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Genomic regions underlying the species-specific mating songs of green lacewings

Katherine L. Taylor[1,2] | Elizabeth J. Wade[3] | Marta M. Wells[4] | Charles S. Henry[1]

[1]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut, USA

[2]Department of Entomology, University of Maryland, College Park, Maryland, USA

[3]Department of Natural Sciences and Mathematics, Curry College, Milton, Massachusetts, USA

[4]Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut, USA

Correspondence
Katherine L. Taylor, Department of Entomology, University of Maryland, College Park, Maryland, USA.
Email: taylork@umd.edu

Funding information
University of Connecticut

Abstract

Rapid species radiations provide insight into the process of speciation and diversification. The radiation of *Chrysoperla carnea*-group lacewings seems to be driven, at least in part, by their species–specific pre-mating vibrational duets. We associated genetic markers from across the genome with courtship song period in the offspring of a laboratory cross between *Chrysoperla plorabunda* and *Chrysoperla adamsi*, two species primarily differentiated by their mating songs. Two genomic regions were strongly associated with the song period phenotype. Large regions of chromosomes one and two were associated with song phenotype, as fewer recombination events occurred on these chromosomes relative to the other autosomes. Candidate genes were identified by functional annotation of proteins from the *C. carnea* reference genome. The majority of genes that are associated with vibrational courtship signals in *other insects* were found within QTL for lacewing song phenotype. Together these findings suggest that decreased recombination may be acting to keep together loci important to reproductive isolation between these species. Using wild-caught individuals from both species, we identified signals of genomic divergence across the genome. We identified several candidate genes both in song-associated regions and near divergence outliers including *nonA*, *fruitless*, *paralytic*, *period*, and *doublesex*. Together these findings bring us one step closer to identifying the genomic basis of a mating song trait critical to the maintenance of species boundaries in green lacewings.

KEYWORDS
Chrysopidae, courtship behaviour, lacewings, Neuroptera, QTL mapping, recombination, reproductive isolation, speciation

INTRODUCTION

Species–specific mating signals are thought to facilitate divergence and speciation (Wilkins et al., 2013). Identifying and characterizing the loci underlying these traits important to reproductive isolation between species is fundamental to understanding how speciation proceeds (Coyne, 1992; Templeton, 1981). Though the genetic underpinnings of traits essential to reproductive isolation have been identified in some organisms, long-standing questions about the number, relative positions, and effect size of barrier loci persist (Nosil & Schluter, 2011; Seehausen et al., 2014; Wu & Ting, 2004).

The genetic basis of acoustic signals key to reproductive isolation between species has been studied extensively in *Drosophila*. Males produce species–specific mating signals with both wing and abdominal vibrations (Fabre et al., 2012; Mazzoni et al., 2013). Many genes have been identified that are necessary for the production of typical wing-produced song phenotype or for the perception of that song in *Drosophila* (Gleason, 2005; Greenspan & Ferveur, 2000). Some of the same genes,

including *fruitless* and *doublesex*, are also associated with courtship abdominal vibration phenotype (Fabre et al., 2012). However, those genes and mutations known to modify mating signals in the laboratory may not necessarily be the genes that drive species–specific differences in songs. The genetic basis of species–specific acoustic mating signals has also been studied in crickets of the genus *Laupala*. Males produce signals using specialized stridulatory organs prior to mating (Mullen & Shaw, 2014). Mating signals in *Laupala* crickets have been associated with quantitative trait loci (QTL) spread across the genome containing multiple promising candidate genes (Blankers et al., 2019; Xu & Shaw, 2021).

When multiple genomic loci are responsible for any complex phenotype, but especially for those traits important to reproduction isolation, strong linkage is expected between loci to keep together these co-adapted genes in the presence of gene flow (Schwander et al., 2014). Signal production and signal preference traits are often closely linked, for example in the wing pattern signalling in *Heliconius* butterflies and in mating song of *Laupala* crickets (Kronforst et al., 2006; Xu & Shaw, 2021). These so-called 'supergenes' might be expected to occur in genomic regions with decreased recombination, such as areas near centromeres, on sex chromosomes, or within chromosomal rearrangements (Noor et al., 2001; Schwander et al., 2014).

