Does hunger lead to hybridization in a genus of sexually cannibalistic insects (Orthoptera: Prophalangopsidae)?

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Allochronic isolation can be a strong mechanism for reproductive isolation and speciation. However, imperfect allochrony and the expression of phenological plasticity can erode temporal barriers to gene flow and result in hybridization between divergent lineages. Here, we combine behavioural ecology and genomics to investigate this scenario in two closely related species of grigs in the genus Cyphoderris. These species exhibit a unique mating system whereby females feed on the fleshy hind wings of the male during copulation, and copulation with conspecific males is more likely in food-restricted females than in well-fed females. In western Canada, Cyphoderris buckelli and Cyphoderris monstrosa are sympatric but largely allochronically separated, with C. buckelli breeding earlier. However, their breeding seasons can overlap, leading to potential for older C. buckelli females to mate with young C. monstrosa males to obtain resources via sexual cannibalism. We used behavioural assays to test whether female feeding status affects the propensity for interspecific mating between C. buckelli females and C. monstrosa males. We then tested for hybridization and gene exchange in wild populations of both species, using morphology, mitochondrial DNA and genome-wide nuclear markers. We found that interspecific courtship and mating can occur, but the relationship between food restriction and increased propensity for hybridization was not significant. Although we observed intraspecific population genetic structure in both species, we found no signatures of hybridization in the morphological or genetic datasets, which suggests that postmating reproductive barriers might be preventing successful hybridization in the wild.


INTRODUCTION

Allochrony, or divergence in breeding time, can be a potent mechanism for assortative mating, reproductive isolation and, ultimately, speciation in sympathy (Hendry & Day, 2005; Taylor & Friesen, 2017). This temporal isolation can act both as an incipient speciation process (Simon et al., 2000; Santos et al., 2007; Yamamoto & Sota, 2009) and in concert with other traits to facilitate divergence (e.g. host preference; Feder et al., 1994). Although assortative mating via temporal isolation can be an automatic route to divergence (e.g. an ‘automatic’ magic trait sensu Servedio et al., 2011), cases exist where allochrony is not perfect and hybridization occurs between lineages that overlap phenologically (Yamamoto & Sota, 2012; Burban et al., 2016). These cases highlight the complex nature of gradual or plastic temporal isolation, whereby allochrony is often interacting with other selective pressures to instigate or maintain genetic divergence (Pashley et al., 1992; Hendry & Day, 2005; Matsubayashi et al., 2010). Our understanding of the dynamics and evolutionary effects...
of allochrony in these systems is greatly dependent on the identification and quantification of interbreeding between divergent lineages or populations, a process that is greatly expedited using genomic approaches (Dupuis & Sperling, 2016; Taylor & Friesen, 2017).

In this study, we assess the potential for breakdown of reproductive isolation between closely related species of hump-winged grigs (Cyphoderris spp., Orthoptera: Prophalangopsidae) in western Canada (Fig. 1A). From an evolutionary ecology perspective, this group is best known for its unique breeding system, whereby females feed on the fleshy hind wings of the males during copulation and ingest both wing tissue and haemolymph from the resulting wound, causing non-fatal, but permanent, damage (Dodson et al., 1983). Simultaneously, males use two pairs of hooks on the dorsal side of their eighth and tenth abdominal tergites, called the gin trap, to clasp the ventral surface of the female’s abdomen during spermatophore transfer (Dodson et al., 1983). In Cyphoderris strepitans Morris & Gwynne, 1978, experimental removal of male hind wings greatly decreases the probability of successful spermatophore transfer (Eggert & Sakaluk, 1994), but gin trap engagement in males lacking hind wings can facilitate successful spermatophore transfer, suggesting a coercive function of the gin trap (Sakaluk et al., 1995).

Furthermore, in both C. strepitans and Cyphoderris buckelli Hebard, 1933, nutritionally deprived females mount males sooner (Johnson et al., 1999) and are more likely to mate, copulate and feed on male hind wings than non-nutritionally deprived females (Judge et al., 2011). Although male C. strepitans can mate successfully with either their fleshy hind wings or gin traps disabled, if both are disabled they are unable to mate (Sakaluk et al., 1995). Virgin males (those with no hind wing damage) are also generally more successful at mating (Morris et al., 1989). Taken together, these observations can be explained if females receive a nutritional benefit from feeding on the hind wings, and males exploit this by using their fleshy hind wings and gin traps, respectively, to entice and coerce females to mate (Sakaluk et al., 1995).

