

**Behavioral mechanisms of reproductive isolation in avian hybrid
zones**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
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DEDICATION

A dissertation is both a generous and selfish endeavor – an indulgent luxury, a celebration of life and learning, and sometimes an oppressive, self-inflicted punishment. I feel eternal gratitude to the loving people who helped me take this whole business less seriously. Thanks to you, it's been an adventure in curiosity, persistence, and discovery. I am especially appreciative of my mother, Deborah Sussman, sister, Sondra Lipshutz, grandparents Sid and Louise Sussman, and uncle cousin Bobby for helping me find the humor.

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ABSTRACT

Sexual signals and mating behaviors influence whether sympatric species interbreed, and can therefore promote or impede behavioral reproductive isolation between species in secondary contact. Traditionally, research on sexual selection and hybridization has focused on the importance of interspecific mate choice and species discrimination from the perspective of choosy females, and competition from the lens of aggressive and indiscriminate males. I examined two different avian systems to compare the role of male and female competition on hybridization: white-crowned sparrows on the west coast of the US, and sex-role reversed jacanas in Panama. Using genomics and experimental field techniques, I tested morphological, behavioral, and historical factors that influence patterns of gene flow between lineages. I found that contrary to traditional expectations, divergence in male competitive signals can promote reproductive isolation, and female competition can facilitate hybridization.

TABLE OF CONTENTS

Introduction: Interspecific competition, hybridization, and reproductive isolation in secondary contact: Missing perspectives on male and females	1
Chapter I: Behavioral response to vocal and genetic divergence in a contact zone between white-crowned sparrow subspecies (<i>Zonotrichia leucophrys</i>)	24
Chapter II: Divergent competitive phenotypes between females of two sex-role reversed species	52
Chapter III: Differential phenotypic and genetic introgression in a hybrid zone between sex-role reversed species	70
Conclusion	89
Vita	90

LIST OF TABLES

Table 1. Sampling information for each locality	29
Table 2. Factor loadings for the first five principal components (eigenvalue > 1) derived from song variables	32
Table 3. Factor loadings for the principal components (eigenvalue > 1) derived from behavioral responses to individual playback experiments and all experiments standardized and combined	34
Table 4. Pairwise Fst matrix based on the 6419 SNP dataset, not including locality 8 .	35
Table 5. AIC values for historical demography models.....	38
Table 6. Maximum likelihood parameter point estimates and 95% bootstrap confidence intervals for the secondary contact model	38
Table 7. Mean $\pm$ standard deviation for behavioral responses to individual playback experiments and all experiments standardized and combined	40
Table 8. Results of statistical comparisons of response to 'own' vs. 'other' for each pairwise comparison using Wilcoxon sign-ranked tests.....	40
Table 9. Sampling information for morphological and behavioral data.....	57
Table 10. Mean and standard error for morphological traits related to aggression of adult male and female <i>Jacana spinosa</i> and <i>J. jacana</i>	57
Table 11. Mean $\pm$ SE behavioral responses to a conspecific, simulated territorial intruder and their principal component score loadings.....	60
Table 12. Rank of models that describe morphological PC1	61
Table 13. Rank of models that describe aggression PC1, PC2, and PC3	62
Table 14. Genetic and morphological sampling scheme and sizes for each locality	76
Table 15. Best supported model and parameters for cline center, width, and shape of genotypic and phenotypic clinal transitions.	84

LIST OF FIGURES

Figure 1. Conceptual framework.....	4
Figure 2. Interpretation of playback experiments	7
Figure 3. Female-female competition in jacanas	12
Figure 4. Sampling map of numbered localities along a transect from the southern subspecies <i>Z. l. nuttalli</i> to the northern subspecies <i>Z. l. pugetensis</i>	28
Figure 5. Probability of assignment to <i>Z. l. nuttalli</i> (black) and <i>Z. l. pugetensis</i> (white) as determined from a STRUCTURE analysis using 6419 SNPs for K = 2 across 17 localities.	36
Figure 6. Interclass principal component analysis of 6419 loci dataset for sampling localities	36
Figure 7. Best supported demographic model of secondary contact	37
Figure 8. Plot of song PC1 for <i>Z.l. nuttalli</i> (black), admixed individuals (grey), and <i>Z.l. pugetensis</i> (white) across hybrid zone transect.	39
Figure 9. Results of territorial playback experiments comparing male responses to their 'own' songs and the songs of the 'other' subspecies in four localities along a hybrid zone transect	41
Figure 10. Individuals discriminated more strongly between songs when songs were more dissimilar.....	42
Figure 11. Individuals discriminated more strongly between their local song and the song of the other subspecies when the pairwise genetic distance was greater.....	42
Figure 12. Multiple matrix regression with randomization (MMRR) analysis performed on genetic and song distance.	43
Figure 13. Sampling map of Costa Rica and Panama.	56
Figure 14. Taxidermic mount used in simulated territorial intrusion.	59
Figure 15. Morphological comparison of competitive phenotype between jacana species and sexes.	63
Figure 16. Response by territory holders to simulated territorial intrusion of a conspecific	64
Figure 17. Genetic sampling map	74
Figure 18. Genetic PC1 and PC2 of 13,339 genome-wide SNPs.	77
Figure 19. A pattern of isolation by distance was found in <i>J. jacana</i> (red) but not <i>J. spinosa</i> (yellow).	78
Figure 20. Classification of hybrid and parental genotypes indicated the presence of hybrid F1s (orange) as well as <i>J. spinosa</i> backcrosses (left center) and <i>J. jacana</i> backcrosses (right center).	78
Figure 21. Best supported cline models for Q (based on STRUCTURE admixture proportions for K=2 from 13,339 genome-wide SNPs) and female body mass	79
Figure 22. Individual variation in female body mass across the hybrid zone	80
Figure 23. Best supported cline models for diagnostic genomic loci.....	80
Figure 24. Best supported cline models for Q (black, genome-wide loci from STRUCTURE for K = 2), competitive traits (purple) and putative species recognition traits (green).	81

INTRODUCTION
INTERSPECIFIC COMPETITION, HYBRIDIZATION, AND
REPRODUCTIVE ISOLATION IN SECONDARY CONTACT: MISSING
PERSPECTIVES ON MALES AND FEMALES

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Abstract

Research on sexual selection and hybridization has focused on female mate choice and male-male competition. While the evolutionary outcomes of interspecific female preference have been well explored, we are now gaining a better understanding of the processes by which male-male competition between species in secondary contact promotes reproductive isolation versus hybridization. What is relatively unexplored is the interaction between female choice and male competition, as they can oppose one another or align with similar outcomes for reproductive isolation. The role of female-female competition in hybridization is also not well understood, but could operate similarly to male-male competition in polyandrous and other systems where costs to heterospecific mating are low for females. Reproductive competition between either sex of sympatric species can cause the divergence and/or convergence of sexual signals and recognition, which in turn influences the likelihood for interspecific mating. Future work on species interactions in secondary contact should test the relative influences of both mate choice and competition for mates on hybridization outcomes, and should not ignore the possibilities that females can compete over mating resources, and males can exercise mate choice.

Introduction

Traditional perspectives on sexual selection and hybridization

Sexual signals and mating behaviors influence whether sympatric species interbreed, and can therefore promote or impede behavioral reproductive isolation (Irwin & Price 1999; see Definitions). Interspecific hybridization is common and is estimated to occur in 10% of animals (Mallet 2005). Traditionally, research on the role of sexual selection in hybridization has focused on the importance of mate choice and species discrimination from the perspective of choosy females, and competition from the lens of aggressive and indiscriminate males (Moore 1987; Grant & Grant 1997; Sætre *et al.* 1997; Parker & Partridge 1998; Wirtz 1999; Randler 2002). This conventional view considers females the gatekeepers of species because of their greater investment in gametes and fewer opportunities for multiple mating relative to males (Bateman 1948; Andersson 1994). In contrast, males are expected to maximize fitness by mating as frequently as possible (Darwin 1871; Bateman 1948; Andersson 1994). The traditional perspective of sexual selection underlays the predictions for evolutionary outcomes in different scenarios of **secondary contact**. For instance when hybridization is maladaptive, lineages in secondary contact are expected to evolve divergence in sexually selected traits and in species recognition of mates to avoid heterospecific mating, a process known as reinforcement (Coyne & Orr 1989; Servedio & Noor 2003). The predictions for reinforcement have been developed from the perspective of females, who face higher fitness costs of heterospecific mating mistakes and are therefore predicted to discriminate more strongly against heterospecifics than males (Sætre *et al.* 1997; Parker & Partridge 1998; Wirtz 1999; Servedio *et al.* 2009; Hudson & Price 2014). An open question is to what extent does male-male competition between lineages influence hybridization outcomes in secondary contact, and when is female mate choice predicted to support or oppose these outcomes?

Definitions

Agonistic character displacement: divergence in competitive signals or traits in sympatry to reduce costly interspecific interactions

Asymmetric introgression: the unidirectional exchange of alleles from one species another

Behavioral reproductive isolation: reduced gene flow due to divergent mating signals and preferences

Competitive asymmetry: the superior competitive ability and/or dominance of one species over another

Heterosis: hybrid vigour, when hybrids are competitively superior to their parental species

Introgressive hybridization: interbreeding between two distinct lineages that results in gene flow

Hybrid swarm: hybridization that erodes parental species boundaries

Interspecific intrasexual conflict: antagonistic coevolution between males and females of interacting species

Reproductive character displacement: divergence in mating signals or traits in sympatry to reduce costly interspecific mating

Interspecific reproductive competition: competition for mates and/or mating resources between species

Reproductive exclusion: sexual interactions between species that cause one to become locally extinct

Secondary contact: Geographic overlap between two genetically distinct lineages that derived from a common ancestor and underwent a phase of allopatric isolation

Social selection: A form of selection resulting from all social interactions in order to gain access to resources, including but not limited to mates

Recent empirical and theoretical work has brought increasing attention to the function of male-male competition in speciation (Doorn *et al.* 2004; Seehausen & Schluter 2004; Dijkstra *et al.* 2007; Qvarnström *et al.* 2012; Drury *et al.* 2016). Reproductive competition, also known as intrasexual selection, is a component of sexual selection that involves fighting over mating resources such as territories and mates. Competition is an important determinant of mating success for many taxa, especially those with polygynous or polyandrous mating systems where reproductive success is highly skewed toward dominant individuals (Emlen & Oring 1977; Clutton-Brock 2007). Interspecific competition is common (Peiman & Robinson 2010), and **interspecific reproductive competition** can occur when species compete for shared territorial and/or signalling space involved in mate attraction and reproduction (Grether *et al.* 2009; Burdfield-Steel & Shuker 2011; Pfennig & Pfennig 2012). Low fitness costs to heterospecific mating for males can facilitate **introgressive hybridization** when males compete over heterospecific mates via male-male competition (Arnqvist & Rowe 2005), but this can result in high reproductive costs for females, termed the so-called “satyr effect” (Ribeiro & Spielman 1986). Interspecific male-male competition is not widely considered to promote reproductive isolation except as it relates to female choice (but see 1B, *Competitive asymmetry and reproductive exclusion*).

Rapid divergence in sexually selected traits between closely related lineages in allopatry can promote reproductive isolation through the maintenance of species-specific signals and recognition when these lineages come into secondary contact (Coyne & Orr 2004; Hudson & Price 2014; Weber & Strauss 2016; Cooney *et al.* 2017). Character shifts in sexual traits can also result from species interactions in secondary contact. These processes have been widely explored in terms of interspecific male-female interactions concerning reinforcement of male traits and female recognition of those traits (see 1A, *Character displacement: ecological, reproductive, and agonistic*). However, interspecific male-male interactions can also impact the evolution of sexual traits, which in turn can influence hybridization outcomes. For instance, when lineages that compete over similar ecological and/or mating resources come into contact, their competitive interactions can cause selection on traits that influence fighting ability and competitor recognition,

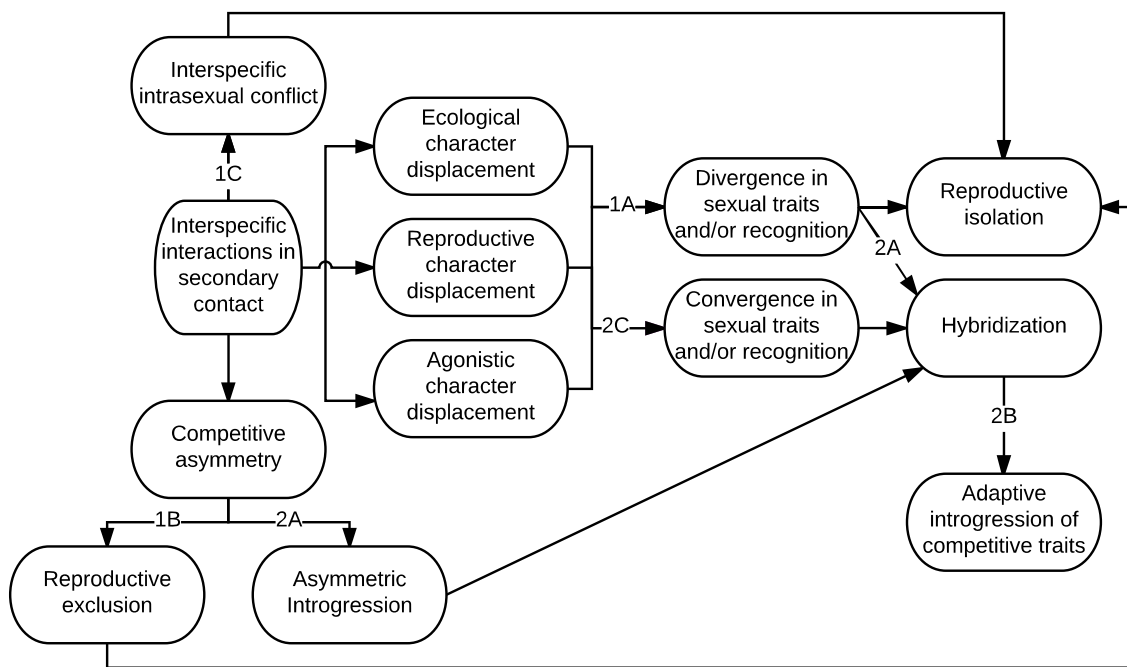


Figure 1. Conceptual framework.

