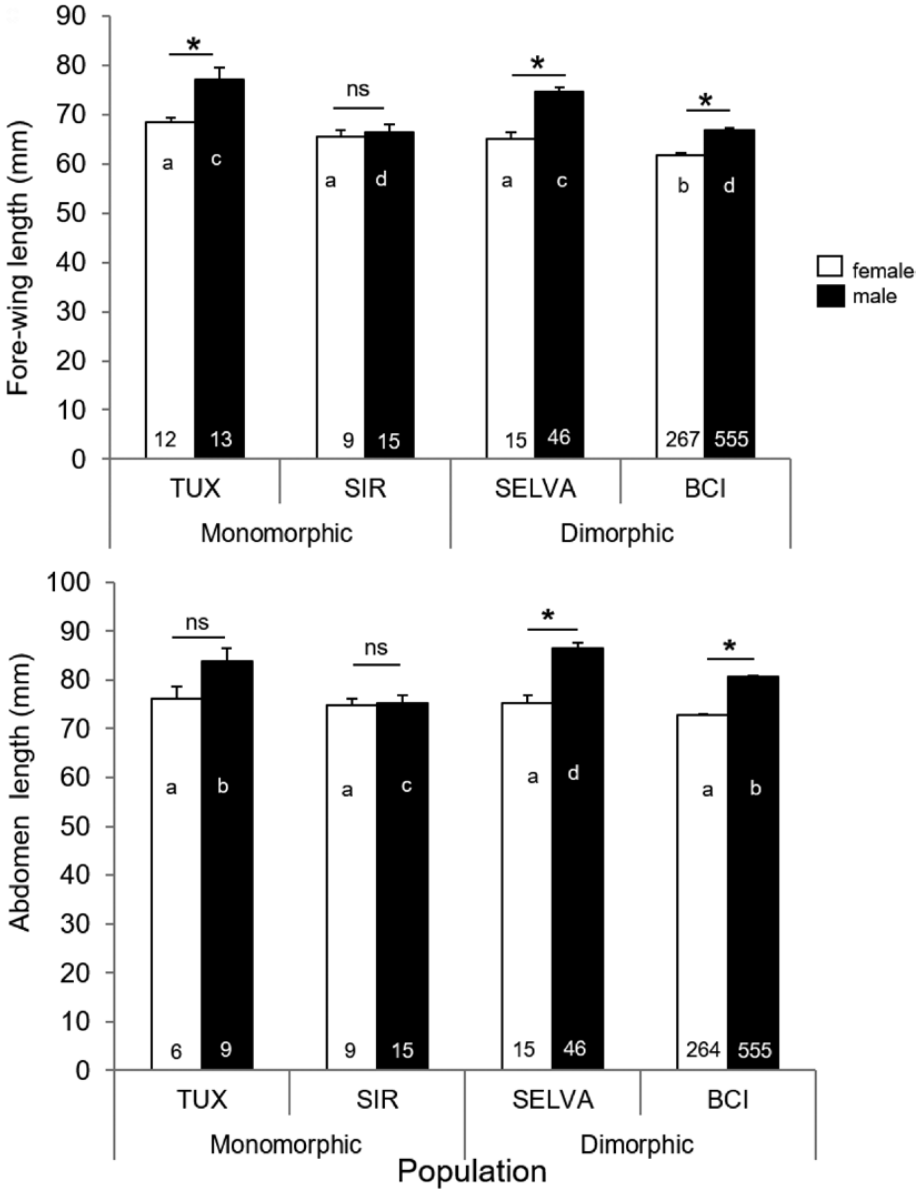

Figure 5. Body size of females and males across four of the study populations: A, forewing length; B, abdomen length. Numbers indicate sample size; letters are the same for populations and sexes that did not differ in size. An asterisk above a bar indicates significant sex differences within populations; ns, not significant.

abdomen length also differed by sex ($F_{1,911}$ = 32.61, P < 0.0001) and population ($F_{1,911}$ = 7.38, P < 0.0001), with an interaction effect between sex and population ($F_{1,911}$ = 3.48, P = 0.016; Fig. 5B). Males had longer abdomens than females at La Selva and BCI, but abdomen length did not differ between the sexes at Los Tuxtlas (t_{13} = –1.93, P = 0.07) or Sirena (t_{22} = –0.24, P = 0.81). Although we had too few measurements from the wing monomorphic El Jaguar population for analysis, in the wing dimorphic Bartola deme, wing length was shorter than in the nearby wing dimorphic La Selva population ($F_{1,34}$ = 6.94, P = 0.01).