Studies of sexual selection in Cyphoderris have, to date, focused on intraspecific mating dynamics. However, in coniferous forests of southern British Columbia and Alberta, the distributions and habitats of C. buckelli and Cyphoderris mostrosa Uhler, 1864 overlap (Buckell, 1924). Cyphoderris mostrosa has a more widespread geographical range in mountainous regions in the Rocky Mountains from central British Columbia and south-west Alberta into Idaho and in the Cascade Mountains from British Columbia into Oregon; Cyphoderris buckelli has a more restricted distribution and is limited to southern British Columbia and Northern Washington and Idaho (Morris & Gwynne, 1978). Morphologically, these species can be distinguished by the subgenital plate on the ventral tip of the abdomen, where C. mostrosa has a characteristic pair of spines shaped like the nail-pulling claw of a hammer that are lacking in C. buckelli (Fig. 1B, C). The mating periods of these species are predominately allochronic, with C. buckelli mating in late spring/early summer and C. mostrosa mating later in the summer; however, there can be overlap in their breeding periods (Supporting Information, Fig. S1). Interestingly, recent surveys have identified some intermediate individuals (Fig. 1D), albeit at a low frequency (~0.5% based on ~1000 scored individuals), from a sympatric location where the species ranges overlap (population NPL; K.A.J., unpublished observation). Given the presence of morphologically intermediate, putative hybrid individuals and the sexually cannibalistic mating dynamics of these species, we postulate the following hypothesis of interspecific interaction: if breeding periods of these species overlap during times when food resources are scarce (e.g. during summer droughts), then older, nutritionally deprived C. buckelli females might choose preferentially to mate with young, virgin C. mostrosa (i.e. with no hind wing damage) in comparison to older C. buckelli with damaged hind wings. Thus, plasticity in allochronic isolation might allow these species to coexist in sympatry.

Figure 1. Male grig morphology. A, habitus of male Cyphoderris mostrosa. B–D, lateral view of distinctive sternal process on the subgenital plate (arrow) in male Cyphoderris buckelli (B, no hook), C. mostrosa (C, prominent hook) and putative hybrid (D, reduced, blunt hook), used for species identification.
of sexual cannibalism to a previous study investigating the dynamics of sexual cannibalism in *C. buckelli* (Judge et al., 2011), we conduct a behavioural assay to evaluate the possibility of such interspecific mating. We then survey populations from across the western Canadian range of these species for morphological, mitochondrial DNA (mtDNA) and genome-wide single nucleotide polymorphism (SNP) variation to identify putative hybrid individuals or signatures of introgression. This is the first investigation of interspecific interactions in *Cyphoderris* and adds to the growing literature of this evolutionarily charismatic group of insects.

**MATERIAL AND METHODS**

**Behavioural assay**

**Study animals and husbandry**

To test the behavioural potential for hybridization between species, we collected 18 female *C. buckelli* from several populations in British Columbia, Canada (Supporting Information, Table S1) in 2011. Male *C. buckelli* and *C. monstrosa* (*N* = 42 and 22, respectively) were also collected in 2011, but from single, separate populations: *C. buckelli* from Dick Hart Recreation Area, British Columbia and *C. monstrosa* near Paul Lake, British Columbia. Males used in the behavioural assays were random subsets (*N* = 18) of non-virgin *C. buckelli* and virgin *C. monstrosa*, as evidenced by their damaged and undamaged hind wings, respectively. Although this difference in male mating status is confounded with the difference in species, it represents the natural situation faced by female *C. buckelli* in a sympatric location at the end of their breeding season and the beginning of the *C. monstrosa* breeding season, when most, if not all, male *C. buckelli* and few, if any, male *C. monstrosa* have mated (K.A.J., unpublished observation). Species identification was based on morphology, following Morris & Gwynne (1978).

All animals were housed individually in plastic deli containers (9 cm in diameter, 8 cm high) with a water-filled, cotton-stoppered microfuge tube for moisture, a piece of cardboard egg carton for shelter and one piece of cat chow (Iams Original with Chicken) and a minimum of five grains of bee pollen for food. This diet corresponds to the high-quality diet described by Judge et al. (2011). Animals were kept in conditions of ambient temperature, humidity and light during the collecting trip, and upon arrival at the University of Lethbridge campus they were housed in a controlled-environment chamber at 20 °C, 70% relative humidity, with a 12 h–12 h light–dark cycle.

**Experimental design**

Our behavioural assay took place in two phases. Phase 1 was an initial intraspecific pairing, in which all females were predicted to reject male mating attempts because they were well fed (Judge et al., 2011). This was followed by a period when half the female *C. buckelli* were starved to induce mating interest. Then phase 2 occurred, in which all *C. buckelli* females were paired with a virgin male *C. monstrosa*. We predicted that: (1) fed females would continue to reject male mating attempts; and (2) starved females would be more likely to mate with a heterospecific male. This experimental design was chosen because: (1) it made maximal use of females, which are difficult to collect in large numbers and so far impossible to rear in the laboratory; and (2) it provided a limited range of experimental conditions (i.e. starved vs. fed) in which to assay the possibility of interspecific copulation. Both phases of the experiment took place in a dark room at ~20 °C during the dark phase of the 12 h–12 h light–dark cycle in which the animals were housed in the laboratory. Illumination was provided by both a single 40 W-equivalent red LED bulb mounted ~50 cm above the trial arenas and a red LED headlamp worn by the observer.