Competition between species in secondary contact can promote reproductive isolation and/or hybridization. Path labels (e.g. 1A) correspond to sections throughout the manuscript.

which can subsequently influence the evolution of reproductive isolation and/or facilitate hybridization. This process, known as **agonistic character displacement**, can result in either divergence or convergence of phenotypic traits involved in competitor recognition and fighting ability, depending on the intensity of resource competition between species (Grether *et al.* 2009). Divergence in competitor signals and recognition is expected to promote reproductive isolation (see Figure 1, conceptual framework). However, even species with diverged competitive traits may hybridize if males of the dominant species (e.g. the lineage that is superior in aggression, body size, and/or competitive ability) monopolize mating resources shared with males of the subordinate species. Convergence in competitive signals is expected to facilitate territorial interactions over shared, limited resources, but can also increase the likelihood of hybridization if those signals also play a role in mate recognition. Alternatively, convergence that results in the exclusion of one species could promote reproductive isolation. In addition to male trait evolution, female mate preferences may diverge or utilize a different sexual trait to avoid hybridization (Hankison & Morris 2003; Seddon & Tobias 2010; Hudson & Price 2014).

Updating perspectives on sexual selection and hybridization

Studies on mating behavior and hybridization often draw a dichotomy between competitive males mating indiscriminately and choosy females limiting heterospecific mating. This dichotomy is oversimplified in several ways. For instance, male mate choice can facilitate mate discrimination within and between *Timema* stick insects (Arbuthnott & Crespi 2009), thereby reducing interspecific gene flow. Additionally, females can prefer heterospecifics when they resemble high quality conspecifics and/or ancestral preferences have not diverged, as in female orange-backed fairy wrens (*Malarus melanocephalus melanocephalus*) that prefer red-backed males resembling another subspecies (*M. m. cruentatus*) (Baldassarre & Webster 2013) and in female tungara frogs (*Physalaemus pustulosus* species group) that prefer call features of heterospecific males (Ryan & Rand 1993). In this review, I propose that

we have overlooked an additional component of sexual selection that could influence hybridization and reproductive isolation in secondary contact: female-female competition.

Despite a growing understanding of male-male competition and speciation, empirical and theoretical studies on the roles of female-female competition as well as male mate choice in hybridization are lacking (but see Wong *et al.* 2005; Servedio 2007; Kozak *et al.* 2009; Roberts & Mendelson 2017). There are many studies demonstrating that females compete over mating resources (reviewed in Rosvall 2011; Cain & Ketterson 2012) and males can be choosy of mates (reviewed in Kraaijeveld *et al.* 2007; Edward & Chapman 2011). Empirical studies across a wide variety of taxa including fish, lizards, and birds suggest that female aggression is adaptive in a number of social contexts (Stockley & Campbell 2013) including territory defense (Woodley & Moore 1999; Desjardins *et al.* 2006; Gill *et al.* 2007; Reedy *et al.* 2017) and reproductive success (While *et al.* 2009). Likewise, adaptive mate choice has been demonstrated for males in several insect species that face high reproductive costs such as sperm limitation and choose among females that vary in quality of signals advertising fecundity (Bonduriansky 2001; Nandy *et al.* 2012). As little attention as female competition and aggression have received in the literature, the role of female-female competition in hybridization has received far less. As a first step to addressing this gap, we need to compare the evolution of competitive traits and recognition in females to those of males, and predict the potential outcomes for hybridization in secondary contact. Future work should also focus on the role of male mate choice in speciation, but the current review will focus on comparing interspecific male-male and female-female competition.

Here I examine the role that interspecific reproductive competition plays in hybridization, specifically between closely related lineages (species, subspecies, and divergent populations) in secondary contact when reproductive isolation is incomplete. Other reviews have focused on the diversifying role of male-male competition in promoting speciation (e.g. Qvarnström *et al.* 2012), but here I expand this perspective to improve our understanding of both male-male and female-female competition and their evolutionary outcomes in secondary contact, which can either facilitate or impede reproductive isolation (see Figure 1, conceptual framework). I review the empirical and theoretical evidence supporting evolutionary scenarios in which 1) competition promotes reproductive isolation, and 2) competition facilitates introgression – the exchange of alleles from one species to another. I also emphasize that the outcomes of interspecific interactions in secondary contact should be considered in the context of both competition and mate choice, as well as from the perspectives of both the signaller and the receiver, and I review what may be the first case of female-female competition promoting hybridization.

1. When competition in secondary contact promotes reproductive isolation

Sexual selection can be a diversifying force in driving the evolution of traits involved in mate choice and competition for mates both within and between species (Lande 1981; Panhuis *et al.* 2001; Coyne & Orr 2004; Ritchie 2007). Closely related lineages are often more divergent in secondary sexual characteristics than other phenotypic traits (West-Eberhard 1983; Allender *et al.* 2003). Sexual characteristics specifically involved in competition include those directly used in fighting, such as body size and weaponry, as well as traits important in signalling dominance, such as coloration and vocalizations (Andersson 1994). Along with divergence in agonistic signals, the visual and auditory sensory systems that receive and recognize these signals may also diverge between heterospecific competitors (Peiman & Robinson 2010; Pfennig & Pfennig 2012; Okamoto & Grether 2013). Because these sexual traits are often used both to attract mates as well as to compete for mating resources (Berglund *et al.* 1996), their divergence between species can have consequences for reproductive isolation. For instance, character divergence that reduces interspecific interactions will limit gene flow between species. Below I describe patterns of divergence in competitive traits and recognition resulting from interspecific interactions, and explore how this divergence can promote reproductive isolation via reproductive exclusion and sexual conflict.

1A. Character displacement: ecological, reproductive, and agonistic

Character shifts in competitive traits and competitor recognition could take place due to different sexual, social and ecological selection pressures on each lineage evolving independently in allopatric

isolation (Rice & Pfennig 2006), or to selection pressures occurring from contact with a heterospecific in sympatry (Grether *et al.* 2009; Pfennig & Pfennig 2009). Three main processes of trait shifts due to interspecific interactions are ecological character displacement, reproductive character displacement, and agonistic character displacement, and they can result in either divergence or convergence in sympatry (Grant 1972). Competitive ecological interactions in secondary contact have been widely explored (reviewed in Pfennig & Pfennig 2012; Weber & Strauss 2016). Ecological character displacement (ECD) is a process that produces greater shifts in ecological niches of species in sympatry than in allopatry. ECD can arise when disruptive selection causes coexisting species to diverge in their ecological niches, thereby reducing interspecific competition over a previously shared ecological resource such as food (Brown & Wilson 1956; Losos *et al.* 2000; Schluter 2001). Both the resource utilized and trait associated with the resource use are expected to change between the sympatric species, (e.g. prey type and jaw morphology in larval feeding of Spadefoot toads *Spea bombifrons* and *S. multiplicata*; Pfennig & Murphy 2003). Divergent ECD is predicted to promote reproductive isolation in several ways. The divergence in resource acquisition traits may reduce contact between species, and therefore impede interspecific gene flow (reviewed in Coyne & Orr 2004; Price 2008). Additionally, ecological divergence between sympatric species can drive divergence in sexual signals, which can lead to reproductive isolation. In Darwin's finches, for example, ecologically adaptive divergence in beak morphology is correlated with divergence in song, which is used in territorial defense and mate choice (Huber & Podos 2006; Podos 2010). In the medium ground finch (*Geospiza fortis*), large and small beak morphs demonstrate positive assortative pairing, and gene flow is reduced between morphs (Huber *et al.* 2007). If offspring produced by matings between these populations are intermediate in phenotype and therefore are competitively inferior in either niche, ecologically dependent postmating isolation can evolve (Pfennig & Rice 2007; Rice & Pfennig 2010) which could initiate the speciation process (Schluter 2001; Pfennig & Pfennig 2009).

For closely related species in secondary contact that have not diverged in their secondary sexual characteristics, similar mating signals can result in species recognition errors and heterospecific mating (Gröning & Hochkirch 2008), which can in turn lead to the evolution of **reproductive character displacement** (RCD). RCD is a process that selects for greater sexual trait divergence and/or species discrimination in sympatry compared to allopatry, and can be indicative of the reinforcement process. Much empirical and theoretical research has investigated how selection resulting from mate misrecognition and maladaptive hybridization can drive divergence in mating signals and/or preferences (Ptacek 2000; Coyne & Orr 2004; Pfennig & Pfennig 2009). Like ECD, RCD can minimize interspecific contact, including reproductive competition, if the traits that diverge also function in competitive interactions. Both ECD and RCD can influence each other, when species that compete for ecological resources also have similar sexual signals (reviewed in Pfennig & Pfennig 2009). Species discrimination between divergent signals can be tested using playback experiments, but their implementation and interpretations can be challenging for both male and female behavior (Figure 2).

To experimentally measure the extent of premating reproductive isolation between two species, studies compare mating signal divergence along with relative responses, i.e. discrimination, between conspecific and heterospecific signals. For paired design playback studies in which males discriminate between conspecific and heterospecific stimuli, this is interpreted as evidence for reproductive isolation because divergent mating signals would reduce heterospecific gene flow (Baker 2001; Slabbekoorn & Smith 2002; Podos 2010; Lipshutz *et al.* 2017). Males whose territorial signals are not recognized by neighboring heterospecifics will face difficulty establishing and defending their territories in sympatry (Searcy & Nowicki 2005), which could promote reproductive isolation if they are forced to set up territories elsewhere. Tests in sympatry often reveal that males do not discriminate between heterospecific and conspecific signals – this is interpreted as lack of a behavioral barrier, which could promote hybridization (Gee 2005; den Hartog *et al.* 2008).

For the majority of playback studies it is common to test only one sex, males, and indirectly infer similar signal discrimination and/or preference by females. This practice is prevalent because it is easier to conduct male playback experiments than female preference experiments in many taxa. However, such an interpretation of discriminatory response is problematic, in that it assumes that the relative salience of conspecific and heterospecific signals to an individual territory holder is a suitable proxy for female discrimination and even female preference.



Figure 2. Interpretation of playback experiments.

Male white-crowned sparrow responding to simulated territorial intrusion during playback experiment.
Photo by Elizabeth P. Derryberry

While male signals can be important for both male-male competition and female choice, the two sexes may not have evolved the same discriminatory abilities, nor should we expect them to respond similarly to a potential heterospecific competitor versus a potential heterospecific mate. We should therefore be interested in the direct value of territorial playback experiments – for understanding species recognition in territorial defense, rather than indirectly interpreting tendency for reproductive isolation between males and females. To understand how male and female discrimination between conspecific and heterospecific sexual signals compare, we should explicitly test responses in both males and females of the same species. One successful example of this is a recent study in the *Ficedula* flycatchers, which found that females discriminate between conspecific and heterospecific sexual signals in sympatry, whereas males did not (Wheatcroft & Qvarnström 2017). Given that sexual signals are often multimodal (e.g. acoustic and visual) and multicomponent (e.g. multiple messages encoded) (Hebets & Papaj 2005), future work should also test the relative salience of specific components of signals for species recognition in males versus females.

Similarity in agonistic signals and competitor recognition can also select for divergence or convergence between species in secondary contact, a process known as agonistic character displacement (ACD). ACD evolves to reduce maladaptive interspecific competition over mating resources (Grether *et al.* 2009), and can change the degree and/or outcome of interspecific interactions (Cody 1969; Grether *et al.* 2013). ACD has received relatively less attention than ECD and RCD, and fewer empirical cases are known. In the rubyspot damselfly genus *Hetaerina*, males of some species use wing coloration for competitor recognition, and similarity in male wing coloration causes misidentification between species (Anderson & Grether 2010a). Observational and experimental studies revealed that interspecific territorial aggression in sympatry selected for shifts in agonistic signals (Anderson & Grether 2010a) and competitor recognition (Anderson & Grether 2010b). Similar patterns have been found in the auditory signal reception of dendrobatid frogs (*Allobates femoralis*) (Amézquita *et al.* 2006) and the male nuptial color of three-spined sticklebacks (*Gasterosteus aculeatus* spp.) (Albert *et al.* 2007). One open question is whether the character shift is expected to occur more often for the competitively inferior species, due to selection for access to resources monopolized by the dominant species.

Divergence in competitive signals also involved in mate recognition can promote reproductive

isolation if females discriminate between these species-specific competitive signals and prefer to mate with conspecifics (Okamoto and Grether 2013). In the hybrid zone between pied flycatchers (*Ficedula hypoleuca*) and collared flycatchers (*F. albicollis*), both ACD and RCD may explain a divergence in male plumage in sympatry, which both reduces heterospecific aggression and heterospecific pairing. Brown morph *F. hypoleuca* males are found in sympatry with competitively dominant and black *F. albicollis*, and they receive less interspecific aggression than *F. hypoleuca* black morphs (Alatalo et al. 1994, Saetre et al. 1993). Female *F. hypoleuca* prefer brown conspecifics in sympatry with *F. albicollis*, but prefer black conspecifics in allopatry (Saetre et al. 1997, Saether et al. 2007). Because the same traits are often used for species recognition by both potential competitors and mates (Berglund et al. 1996), disentangling ACD from RCD and ECD can be difficult and these processes may not be mutually exclusive (Grether et al. 2009, Okamoto and Grether 2013). For instance, character displacement in bill morphology, male song, and response to song have been demonstrated between sympatric species of African tinkerbirds *Pogoniulus bilineatus* and *P. subsulphureus* (Kirschel et al. 2009), but the mechanism driving character displacement is not known. Clear cases of ACD must demonstrate that divergence in male traits is due to competition over mating resources, and not due to selection for species-specific mate recognition by females (Okamoto and Grether 2013), which has been shown in the *Hetaerina* damselflies (Drury and Grether 2014). The traditional perspectives of sexual selection emphasize RCD on male sexual traits and female recognition, and ACD on male agonistic traits and recognition (see Figure 1, pathway 1A of conceptual framework). An apparent knowledge gap is whether RCD can occur on female sexual traits and male recognition, and ACD can occur for female agonistic traits and competitor recognition. Evidence is likely to be found in systems where male exercise mate choice and females compete over shared mating resources.

1B. Competitive asymmetry and reproductive exclusion

Divergence in competitive morphology and behavior of lineages in allopatry can result in the superior competitive ability of one lineage over the other upon secondary contact. A recent review found that most aggressive interactions between closely related bird species were asymmetric (Martin et al. 2017). **Competitive asymmetry** that reduces interactions between species can lead to reproductive isolation. For instance, if one of the species is a superior competitor and resources are limited, the dominant species may displace the subordinate species via competitive exclusion (Gause 1934; Hardin 1960). The expectations for ecological competitive exclusion are similar to those for **reproductive exclusion**, also known as sexual exclusion – when the dominance of one species in monopolizing territories and mates displaces the less competitive species and excludes them from establishing residence in sympatry (Kuno 1992; Hochkirch et al. 2007; Gröning & Hochkirch 2008). As the outcome of both ecological competitive exclusion and reproductive exclusion is that the species cannot coexist (Pfennig & Pfennig 2012), local extinction that reduces interspecific interactions could promote reproductive isolation between populations. For example, an experiment with *Callosobruchus maculatus* and *C. chinensis* weevils demonstrated that indiscriminate male mating attempts towards heterospecifics, linked with intolerance by female *C. maculatus* females, resulted in reduced reproduction, population decline, and local extinction of *C. maculatus* (Kishi et al. 2009). The expansion of a more dominant and/or invasive species' range, exacerbated by anthropogenic changes such as habitat modification and climate change, can accelerate the geographical displacement of a less dominant species (Rhymer & Simberloff 1996; Krosby et al. 2015). When the species co-occur throughout their distribution, or the more dominant species expands its range (Canestrelli et al. 2016), the less dominant species could become locally extinct (Duckworth 2008; Pfennig & Pfennig 2012). In a simulation study, the competitive ability of a native plant species via faster pollen-tube growth rates and enhanced seedling competition was predicted to prevent the risk of extinction due to both natural hybridization with invading plant species and competition with hybrids and invasives (Wolf et al. 2001). Species that are already rare are more vulnerable to extinction by hybridization (Levin et al. 1996). Reproductive exclusion is expected to promote reproductive isolation, but examples are limited. Evidence of this process is likely difficult to observe in nature because it does not leave a genetic trace, as is the case with hybridization.