In the rapid radiation of *carnea*-group green lacewings, species–specific premating duets isolate more than twenty morphologically cryptic species (Henry et al., 2013). Both male and female lacewings in the *carnea*-group produce signals that are exchanged in an intricate duet prior to copulation. The signals are sexually monomorphic, with males and females producing nearly identical songs. These signals are thought, at least in part, to have driven the recent and rapid radiation of species in this group (Henry et al., 2013). Phenotypic information from laboratory crosses suggests that mating song phenotypes in the *carnea*-group are genetically controlled by a few loci of large effect (Henry et al., 2002), but to date, no loci underlying mating song phenotypes have been identified.

There are multiple pairs of sympatric species that vary primarily in their courtship signals in the *Chrysoperla carnea*-group. One such species pair is *Chrysoperla plorabunda* and *Chrysoperla adamsi*, which perform courtship songs that are similar in structure except for a consistent major difference in one critical song feature, the volley period (Henry et al., 1993, 2002). The signals are composed of multiple bursts of low-frequency substrate-borne sound which are produced by vigorous but highly controlled shaking of the thorax and abdomen. Volley period is the length of a single burst of sound (Figure 1). These two species lack strong morphological or ecological differences and co-occur in western North America. (Henry et al., 1993, 2002). As larvae, both species are generalist predators of invertebrates, while as adults they feed on nectar and honeydew. *Chrysoperla plorabunda* is largely found on herbaceous plants, while *C. adamsi* can be found on herbaceous plants, shrubby vegetation, and fruit trees. These authors have collected individuals of both species from the same fields at the same time and they will hybridize in laboratory conditions, yet field-caught hybrids have not been identified.

Herein, we used quantitative trait mapping and genome scans to identify the genomic architecture underlying the volley period phenotype in two sympatric species in the *Chrysoperla carnea*-group. We identified genomic regions associated with this trait important to reproductive isolation using an F_2 laboratory cross. With this approach, we were able to identify regions truly related to the phenotype; however, fine mapping to small genomic windows was limited by number of individuals in the cross, recombination frequency, marker density, and strength of the effect of the loci (Broman, 2001; Mackay, 2001). We narrowed our search based upon signals of genomic divergence between wild members of the same two species to reveal patterns related to selection or a lack of introgression near these traits of interest. We then used functional annotation to identify candidate genes and found patterns suggesting that decreased recombination in these song-associated regions may help to keep song-associated loci together.