Wing ratio also differed between the sexes ($F_{1,47}$ = 19.11, P < 0.001), and across populations ($F_{3,47}$ = 43.91, P < 0.001), with no interaction effect between sex and population ($F_{3,44}$ = 0.32, P = 0.81). At all four sites, females had broader wings than males (Fig. 6). Individuals at Los Tuxtlas had the broadest wings and those at Sirena had the narrowest wings. In contrast, the two dimorphic wing populations, which did not differ in wing ratio, had wings that were intermediate in breadth relative to the two wing monomorphic populations. Consistent with the above analyses, a mixed model with population as a random variable indicated that

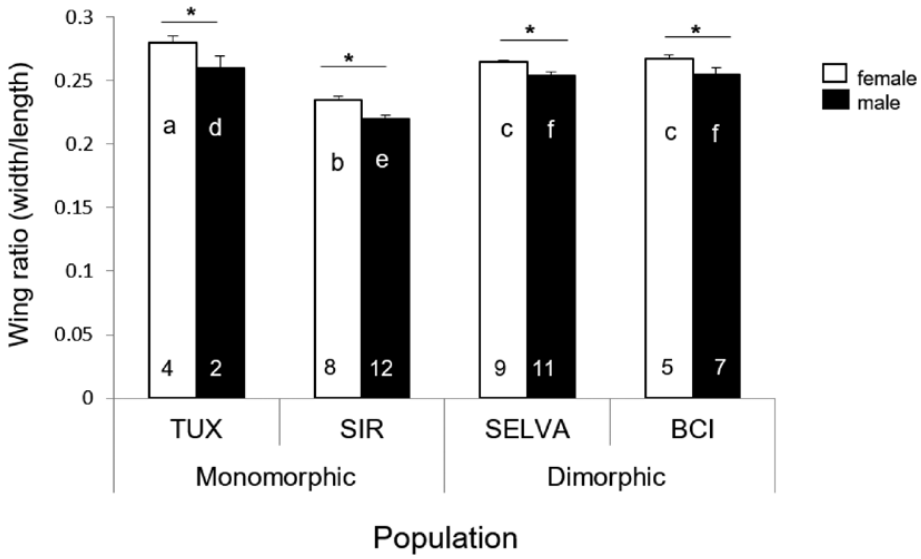

Figure 6. Wing ratio (width/length) of females and males across four of the study populations. Numbers indicate sample size; letters are the same for populations and sexes that did not differ in wing shape. An asterisk above a bar indicates significant sex differences within populations.

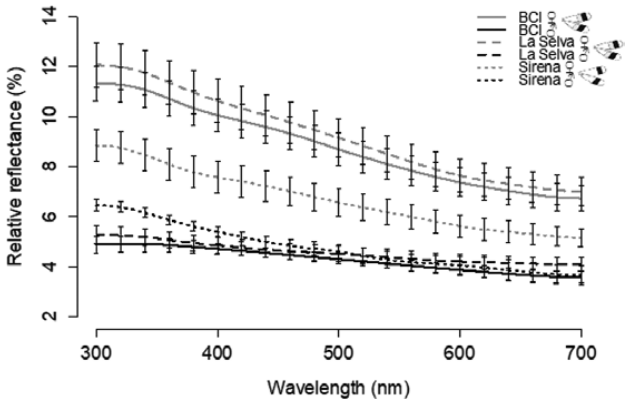

Figure 7. Relative reflectance of males and females from two dimorphic populations (BCI and Le Selva), and one monomorphic population (Sirena). Solid lines represent males, dashed lines represent females and whiskers represent standard errors.

wing type alone failed to explain differences in wing length ($F_{1,2} = 1.50$, $P = 0.73$; $F_{1,2} = 2.22$, $P = 0.27$, males and females, respectively), abdomen length ($F_{1,2} = 0.07$, $P < 0.82$; $F_{1,2} = 0.07$, $P < 0.82$) or wing ratio ($F_{1,2} = 0.07$, $P < 0.82$; $F_{1,2} = 0.07$, $P < 0.82$).

Mean spectra for male and female white wing tips from the three populations measured are shown in Figure 7. Despite the low sample sizes, the mean spectrum of Sirena females had almost no overlap with those of La Selva and BCI females.