The consequences of male-male competition for reproductive exclusion and reproductive isolation are likely to be similar in female-female competition, if females of one species outcompete

another for mating resources, for example territories for breeding. Female-female agonistic interactions that occur within species have been predicted to promote diversification and incipient speciation. Females of some haplochromine cichlid species with bright coloration are territorial and aggressive, and use colour as a cue in social interactions (Seehausen *et al.* 1999). An experimental assay in the cichlid species *Neochromis omnicaeruleus* demonstrated that females bias aggression toward females of their own color morph (Dijkstra *et al.* 2009). *N. omnicaeruleus* exhibits mutual mate choice (Seehausen *et al.* 1999), and females compete for males of the same morph. Furthermore, female coloration is associated with behavioral dominance among female morphs (Dijkstra *et al.* 2009). How competitive interactions between females of the same species compare to female competition between two species, and whether both of these processes are expected to contribute to diversification and reproductive isolation, is an exciting avenue for future research.

1C. Interspecific intrasexual conflict

Agonistic interactions can occur not only between the same sex of different species (e.g. male-male and female-female interspecific interactions), but also between males and females of different species (e.g. female-male interspecific interactions). Female aggression against male heterospecifics can promote reproductive isolation. For example, females at risk of interspecific pairings between salmon and brown trout showed higher rates of aggression against heterospecific males and reduced the number of eggs available for spawning (Beall *et al.* 1997). In other cases, however, females are unable to exert conspecific mate choice, for example in insect and waterfowl species where males force copulations (McKinney *et al.* 1983; Arnqvist & Rowe 2002). This antagonistic coevolution between females and males is known as sexual conflict, when the two sexes have different evolutionary interests (Parker & Partridge 1998). Within species, sexual conflict can be a driver of speciation, and can promote rapid evolutionary divergence of reproductive traits (Arnqvist *et al.* 2000; Martin & Hosken 2003). For instance, sexual conflict can result in antagonistic coevolution of genital morphology as well as color signalling and perception, resulting in sexual polymorphisms (Hosken & Stockley 2004; Brennan *et al.* 2010; Gavrillets 2014). Female *Ischnura ramburii* have evolved male visual mimicry to resist male harassment, which can promote mate recognition errors by males (Gering 2017). A color polymorphism in the wing patterns of *Colias* butterflies allows females with the rare 'alba' morph to avoid reproductive interference, as a means of resistance to interspecific male mating harassment (Nielsen & Watt 2000). Female sexual polymorphisms due to variation in resistance or toleration of unwanted mating could lead to speciation, but this has largely been explored within a species (Svensson *et al.* 2009). **Interspecific sexual conflict**, between males and females of different species, could occur if heterospecific mating promoted by indiscriminate males is opposed by female preference for conspecifics. There are several cases of forced copulations resulting in hybrids (Randler 2005; Rohwer *et al.* 2014) but it is unknown whether females have evolved postmating divergence in genital morphology or other traits to avoid coercive heterospecific mating. To what extent does interspecific sexual conflict, involving the opposition of competition and mate choice, promote reproductive isolation between species?

2. When competition in secondary contact facilitates introgressive hybridization

Reproductive competition between sympatric lineages can also promote hybridization, if interspecific interactions over shared mating resources occur and reproductive isolation is incomplete. The previous section explained how divergence in competitive traits between lineages could lead to reproductive exclusion, but competitive asymmetry can also facilitate a dominant lineage's monopolization of breeding with both conspecific and heterospecific mates. Some patterns indicating these processes include asymmetric introgression of genetic loci and phenotypic traits, as well as moving hybrid zones. Hybridization itself can result in the superior competitive ability of hybrids relative to their parental taxa, which can further promote backcrossing. While one outcome of interspecific reproductive competition is divergence in sexual traits, competitive signals that facilitate territorial interactions can also converge between species, which can also promote hybridization. The majority of evidence for these process has been found between males of species that compete for mating resources, but recent evidence suggests that female-female competition can also promote hybridization.

2A. Competitive asymmetry and directional hybridization

Competitive asymmetry can lead to **asymmetric introgression**, in which loci and traits that confer a reproductive advantage and are inherited from a competitively superior parental species progress into the hybrid zone farther than background neutral loci (Barton 1979; Piálek & Barton 1997). For example, an asymmetry in male-male competition between two lineages of common wall lizards (*Podarcis muralis*) may be promoting directional hybridization (While *et al.* 2015). The lineages are divergent in competitive morphology – males of the northern Italian subspecies *P.m. nigriventris* have larger heads, stronger bite force, and greater testes mass compared to the Western Europe subspecies *P.m. brogniardii*. *P.m. nigriventris* males are more aggressive and dominant to *P.m. brogniardii* males in territorial interactions, which allows them to monopolize high quality territories and courtship of both conspecific and heterospecific females (MacGregor *et al.* 2017). Sexual traits associated with *P.m. nigriventris* males, including head size and dorsal and ventral coloration, are introgressing into the hybrid zone (While *et al.* 2015).

Directional hybridization can occur particularly when male-male competition is a stronger determinant of mating than female mate preferences (e.g. Reichard *et al.* 2005). For example, in experimental secondary contact among *Tropheus* cichlid fish of different color morphs, dominance of the red male morph interfered with positive assortative mating preferences by females and promoted asymmetric hybridization (Sefc *et al.* 2015). When males of a dominant lineage displace lower-ranked males of the subordinate lineage from breeding territories, their conspecific females are left with no choice but to join the territory of a heterospecific in order to reproduce (Wirtz 1999). However, particularly when hybridization is maladaptive, females could still exercise choice for conspecifics through extra-pair mating with nearby conspecific males. This happens, for example, in fur seals that pursue extra-territory inseminations when their phenotype did not match that of territorial mates (Goldsworthy *et al.* 1999). The outcomes of interspecific male-male competition for hybridization in the *Podarcis* wall lizards may be influenced by weak female preference as well as by male mate choice for conspecifics (Heathcote *et al.* 2016). Although *P.m. nigriventris* males outcompete *P.m. brogniardii* males for mating opportunities in the hybrid zone, *P.m. nigriventris* males prefer to mate guard the largest females, which are typically also *P.m. nigriventris*, thereby promoting assortative mating and reducing gene flow between the two lineages (Heathcote *et al.* 2016). These examples demonstrate some of the ways competition and mate choice can interact to promote similar or opposing outcomes for hybridization. When possible, empirical studies on the behavioral mechanisms of hybridization should investigate the contributions of both male and female behavior separately, to understand the interactions between competition and mate choice (Wong & Candolin 2005).

Unidirectional hybridization resulting from competitive asymmetries can yield increased prevalence of one heterospecific cross – for example mating between females of one species with males of the competitively dominant species, but the reciprocal cross is rare. A pattern of mitochondrial DNA (mtDNA) of only one parental species found in hybrids can suggest unidirectional hybridization. For example in hybridizing macaques, the Tonkean macaque (*Macaca tonkeana*) has more intense male-male competition for mates, and may be outcompeting the Moor macaque (*M. maura*) for *M. maura* females (Supriatna 1991; Bergman & Beehner 2003). Genetic patterns of introgression for autosomal loci and mtDNA suggests that hybridization occurs between *M. tonkeana* males and *M. maura* females (Evans *et al.* 2001). Unidirectional introgression of mtDNA, autosomal loci, and/or phenotypic traits can be explained by sexual selection, either due to the competitive dominance of one species, or to mate choice favoring one species. It can also be found between females of a rare species and males of a common species in sympatry (Wirtz 1999). Patterns suggesting unidirectional hybridization can additionally be explained by the reduction in fitness from one cross type due to deleterious epistatic interactions – so-called “Darwin’s Corollary to Haldane’s Rule” (Turelli & Moyle 2007). Studies testing whether pre-mating behaviors can explain patterns of asymmetric introgression should also consider alternative, but not necessarily mutually exclusive hypotheses of post-mating and postzygotic reproductive isolation (e.g. Carling & Brumfield 2008). For example, unidirectional hybridization between two sunfish species *Lepomis macrochirus* and *L. gibbosus* was explained by both asymmetric conspecific sperm precedence and hybrid inviability of one cross (Immler *et al.* 2011).

Asymmetric introgression can also lead to a moving hybrid zone. Moving hybrid zones can occur between sympatric species with asymmetric competitive interactions that result in the geographic and/or genetic displacement of the inferior competitor via hybridization. Especially when an aggressive phenotype is linked with greater dispersal (Duckworth & Badyaev 2007; Canestrelli *et al.* 2016), range expansion of the superior competitor can cause a hybrid zone to move over time. In the *Setophaga* hybrid zone between hermit (*Setophaga occidentalis*) and Townsend's (*S. townsendi*) warblers, *S. occidentalis* are superior competitors over breeding territories and mates, and hybrids are intermediate to parentals in aggression (Pearson 2000; Owen-Ashley & Butler 2004). While hybridization is restricted to narrow zones, *S. townsendi* mtDNA is found in a phenotypically pure *S. occidentalis* population (Krosby & Rohwer 2009), and a resampling of hybrid zone sites 10-20 years later indicated they have become more *townsendi*-like over time (Krosby & Rohwer 2010). This geographic replacement of the competitively inferior *S. occidentalis* (Krosby & Rohwer 2010) could ultimately result in its extinction. Hybridization between species with asymmetric competitive abilities can have important conservation implications – resulting in the extirpation of the less competitive lineage through genetic or demographic swamping, but also facilitating genetic rescue (reviewed in Allendorf *et al.* 2001; Mooney & Cleland 2001; Todesco *et al.* 2016; vonHoldt *et al.* 2017). Female choice in conjunction with male-male competition can also facilitate hybrid zone movement. For example, females of both Black-capped (*Poecile atricapillus*) and Carolina (*P. carolinensis*) chickadees display mate choice for dominant males, which are typically *P. carolinensis* (Bronson *et al.* 2003). The dominance of *P. carolinensis* males over territories and mates can explain its northward range expansion and the northern movement of the hybrid zone, but climate change can also explain this movement (Taylor *et al.* 2014). Because hybrid zone movement can be explained by many other drivers including mate choice, postzygotic genetic incompatibilities, and environmental change (Buggs 2007), hypotheses for competition as a driver of asymmetric introgression and hybrid zone movement should be explicitly tested, for example by comparing aggression to simulated territorial intrusion (e.g. Billerman & Carling 2017; Lipshutz 2017). These are not mutually exclusive processes, however, as the presence of competitive asymmetries is a necessary but not sufficient demonstration that competition is a key driver of hybrid zone movement.

When mate choice is based on an evaluation of traits also involved in competitive interactions, it can be difficult to disentangle the effects of reproductive competition from mate choice on hybridization (e.g. Mennill *et al.* 2002). In the golden-collared (*Manacus candei*) and white collared (*Manacus vitellinus*) manakin hybrid zone, male-male competition may be driving asymmetric introgression of gold plumage across the hybrid zone, as *M. candei* males are more aggressive than *M. vitellinus* males and plumage color is associated with aggression (McDonald *et al.* 2001). However, this pattern may also be driven by female preference for *M. candei* males in mixed leks (Brumfield *et al.* 2001; Stein & Uy 2006). As with identifying the drivers hybrid zone movement and distinguishing between ACD and RCD, we should test alternative hypothesis for competition versus mate choice in driving asymmetric introgression, for example with experimental tests of interspecific competition (While *et al.* 2015) and mate choice (Heathcote *et al.* 2016) in the same system.

Female-female competitive asymmetry

Could competitive asymmetries between females of sympatric species promote hybridization, in a similar fashion to males? Within a species, competitive phenotypes in females can influence mating success. In the social lizard *Egernia whiti*, more aggressive females have more extra-pair offspring (While *et al.* 2009). Between species, female-female competition for mating opportunities is less understood. Interspecific female-female competition for male sperm has been documented between mollies *Poecilia latipinna* and a unisexual species of hybrid origin *P. formosa* from crossings of *P. latipinna* and *P. mexicana* (Riesch *et al.* 2008). In order to trigger embryogenesis, hybrid female *P. formosa* require sperm from either parental species, known as sexual parasitism (Schlupp 2009). While *P. formosa* was more aggressive towards *P. latipinna* than vice versa, it is unknown what role interspecific female competition plays in maintaining the *Poecilia* species complex (Makowicz & Schlupp 2015). That aggressive females are more promiscuous could influence their likelihood of mating with a heterospecific. Costs of heterospecific mating may be higher in females because of gametic and parental investment (Wirtz 1999), but these costs may be lowered if females mate with multiple males. For example, one

experimental study of *Gryllus* crickets demonstrated that mating barriers between hybridizing species were weakest among females of the more polyandrous species (Veen *et al.* 2011). Females of the more polyandrous species, *G. bimaculatus* discriminated less and mated more with heterospecific males. Therefore, we might expect females in polyandrous systems, especially those that compete for mates, to mate less discriminately than females in monogamous mating systems.

Interspecific female-female competition in polyandrous mating systems, in which females compete for access to male mates, may be analogous to interspecific male-male competition. Because polyandrous females have multiple opportunities to breed, they may face lower costs of heterospecific mating (Arnqvist *et al.* 2000). One example is a hybrid zone between two polyandrous sex-role reversed bird species, the Wattled Jacana (*Jacana jacana*) and the Northern Jacana (*Jacana spinosa*) (Miller *et al.* 2014; Figure 3). Female jacanas of both species control access to mates by competing for territories encompassing a harem of males. Females are under stronger selection for increased aggression and larger body size and spur weaponry, while males provide parental care (Jenni & Collier 1972; Emlen & Wrege 2004a; b). There is an asymmetry of hybridization – phenotypic hybrids only had *J. spinosa* mtDNA haplotypes, suggesting predominant crosses between *J. spinosa* females and *J. jacana* males (Miller *et al.* 2014). Unidirectional introgression of *J. spinosa* mtDNA across the hybrid zone may be explained by interspecific female-female competition for mates, whereby the larger body size, spur length, and higher aggression of female *J. spinosa* allows them to exclude female *J. jacana* from obtaining territories in mixed-species populations (Lipshutz 2017).