**Phase 1: intraspecific pairings**

Female *C. buckelli* were each paired with a randomly assigned male *C. buckelli*. Before initiating each pairing, males and females were held under separate overturned plastic cups (4.5 cm in diameter) on a paper towel that was used as substrate during the experiment. After a 2 min acclimation period, the cups were raised gently, and an overturned plastic deli container (9 cm in diameter, 8 cm high), which then served as the arena, was placed over the male–female pair. The 18 pairings were initiated in a randomly determined order, staggered by 5 min intervals and lasted 3 h. During the experiment, the observer watched each active pair in sequence (maximum *N* = 18) for ~1 s. Thus, each trial was observed as often as once every 18 s, although in practice this interval was longer (1–2 min) given that behavioural note-taking took a few seconds. Resulting behavioural observations can be considered accurate to within 1 min, and that is the precision with which we noted the timing of the following events: (1) first contact; (2) initiation of courtship singing by the male; (3) initiation of mounting of males by females;
(4) initiation and termination of hind wing feeding; (5) initiation and termination of gin trap engagement; and (6) initiation and termination of spermatophore transfer. From these observed times, the following variables were calculated: (1) latency to contact (time of first contact minus time of pairing initiation); (2) latency to court (time of first courtship minus time of first contact); (3) total number of mountings; (4) total number and average duration of hind wing feedings; (5) total number and average duration of gin trap engagements; and (6) duration of spermatophore transfer. If the male was completely unresponsive for the first 5 min of the assay, he was replaced with a new male. Finally, if any hind wing feeding was observed, we photographed the hind wings of the male 24 h after the trial to assess the amount of hind wing damage inflicted, using the same methods as in the study by Judge et al. (2011).

Dietary manipulation
Immediately after pairing with a C. buckelli male, each female was assigned randomly to one of two dietary treatments: (1) a high-quality diet, which was a continuation of the diet all females received before phase 1; or (2) a starvation treatment, in which females received no food but ad libitum water as before. Dietary manipulation continued for 8 days. We measured the mass of all females before dietary manipulation and on days 5 and 7 after the dietary manipulation. The change in mass was assessed after each measurement of mass, and the dietary manipulation was validated by body mass loss of ~15%. Phase 2 of the behavioural assay took place 8 days after phase 1.

Phase 2: interspecific pairings
Phase 2 of the mating experiment proceeded exactly as phase 1 except that females were paired with a virgin male C. monstrosa, assigned randomly. The same observations were recorded as in phase 1, with one addition. In phase 1 we noticed that females tended to walk around the margins of the container almost continuously. To test whether the dietary manipulation had an effect on female activity, we recorded the amount of time spent walking during a 2 min time span at ~80 min into each trial. All individuals were housed individually after the experiment until they died and were then preserved in 70% ethanol for subsequent measurement of morphology.

Statistical analysis for behavioural assays
We used permutation tests (Legendre & Legendre, 1998) as described by Judge et al. (2011) to test for differences between dietary treatment and female feeding rate, male hind wing damage, and female mating rate. Unlike parametric statistical tests, permutation tests do not assume underlying distributions and are therefore useful for datasets where parametric assumptions are likely to be violated (e.g. small sample sizes, as in our data). Briefly, we randomly shuffled (without replacement) the treatment values (starved or fed) among the observed values for each response variable and then calculated a difference between the permuted treatment means. We replicated this process 10 000 times and calculated the proportion of times the permuted difference exceeded the observed difference, which is equivalent to a P-value in a two-tailed test. We also calculated 95% confidence intervals from 10 000 bootstrap replicates for starved and fed females for all response variables. These calculations were conducted using the PopTools add-in (Hood, 2009) in Microsoft Excel.

Preparation of morphological and molecular specimens
We collected specimens of Cyphoderris during the summer of 2013 from several populations across British Columbia and western Alberta, Canada (Table 1; Supporting Information, Table S2). Individuals were hand-captured and either euthanized by freezing at ~20 °C and preserved in 100% ethanol or kept alive for behavioural experiments. After these behavioural experiments were finished, individuals either died and were preserved in 70% ethanol or were euthanized by freezing at ~20 °C and preserved in 100% ethanol.

For morphological measurements, we dissected individuals and removed pertinent body parts that were then photographed in standardized orientation submerged in 100% ethanol. For molecular datasets, DNA was extracted from a single leg for live-frozen specimens using DNeasy Blood and Tissue kits (Qiagen), with the optional RNase A (Sigma-Aldrich) treatment, following the manufacturer’s recommendations. We ethanol precipitated the resulting DNA extracts, eluted them in 50 µL Millipore water, and quantified and qualified the DNA using a Qubit 1.0 dsDNA BR assay kit (Invitrogen) and a Nanodrop ND-1000 (Thermo Scientific), respectively.