Mean brightness of the white wing tips differed among populations ($F_{2,24} = 5.44$, $P = 0.01$), and between the sexes ($F_{1,24} = 55.81$, $P < 0.0001$); there was also an interaction between population and sex ($F_{2,24} = 3.74$, $P = 0.04$). Specifically, the difference between female and male wing tip brightness was smaller at Sirena than in the other two populations (Fig. 8). Post-hoc comparisons revealed that females on average had higher brightness than males (adjusted $P < 0.0001$). The wing tips of Sirena females were significantly less bright than those of La Selva females (adjusted $P = 0.04$) but wing tip brightness did not differ between Sirena and BCI females ($P = 0.13$) nor between La Selva and BCI females (adjusted $P = 1.00$). In contrast, wing tip brightness did not differ between any pair of sites for males (La Selva vs. BCI: $P = 0.99$, Sirena vs. BCI: $P = 0.93$, Sirena vs. La Selva: $P = 1.00$).

Cues of sex and competitors for wing monomorphic males

In our experiment on mate and competitor recognition cues for males in the wing monomorphic population of Sirena, we lacked balanced results from all six focal males. In female presentations, the four focal males all reacted sexually to control females. Blackening the female tips elicited a non-sexual reaction from three of the four males, but this was not a significant behavioural change (Wilcoxon signed rank test, $V = 6$, $P = 0.15$). Similarly, in male presentations, controls elicited non-sexual reactions from two of three focal males, and all three reacted sexually after the wing tips were whitened, but this was not a significant change ($V = 0$, $P = 0.35$).

Relaxing the requirement of data independence to explore all 25 of the elicited reactions from all

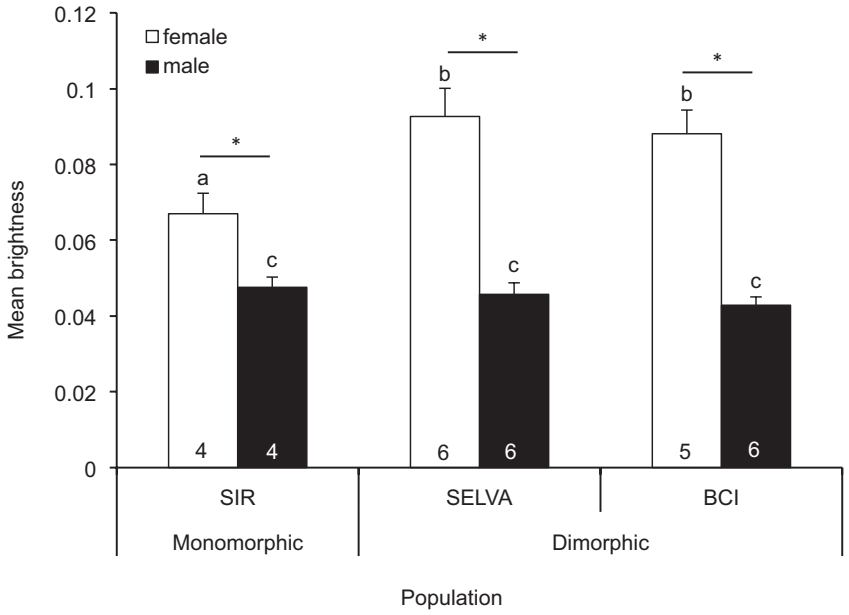

Figure 8. Relative brightness of the wing tips of males and females from two dimorphic populations (BCI and La Selva), and one monomorphic population (Sirena). Whiskers represent standard errors. Numbers indicate sample sizes; letters indicate significant differences among groups.

six focal males revealed suggestive trends. Pooling control females with those whose wing tips were subsequently whitened after being painted black suggested that white wing tips of females tended to elicit more sexual reactions than male controls (Fig. 9A, Fisher's exact test, $P < 0.05$). White female wing tips elicited relatively more sexual reactions than blackened tips of females (Fig. 9A, $P < 0.05$). Similarly, males manipulated with a whiter wing tip elicited relatively more sexual responses than male controls ($P < 0.05$). Sirena males with white wing bands were as likely as control males lacking the white band to elicit an aggressive response from resident males ($P = 1.0$, Fig. 9B). Indeed, the presence of a white wing band failed to prevent a doubly modified male with female-like wing tips (male number 10, Fig. 1H) from being taken in tandem by a resident male. After the manipulated male was un-tethered, the resident male maintained tandem and transferred sperm to his pene in preparation for copula. He then periodically 'jerked' the modified male (which typically stimulates a willing female to form a copula) for more than 55 min before finally releasing his unresponsive 'mate' (Video S1). Although more data are needed for statistical inference, the trend in our collective results tentatively supports the hypothesis that differential wing tip reflectance of females and males cues Sirena males to both potential mates and competitors, as was found in the wing dimorphic BCI study.