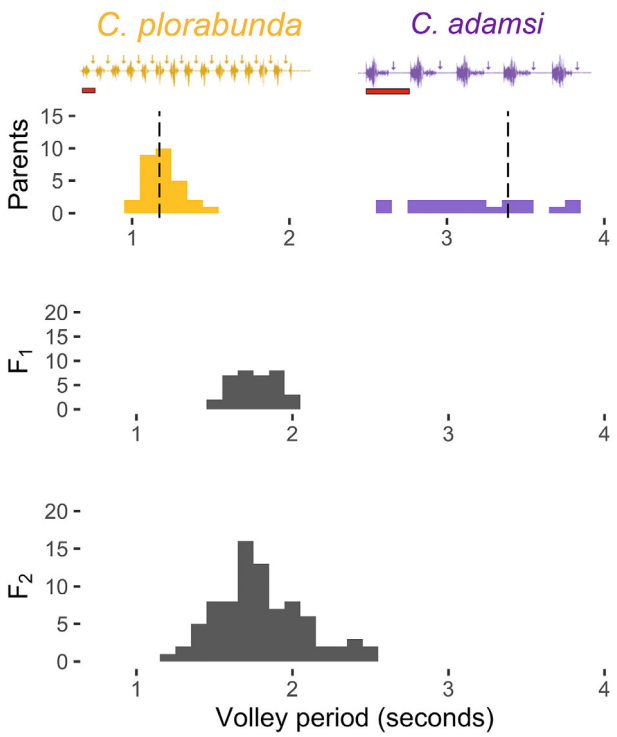

FIGURE 1 F_2 hybrid cross phenotypes. At the top of the figure are 12-second oscillograms of vibrational mating signals of the two parent species, with *Chrysoperla plorabunda* in yellow and *Chrysoperla adamsi* in purple. A single volley period for each of the parental species is indicated with a red bar under the oscillogram. Arrows on the oscillograms indicate where a duetting partner would insert a response. Below are the volley period histograms for the *Chrysoperla plorabunda* ($n = 29$), *Chrysoperla adamsi* ($n = 20$), F_1 ($n = 35$), and F_2 ($n = 83$) individuals from the laboratory cross. The phenotypes of the individual *C. plorabunda* and *C. adamsi* grandparents that founded the cross are indicated with dashed vertical lines.

METHODS

Collecting, laboratory rearing, phenotype recording

Chrysoperla plorabunda (Fitch) and *Chrysoperla adamsi* Henry et al. cross parents were collected in western Oregon, United States in 2015. The wild-caught *Chrysoperla adamsi* female was collected from

shrubby vegetation near the top of Mary's Peak, OR. The wild-caught *Chrysoperla plorabunda* male was collected from a clover field along a road in Benton, OR. Six additional wild-caught individuals of *Chrysoperla adamsi* and *Chrysoperla plorabunda* were collected from North Winters and Gates Canyon California, Guilford Connecticut, Moscow Idaho, and Benton and Marion Oregon between 2008 and 2016. All lacewings were laboratory-reared on a long day light cycle (16 hours light, 8 hours dark) at 25 ± 1°C. Adults were kept in clear plastic 8 oz cups with a hole in the bottom for a cotton wick. Rearing cups were stacked inside a second clear plastic cup containing a water reserve and covered by a petri dish lid. A 2:1:1 mixture of Wheast, honey, and water was provided ad libitum for adults. Larvae from the family cross were reared in clear plastic 1 oz cups with snap-on lids. Sterile *Ephestia kuehniella* eggs were provided to the larvae ad libitum.

Mating signals for all individuals were recorded in the laboratory at 25 ± 1°C using an optical microphone and waveform software (see Henry et al., 2013). The wild-caught individuals were identified to species based upon mating signals. A single pair of wild-caught parents were induced to mate by confinement together in a rearing container. F_1 hybrid offspring were reared to adulthood and brother–sister mated to produce F_2 hybrids. The *C. adamsi* by *C. plorabunda* cross produced 78 F_2 offspring that were successfully sequenced.

Family cross-sequencing and bioinformatic analysis

DNA was extracted using a Qiagen DNeasy blood and tissue kit from the two parents founding the line, 13 F_1 individuals, and 78 F_2 individuals. A reduced representation sequencing library of the family cross individuals was prepared by Floragenex with the PstI cutting enzyme and sequenced on an Illumina HiSeq at the University of Oregon. Single-end 150 base pair reads were demultiplexed with Stacks (v. 2.2; Rochette et al., 2019) and mapped against the reference assembly for the closely related species *Chrysoperla carnea* (GCF_905475395.1; Crowley, 2021) with BWA (v. 0.7.17; Li & Durbin, 2009), and genotypes were called with bcftools (v. 1.9; Danecek et al., 2021) mpileup. Genotypes were filtered with bcftools to include only biallelic single nucleotide polymorphisms (SNPs) with a quality score >30, a minor allele frequency >0.05, missing in less than 80% of individuals, lacking significant deviations ($p < 0.01$) from an X^2 normality test (1:2:1), fixed in the cross founding parents, and heterozygous in the female F_1s.

The association of song period with genomic variants was measured using a linear mixed model (LMM) in Gemma (v. 0.98.4; Zhou & Stephens, 2012). For this analysis, missing genotypes were imputed using LinkImpute (v. 1.1.5; Money et al., 2015). The effect size of the variants was estimated as β from the LMM and significance was assessed by likelihood ratio test (alpha = 0.01) after a Bonferroni correction for multiple tests. A classical approach to quantitative trait mapping was also completed using the scanone function of the package qtl (v. 1.5; Broman et al., 2003) in R software (v. 4.1.1; R Development Core Team, 2022). The logarithm of odds (LOD) score was used to measure association with a significance threshold of 4.08 determined by a genome wide permutation test (alpha = 0.01).