While interspecific female-female competition over territories and mates may be more likely to influence hybridization outcomes in species with polyandrous mating systems, to what extent does female-female competition impact the likelihood of hybridization in other mating systems?

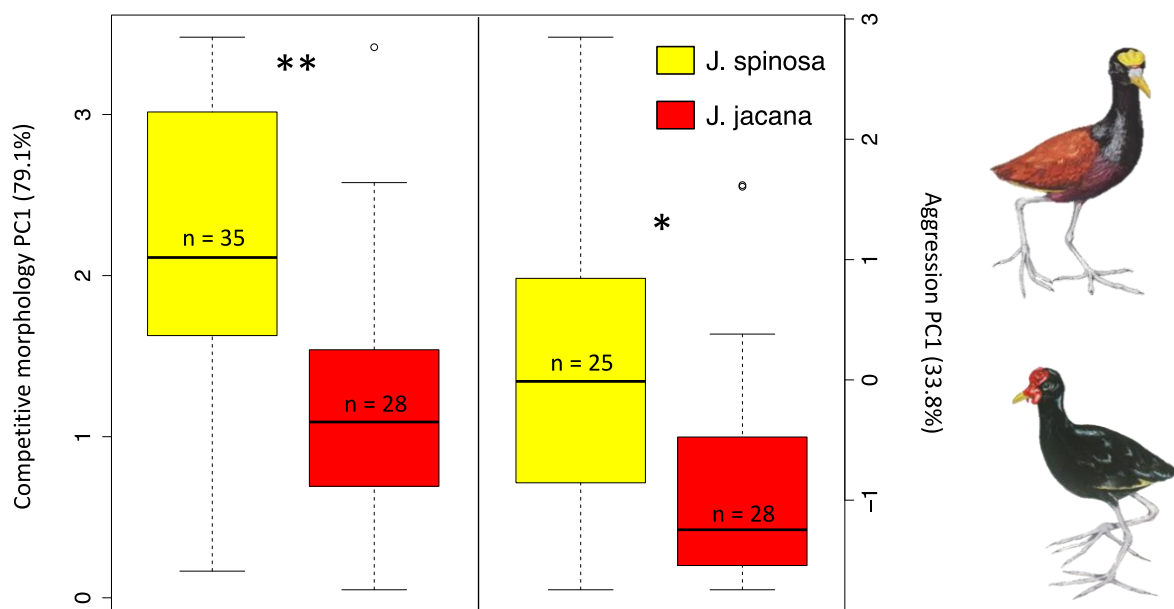


Figure 3. Female-female competition in jacanas.

Females of two polyandrous, sex-role reversed shorebird species that hybridize in Panama show competitive asymmetries in morphology (left panel) and aggressive behavior (middle panel). *J. spinosa* females (right panel top) have larger body mass, longer wing spurs used for fighting, and are more aggressive than *J. jacana* females (right panel bottom), which may explain the asymmetric introgression of mitochondrial DNA in the hybrid zone. Figure adapted from Lipshutz 2017. Illustrations by Stephanie McClelland.

2B. Adaptive introgression of competitive traits

Heterospecific mating is often considered an accidental byproduct of incomplete species recognition, which reduces fitness due to wasted time, energy, and gametes. However, hybridization can also be adaptive (Willis 2013). While this review has thus far examined how competition influences the likelihood for hybridization, heterotypic mating can also increase competitive ability. For example, hybrid tadpoles between *Spea bombifrons* and *S. multiplicata* develop more rapidly and are more likely to achieve metamorphosis than *Spea bombifrons* tadpoles, which can facilitate survival in ephemeral ponds. *S. bombifrons* females become more likely to hybridize with *S. multiplicata* males when water levels are low, (Pfennig *et al.* 2002; Pfennig & Rice 2007), suggesting that unidirectional hybridization is adaptive in certain environments. Inheritance of competitive traits from the dominant parental lineage could also provide hybrids with a selective advantage over the competitively inferior lineage.

Heterosis, or hybrid vigor, occurs when hybrids are competitively superior to their parental species (Birchler 2003), and can also result in reduction or extinction of parental species. A pattern of hybrids outcompeting their parental taxa is particularly associated with invasive species (Pyšek *et al.* 2003; Suehs *et al.* 2004). Hybrids between two morphs of invasive Thiarid snail *Melanoidea tuberculata* are produced sexually, but the hybrid morphs reproduce asexually via apomictic parthenogenesis (Samadi *et al.* 1999). Hybrid morphs are superior competitors to their parental taxa in natural habitats by having greater colonization ability and larger bodied offspring (Facon *et al.* 2005, 2008), and are mostly female (Facon, pers. comm.). There are several other examples where hybrids are superior competitors to parentals, for example in several crosses of Darwin's finches (*Geospiza sp.*) where hybrids have higher breeding success (Grant & Grant 1992), and in hybrid gulls between *Larus occidentalis* and *L. glaucescens* because of the combination of adaptive traits from parentals in an intermediate environment (Good *et al.* 2000). Heterosis can also be a mechanism of speciation if hybrids are reproductively isolated from their parental species. This can occur due to an inversion (Lowry & Willis 2010) or allopolyploidy (Comai 2005; Van de Peer *et al.* 2017), which is more common for plants (Abbott *et al.* 2016) but also documented in animals (Mable *et al.* 2011). Heterosis can also be associated with a **hybrid swarm** because of the production of highly fit recombinant genotypes that erode parental genetic boundaries, for example in the copepod *Tigriopus californicus* (Hwang *et al.* 2011). In a hybrid swarm between Pecos pupfish (*Cyprinodon pecosensis*) and sheepshead minnow (*C. variegatus*), male-male competition is asymmetric (Rosenfield & Kodric-Brown 2003). Male *C. variegatus* as well as F1 hybrids outcompeted male *C. pecosensis* for mates, suggesting hybrid vigour can promote extensive hybridization via competition. The adaptiveness of hybridization is based on the fitness of hybrids relative to parental species, and this can be challenging to quantify but useful for understanding how and why hybridization occurs. For species in which hybridization is maladaptive, introgression of traits that increase a hybrid individual's competitive advantage may be undermined by lower survival due to incompatibilities for other loci.

2C. Convergence in agonistic signals

Although studies of interspecific competition typically focus on the evolution of trait divergence, competition over shared mating resources can actually drive convergence in signals and signal recognition involved in territorial defence to facilitate aggressive interactions between heterospecifics (Cody 1969; Tobias & Seddon 2009; Vokurková *et al.* 2013). Convergence in competitive signals has been found within an avian radiation of ovenbirds (Furnariidae), whereby species coexistence predicted convergence in male song (Tobias *et al.* 2013). Agonistic signal convergence could evolve due to direct interactions in competing over shared ecological or mating resources (Grether *et al.* 2009; Dufour *et al.* 2015; Laiolo 2017), or because of acoustic adaptation to a shared environment (e.g. Cardoso and Price 2010). Convergence in competitive signals can also occur due to hybridization (Grant *et al.* 2004; Secondi *et al.* 2011), either if signals are genetically determined and are intermediate to parental signals (e.g. de Kort *et al.* 2002; Gee 2005), or due to learning if offspring imprint on the songs of heterospecifics (e.g. Secondi *et al.* 2003; Haavie *et al.* 2004).

While signal convergence between sympatric species is expected to facilitate competitor recognition and interspecific territoriality (Grether *et al.* 2009), it could also increase the probability of

heterospecific pairing and hybridization, especially in species that use the same signals to both defend territories and attract a mate (Berglund *et al.* 1996; Wong & Candolin 2005). For example, in sympatric *Ficedula* flycatchers, the pied flycatcher (*F. hypoleuca*) song converges with the song of the more dominant collared flycatcher (*F. albicollis*) by incorporating learned parts of its song repertoire (Haavie *et al.* 2004). This mixed singing leads to heterospecific pairing and increases the likelihood of hybridization (Qvarnström *et al.* 2006). However, the convergence of male song and song discrimination to facilitate territorial competition is opposed by stricter female choice in sympatry (Wheatcroft & Qvarnström 2017). These findings, that divergence in species recognition can evolve in females along with convergence in male sexual signals, provide a more inclusive understanding of reproductive isolation in the flycatcher system. This study adds to an emerging understanding that signal discrimination may diverge between the sexes, based on different selective pressures of mate and competitor recognition. In another example, a study of two sympatric *Hypocnemis* antbird species found that females discriminate between conspecific and heterospecific males in sympatry, despite convergence in male song (Seddon & Tobias 2010). Concerning interspecific communication in secondary contact, the evolution of signal recognition is expected to facilitate competition over a shared mating resource in males and to avoid maladaptive hybridization in females. Both convergent and divergent character displacement on the same sexual signals and their recognition can therefore have opposing outcomes for reproductive isolation in males versus females (see Figure 3). When this tension exists, the selective pressures resulting divergent RCD dominate those favouring convergent ACD, due to the costs of reproduction outweighing the costs of aggression (Okamoto & Grether 2013).

When females compete, is the evolution of competitive signals and recognition in females predicted to have similar outcomes for hybridization as those found in males? For species in which both males and females defend territories, we might expect the sexes to have similar patterns of agonistic signal evolution. This can depend on whether the agonistic signals are also used in mate choice decisions for either sex (Wong & Candolin 2005). If male signals are under selection in both choice and competition contexts, but female signals are not, then we might predict fewer constraints on the direction of evolution of female signals. In a scenario where convergence in agonistic signals facilitates interspecific territorial interactions, female agonistic signals may be more likely to converge in secondary contact, whereas male signals may be expected to be more divergent to facilitate species recognition. However, if males use female agonistic signals to select a mate, then we should see similar patterns of convergence in the agonistic signals of both sexes. In a sympatric species pair of Neotropical antbirds, *Hypocnemis peruviana* and *H. subflava*, both males and females sing to defend territories, and interspecific aggression is intense (Tobias & Seddon 2009). Both male and female songs converged in sympatry, likely due to **social selection**, which includes competition for ecological resources in addition to mate acquisition (West-Eberhard 1983; Tobias *et al.* 2012). Interestingly, female songs showed greater similarity in acoustic structure in sympatry than male songs, potentially because of selection on male song for females to discriminate between conspecifics and heterospecifics and avoid hybridization (Searcy & Brenowitz 1988). Although hybridization does not occur between these species, this study can provide insight for female versus male agonistic signal evolution resulting from interspecific interactions. Female territorial signals may be less constrained by conspecific mate recognition than male signals, and can therefore evolve more strongly in response to interspecific competition than male signals. Currently, there are no known studies of agonistic character displacement in female competitive traits and/or species recognition. Are female agonistic signals more likely to converge or diverge in secondary contact with closely related competitors, in comparison to male signals?

Conclusions and next steps

This review has examined the processes by which reproductive competition between species in secondary contact promotes reproductive isolation versus hybridization. When possible, I have compared the evidence for male-male competition to that of female-female competition, but thus far both theoretical and empirical studies are rare for female competition. Interspecific competition that promotes the divergence of sexual traits and/or recognition between species via character displacement, as well interspecific interactions that result in reproductive exclusion, can promote reproductive isolation (Figure 2: Conceptual framework). While evidence for ECD, RCD, and ACD includes the involvement of both

males and females, reproductive exclusion has only been documented in males. Competition between species in secondary contact can also promote hybridization, for instance when a dominant species monopolizes mating resources, sometimes leading to asymmetric introgression. Convergence in sexual traits and recognition due to competition can also increase the likelihood for hybridization if the same traits are involved in mate choice. Hybridization itself can cause the introgression of competitive traits, which can facilitate further hybridization. Evidence for the involvement of both males and females has been found in all of these processes, though the male examples are strikingly more prevalent.

Our understanding of how male-male competition influences hybridization outcomes is solidifying. Still, the predictions for how female choice can reinforce reproductive isolation via selection for male trait divergence are more clearly developed than the predictions for how male-male competition can influence hybridization. This is paradoxical, because most empirical studies examining whether sexual trait divergence promotes reproductive isolation are carried out by testing male-male interactions and not male-female interactions, due to logistical challenges (see Figure 3: Playback Experiments). Only by testing both competition and mate choice within the same study systems can we disentangle whether the mating behavior of males and females impedes or promotes the evolution of reproductive isolation.

Does taking a non-traditional perspective change our understanding of how sexual selection impacts the process of reproductive isolation? For those systems in which females of different species compete for shared mating resources, the likelihood for female-female competition to promote reproductive isolation versus facilitate hybridization depends on the cost of mating with a heterospecific. Mating behavior is just one component of species interactions that influences the potential for hybridization between lineages in secondary contact, and the evolutionary context of interacting lineages is important to consider. The outcomes for reproductive isolation depend not only on interspecific competition and mate choice, but also the fitness costs to hybridization, which can be related to the age of divergence between the interacting lineages and accumulation of genetic incompatibilities (Pfennig 1998; Ord *et al.* 2011; Drury *et al.* 2015). For instance, the accumulation of intrinsic genetic incompatibility over time is likely to select for species recognition traits to avoid heterospecific mating. As females typically have higher gametic and parental investment and fewer opportunities for multiple mating attempts, one prediction is that male competition is more likely to result in hybridization than female competition. Future empirical and theoretical work should explicitly test this prediction on the outcome of intraspecific competition for hybridization in males versus females, in the context of the strength of intrinsic incompatibilities between sympatric lineages.

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CHAPTER I
BEHAVIORAL RESPONSE TO VOCAL AND GENETIC DIVERGENCE IN
A CONTACT ZONE BETWEEN WHITE-CROWNED SPARROW
SUBSPECIES (*ZONOTRICIA LEUCOPHRYS*)

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Abstract

Divergence in sexual signals may drive reproductive isolation between lineages, but behavioral barriers can weaken in contact zones. Here, we investigate the role of song as a behavioral and genetic barrier in a contact zone between two subspecies of white-crowned sparrows (*Zonotrichia leucophrys*). We employed a reduced genomic dataset to assess population structure and infer the history underlying divergence, gene flow and hybridization. We also measured divergence in song and tested behavioral responses to song using playback experiments within and outside the contact zone. We found that the subspecies form distinct genetic clusters, and demographic inference supported a model of secondary contact. Song phenotype, particularly length of the first note (a whistle), was a significant predictor of genetic subspecies identity and genetic distance along the hybrid zone, suggesting a close link between song and genetic divergence in this system. Individuals from both parental and admixed localities responded significantly more strongly to their own song than to the other subspecies song, supporting song as a behavioral barrier. Putative parental and admixed individuals were not significantly different in their strength of discrimination between own and other songs; however, individuals from admixed localities tended to discriminate less strongly, and this difference in discrimination strength was explained by song dissimilarity as well as genetic distance. Therefore, we find that song acts as a reproductive isolating mechanism that is potentially weakening in a contact zone between the subspecies. Our findings also support the hypothesis that intra-specific song variation can reduce gene flow between populations.