Morphology of secondary genitalia

Ancestral state reconstruction of male genitalia suggested that right-orientated ligula of the pene (for sperm transfer to females) was the ancestral condition (Fig. 10A). Among male claspers, which engage the female during tandem formation, long cerci were ancestral, with short ones being derived. The thick, hooked paraprocts were ancestral and the thin straight ones were derived (Fig. 10B). Within the western Costa Rican samples (SIR), cerci ranged from long and thin, to short and stout. Individuals collected from other regions additionally exhibited cerci variation in the roundness of the base, length, thickness and orientation (upward or downward). We found no difference across populations in cerci length ($F_{4,11} = 1.25$, $P = 0.35$), width ($F_{4,11} = 0.96$, $P = 0.47$) or paraproct length ($F_{4,11} = 0.78$, $P = 0.56$). Paraproct structure was less variable than cerci across all locations, with a large, hooked paraproct, varying only in the angle of the hook. Nevertheless, paraproct hook angle differed among populations ($F_{4,11} = 8.82$, $P = 0.002$). Post-hoc tests revealed that with the exception of Chiriqui, the four populations all differed from each other. LS mean angles were: Los Tuxtlas, 164.9°; Sirena, 159.7°; BCI, 158.9°; and La Selva, 144.9, suggesting that some mechanical incompatibility may have helped to isolate these populations.

DISCUSSION

Isolation-by-distance, a proxy for neutral divergence, explained only 18% of the genetic variation that we

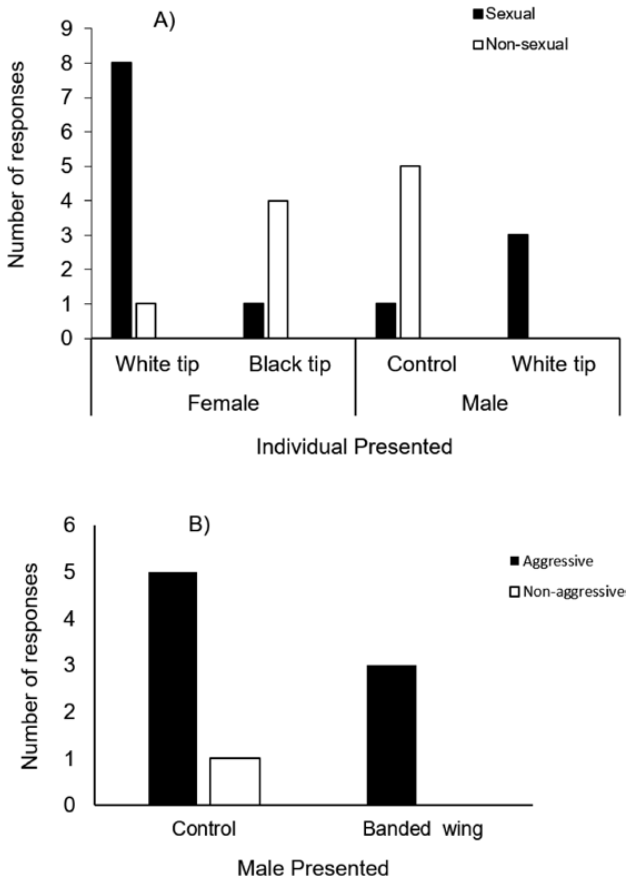

Figure 9. Binary reactions of six unique Sirena resident males in wing manipulation experiments. A, sexual cue experiment. 'White tip' consisted of five controls and the three treatments in which previously blackened female wings were re-painted white. B, competitor recognition experiment (control males are the same as in A).

found among populations, suggesting that additional factors were influencing the structure we observed. We documented a non-random pattern suggesting that relative to wing monomorphic demes, wing dimorphic populations are under stronger sexual selection on males. Collectively, our data suggested that body morphology can evolve across demes, independent of the state of the wing band. Wing monomorphic Sirena males tended to react to wing manipulated individuals in ways consistent with results from the BCI study, a conclusion that awaits more data.