Morphology
Owing to small sample sizes of females and juveniles and high levels of morphological sexual dimorphism in these species (K.A.J., unpublished observation), we measured morphological characters for adult males only. In preparing specimens for morphological measurement, we removed the head and hind legs and then took photographs of three different body parts/regions from standardized orientations (the posterior
head, the lateral surface of both hind legs, and the posterior dorsum) using a digital camera (Lumenera INFINITY 1-3C) mounted on a stereomicroscope (Wild M5). Images were captured using INFINITY CAPTURE v.5.0.2 (Teledyne Lumenera), saved as jpegs and analysed using software from SB Morphometrics (Rohlf, 2016). Specifically, image files were bundled together using tpsUtil v.1.70, and landmarks were placed in the resulting TPS file with tpsDig2 v.2.26. We measured five linear dimensions from the collected photographs: (1) head width (HW; maximal distance perpendicular to the frontal plane); (2) maxillae span (MS; distance between the cardo-stipes articulations of the left and right maxillae); (3) hind femur length (FL); (4) hind femur depth (FD; maximal depth perpendicular to femur length); and (5) gin trap span (GTS; distance between the left and right hooks on the eighth abdominal tergite). These dimensions are all highly repeatable (data not shown), represent characteristics spread across all three body tagmata, and are all predicted to be related directly to male mating success (e.g. Sakaluk et al., 1995; Judge & Bonanno, 2008).

We used principal components analysis (PCA) to reduce the morphological dataset to a few uncorrelated composite dimensions that could be used to visualize the morphological similarity between populations and species. This analysis was carried out using IBM SPSS Statistics v.25 (IBM Corporation, 2017).

**Genotyping by sequencing**

Genotyping by sequencing (GBS) libraries were prepared (with DNA normalized to 20 ng/µL) following Poland et al. (2012), using the restriction enzymes PstI and MspI. Duplex-specific nuclease (Zhulidov et al., 2004) and complexity reduction following Sonah et al. (2013) (a C added to the reverse primer) were implemented in the library preparation, and 96 individual libraries were pooled into a single lane of 100 bp single-end sequencing on a HiSeq 2000 (Illumina). Some individuals yielded poor-quality DNA in the first round of extractions, and for these we generated and sequenced GBS libraries from DNA extracted from a second leg.

We used Stacks v.2.1 (Catchen et al., 2011, 2013) to process the raw GBS data and call SNPs using default parameters and settings unless otherwise noted. First, process_radtags was used to demultiplex and filter raw data files (removing reads with low quality scores/uncalled bases and rescuing reads with barcode/cutsite errors). We then used denovo_map.pl to execute the Stacks pipeline, allowing three mismatches between stacks within and between individuals (-M and -n, respectively). We called the final SNP datasets using...
populations with a population map assigning all individuals to one population and used alternative filtering strategies to create two datasets, one tailored to population genetic analyses and one to phylogenetic analyses. For the population genetic dataset, we required loci to be present in one population and 1% of individuals to be processed in populations and generated a vcf output with a single SNP per catalogue locus. We then identified poor-quality individuals from this vcf file using the individual missingness output in VCFtools v.0.1.15 (Danecek et al., 2011) (those with > 70% missing data after removal of loci with > 50% missing genotypes). We used VCFtools to remove poor-quality individuals and duplicate individuals (resulting from multiple DNA extractions; see above) and removed loci with > 30% missing data and a minor allele frequency < 5% to generate the final population genetic dataset. For the phylogenetic dataset, we removed the same individuals as for the population genetic dataset and used populations to remove loci with > 50% missing data and a minor allele frequency <5% and generate a phylip-formatted output with a single SNP per catalogue locus.

We used individual-based Bayesian clustering in STRUCTURE v2.3.4 (Pritchard et al., 2000) to assess genetic structure (i.e. the number of unique genetic clusters, K) in the population genetic dataset. We assessed K = 1–20, each with 20 replicates of 250 000 sampled generations (after 50 000 burn-in generations), correlated allele frequencies (Falush et al., 2003) and the admixture model. To determine the optimal value of K, we calculated LnPr(X|K) natural logarithm of the probability of the data (Pritchard et al., 2000) and ΔK (Evanno et al., 2005) with CLUMP v1.1 (Kopelman et al., 2015), and MedMed/MaxMed K and MaxMed/MaxMed K (median of medians, median of means, maximum of medians, maximum of means of K, respectively) (Puechmaille, 2016) with STRUCTURESELECTOR (Li & Liu, 2018) (using a threshold of 0.5, we refer to these as the Puechmaille statistics). To assess fine-scale substructure, we conducted hierarchical analysis, following Pritchard & Wen (2004) and Vähä et al. (2007). For the phylogenetic dataset, we conducted maximum likelihood (ML)-based tree searching in IQ-TREE v.1.6.7 (Nguyen et al., 2015) using the best-fitting model of evolution predicted by MODELSELECTOR (Kalyaanamoorthy et al., 2017), including an ascertainment bias appropriate for SNP-based datasets with no invariable sites (Lewis, 2001). We assessed node support using 10 000 replicates of both ultra-fast bootstrap (ufBS; Hoang et al., 2018) and the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon et al., 2010).