Song phenotype genomic architecture

The minimum number of genetic components underlying the volley period phenotype was estimated from the variance in the phenotype data (Lande, 1981). Additionally, hyperparameters related to the genomic architecture of the trait were estimated by a Bayesian sparse linear mixed model in Gemma (Zhou & Stephens, 2012). Mean estimates were calculated from five independent runs; each run was five million sampling steps and the first 500,000 were discarded as burn-in.

Linkage disequilibrium

Linkage between variants was measured by Plink (v. 1.9; Chang et al., 2015). Variants were binned into 10 Mb distance bins and the mean R^2 was plotted across each autosome for all bins with at least 5,000 SNP comparisons.

Genome scan

DNA was extracted from wild-caught *C. adamsi* and *C. plorabunda* using a Qiagen DNeasy blood and tissue kit. A reduced representation sequencing library was prepared by the Center for Genome Innovation at the University of Connecticut with the SbfI cutting enzyme and sequenced on an Illumina HiSeq at the University of Connecticut. Alignment and variant identification were performed as described above for the F_2 cross data set, except without filtering based upon X^2 normality test or cross-parent genotypes. Weir and Cockerham's windowed F_{ST} was calculated by VCFtools (v. 0.1.6; Danecek et al., 2011) across 50 kb windows with a 5 kb step. Significance threshold was set as z transformed F_{ST} >3. No windows rose above the more conservative threshold of zF_{ST} >6.

Candidate gene identification

Proteins from the reference genome assembly for *Chrysoperla carnea* (GCF_905475395.1) were functionally annotated by EnTAP (v. 0.10.8; Hart et al., 2020) based upon similarity to proteins in the UniProt database and gene family assignment by eggNOG. We identified genes annotated with the gene ontology term "male courtship behaviour, veined wing generated song production" (GO:0045433) as candidate genes.

RESULTS

Song phenotype genomic architecture

Volley periods for hybrid F_1 and F_2 offspring were intermediate to the parental phenotypes, showing greater phenotypic range in the F_2 offspring (Figure 1). The average volley period phenotype for the *C. adamsi* and *C. plorabunda* parents that founded the cross were 3.39