Compared to wing dimorphic taxa, wing monomorphic clades, regardless of geographical origin, shared more genetic haplotypes and morphological characters across their geographical range from the TUX population in Mexico to the SIR/CNP deme in Costa Rica and EJ populations in Nicaragua (Fig. 2). There was strong phylogenetic distinction between the three wing monomorphic clades and the wing dimorphic populations (BCI and SELVA) despite close proximity of the alternative wing types in Costa Rica and Nicaragua (Fig. 3). Our results suggested little gene flow among countries, but low genetic structure among individuals sharing the same wing type, suggesting greater gene flow within, than between, morphotypes.

Our analyses suggested that wing monomorphic forms are ancestral, whereas the male's white wing band is derived (see also Svensson & Waller, 2013, for *Calopteryx* damselflies). This result is consistent with the lack of a male-specific wing band in three of the four subspecies, as well as in *Microstigma*, the sister genus of *Megaloprepus*. Although all of our study populations exhibited a territorial defence mating system, relative to our wing monomorphic demes (TUX, SIR, EJ), the wing dimorphic ones (BCI, SELVA) experienced higher male–male competition, inferred from their higher population density, higher probability that new holes were defended and greater frequency of territorial fights.

In high-density populations, the benefit of the waxy white wing band to a male in assessing rivals during agonistic encounters is likely to be worth the signal's production cost (Xu, 2014). Given the low probability of encountering rivals in the wing monomorphic, low-density populations, selection should not favour this expensive signal that indicates male size to rivals. Similarly, in *Mnais* damselflies, which exhibit wing polymorphisms within species, populations that are sympatric with a congeneric competitor are more likely to have males with a wing band (Tsubaki & Okuyama, 2016).

As expected given the greater degree of male–male competition in wing dimorphic demes, wing dimorphic demes were sexually dimorphic in body size and wing tip brightness. In contrast, in the monomorphic Sirena deme, males and females were much more similar with respect to body size (wing and abdomen length), wing tip brightness and wing ratio (our proxy for shape) (Fig. 6). In all other populations, regardless of wing type, female wings were broader than male wings, probably resulting from natural selection for sufficient lift for females carrying egg loads. Curiously, however, in the wing monomorphic Los Tuxtlas population, males were larger than females. That population might have experienced a relatively higher density and greater sexual selection on males in the past, with the loss of the wing band being a relatively recent event, evolving faster than body size. However, such a scenario is inconsistent with the phylogenetic pattern (Fig. 2), which suggests that the dimorphic wing state evolved only once among our study populations. More extensive sampling in Central and South America might provide examples of wing band loss.