MITOCHONDRIAL DNA

We amplified ~680 bp of cytochrome c oxidase subunit I (COI) using the primers LepF1 (5′-ATTCAACCAA TCATAAGATATTGG-3′) and LepR1 (5′-TAAACTT C7GGATGTCAAAAATA-3′); polymerase chain reaction conditions, clean-up and sequencing followed Dupuis & Sperling (2015). We combined forward and reverse reads with CHROMASEQ v.1.31 (Maddison & Maddison, 2018a) in MESQUITE v.3.51 (Maddison & Maddison, 2018b), checked for erroneous base calls by eye, aligned sequences using MAFFT v.7.305b (Katoh & Standley, 2013), and trimmed 5′ and 3′ ends of the alignment to remove variably missing data between individuals. We conducted ML tree searches on the trimmed mtDNA alignment using IQ-TREE as above, except that we did not use a model incorporating an ascertainment bias as we did for the SNP-based analysis. All trees were visualized with FigTREE v.1.4.4 (Rambaut & Drummond, 2010) and compared with the ape v.5.3 (Paradis et al., 2004) and phytools v.0.6.99 (Revell, 2012) packages in R v.3.4.4 (R Core Team, 2018).

RESULTS

BEHAVIOURAL ASSAYS

In phase 1 of the behavioural assay (intraspecific pairings), all female C. buckelli experienced vigorous courtship singing by conspecific males, but no mating (hind wing feeding or spermatophore transfer) resulted from these interactions. However, at least two males were observed using their gin traps successfully to clasp their paired female as she circled the experimental arena (Sakaluk et al., 1995) (Supporting Information, Video S1). Both females pulled away from the male after being gin trapped (Supporting Information, Video S1), and one of these females died a few days after phase 1 ended (Supporting Information, Table S1).

In phase 2 (interspecific pairings), all male C. monstrosa courted female C. buckelli vigorously (Supporting Information, Video S2), and either gin trap contact, hind wing feeding or spermatophore transfer was observed in 12 of 17 pairings (Supporting Information, Table S1). In all of the instances where gin trap contact was observed, it seemed to be initiated by the male as the female walked past or over his abdomen (Sakaluk et al., 1995). When this occurred, females usually spent some time feeding on the hind wings of the male, but also appeared to struggle to free themselves, sometimes by biting the hind legs and dorsal abdomen of the male (Supporting Information, Video S3). In two cases, females did not successfully extricate themselves, and a spermatophore was transferred (Supporting Information, Video S4). Two pairings were stopped early: one because the female pulled away part of her abdominal cuticle during a struggle to free herself from the male’s gin trap, and
the second because the male attacked the female. As predicted by our hypothesis, starved females were more likely to feed on male hind wings, did more damage to male hind wings when feeding and were more likely to mate than fed females. However, none of these relationships was statistically significant (all \( P > 0.191 \); Fig. 2).

**MORPHOLOGY**

Eighty-three individuals (including juveniles and adult females used for GBS but not morphology) were collected for morphological and genetic analysis, with the collection localities for these populations centred around an area where both species are present in close proximity, including the locality where intermediate individuals have previously been collected (Fig. 3 inset, population NPL). Sixty-four adult males were included in the final morphological dataset (Table 2; Supporting Information, Table S3).

Principal components analysis of this adult male dataset using five morphological traits (FD, FL, HW, MS and GTS) resulted in two principal components (PCs) that explained 92.82% of the variation in the original traits (Supporting Information, Table S4). GTS loaded negatively on PC1 and in strongly positive manner on PC2, whereas all four other traits loaded in a strongly positive manner on PC1 and a weakly positive manner on PC2 (Supporting Information, Table S4). Principal component 1 separated the two species, with only two individuals having relatively intermediate placement (Fig. 4), neither of which came from populations at or near the location where individuals with intermediate genitalia have been found (NPL), nor did they show genetic intermediacy (see below, genotyping by sequencing). The second principal component showed some spread of individuals, but finer-scale groupings did not correspond to any geographical populations (Fig. 4).

**GENOTYPING BY SEQUENCING**

The GBS sequencing resulted in 145.2 million reads, of which 110.4 million reads (per individual maximum, minimum and average: 3.2 million, 48.5 thousand and 1.2 million, respectively) were retained after initial data filtering. The SNP calling resulted in 244 229 SNPs, and after we removed low-quality and duplicated individuals (resulting from multiple DNA extracts; see Material and Methods) and conducted final filtering, our population genetic and phylogenetic datasets consisted of 78 individuals and 3042 and 16 794 SNPs, respectively.

In STRUCTURE analysis of the population genetic dataset, we found mostly consistent support for the best value of \( K \) (for all STRUCTURE results and best \( K \) support, see Supporting Information, Fig. S2): \( \text{LnPr}(X|K) \) plateaued at \( K = 3 \), \( \Delta K \) supported \( K = 2 \) and \( K = 3 \), and the Puechmaille statistics generally supported \( K = 4 \). Clustering at \( K = 2 \) separated \( C. \) buckelli from \( C. \) monstrosa (Fig. 3A), and at \( K = 3 \) a unique cluster of \( C. \) monstrosa from populations FT and GDUR was observed. Owing to sample size constraints, we analysed the two clusters from \( K = 2 \) hierarchically, and both had support for \( K = 2 \) and \( K = 3 \) as the best value of \( K \). For the \( C. \) buckelli cluster, \( \text{LnPr}(X|K) \) supported \( K = 3 \), \( \Delta K \) supported \( K = 2 \) and \( K = 3 \), and the Puechmaille statistics supported \( K = 4 \) and \( K = 5 \). For the \( C. \) monstrosa cluster, \( \text{LnPr}(X|K) \) supported \( K = 3 \), \( \Delta K \) supported \( K = 4 \), and the Puechmaille statistics supported \( K = 3 \). Given the sample sizes of these clusters and their biological