Introduction

Discrimination between diverged sexual signals can contribute to reproductive isolation by reducing gene flow between populations (Dobzhansky 1940; Mayr 1963; West-Eberhard 1983; Coyne & Orr 2004). Behavioral responses to a sexual signal provide a measure of the salience of that signal in mate choice (Searcy 1992) and in territorial interactions (Kroodsma 1986; Nowicki *et al.* 1998). Numerous empirical studies suggest that mating signals used to attract mates and repel competitors can also promote behavioral isolation among closely related populations (reviewed in West-Eberhard 1983; Andersson 1994; Price 1998; Panhuis *et al.* 2001). Concordance in geographical patterns of sexual signal and genetic variation occurs in a number of taxa (e.g. crickets, Shaw *et al.* 2007; mice, Campbell *et al.* 2010; gibbons, Thinh *et al.* 2011; and frogs, Warwick *et al.* 2015), supporting the hypothesis that divergence in mating signals between populations can act as a behavioral reproductive isolating mechanism.

Much emphasis has been placed on the importance of song, and in particular learned song, in facilitating speciation in birds (Marler & Tamura 1964; Nottebohm 1969; Baker & Cunningham 1985; Grant & Grant 1996; Martens 1996; Price 1998, 2008; Slabbekoorn & Smith 2002; Podos & Warren 2007). As a long-distance signal, song is often the first aspect of the phenotype that can be assessed by would-be mates or competitors (Catchpole & Slater 2008), and so has potential as a behavioral barrier to gene flow (Coyne & Orr 2004). Many birds produce distinct songs, and birds typically respond strongest

to the song of their own species (reviewed in Andersson 1994; Martens 1996), supporting a role for song as an isolating mechanism. Within a species, there is also substantial geographic variation in song (reviewed in Podos & Warren 2007). Both male and female receivers often discriminate between songs of different cultural populations (Searcy *et al.* 1997; Derryberry 2007; Seddon & Tobias 2007), which should reduce gene flow between populations with diverged signals. However, there is little evidence for song acting as an intra-specific barrier to gene flow between cultural populations (reviewed in Slabbekoorn & Smith 2002). The majority of empirical studies that focus within a species do not find genetic substructuring based on song divergence for songbirds (oscines) (e.g. Payne & Westneat 1988; Loughheed & Handford 1992; Soha *et al.* 2004; Ruegg *et al.* 2006; Leader *et al.* 2008; Ortiz-Ramírez *et al.* 2016), nor for non-oscines in which vocal learning evolved independently (e.g. Wright & Wilkinson 2001; Saranathan *et al.* 2007; Gonzalez & Ornelas 2014). Thus, although there is abundant evidence that song acts as an isolating mechanism between bird species, when and how intra-specific song variation facilitates reproductive divergence is less clear (Slabbekoorn & Smith 2002; Lachlan & Servedio 2004).

Interactions between hybridizing lineages present an opportunity to investigate how intra-specific song divergence affects the process of mate selection and resource acquisition. Hybrid zones are natural laboratories for studying the speciation process because they facilitate the testing of behavioral and genetic barriers between differentiated lineages (Endler 1977; Barton & Hewitt 1985; Hewitt 1988; Harrison 1993). Although studies of hybridizing lineages typically find song divergence in allopatry (e.g. Halfwerk *et al.* 2016), songs are often more convergent in sympatry, due to local acoustic adaptation or interspecific learning (Secondi *et al.* 2003; Haavie *et al.* 2004; Qvarnström *et al.* 2006; Kenyon *et al.* 2011), or hybridization itself (de Kort *et al.* 2002). Likewise, in many cases individuals in hybrid zones discriminate between diverged songs (Patten *et al.* 2004; Turčoková *et al.* 2011; Greig & Webster 2013), while in other cases, individuals do not discriminate between non-local and local songs (Matessi *et al.* 2000; Gee 2005; den Hartog *et al.* 2008), or there is asymmetric song recognition (Kershner & Bollinger 1999; Dingle *et al.* 2010; Ruegg *et al.* 2012; McEntee 2014; Pegan *et al.* 2015). Concordance between 1) genetic and 2) song divergence, along with 3) behavioral discrimination between lineage-specific songs suggests that songs have the potential to maintain, if not drive reproductive isolation. However, few studies test for an association among all three components, and fewer studies place these patterns in the context of evolutionary history. This context can help us understand how behavioral isolating barriers function in the transition from populations to species (Coyne & Orr 2004; Edwards *et al.* 2005).

Although recently diverged lineages offer a window into the speciation process (Hewitt 1988), their evolutionary history can be problematic to determine (Durrett *et al.* 2000; Pettengill & Moeller 2012). Historical demographic inference based on the coalescent can be used to estimate the relative time since divergence between lineages, which may indicate the role of historic ecological or biogeographic processes (e.g. Hickerson *et al.* 2006), as well as to calculate the degree of historical gene flow between current lineages (e.g. Carling *et al.* 2010; Field *et al.* 2011). Model-based approaches employing coalescent-based analyses of multilocus sequence data can also test alternative hypotheses of evolutionary histories (Rosenberg & Nordborg 2002; Gutenkunst *et al.* 2009; Excoffier *et al.* 2013), which can provide a framework for interpreting reproductive barriers. Examples of evolutionary histories for recently diverged lineages include primary divergence with either strict isolation or ongoing symmetrical or asymmetrical migration, migration after a period of allopatric divergence (e.g., secondary contact), and panmixia. Differentiating between primary divergence and secondary contact can provide information on whether behavioral divergence could have occurred in allopatry. If evidence of asymmetric gene flow between taxa coincides with a pattern of asymmetric recognition of songs, then this pattern would provide support for song as an incomplete behavioral barrier (e.g. Halfwerk *et al.* 2016). In comparison, concordance between symmetric song discrimination and secondary contact with reduced gene flow would suggest song acting as a behavioral barrier. A supported model of panmixia, on the other hand, would indicate a limited role for reproductive isolation between lineages (e.g. Oomen *et al.* 2011).

Here, we investigate whether song is a reproductive isolating mechanism in an oscine species widely studied for song evolution: the white-crowned sparrow (*Zonotrichia leucophrys*). The question of whether song is a behavioral barrier to gene flow has been asked in the white-crowned sparrow for decades (Baker 1975; Baker *et al.* 1984; Baker & Cunningham 1985; MacDougall-Shackleton & MacDougall-Shackleton 2001; Soha *et al.* 2004), because of a strong pattern of discrete song types, i.e. dialects, across small geographic scales (Marler & Tamura 1964) with male (Nelson & Soha 2004) and

female (Petrinovich & Patterson 1981) discrimination between dialects. Empirical data for genetic differentiation between cultural dialect populations within subspecies of white-crowned sparrows is mixed (Baker *et al.* 1982 as revisited by Soha *et al.* 2004, MacDougall-Shackleton & MacDougall-Shackleton 2001). However, behavioral studies suggest that examining this question in the context of hybridization between subspecies may lend insight into this question. Experiments with the white-crowned sparrow demonstrate that males have a genetic predisposition to learn the song of their own subspecies, and females have a genetic predisposition to pay closer attention to the song of their own subspecies (Whaling *et al.* 1997; Nelson 2000), suggesting an innate behavioral barrier to gene flow between subspecies despite learned song.

We focus on two subspecies distributed along the western coast of North America: the Puget Sound subspecies (hereafter *Z. l. pugetensis*), and the Nuttall's subspecies (hereafter *Z. l. nuttalli*). *Z. l. pugetensis* is migratory and breeds from northern California to southern British Columbia, whereas *Z. l. nuttalli* is a year-round resident that breeds in coastal central and northern California (Grinnell 1928; Blanchard 1941; Banks 1964). The subspecies are hypothesized to have diverged in glacial refugia during the Pleistocene (Banks 1964; Baker *et al.* 1984), and a putative contact zone exists in northern California (Banks 1964; Mewaldt *et al.* 1968; Corbin & Wilkie 1988). Previous studies on *Z. l. nuttalli* and *Z. l. pugetensis* documented cultural differences (Baker 1987) as well as behavioral discrimination between subspecific songs (Lampe & Baker 1994), but found limited genetic divergence based on allozymes (Corbin 1981; Corbin & Wilkie 1988) and mitochondrial haplotypes (Weckstein & Zink 2001). If the two subspecies are distinct and hybridizing, we expect to find (1) two genetic clusters with admixture between them, as well as (2) support for a historic demographic model of secondary contact. If song functions as an isolating mechanism between the subspecies, we expect to find (3) song divergence between the subspecies, (4) differential male response to subspecific songs, and (5) an association between song divergence, genetic divergence and the strength of discrimination between songs.

Methods

Genetic sampling and sequencing

We sampled 190 individuals from 17 localities along a coastal transect spanning the ranges of *Z. l. nuttalli* and *Z. l. pugetensis* (Figure 4; Table 1). We collected blood samples (20 μ L) by brachial venipuncture from 132 mist-netted males in 2004 and 2005 and released birds after metal banding. We transferred blood to EDTA-saturated filter paper, and stored in airtight containers on DrieRite at room temperature. Our sample also included tissues from 51 vouchered specimens collected in 2010. Voucher specimens are deposited in the Museum of Natural Science at Louisiana State University. We also collected vocal data for these 183 males (see Song recording and analysis). Seven additional samples, including four females, were provided as tissue loans from the Museum of Vertebrate Zoology and the Burke Museum. We extracted total genomic DNA using a DNeasy blood and tissue extraction kit following the manufacturer's recommended instructions (Qiagen, Valencia, CA).

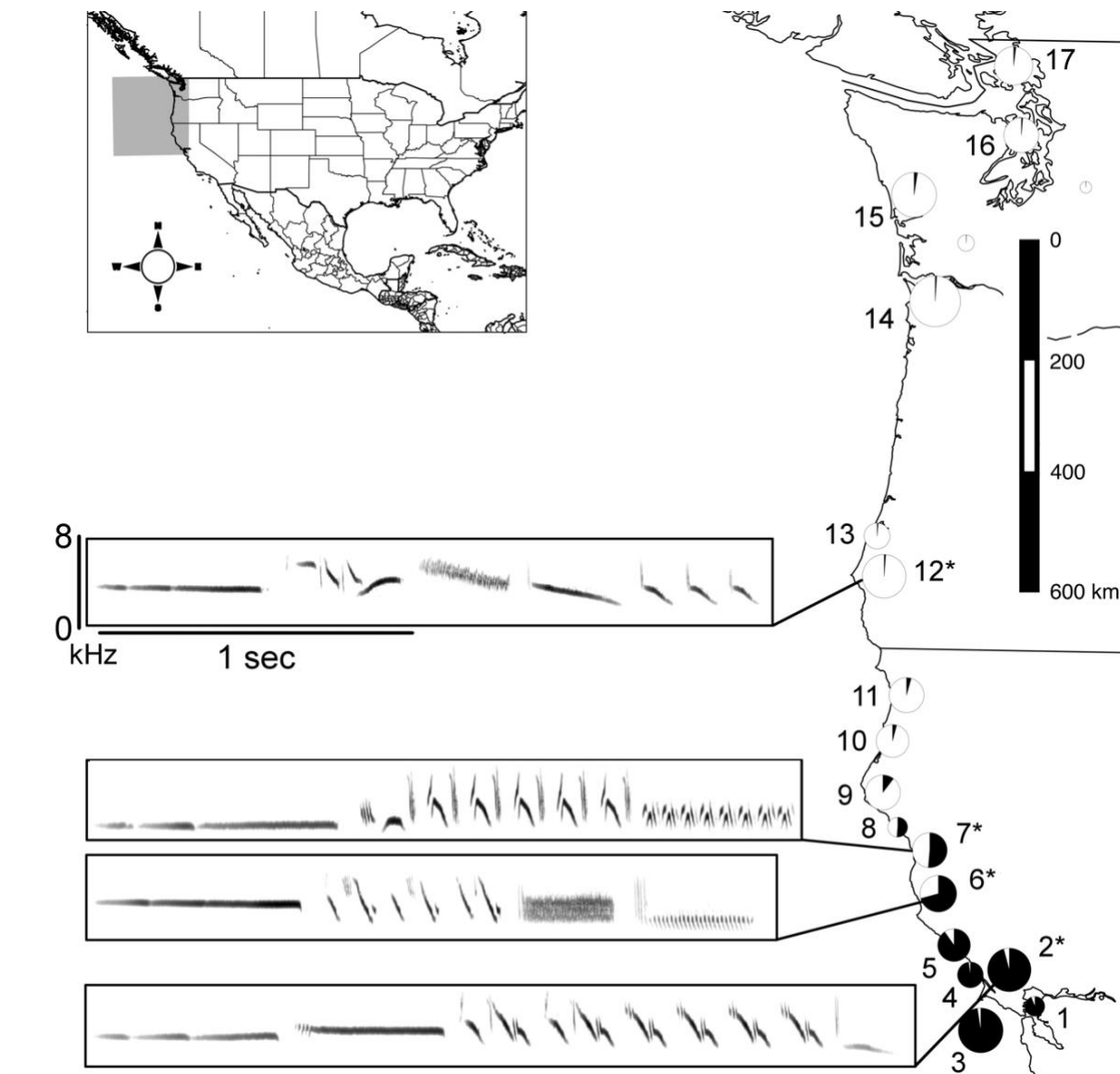


Figure 4. Sampling map of numbered localities along a transect from the southern subspecies *Z. l. nuttalli* to the northern subspecies *Z. l. pugetensis*.

Song spectrograms correspond to the sites of behavioral playback experiments. Pie charts represent admixture proportions from STRUCTURE for each locality and circle size corresponds to the number of individuals selected for sequencing at each site. Gray pie charts indicate two samples collected outside of main localities. Asterisks indicate localities for territorial playback experiments.

Table 1. Sampling information for each locality.

Q values from STRUCTURE refer to mean admixture proportions for individuals in each locality.