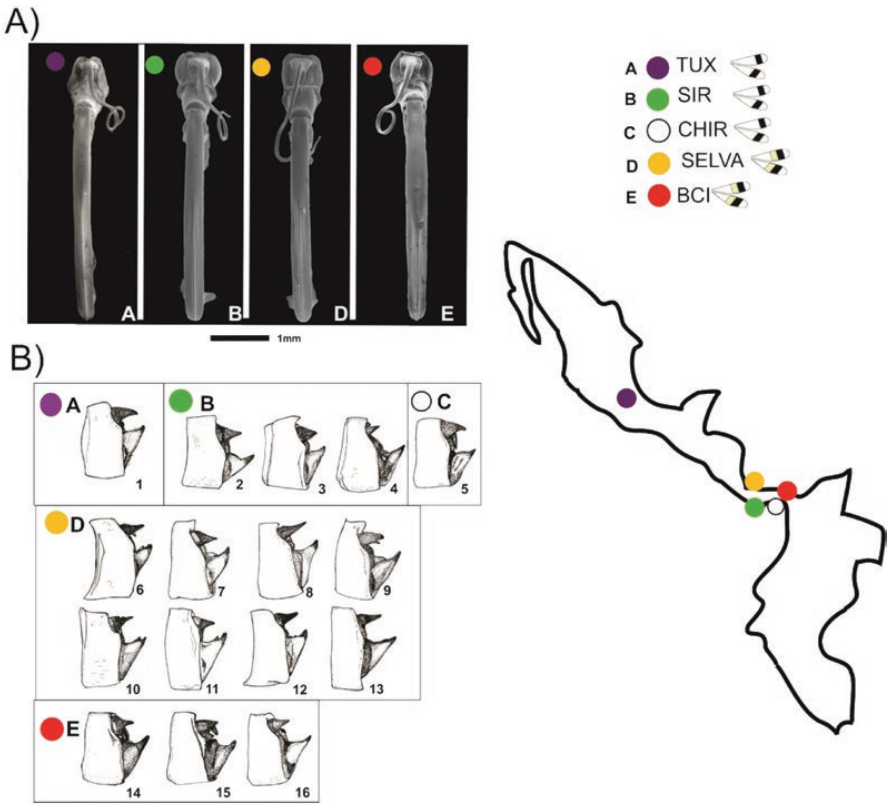

Figure 10. Male *Megaloprepus caerulatus* genital morphology as it corresponds to wing type. A, SEM images of pene and ligula. B, illustrations of male *Megaloprepus caerulatus* genital morphology (claspers and paraprocts) demonstrating variation within populations between geographical regions. The key refers to our study populations that fall within the same region as collection sites (see Supporting Information) with the exception of CHIR, which refers to Chiriqui, Panama, not one of our study populations.

Our wing manipulation experiment using wing monomorphic Sirena males lacked statistical power to draw any firm conclusions about cues to sex and competitors. Nevertheless, the trends identified here, coupled with earlier results that indicated differential wing tip reflectance cues both sex and competitors to the wing dimorphic BCI population (Schultz & Fincke, 2009), are parsimoniously consistent with the null hypothesis that males of both wing types do not differ in these signalling cues. Thus, we suspect that reproductive isolation among *Megaloprepus* subspecies is unlikely to be mediated via differences in male mate preference for female wing tips or by differential aggression towards unbanded or banded males. Even the relatively small difference in wing tip brightness of Sirena females and males (Figs 7, 8) was sufficient to elicit different sexual responses in most of the resident males (Fig. 9). Moreover, Sirena males did not discriminate against females with a more prominent white, painted tip (Fig. 9A), suggesting they would not discriminate against females of wing dimorphic BCI damselflies, whose wing tips are brighter (Figs 1, 7; and BCI study). Given that the two wing types are not known to occur sympatrically, and that male wing bands are a derived state, which may have evolved only once, it is not surprising that all three of the wing monomorphic Sirena males tested reacted aggressively to novel rivals with a white band (Fig. 9B). Similarly, wing dimorphic BCI males reacted aggressively to novel males whose white wing bands had been blackened.

Low population density precluded an explicit test of female mate choice in Sirena. Nevertheless, on BCI, females did not reject as a mate a free flying resident male whose white wing band had been blackened (O. M. Fincke, pers. observ.). In contrast, in territorial *Calopteryx* damselflies, female discrimination among male wing patterns has led to genetic divergence (Svensson & Waller, 2013).

In contrast to our results and those of the BCI study are results of a relevant laboratory study of darters (Moran *et al.*, 2017), the females of which, like those of *Megaloprepus*, do not exhibit mate preference, and males of which exhibit variation in coloration that functions in male–male competition, similar to the white wing band of *Megalorpepus* males. Relative

to their reactions to heterospecifics, male darters exhibited a mating preference towards female conspecifics, and were more aggressive towards conspecific males. The difference between the darters and our damselflies may lie in the nature of the sexual signal. In *Megaloprepus*, the conspecific signal difference seems to be only in degree for recognition of females and males, whereas in darters, both the sexes and heterospecifics differ in hue, not unlike *Heterina* damselflies males of which discriminate against heterospecifics based on hue or amount of wing coloration (Anderson & Grether, 2010).

Both dimorphic wing patterns and our genetic data that revealed monophyletic, dimorphic populations (La Selva, BCI and Canandé) were consistent with de Selys Longchamps' (1886) subspecies designation of *caerulatus caerulatus*. For the wing monomorphic Los Tuxtlas population our results were consistent with his designation of *caerulatus latipennis*. However, the wing monomorphic populations at El Jaguar (Nicaragua) and Sirena (Costa Rica) formed a monophyletic clade distinct from *M. caerulatus latipennis* not only genetically, but also morphologically (Figs 5, 6), strengthening the earlier call of Feindt *et al.* (2014) for subspecies or even species status, based only on genetic data. Indeed, our more robust molecular study revealed a high haplotype diversity among countries (i.e. H = 0.78) relative to 16S-based H for reduviid hemipterans, which ranged from 0.219 to 0.840 within Argentina (Garcia *et al.*, 2003), and for freshwater bryozoans across Europe (H = 0.27–0.778; Freeland, 2003).