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Figure 2. The effect of dietary treatment on interactions between starved or high-food female *Cyphoderris buckelli* and male *Cyphoderris monstrosa*. Error bars represent 95% confidence intervals generated by bootstrapping; \( P \)-values are the result of permutation tests.

plausibility, we considered $K = 3$ as the best value of $K$ for each species, which resulted in geographically associated clustering within each species (Fig. 5). Most of the population samples showed very little admixture between each of the $K = 3$ intraspecific genetic clusters, except for populations in the south-west portion of our sampling range.

For direct comparison between the GBS and mtDNA datasets, mid-rooted ML consensus trees in Figure 5 have had individuals removed for which we failed to obtain either GBS or mtDNA data (four individuals not shown for GBS and five for mtDNA; full trees are provided in Supporting Information, Figs S2, S3). The GBS ML consensus resulted in well-supported reciprocal monophyly of *C. buckelli* and *C. monstrosa* (Fig. 5; Supporting Information, Fig. S3). Many of the same intraspecific clusters from STRUCTURE analyses were also recovered, although if admixed individuals were observed in STRUCTURE, those clades were generally not well supported in the ML analysis.

**Mitochondrial DNA**

The final mtDNA dataset consisted of 641 bp (77 parsimony informative sites) for 79 individuals (34 *C. buckelli* and 45 *C. monstrosa*). As with the GBS data, we observed reciprocal monophyly between *C. buckelli* and *C. monstrosa* (Fig. 5; Supporting Information, Fig. S4), and some of the most divergent of the
Table 2. Mean adult male trait values for morphological traits measured in populations of *Cyphoderris*

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>N</th>
<th>FD (mm)</th>
<th>FL (mm)</th>
<th>HW (mm)</th>
<th>MS (mm)</th>
<th>GTS (mm)</th>
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Numbers in parentheses are standard deviations.
Abbreviations: FD, femur depth; FL, femur length; GTS, gin trap span; HW, head width; MS, maxillae span; N, sample size.

*Population where both species are found and where intermediate, putative hybrid individuals have been observed.*
intraspecific clusters/clades identified with the GBS data were recovered as well-supported clades with the mtDNA data (e.g. the *C. monstrosa* cluster of GDUR and FT populations). However, in general there was much less intraspecific resolution with this dataset and short intraspecific branch lengths (see scales in Fig. 5); divergence between most of the closely related haplotype pairs/groups were the result of one or two base differences. This lack of resolution explains most of the apparent fine-scale conflict when compared with the GBS-based phylogeny (Fig. 5).

**DISCUSSION**

Our study used behavioural assays to investigate the potential for interspecific mating between older, food-starved, female *C. buckelli* and young, male *C. monstrosa*, and surveyed populations for hybrid individuals using both morphological and genetic analyses. We found that courtship and mating can occur between these species in the aforementioned conditions (older, food-starved, female *C. buckelli* and young, male *C. monstrosa*). Although it appears that food restriction might increase the propensity for this hybridization, none of these trends were statistically significant. Mitochondrial diversity was low in both species, but intraspecific population structure, particularly in the genome-wide SNP dataset, was apparent in both *C. buckelli* and *C. monstrosa*. Despite this, we found no morphological or genetic signatures of hybridization with any of the population-level datasets. Taken together, we were able to find support for only part of the prediction laid out in our original hypothesis. Our results suggest that hybrid matings between *C. buckelli* and *C. monstrosa* can occur in the laboratory, but that if they do in the wild, some factor might be preventing the persistence of hybrids. It is unclear whether this is the result of pre- (e.g. unsuccessful fertilization after hybrid mating) or postzygotic (e.g. hybridization producing infertile offspring) barriers, or whether the intermediate, putative hybrid individuals are simply cases of anomalous morphology.

Both *C. buckelli* and *C. monstrosa* tend to mate on vegetation, but during dusk and the first few hours after dark during the breeding season, males can both be found singing on the ground and low on shrubs and trees. As the night progresses, male *C. monstrosa* move higher up trees, predominantly lodgepole pine, and aggressively defend territories (Mason, 1996), whereas male *C. buckelli* stay in the understory and undergrowth, moving often and without the same degree of territoriality (Morris et al., 2002). Thus, in locations where breeding of both species overlaps (e.g. locality NPL, Fig. 3) there is opportunity for young male *C. monstrosa* and older *C. buckelli* females to encounter each other when both are actively seeking a mate. Using behavioural assays, we were able to demonstrate that interspecific courtship and mating between female *C. buckelli* and male *C. monstrosa* is possible, and that, with respect to male *C. monstrosa*, there is no behavioural isolation between these species. Although our data trended toward a relationship between food deprivation and increased interspecific mating, this relationship was not statistically significant, unlike previous studies examining the effects of diet on interspecific mating in *C. strepitans* (Johnson et al., 1999) and *C. buckelli* (Judge et al., 2011). If there is some biological basis for the trend, the lack of significance in our present study could be the effect of: (1) the small sample size constrained by the difficulty of collecting adult females; (2) insufficiently severe manipulation of nutritional status; or (3) other factors besides female nutritional status driving interspecific pairings. Density, which is predicted to affect encounter rates between the species, is one such factor that has received relatively little attention in studies of mating dynamics, despite its theoretical importance (Kokko & Rankin, 2006). More comprehensive evaluation of the activity patterns, local densities and proportions of mated and virgin individuals of both species would be invaluable for understanding the potential for interspecific mating interactions in *Cyphoderris*.