Site	Locality	County	State	Latitude	Longitude	Year	Q Value	Song N	Genetic N	Subspecies
1	San Francisco	San Francisco	CA	37.803	-122.478	2005	0.061	12	3	nuttalli
2	Bolinas	Marin	CA	37.908	-122.722	2005	0.024	34	15	nuttalli
3	Schooner Bay	Marin	CA	38.083	-122.914	2004	0.041	9	14	nuttalli
4	Abbotts Lagoon	Marin	CA	38.122	-122.953	2010	0.023	9	5	nuttalli
5	Sonoma	Sonoma	CA	38.419	-123.105	2010	0.098	11	8	nuttalli
6	Manchester	Mendocino	CA	38.981	-123.702	2010	0.292	5	10	admixed
7	MacKerricher	Mendocino	CA	39.489	-123.8	2005	0.487	15	9	admixed
8	Sinkyone	Mendocino	CA	39.832	-123.85	2010	0.487	0	3	admixed
9	Ferndale	Humboldt	CA	40.544	-124.358	2010	0.896	10	9	pugetensis
10	Eureka	Humboldt	CA	40.742	-124.239	2005	0.96	12	8	pugetensis
11	Trinidad	Humboldt	CA	41.258	-124.099	2010	0.957	10	9	pugetensis
12	Bandon	Coos	OR	43.071	-124.435	2005	0.989	12	14	pugetensis
13	Bullards Beach	Coos	OR	43.127	-124.416	2005	0.986	6	5	pugetensis
14	Nehalem	Tillamook	OR	45.684	-123.938	2005	0.99	16	19	pugetensis
	Frances	Pacific	WA	46.557	-123.4		0.995	0	2	pugetensis
15	Ocean Shores	Grays Harbor	WA	46.928	-124.17	2005	0.975	7	15	pugetensis
	Enumclaw	King	WA	47.248	-122.013		0.996	0	1	pugetensis
16	Dosewallips	Jefferson	WA	47.692	-122.895	2005	0.987	6	9	pugetensis
17	San Juan Island	San Juan	WA	48.461	-123.014	2004	0.98	34	11	pugetensis

We sent DNA extracts to the Institute of Genomic Diversity at Cornell University in Ithaca, NY, USA, for genotyping-by sequencing (GBS). GBS reduced-representation libraries were prepared and analyzed according to the methods of Elshire *et al.* (2011) using the restriction enzyme PstI (CTGCAG) for digestion and creating a library with 95 unique barcodes, one for each individual for each plate. Samples were sequenced on two lanes of the Illumina HiSeq platform, generating 551,083,045 reads.

Single-nucleotide polymorphisms (SNPs) were called by processing the raw 100bp single-end sequence reads using the UNEAK pipeline (Lu *et al.* 2013), an extension of the Java program of TASSEL 4.0 (Bradbury *et al.* 2007). Reverse complement tag-pairs were collapsed, and loci with greater than 20% missing data, as well as minor allele frequency less than 1%, were excluded. Samples were defined as failed if the number of sequences produced for that sample was less than 10% of the mean number of sequences for all samples sequenced in that flow cell lane – four failed samples were excluded from further analysis.

The UNEAK pipeline identified a total of 79,130 biallelic SNPs. After filtering in the pipeline, the final data matrix for all 186 individuals contained 1583 SNPs. This reduction in loci was due in part to a handful of individuals with very few reads overall. To address this, a restricted dataset was created by removing individual samples that produced fewer than 500,000 reads. The restricted dataset resulted in 6419 SNPs for 169 individuals, with total missing data (number of missing genotypes per locus per sample) equal to 16.9%. All subsequent analyses were conducted with this restricted dataset.

Population structure analysis

To characterize patterns in genetic structure and assign individuals to populations, we used the program STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000). Structure analyses were performed using a burn-in length of 200,000, and 500,000 MCMC repetitions to test clusters ranging from K=1 to K=20 to allow for sub-structuring within sampling sites, with 10 independent replicates, after which all parameters converged. Parameter settings also included an admixture model of ancestry and correlated allele frequencies. An individual was classified as parental *Z. l. nuttalli* if $Q \leq 0.1$, parental *Z. l. pugetensis* if $Q \geq 0.9$, and admixed if $0.1 < Q < 0.9$. The optimal number of clusters (K value) was calculated using ΔK likelihood evaluations (Evanno *et al.* 2005) in Structure Harvester (Earl & vonHoldt 2012). We used CLUMPP (Jakobsson & Rosenberg 2007) to identify potential multi-modality and account for label switching among replicates and Distruct (Rosenberg 2004) to visual admixture proportions. We recognize that STRUCTURE is merely an exploratory starting point for downstream population-based analyses, and it can be hazardous to read its results as actual inference (Falush *et al.* 2016). Therefore, we also assessed population structure with a model-free method based on multidimensional statistics: principal component analysis (PCA) implemented in the R (R-Core-Team 2015) package adegenet v.2.0 (Jombart & Ahmed 2011). We used the function scaleGEN to scale allele frequencies and replace missing genotype data with the mean allele frequencies. We performed the PCA with the function dudi.pca. For visualization each individual was labeled according to sampling location, allowing us to examine relationships among individuals without *a priori* assumption about subspecies assignment. Alternative methods of analysis (e.g. fastStructure (Raj *et al.* 2014) and DAPC (Jombart *et al.* 2010)) showed qualitatively similar findings and are not presented here.

Inference of historical demographic parameters

To distinguish the demographic history of these populations and to estimate key parameters of interest, such as divergence time and effective population size, we used the composite-likelihood simulation-based approach of fastsimcoal2 (Excoffier *et al.* 2013). We generated the observed joint, folded site frequency spectrum (SFS) using custom python scripts and *∂a∂i* (Gutenkunst *et al.* 2009). *∂a∂i* provides a facility for projecting an SFS from a larger sample size to a smaller sample size. For RAD-like datasets, which often contain significant missing data, projecting down can increase the number of usable SNPs by averaging over resamplings of the larger dataset. We explored multiple possible values for projection, and selected two values for full downstream analysis: one larger (75 x 75) and one smaller (20 x 20), as measured in number of individuals per population. The larger projection was selected to maximize the number of segregating sites. The smaller projection was selected to minimize total size of the SFS without sacrificing demographically important signal in the data, in an effort to explore the tradeoff between computation time and accuracy of inference. We tested five different historical

demographic models for goodness of fit to the observed data: (1) primary divergence with strict isolation, (2) divergence with ongoing symmetrical and (3) asymmetrical migration, (4) migration after a period of allopatric divergence (e.g. secondary contact), and (5) panmixia. For all models we explored two partitions of the data: (1) admixed individuals assigned to subspecies based on admixture proportions from the aggregated STRUCTURE runs for $K = 2$ and (2) only parental individuals by excluding those with admixture proportions between 0.1 and 0.9. We selected wide, uninformative, uniform search ranges for all estimated parameters. We performed 50 independent runs per model, per data partition to obtain likelihood values for the observed data under each given model. For each run we performed 100,000 simulations and 40 expectation-conditional maximization (ECM) cycles. We evaluated model fit with both information theoretic (AIC) and likelihood (LRT) based methods. We generated 95% confidence intervals for demographic parameters of interest using 100 parametric bootstrap replicates. For each bootstrap replicate we simulated a new SFS with the same number of SNPs as our observed data using the maximum likelihood parameters and the best fitting model from the model selection step. We re-estimated parameters across replicates for the simulated SFS and aggregated maximum likelihood parameters. We generated bootstrap confidence intervals using the python package Scikits-Bootstrap (<https://github.com/cgeevans/scikits-bootstrap>).

Song recording and analysis

Within these two subspecies, males produce one stereotyped song type (e.g. dialect). Most males in each location produce the same dialect, and males in different locations produce different dialects. We recorded 208 males defending territories during the breeding season in 16 different localities (we did not have songs for site 8), each with their own unique song dialect, in 2004, 2005, and 2010, with an average of 12 individuals (range = 5–34) per site (Table 1). Song dialects in these localities have been stable over 30 years (Derryberry 2009; Luther & Derryberry 2012), so have not likely changed across this six-year spread in sampling. Recordings were made using a Sony TCM-5000EV cassette recorder, a PRO-302 Unidirectional Dynamic microphone, and a Sony PBR330 parabolic reflector. All songs were digitized with 16-bit precision at a 25 kHz sampling rate using Syrinx 2.2b (Burt 2001) and an Echo Digital Audio sound card. All songs were high pass filtered to eliminate noise below 1500 Hz. We measured 8 acoustic parameters shared by all song types: song maximum and minimum frequencies (Hz); the dominant (peak) frequency of the whistle; the duration of the whistle, the introduction, and the average syllable duration; and the rate of trill note delivery and frequency bandwidth of the trill. All measurements were taken using Signal version 3.1 or 5 (Beeman 1999). We took minimum and maximum frequency measurements at -36 dB relative to the peak amplitude frequency in the song from digital spectrograms (256 pt transform, frequency resolution = 97.7 Hz). We calculated frequency bandwidth as the difference between the maximum and minimum frequencies. We measured dominant frequency as the frequency at which the most sound energy was transmitted during production of the relevant song section from a smoothed power spectrum (smoothing resolution 100 points). Temporal variables were measured from oscillograms (time waveforms). Trill rate was calculated as the number of notes produced per second. Following Podos (2001), we calculated a ninth acoustic parameter, vocal performance, as the orthogonal distance between each song and an upper-bound regression for the plot of trill frequency bandwidth as a function of trill rate for 375 white-crowned sparrow songs from 15 different dialects that has been shown to be robust to different methods for estimating the performance trade-off between bandwidth and trill rate (Derryberry 2009; Wilson *et al.* 2014). Songs closer to the limit are higher performance. All raw song data were transformed to a scale with a mean of 0 and a standard deviation of 1 (a z-score) to allow for scale free comparisons.

We assessed if song acoustic parameters could be used to distinguish the two subspecies using two approaches. First, we ran a discriminant function analysis (DFA) of the individual song variables using JMP v.12 (Sall 2015). Songs from genetically admixed individuals were classified to subspecies based on their admixture proportions from STRUCTURE. Next, we summarized 7 of the acoustic parameters using a PCA in JMP. We excluded trill rate and frequency bandwidth from the PCA, as these were used to calculate vocal performance. This yielded four independent factors with an eigenvalue greater than 1, explaining a total of 75% of the original song variation (Table 2). To assess whether songs have diverged between subspecies, we used a linear mixed model approach with locality ($n=16$) as a random effect and subspecies as the predictive factor.

Table 2. Factor loadings for the first five principal components (eigenvalue > 1) derived from song variables.

Bold values indicate those with high loadings.

Song Parameter	PC1	PC2	PC3	PC4
Eigenvalue	1.27	1.21	1.07	1.03
Percent variation	23	21	16	15
Whistle length (ms)	0.59			
Avg. note length (ms)	-0.59	0.24	0.15	
Song maximum frequency (Hz)	0.18	0.66	-0.17	-0.16
Song minimum frequency (Hz)	0.2	-0.45	-0.52	-0.39
Whistle dominant frequency (Hz)	-0.41		-0.21	-0.69
Introduction length (ms)	-0.26	-0.28	-0.46	0.56
Vocal performance		-0.46	0.65	-0.17

Territorial playback experiment

To test subspecies-specific discrimination among songs, we measured the response of free-living, territorial adult males using territorial playbacks, a standard experimental design that quantifies male response to simulated intrusion on their territories (McGregor *et al.* 1992). We conducted playback experiments in two parental localities (sites 2 and 12) and two admixed localities (sites 6 and 7), hereafter referred to as playback localities. Playbacks were conducted between May and June in 2010 (sites 2, 7, 12) and in 2013 (site 6), when males were actively defending breeding territories. Focal males were not genotyped.

Stimuli were presented in a paired, balanced design. In each parental locality we assessed male response to songs from their own location ('own') and to songs from a parental locality of the other subspecies ('other'), and in each admixed location we assessed male response to 'own' local song versus 'other' for each of the two subspecies. We used 10 exemplars for each song category and tested 10 males for each comparison. Each male heard a different pair of exemplars to avoid pseudoreplication (Kroodsma 1990; Kroodsma *et al.* 2001). Order of presentation and selection of exemplars were randomized across males. Stimuli from site 2 were selected from recordings made in 2004, sites 7 and 12 from 2005, and site 6 from 2010. Adult males in the wild live an average of 16 months (Cortopassi & Mewaldt 1965). Thus, it is unlikely that males tested were familiar with the males that produced the stimulus songs.

We separated treatments by 48 hours to minimize habituation and did not test neighbors on the same day. Songs were amplitude normalized and broadcast at a constant level (80 – 82 dB SPL 1m) and at a natural rate of six songs per minute from a speaker (Altec Lansing IMT320 inMotion) near the center of the focal male's territory as determined by behavioral observations. We measured four response variables: mean distance from the speaker during the (1) 3-min playback period and the (2) 3-min post-playback period, (3) number of flights over the speaker during the playback period and (4) song rate (songs/min) during the playback period. To facilitate accurate measures of distance, we placed markers at 4 meters and 8 m on either side of the speaker before the trial began. Distances and observed behaviors were recorded at 10-second intervals. Males were considered to have a stronger response to the stimulus when they approached the speaker more closely (Searcy *et al.* 2006), flew over the speaker more often, and produced songs at a higher rate. One observer narrated observations while another recorded the observations onto datasheets. Experiments were not blind to stimulus type.

For each experiment, we reduced the four behavioral response measures using PCA and used the PC scores in statistical testing in R (R-Core-Team 2015). The original behavioral variables were not

statistically independent (Rice 1989) and separate tests would not reflect the multivariate nature of the males' responses (McGregor *et al.* 1992). Playback and post-playback distance were highly correlated, so we calculated an average distance to include in PCAs. We retained PCs with an eigenvalue greater than 1 for analyses, or the minimum number of PCs required to explain 50% or more of the variance in the original variables (Table 3). To test whether males discriminated between 'own' and 'other' within each locality, we analyzed paired comparisons separately using Wilcoxon signed-rank tests on the respective PCs. Repeated measures MANOVAs were then used to test for differences in male response due to (1) locality and (2) genetic status (putative parental vs. putative admixed). Joint analyses standardized the post-playback data to the first 3-min. For combined analyses using MANOVAs, male response was normally distributed (all Shapiro-Wilk $P > 0.25$) and equal in variance (all Leven's $P > 0.18$). Effect size (Cohen's d) and power to reject a false null hypothesis were also determined (Cohen 1988). We calculated the power for our given N , effect sizes (estimated from means and standard deviations), and alpha level of 0.05 using the G* Power 3.1 (Faul *et al.* 2009) for Wilcoxon signed-rank tests (matched pairs).

Song dissimilarity, behavioral response, and F_{st}

We asked whether variation in male response to 'own' versus 'other' song was explained by acoustic dissimilarity, as well as pairwise F_{st} between the parental (2 and 12) and admixed (6 and 7) playback locations. We calculated a dissimilarity score between all songs used for playback analyses using the dynamic time warping function in Luscinia v.2.02.10.15 (Lachlan 2007). This function searches for the optimal alignment of two signals and then calculates a dissimilarity score based on temporal and spectral characters. For each playback experiment, we averaged song dissimilarity scores and the response difference to 'own' vs. 'other'. We calculated genetic distance among the playback localities using a pairwise F_{st} matrix in Arlequin v3.5 (Excoffier & Lischer 2010) based on the 6419 SNP dataset (Table 4). We then used linear regression to compare male response to song dissimilarity and male response to genetic distance in R (R-Core-Team 2015).