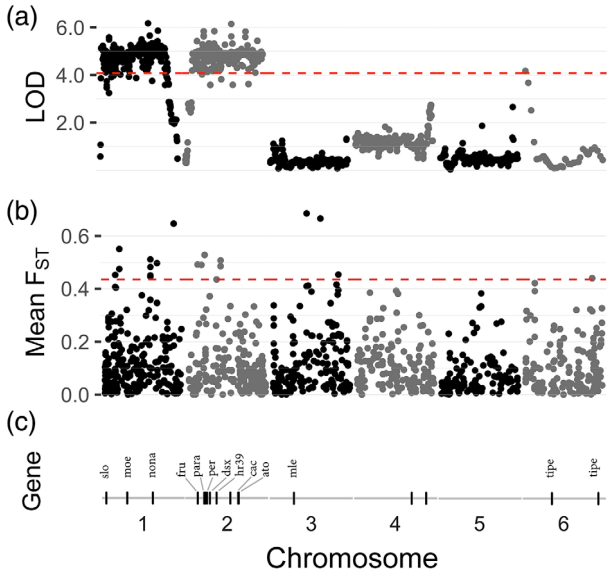

FIGURE 2 The genomic basis of volley period song phenotype. Variants are plotted across the six chromosomes with points in alternating light and dark grey. (a) QTL identified from an F_2 cross between *Chrysoperla plorabunda* and *Chrysoperla adamsi* by the association of volley period with genomic markers in F_2 offspring ($n = 78$). Association is measured by LOD and a red line indicates the significance threshold determined by a permutation test. (b) Divergence between wild-caught *C. plorabunda* ($n = 6$) and *C. adamsi* ($n = 6$) measured by Weir and Cockerham's windowed F_{ST} calculated across 50 kb windows with a 5 kb step. The mean F_{ST} for all windows with >5 variants is plotted and the zF_{ST} significance threshold is indicated by a dashed red line. (c) Positions of genes associated with wing-generated song production. Genes are labelled when identity was determined; unlabelled genes were not identified. Candidate genes within song QTL and in close proximity to QTL peaks are *nona* (no-on-transient A), *para* (paralytic), *per* (period), and *dsx* (doublesex).

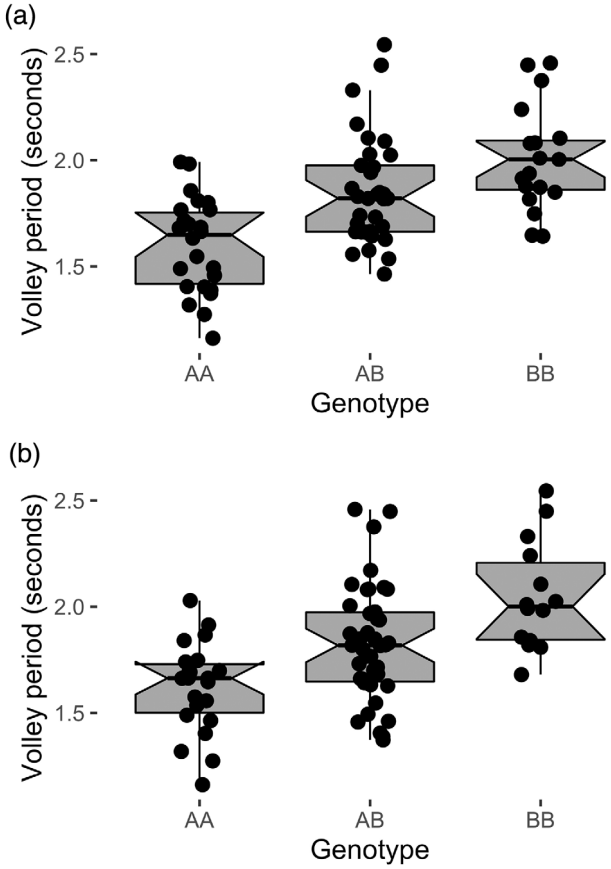

FIGURE 3 Volley period of F_2 hybrid songs from a *Chrysoperla plorabunda* and *Chrysoperla adamsi* cross. Song phenotypes are plotted for F_2 offspring of each possible genotype, AA homozygous for the *C. plorabunda* grandparent genotype, AB heterozygous, and BB homozygous for the *C. adamsi* grandparent genotype at the top variant by LOD on (a) chromosome one and (b) chromosome two. Individual phenotypes are plotted as points over boxplots for each group.

and 1.17 s respectively. The average volley period was 1.76 s (SD = 0.13) for F_1 and 1.80 s (SD = 0.29) for F_2 offspring. The minimum number of genetic factors estimated from variance in the phenotype data to underlying volley period phenotype was 2.5. Estimation of the genetic architecture by trait hyperparameter estimates from the Gemma Bayesian sparse linear mixed model (BSLMM) showed that our complete set of genetic variants explained about 70% of phenotypic variance (PVE = 0.699 [0.506, 0.888]), while approximately 66 major effect variants explained about 40% of the variance in phenotype ($N = 66.191$ [0, 269], PGE = 0.403 [0.000, 0.968]). The top 1% of SNPs according to sparse effect size from our BSLMM were found across all six chromosomes.