The results of our behavioural experiment both highlight the extent of conflict between the sexes...
in the genus *Cyphoderris* and shed new light on a previous experiment that supported a material benefits explanation for hind wing feeding in *C. buckelli* (*Judge et al., 2011*). Female *C. buckelli*, all well fed, never mounted a conspecific male in phase 1 of our behavioural experiment. However, two of these
18 females were gin trapped by conspecific males and escaped (e.g. Supporting information, Video S1). Likewise, in phase 2, nine females were gin trapped by *C. monstrosa* males (four of nine well fed; five of eight starved), some up to three times. All but two of the gin-trapped females freed themselves without mating, both of which were in the starved treatment. Although noted for *C. strepitans* (Sakaluk et al., 1995), previous work on *C. buckelli* failed to observe gin trapping as a coercive male strategy (Judge et al., 2011). This is likely to be attributable to the fact that Judge et al. (2011) made focal observations of their mating pairs of *C. buckelli* only once every 20 min, whereas we observed mating pairs continuously. If male *C. buckelli* use gin trapping regularly as a coercive mating strategy then females in poor nutritional condition in their experiment might simply have been unable to extricate themselves from a male’s gin trap compared with females in good condition. Furthermore, if, as our data suggest, *C. monstrosa* males use gin trapping regularly, then their overall larger size (Fig. 4), and therefore strength, might greatly increase the likelihood of: (1) gin trapping any encountered female *C. buckelli*; and (2) clasping that female until after successful spermatophore transfer, thus facilitating hybrid mating. Several females in our experiment sustained damage to their abdominal cuticles as a result of gin trapping (see Supporting Information, Fig. S5), suggesting both high costs to interspecific pairings and that it might be possible to identify female *C. buckelli* that experience attempted interspecific mating in the field. In the presence of high costs to females of resisting male mating attempts, females are predicted to increase their mating rate beyond their preferred optimum. Such mating systems (e.g. water striders, family Gerridae) are characterized by convenience polyandry (Thornhill & Alcock, 1983; Rowe, 1992; Arnqvist & Rowe, 2005). We speculate that in conditions of nutritional stress, *C. buckelli* females might engage in convenience interspecific mating.

Although we found no signatures of hybrid individuals with either the mtDNA or GBS datasets, we did observe genetic population structure in both species. This structure was most pronounced in the GBS dataset, where we found *K* = 3 to be well supported in both species (Fig. 3B). Populations in the eastern part of the sampled distribution were more highly differentiated in STRUCTURE analysis (Supporting Information, Fig. S2) and sister to the other clusters in phylogenetic analyses (Fig. 5), suggesting that the Rocky Mountains might be a stronger isolating factor than geographical (Euclidean) distance (Fig. 3C). The distribution of admixture between the other two clusters in each species supports this inference, although quantitative evaluation of the effect of landscape features would be required to substantiate this hypothesis (Bull et al., 2011). In contrast to the GBS dataset, few populations formed clusters or monophyletic groups with the *COI* dataset (Fig. 5). This seemed mostly attributable to a lack of information in this dataset, which is apparent from the short intraspecific branch lengths in the main clades of both species. Again, the eastern populations showed the most phylogenetic distinction in the entire dataset, supporting a hypothesis of strong isolating pressures of the Rocky Mountain range. The higher information content of the GBS dataset is unsurprising given the proven utility of this style of genomic dataset for population genetics and phylogenetics (e.g. Andrews et al., 2016). Our sampling centred around an area where both species are in close proximity and where intermediate individuals have been observed (Fig. 3 inset, population NPL); therefore, more geographically comprehensive sampling across the ranges of these species should provide a finer-scale assessment of population structure in both species.

These results serve as a foundation for future research in this system, with two aspects of the present study being important to address in future efforts. First, although we sampled populations where intermediate individuals have been observed, we were unable to generate genetic data from any intermediate individuals. Increased sampling of these populations or adoption of genomic techniques more amenable to museum specimens (e.g. whole-genome shotgun sequencing; Sproul & Maddison, 2017) will be needed to characterize the genetics of these putative hybrids. Second, although we have demonstrated interspecific copulation between *C. buckelli* and *C. monstrosa*, this does not translate directly to the viability and fecundity of hybrid individuals. Rearing hybrid broods to both evaluate hybrid viability and confirm that intermediate morphology results from hybrid mating would provide strong evidence to substantiate these hypotheses. Given that *Cyphoderris* has never been cultured successfully in the laboratory, development of rearing methods would greatly increase our ability to investigate the viability of hybridization in this system and address many potentially confounding variables in the study of sexual conflict between these species (e.g. individual age, body condition, mating status).