Multiple matrix regression with randomization

To quantify the independent contributions of song and geographic distance on genetic differentiation, we used a multiple matrix regression with randomization (MMRR) (Wang 2013). Unlike a partial Mantel, MMRR uses a randomized permutation procedure to account for non-independence between variables (Wang 2013). We quantified isolation by song (IBS) and isolation by distance (IBD) with the "MMRR" function in R for 10,000 permutations, using pairwise genetic distance as the response variable and geographic and song distances as the explanatory variables. We calculated genetic distance among locations using a pairwise F_{st} matrix in Arlequin v3.5, based on the 6419 SNP dataset (Excoffier & Lischer 2010). We calculated the geographic distance matrix from GPS coordinates using the "earthdist" function in the R package fossil (Vavrek 2011). We calculated the song distance matrices for each of the 9 song parameters as well as song PC1 using the "write.matrix" function in the R package MASS (Venables & Ripley 2002). We excluded Sinkyone (site 8; Table 1) from analyses because we did not have song data.

Results

Population structure

The optimal number of populations in the Bayesian assignment probability analysis using STRUCTURE for all replicates was $K=2$, based on the Evanno method, with $\Delta K = 922.63$ (Figure 4). Increasing K did not provide a better explanation of the data as measured by log likelihood. Based on admixture proportions (Q values), individuals from localities 1 – 5 were confidently assigned to the southern subspecies (*Z. l. nuttalli*), localities 6 – 8 were admixed, and localities 9 – 17 were assigned to the northern subspecies (*Z. l. pugetensis*). For the PCA, we found two separate clusters corresponding to the two subspecies (Figure 5). The admixed MacKerricher (site 7) and Sinkyone (site 8) samples were distributed between the two clusters, whereas Manchester (site 6) samples did not cluster with the rest of the *Z. l. nuttalli* samples.

Table 3. Factor loadings for the principal components (eigenvalue > 1) derived from behavioral responses to individual playback experiments and all experiments standardized and combined.

Experiments (Own vs. Other)	Response variables	PC1	PC2
Bolinás (2) vs. Bandon (12)	Average distance	-0.76	
	Song rate	0.87	
	Fly overs	0.65	
	Eigenvalues	1.75	
	Cum. Percent Variance	58.5	
Manchester (6) vs. Bolinás (12)	Average distance	0.02	0.99
	Song rate	-0.76	0.05
	Fly overs	0.76	0.01
	Eigenvalues	1.17	1
	Cum. Percent Variance	39	72.4
Manchester (6) vs. Bandon (12)	Average distance	-0.78	0.47
	Song rate	0.89	0.1
	Fly overs	0.3	0.92
	Eigenvalues	1.5	1.08
	Cum. Percent Variance	50	86
MacKerricher (7) vs. Bolinás (2)	Average distance	-0.83	
	Song rate	0.63	
	Fly overs	0.73	
	Eigenvalues	1.62	
	Cum. Percent Variance	54	
MacKerricher (7) vs. Bandon (12)	Average distance	-0.83	
	Song rate	0.8	
	Fly overs	0.71	
	Eigenvalues	1.8	
	Cum. Percent Variance	61	
Combined Own vs. Other	Average distance	-0.8	
	Song rate	0.79	
	Fly overs	0.63	
	Eigenvalues	1.7	
	Cum. Percent Variance	55	

Table 4. Pairwise Fst matrix based on the 6419 SNP dataset, not including locality 8.

Site	Locality	1	2	3	4	5	6	7	9	10	11	12	13	14	15	16	17
1	San Francisco	0															
2	Bolinas	-0.035	0														
3	Schooner Bay	-0.023	-0.019	0													
4	Abbotts Lagoon	-0.051	-0.028	-0.002	0												
5	Sonoma	-0.046	-0.029	-0.026	-0.027	0											
6	Manchester	-0.035	-0.022	-0.008	-0.028	-0.028	0										
7	Mac-Kerricher	0	-0.017	-0.001	0.009	-0.003	-0.015	0									
9	Ferndale	-0.058	-0.068	-0.045	-0.069	-0.058	-0.055	-0.07	0								
10	Eureka	0.008	-0.008	0.007	0.008	-0.008	-0.003	-0.005	-0.081	0							
11	Trinidad	0.005	-0.016	-0.001	-0.01	-0.011	-0.014	-0.001	-0.075	-0.032	0						
12	Bandon	-0.001	-0.002	0.024	-0.003	0	-0.002	0.015	-0.07	-0.027	-0.032	0					
13	Bullards Beach	0.006	0	0.017	-0.005	-0.002	0.002	0.007	-0.064	-0.018	-0.015	-0.017	0				
14	Nehalem	0.014	0.011	0.022	0.014	0	-0.004	0.008	-0.055	-0.023	-0.018	-0.012	-0.008	0			
15	Ocean Shores	-0.005	-0.012	-0.001	0.006	-0.008	0.003	-0.004	-0.064	-0.033	-0.028	-0.016	-0.008	-0.004	0		
16	Dose-wallips	0.007	0.002	0.021	0.005	0.002	0.02	0.021	-0.03	-0.01	-0.012	-0.004	0	-0.003	-0.011	0	
17	San Juan Island	-0.003	-0.013	-0.003	0.001	-0.008	-0.003	0.014	-0.06	-0.021	-0.023	-0.01	-0.001	-0.006	-0.011	-0.01	0

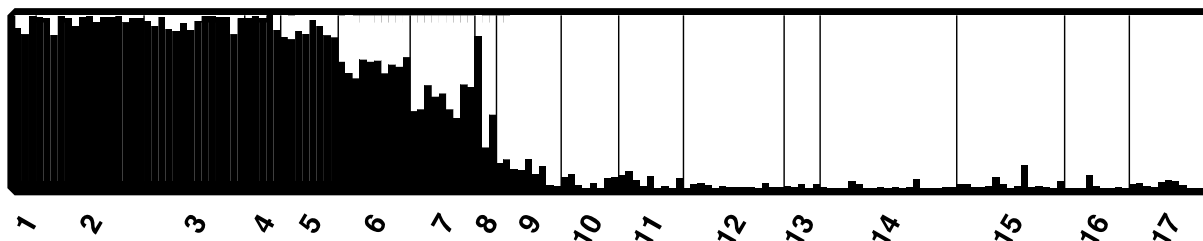


Figure 5. Probability of assignment to *Z. l. nuttalli* (black) and *Z. l. pugetensis* (white) as determined from a STRUCTURE analysis using 6419 SNPs for K = 2 across 17 localities.

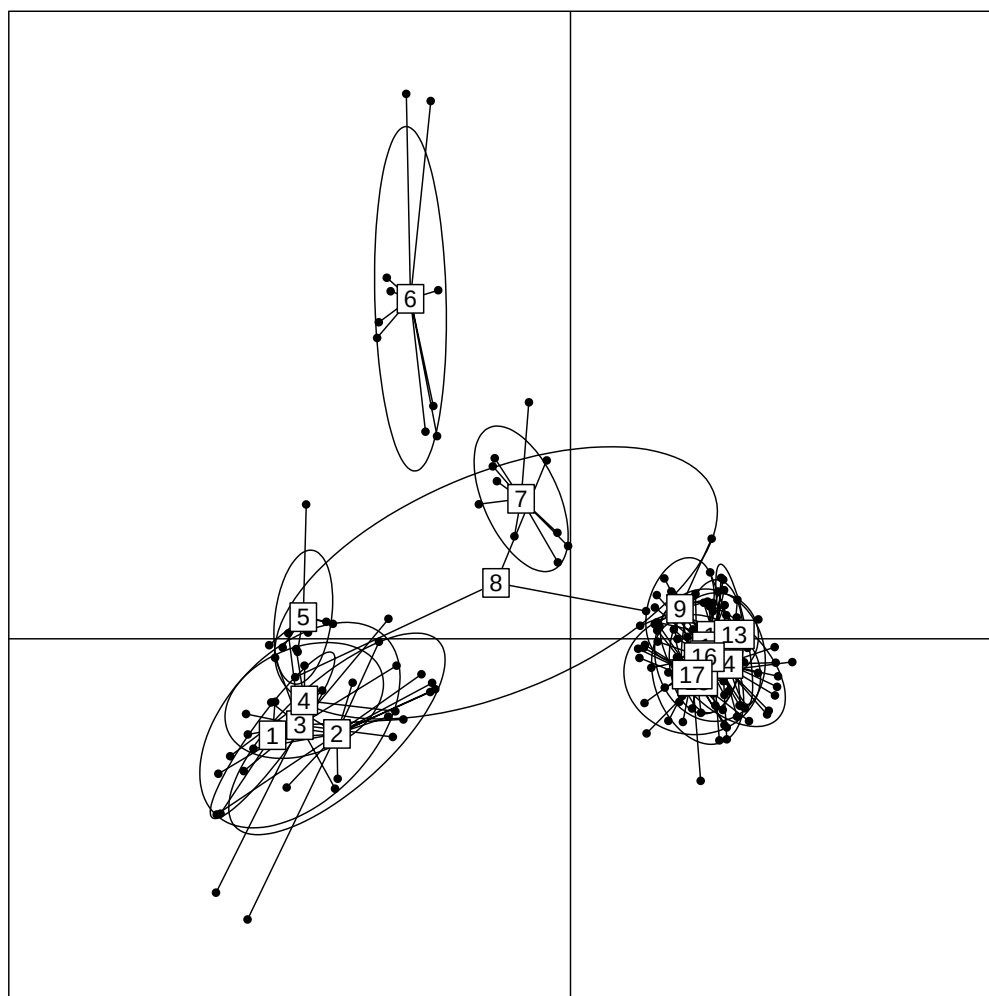


Figure 6. Inter-class principal component analysis of 6419 loci dataset for 17 sampling localities.

Historical demographic inference of secondary contact

Including admixed individuals in the dataset consistently increased the fit of all models; therefore, we report only the results including admixed individuals in the data matrix. Likewise, the reduced projection dataset provided a poor fit for all the models, so we report results only of the larger projection. All model comparison criteria decisively supported the secondary contact model over models of continuous symmetric or asymmetric migration, indicating that some period of isolation was important in establishing the divergence between these subspecies (Figure 7, see Table 5 for a comparison of all models). However, we caution here that we could not possibly evaluate all historical scenarios, and therefore cannot fully reject a model of primary differentiation. Our parameter estimates indicate incomplete isolation beginning during the last glacial cycle (~45kya), followed by a short period of complete isolation after the Last Glacial Maximum (LGM) (~9kya), and secondary contact only relatively recently (~2kya). Recent migration rates (~10 individuals per generation) were on the order of 5x higher than the migration rate between the time of initial divergence and isolation (~2 individuals per generation), indicating a period of drastically reduced connectivity (Table 6).

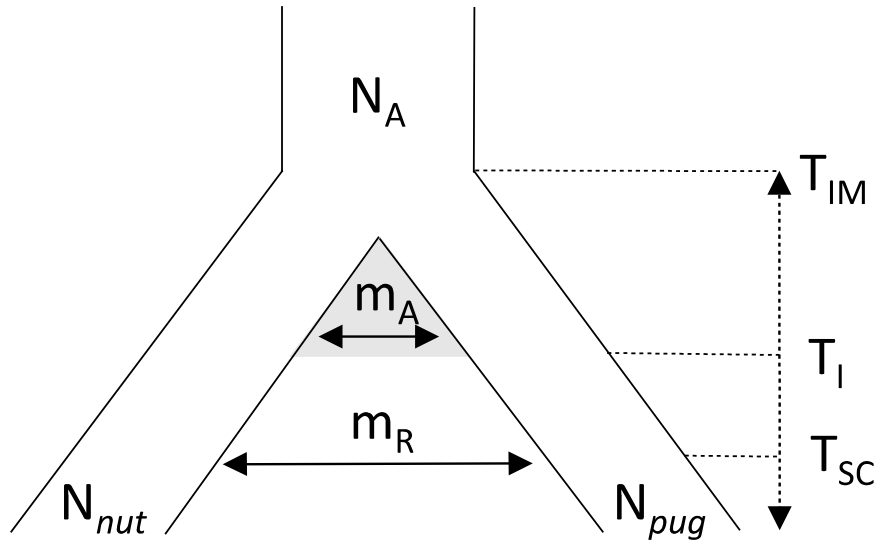


Figure 7. Best supported demographic model of secondary contact.

T_{IM} indicates time of isolation with migration, T_I indicates time of isolation, and T_{SC} indicates time of secondary contact. N_A indicates effective population size of ancestral population, N_{nut} indicates effective population size for *Z. l. nuttalli*, and N_{pug} indicates effective population size for *Z. l. pugetensis*. m_A indicates ancestral migration rate and m_R indicates recent migration rate.

Table 5. AIC values for historical demography models.

Model	Free Parameters	Best Likelihood	CLR	AIC	Δ AIC	Relative Likelihood	AIC Weight	AICc
Secondary Contact	9	-14202.489	0.006	28422.978	0	1	1	28242.978
Symmetric Migration	6	-14311.629	0.009	28635.258	212.28	0	0	28551.258
Asymmetric Migration	5	-14344.907	0.01	28699.814	276.836	0	0	28639.814
No Migration	2	-14470.354	0.014	28944.708	521.73	0	0	28932.708
Panmixia	1	-15579.421	0.046	31160.842	2737.864	0	0	31156.842

Table 6. Maximum likelihood parameter point estimates and 95% bootstrap confidence intervals for the secondary contact model.

Parameter	Max Likelihood Point Estimate	Bootstrap Values		
		Median	Lower 95%	Upper 95 %
Ancestral N_e	74422	54872	49553	67125
<i>Pugetensis</i> N_e	7478	35096	15114	63980
<i>Nutalli</i> N_e	8802	17785	7779	31860
Ancient migration rate	0.00013	0.00006	0.00003	0.00013
Recent migration rate	0.00078	0.00374	0.00289	0.00506
Time of initial isolation with migration	45372	45324	40585	63789
Time of full isolation	9142	6225	8447	37283
Time of secondary contact	2281	6139	8431	36695

Song variation between subspecies

Songs have diverged between subspecies, although not in all parameters (see factor loadings for the first four PCs derived from song parameters in Table 2). In general, *Z. l. nuttalli* produce songs with longer whistles and shorter other notes. Using all acoustic parameters, all but two songs (1%) were correctly classified to subspecies by a DFA. A forward, stepwise DFA revealed that the best variable to distinguish between the subspecies was whistle length. Whistle length had a jack-knifed classification accuracy of 80%; adding song minimum frequency and note length increased accuracy to 90%. Using DFA, songs from admixed individuals in Manchester (site 6) and MacKerricher (site 7) were classified as *Z. l. nuttalli*. A linear mixed model revealed that genetic subspecies identity was a significant predictor of song structure for PC1 ($F=22$, $DF=1$, $P<2.29\times10^{-6}$) but not for PC2-4 (PC2: $F=0.1$, $DF=1$, $P=0.8$; PC3: $F=0.6$, $DF=1$, $P=0.44$; PC4: $F=0.4$, $DF=1$, $P=0.5$). Plotting song PC1 against geographic distance illustrates that the subspecies have diverged in song, and the songs of admixed individuals group with *Z. l. nuttalli* (Figure 8).