Genotype-phenotype association

After filtering, described in the methods, 9,733 variant loci remained with an average site sequencing depth of ~26×. Using two QTL mapping approaches we determined that genetic markers spanning chromosomes one and two were significantly associated with volley period phenotype. In our traditional QTL mapping approach using R qtl, we found that markers across much of chromosomes one and two and a small window of chromosome six rose above the genome-wide significance threshold (Figure 2a). Our LMM-based approach with Gemma yielded very similar results: the markers on chromosomes one and two had the largest estimated effect size and were the only markers significantly associated with song phenotype. The phenotypes of F_2 offspring are plotted by genotype at the top SNP by the LOD on chromosomes one and two, showing that for both chromosomes, F_2 offspring homozygous for the allele from the *C. plorabunda* parent had the shortest volley period, heterozygotes had an intermediate volley period, and those homozygous for the allele from the *C. adamsi* parent had the longest volley period (Figure 3). We focus on the QTL on chromosomes one and two, as they were identified as the regions of the largest effect and were recovered as significant in both QTL mapping approaches.

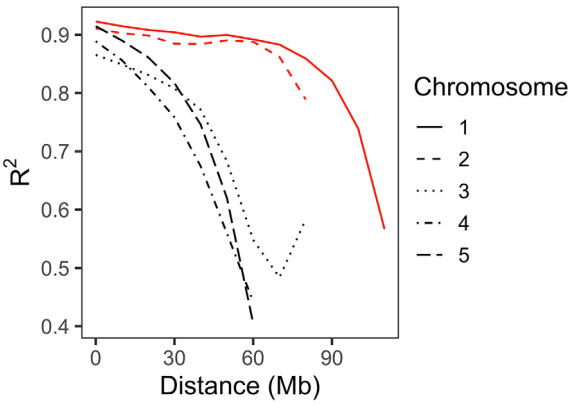

FIGURE 4 Linkage across the five *Chrysoperla* autosomes in the F_2 cross progeny with line type indicating chromosome. The R^2 for SNPs is plotted against the physical distance between those SNPs in the reference genome. Chromosomes 1 and 2, which both contain large QLT for volley period phenotype, are coloured red.

Linkage disequilibrium (LD)

Estimates of LD across the genome in the F_2 cross progeny revealed fewer recombination events on chromosomes one and two, which contain major QTL for song phenotype (Figure 4). We binned SNPs by physical distance in the reference genome and found that distant SNPs show higher estimated R^2 on chromosomes one and two compared to the other three autosomes.

Genome scan

Using a sample of six individuals from across the ranges of each of these two species, significant divergence peaks, identified by F_{ST}, were found on chromosomes 1, 2, 3, and 6 that rose above the zF_{ST} threshold of three (Figure 2b). We identified the F_{ST} peaks found on chromosomes 1 and 2 as being within the large QTL identified for the volley period, suggesting that divergence between species in these regions of the genome may be related to song phenotype.

Candidate genes

Thirty-two proteins from the *Chrysoperla carnea* genome were annotated with the gene ontology term "male courtship behavior, veined wing generated song production" (GO:0045433). The number of proteins annotated with GO:0045433 from chromosome one to six, respectively, were 5, 19, 1, 3, 0, 4. The majority of those proteins were mapped to chromosomes one and two, which contained large QTL for the volley period (Figure 2c). Gene identity was determined by EggNOG and when unidentified was assigned the identity of the top UniProt similarity search hit to an arthropod.

Correspondence between QTL, F_{ST} peaks, and the positions of candidate genes was assessed. The outliers on chromosome one between 18.6 and 26.0 Mb were more than 1 Mb from all identified candidate gene; the two closest candidates were *slowpoke* (0.8 Mb) and *moesin* (39.9 Mb). Several significant F_{ST} outlier windows fall between 81 and 94 Mb on chromosome one. This region contains the gene *nonA* (87 Mb), as well as other non-candidate genes. On chromosome two, multiple F_{ST} outlier windows were found between 11.3 and 40.1 Mb; this region included the candidate genes *fruitless* (11.6 Mb), *paralytic* (20.3 Mb), *period* (23.9 Mb), *doublesex* (27.9 Mb), one unidentified candidate gene (36.8 Mb), and other non-candidate genes. In all cases, there were non-candidate genes closer to significant F_{ST} peaks than the candidate genes. Both candidate genes and non-candidate genes may be involved in song phenotype.