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for assistance in specimen collection, Taylor Becker for assistance with wet laboratory procedures, and Bill Cade, Paul De Luca, Andrew Mason and Glenn Morris for helpful discussions. Thank you to Thor Veen and two anonymous reviewers for helpful comments on the manuscript. This research was supported by Natural Sciences and Engineering Research Council of Canada Discovery Grants to F.A.H.S. (RGPIN-217174) and K.A.J. (RGPIN-2017-04674), a University of Lethbridge Postdoctoral Fellowship to K.A.J., and a MacEwan University Faculty of Arts and Science Research Fund to K.A.J. Figures were created using R v.3.1.1 (R Core Team, 2018) and INKSCAPE v.0.91 (The Inkscape Team, 2017). Raw GBS sequencing reads are deposited in NCBI, BioProject PRJNA601897, SRA accessions SRR10907472–SRR10907567 and mtDNA GenBank accessions MT196142–MT1966220. Input files for genetic datasets and raw morphological data are provided in the Supporting Information (Files S1–S3 and Table S3, respectively).

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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. Results of behavioural assays.
Table S2. Detailed specimen information.
Table S3. Raw morphological data.
Table S4. Eigenvalues and variance of principal components analysis.

Figure S1. Phenogram of historical field collections (K.A.J., unpublished information) of *Cyphoderris buckelli* (blue) and *Cyphoderris monstrosa* (red) from the populations assessed in the present study. The number of males captured is displayed on a logarithmic scale owing to disproportionately large sample sizes in 2009.

Figure S2. All STRUCTURE results, including LnPr(X | K), ΔK, and Puechmaille statistics and barplots for all values of K for analyses of all individuals and the hierarchical analysis of the *Cyphoderris buckelli* and *Cyphoderris monstrosa* clusters.

Figure S3. Maximum likelihood (ML) consensus tree for single nucleotide polymorphism (SNP) dataset for all individuals. Branch support is Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT)/ultra-fast bootstrap (ufBS), with good support indicated by > 0.8/0.95.

Figure S4. Maximum likelihood (ML) consensus tree for mitochondrial DNA dataset for all individuals. Branch support is Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT)/ultra-fast bootstrap (ufBS), with good support indicated by > 0.8/0.95. This tree is rooted with a sequence from *Pseudorhynchus acuminatus* (Orthoptera: Tettigonidae) retrieved from GenBank (accession NC_033992.1).

Figure S5. Photograph of the ventral abdomen of one of two female *Cyphoderris buckelli* that received a spermatophore from a male *Cyphoderris monstrosa* during phase 2 of the behavioural experiment. Arrows indicate melanization, an insect immune response, presumably as a result of puncture wounds caused by the male’s gin trap.

Video S1. An intraspecific interaction from phase 1 of the behavioural experiment, in which a male *Cyphoderris buckelli* uses his gin trap to clasp a female *C. buckelli* by her ventral abdomen as she walks over his dorsum. Note that before the female is gin trapped, the male appears at several points to be attempting to use his gin trap to clasp the edge of the plastic arena. The damaged hind wings of the male can be seen whenever he faces away from the camera. Video was recorded using a hand-held Canon PowerShot SD790 IS with a red LED headlamp for illumination.

Video S2. An interspecific interaction from phase 2 of the behavioural experiment, in which a male *Cyphoderris monstrosa* vigorously courts a female *Cyphoderris buckelli*. Courtship consists of stridulation (rubbing together of his fore wings) and periodically turning to present his posterior abdomen to the female. Note that whenever the female passes his posterior, there is a short motion of the distal abdomen of the male, where his gin trap is located. The undamaged hind wings of the male can be seen whenever he faces away from the camera. Video was recorded using a hand-held Canon PowerShot SD790 IS with a red LED headlamp for illumination.

Video S3. An interspecific interaction from phase 2 of the behavioural experiment, in which a female *Cyphoderris buckelli* is mounted on the dorsum of a male *Cyphoderris monstrosa*, apparently clasped by his gin trap. The female periodically feeds on the hind wings of the male and also appears to struggle to free herself by occasionally biting his dorsal abdomen and hind legs. The male appears to be trying actively to engage his genitalia. Video was recorded using a hand-held Canon PowerShot SD790 IS with a red LED headlamp for illumination.

Video S4. An interspecific interaction from phase 2 of the behavioural experiment, in which a female *Cyphoderris buckelli* receives a spermatophore from a male *Cyphoderris monstrosa* and is then released by his gin trap. Video was recorded using a hand-held Canon PowerShot SD790 IS with a red LED headlamp for illumination.

File S1. Population genetic dataset [3042 single nucleotide polymorphisms (SNPs)]. Genotypes are in STRUCTURE format.
File S2. Phylogenetic dataset [16 794 single nucleotide polymorphisms (SNPs)]. DNA alignment is in fasta format.
File S3. Mitochondrial DNA dataset (641 bp). DNA alignment is in fasta format.