We found a few 16S haplotypes in common between the Los Tuxtlas and Sirena populations (Fig. 3), unlike Feindt *et al.* (2014) who found none. Our Sirena population was genetically distinct from the same population (i.e. CNP) collected by Feindt *et al.* (2014), even though their samples were taken during a similar time of year. Although our SIR samples were from adults and their samples were mainly from larvae, the monophyly of the Costa Rica clade (Fig. 2) suggested that all larvae were correctly identified as *Megaloprepus*. Post-sequencing base calling and contig assembly may have differed between our respective labs. Alternatively, alignment differences may have resulted in differences in haplotype diversity estimates. Unlike Feindt *et al.* (2014), we aligned the 16S large ribosomal subunit with reference to its secondary structure, which facilitates homology assessment and reduces ambiguity.

Despite considerable geographical separation between Los Tuxtlas and Sirena/El Jaguar, the two wing monomorphic subspecies from Mexico and Costa Rica shared more genetic signal with each other than either did with wing dimorphic demes separated by only 240 km between eastern and western Costa Rica, or by 290 km between eastern and western Nicaragua. Interestingly, the wing monomorphic deme at El Jaguar is just 118 km from a wing dimorphic population in the slightly higher cloud forests of Reserva Bosawás. Although the bandless males of both *latipennis* and the new subspecies should be less successful in invading populations of banded males, where they presumably would lose territorial fights, the white-banded, larger La Selva males should be able to invade wing monomorphic demes. No *Megaloprepus* subspecies are known to occur sympatrically.

Differences in male secondary genitalia among *Megaloprepus* subspecies, such as the significant variation we found across four of five populations in the angle of the paraproct hook, offer a possible reproductive isolating mechanism that may diverge due to drift alone during geographical isolation. Variation in the male ligula is often species specific in damselflies, but is known to converge for the function of sperm displacement among dragonfly species (Miller, 1991). Nevertheless, even within a given subspecies, we found that right- and left-curling ligula varied, as did the cerci, morphology that is critical in taking a female in tandem (Fig. 10). In contrast, McPeek *et al.* (2011) found little variation in secondary genitalia among males of *Enallagma* populations in North America. Our samples were biased towards *M. caerulatus caerulatus*; evaluation of secondary genitalia of all subspecies across their geographical range is needed, as are direct tests of mating capability between them.

Although many insects are known to differ in their response to abiotic factors such as thermal stress and dehydration that often differ by geographical location (e.g. Sørensen *et al.*, 2003; Tine *et al.*, 2010), *Megaloprepus* wing types did not assort to habitats by rainfall or temperature extremes (Table 1). The central highlands that separate the Pacific and Atlantic coasts of Mexico and Central America may pose other physiological challenges to migration. What seems more certain is that on-going, anthropogenic habitat fragmentation of old-growth tropical forests threaten *Megaloprepus* populations (Fincke & Hedström, 2008; Escoto-Moreno *et al.*, 2018) as it does other tropical taxa (reviewed by Didham *et al.*, 1996; Frankie *et al.*, 2004). Thus, we expect isolation by distance to play an increasing role in the contemporary divergence of *Megaloprepus* populations.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Accession numbers for sequences from this study.
Table S2. Results from the wing manipulation experiment, colour coded to show data used in the Wilcoxon signed rank test on binary data (sexual vs. non-sexual; aggressive vs. non-aggressive). FID = focal male ID, TRT = treatment: C = control, WT = white wing tip, WB = white band, WBT = white band and tip; SX = sex of presented individual, PID = presented individual ID, RES = response by focal male: A = aggressive, S = sexual, N = neutral.
Table S3. Site locality information for illustrations featured in Figure 10.
Fig. S1. Measurements taken of secondary genitalia: A, male clasper morphology: cerci length (cl) and width (cw), paraproct length (pl), angle of paraproct hook (pha); B, male flagella length (fl), in addition to its orientation to the left or right. The flagella is part of the ligula complex.
Video S1. A male *Megaloprepus* from Sirena that took a doubly-manipulated male in tandem and continued to try to mate with him.