DISCUSSION

We explored the genomic basis of reproductive isolation and speciation in a rapidly evolving lacewing group. Using association mapping for an F_2 cross between *Chrysoperla plorabunda* and *Chrysoperla adamsi*, we identified two large genomic regions associated with mating song phenotype on chromosomes one and two. The majority of genes associated with *Drosophila* vibrational courtship signals are found within the song-associated regions for *Chrysoperla*, suggesting that the genomic basis of these traits may be shared across multiple insect orders. Though these results align with our expectations, we cannot rule out the role of non-candidate genes in song-associated regions of the genome.

Using small samples of wild-caught lacewings, we identified signals of divergence that may be related to selection or a lack of introgression near our trait of interest. As genome-wide signals of divergence between species may also be related to selection for other traits or other demographic processes, we identified divergence signals most likely to be related to song phenotype by narrowing our search to only include regions with elevated divergence in our volley period QTL. Using this targeted approach we identified several song-associated regions with elevated F_{ST} containing candidate genes. Some of these candidates are the *period* gene, plus several genes known to modify the abdominal vibration in *Drosophila*: *fruitless*, *doublesex*, and *nonA*, which is responsible for species–specific differences in vibrational signals in *Drosophila* (Campesan et al., 2001; Fabre et al., 2012; Gleason, 2005). Though these candidate genes may be responsible for differences in volley period between these species, other non-candidate genes in these regions may be what truly underlies song phenotype. Functional validation will be necessary to confirm the relationship between genes and song phenotype and to identify how they might modify song. RNAi for these candidates would be a clear next step, as it is already being successfully used on closely related insects in the genus *Chrysopa* (Zhang et al., 2022).

We identified two major effect regions in our association of genotype and phenotype, but it is likely that other loci of small effect related to volley period are spread throughout the genome and are undetected in this analysis. Our estimation of trait architecture identified that only about 40% of phenotypic variance can be explained by

large effect loci, while up to 70% of the variance can be explained by the total panel of genomic variants, though we note that there were wide credible intervals around these estimates. It is unsurprising that more genomic regions are involved in the phenotype than those identified by quantitative trait mapping, as we expect a bias towards identifying the loci of large effect with this type of approach (Broman, 2001). The effect size estimates for the QTL we are able to detect could be inflated by these undetected regions of small effect (Beavis, 1998). Additionally, because we mapped a polygenic trait using a single cross, it is possible that we have failed to capture other song-associated variants present in the population but not fixed between species and the parents founding this cross (Muranty, 1996). Despite these limitations, we can say with confidence that the regions identified on chromosomes one and two likely have the largest effect on volley period differences between *C. plorabunda* and *C. adamsi*. Future analysis with replicated crosses, larger numbers of offspring, or a greater number of generations will be necessary to identify the precise genomic basis of mating song phenotypes in these species and other *carnea*-group species.

We also observed lower rates of recombination on chromosomes 1 and 2 compared to the other autosomes in our laboratory cross. One potential explanation for decreased recombination in this context could be chromosomal rearrangements between species. We do not expect that selection on song phenotype could explain decreased recombination, as we did not select for this in our laboratory experiment. Potentially, selection for other traits related to survival in our laboratory setting in the same areas of the genome could explain decreased recombination, or other features of the genomic landscape such as of repetitive content or gene density (Stapley et al., 2017). Whatever the source, the decreased rates of recombination observed here could function to keep together multiple genomic loci important to song phenotype or song preference, especially in the presence of gene flow.

Overall, we have made significant steps towards characterizing the genetic architecture of song volley period phenotype, a mating song feature critical to reproductive isolation in the *Chrysoperla carnea*-group. We identified major effect loci on chromosomes one and two containing candidate genes and found evidence of decreased recombination in song-associated regions of the genome.

AUTHOR CONTRIBUTIONS

EJW, MMW, and CSH conceived of the project and secured funding, KLT, MMW and CSH conducted the experiments, KLT analysed the data and wrote the manuscript, all authors edited the manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/bioproject/, reference number PRJNA880641.

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