Behavioral response to playbacks

Coding of playback populations as parental in Bolinas (site 2) and Bandon (site 12), and as admixed in Manchester (site 6) and MacKerricher (site 7), was corroborated by genetic clustering analyses (see *Results* Population structure, Table 1). Note that admixture proportions in Manchester (site 6) are 71% *Z. l. nuttalli* and in MacKerricher (site 7) are 51%. Within the two parental playback localities (sites 2 and 12), males responded more strongly to their local song than to the song of the other subspecies (site 2: PC1: $S=-25.5$, $P<0.0059$, effect size Cohen's $d=1.5$; site 12: PC1: $S=-23.5$, $P<0.0137$, $d=0.92$; Table 7, Table 8). In the two admixed playback localities (sites 6 and 7), males gave equal responses to local and non-local songs of their more genetically similar subspecies, *Z. l. nuttalli* (site 6: PC1: $S=3.5$, $P<0.78$, $d=0.27$, PC2: $S=1.5$, $P<0.92$, $d=0.22$; site 7: PC1: $S=-12.5$, $P<0.23$, $d=0.22$; Figure 9, Table 5), but responded more strongly to local song than to *Z. l. pugetensis* song (site 6: PC1: $S=-21.5$, $P<0.0273$, $d=0.86$, PC2: $S=1.5$, $P<0.92$, $d=0.09$; site 7: PC1: $S=-21.5$, $P<0.0273$, $d=0.99$; Table 7, Table 8).

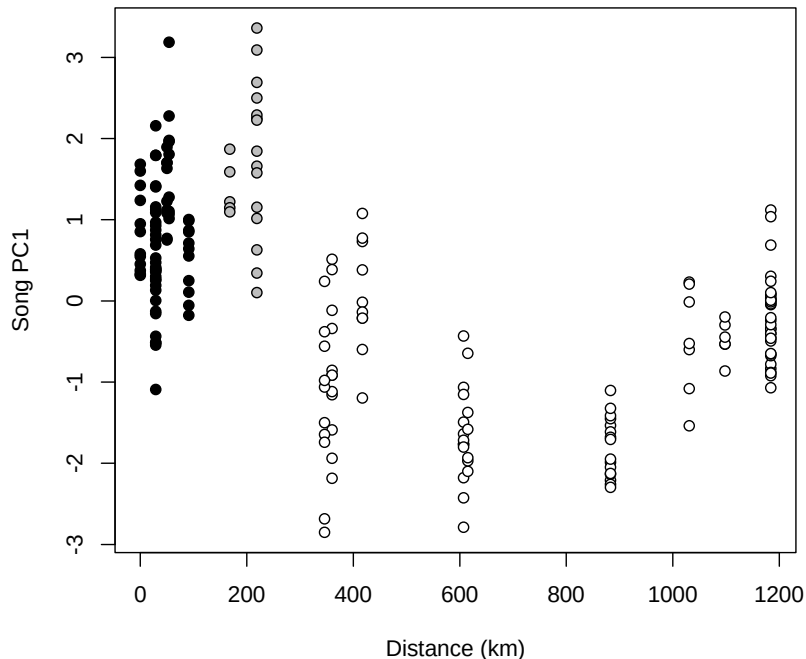


Figure 8. Plot of song PC1 for *Z.l. nuttalli* (black), admixed individuals (grey), and *Z.l. pugetensis* (white) across hybrid zone transect.

Table 7. Mean $\pm$ standard deviation for behavioral responses to individual playback experiments and all experiments standardized and combined.

Experiments (Own vs. Other)	Response variables	Mean $\pm$ SD	
		Own songs	Other songs
Bolas (2) vs. Bandon (12)	Average distance	11.8 $\pm$ 7.0	18.1 $\pm$ 9.2
	Song rate	6.9 $\pm$ 2.9	4.1 $\pm$ 2.3
	Fly overs	6.2 $\pm$ 4.8	1.5 $\pm$ 1.3
Manchester (6) vs. Bolas (2)	Average distance	6.6 $\pm$ 3.7	7.5 $\pm$ 3.9
	Song rate	7.1 $\pm$ 2.3	5.0 $\pm$ 2.5
	Fly overs	7.4 $\pm$ 4.2	5.9 $\pm$ 3.5
Manchester (6) vs. Bandon (12)	Average distance	6.3 $\pm$ 5.5	10.9 $\pm$ 8.1
	Song rate	7.5 $\pm$ 1.9	5.1 $\pm$ 3.3
	Fly overs	5.8 $\pm$ 3.7	5.4 $\pm$ 3.1
MacKerricher (7) vs. Bolas (2)	Average distance	6.5 $\pm$ 9.2	9.6 $\pm$ 8.9
	Song rate	2.3 $\pm$ 2.1	4.2 $\pm$ 1.8
	Fly overs	4.7 $\pm$ 4.3	2.2 $\pm$ 2.5
MacKerricher (7) vs. Bandon (12)	Average distance	3.3 $\pm$ 2.1	12.7 $\pm$ 10.6
	Song rate	6.5 $\pm$ 3.7	4.5 $\pm$ 3.6
	Fly overs	5.5 $\pm$ 3.9	3.3 $\pm$ 3.6
Combined Own vs. Other	Average distance	8.0 $\pm$ 6.4	11.26 $\pm$ 7.61
	Song rate	7.1 $\pm$ 5.4	5.4 $\pm$ 3.4
	Fly overs	5.2 $\pm$ 4.1	3.0 $\pm$ 3.0

Table 8. Results of statistical comparisons of response to 'own' vs. 'other' for each pairwise comparison using Wilcoxon sign-ranked tests.

Significant *P* values indicated by a (*), and *d* is the post hoc calculated effect size.

Experiments (Own vs. Other)	Response variable	N	S	P	d
Bolas (2) vs. Bandon (12)	PC1	10	-25.5	0.0059*	1.5
Manchester (6) vs. Bolas (12)	PC1	10	3.5	0.78	0.27
	PC2	10	1.5	0.92	0.22
Manchester (6) vs. Bandon (12)	PC1	10	-21.5	0.0273*	0.86
	PC2	10	1.5	0.92	0.09
MacKerricher (7) vs. Bolas (2)	PC1	10	-12.5	0.23	0.22
MacKerricher (7) vs. Bandon (12)	PC1	10	-21.5	0.0273*	0.99
Bandon (2) vs. Bolas (12)	PC1	10	-23.5	0.0137*	0.92

In testing whether male response to own versus other song varied among playback localities, the locality main effect was significant ($F_{3,56} = 4.16$, $P = 0.0099$), indicating that males from the four localities (sites 2, 6, 7 and 12) varied in their overall level of response to simulated intruders. The song main effect was also significant ($F_{1,56} = 40.1$, $P = 0.0001$), indicating that males responded more strongly to simulated territorial intruders with their own song than intruders with songs from other locations. The interaction term for this test was not significant ($F_{1,56} = 0.42$, $P = 0.42$), indicating that males in all locations gave a stronger response to 'own' than to 'other' songs.

In testing whether male response to 'own' vs. 'other' varied according to whether the subject males are from parental or admixed populations, the genetic status main effect was significant ($F_{1,58} = 10.35$, $P = 0.0021$), indicating that putative admixed males responded more strongly to territorial intruders than did putative parental males (Figure 9). Consistent with the previous model, the song main effect was also significant ($F_{1,58} = 41.3$, $P = 0.0001$), indicating that overall males responded more strongly to their 'own' than to the 'other' song phenotype. The interaction term for this test was not significant ($F_{1,58} = 2.69$, $P = 0.11$), indicating that both putative parental and putative admixed males showed similar levels of discrimination between 'own' and 'other' songs, although there was a trend towards weaker discrimination among putative admixed males (Figure 9).

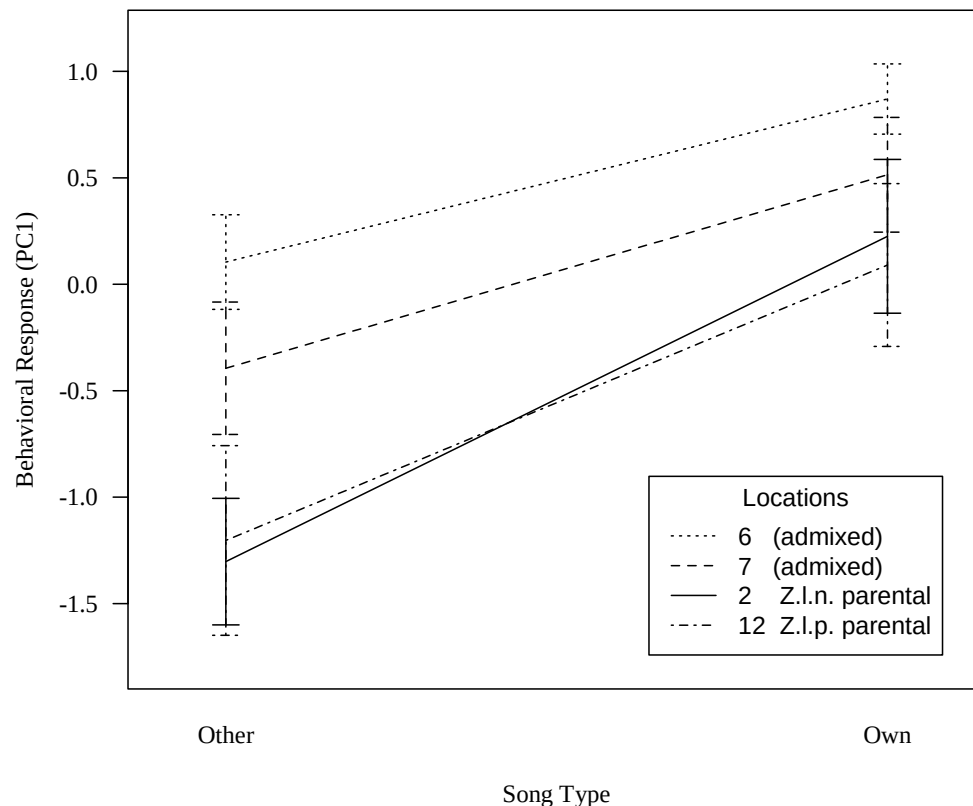


Figure 9. Results of territorial playback experiments comparing male responses to their 'own' songs and the songs of the 'other' subspecies in four localities along a hybrid zone transect.

Localities (from south to north): 2. Bolinas (solid line), 6. Manchester (dotted lines), 7. MacKerricher (dashed lines), and 12. Bandon (dash dot lines). Larger values of PC1 indicate a stronger behavioral response to simulated territorial intrusion. A steeper slope indicates a stronger discrimination between local and foreign stimuli. Bars represent standard error.

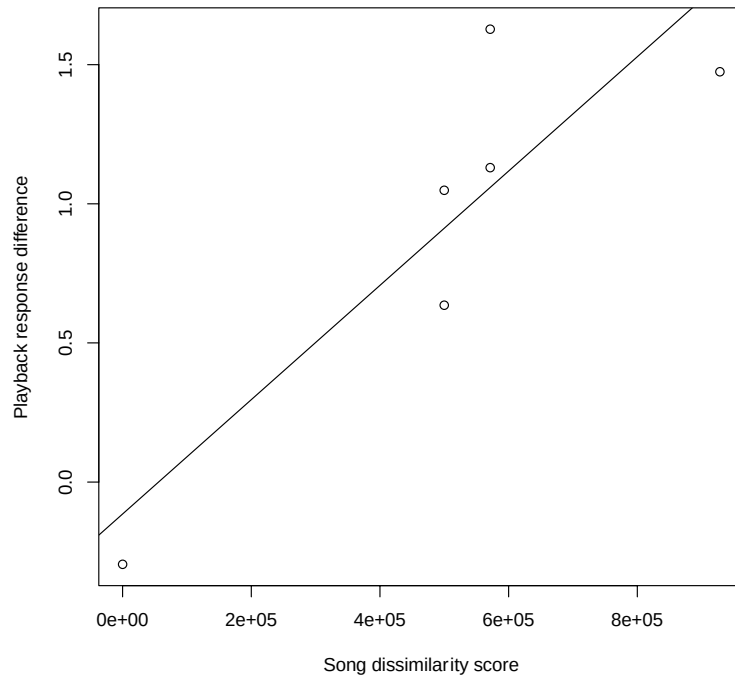


Figure 10. Individuals discriminated more strongly between songs when songs were more dissimilar.

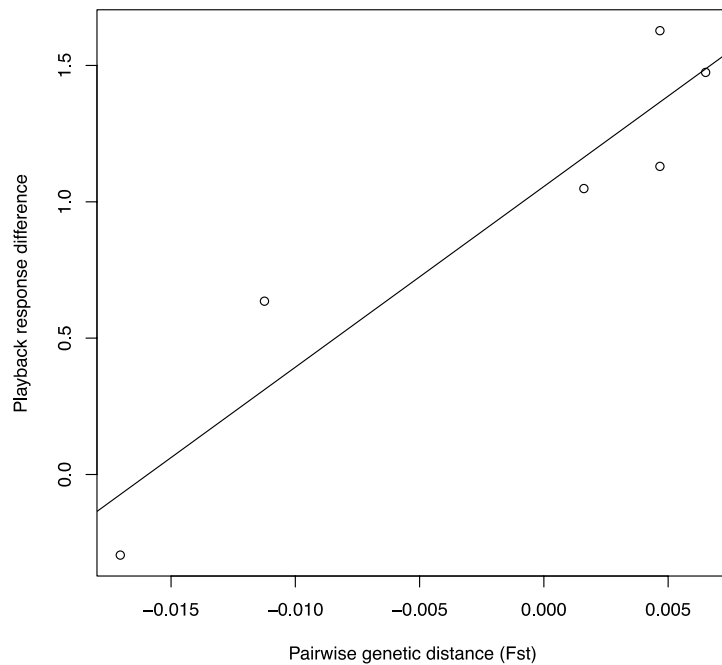


Figure 11. Individuals discriminated more strongly between their local song and the song of the other subspecies when the pairwise genetic distance was greater.

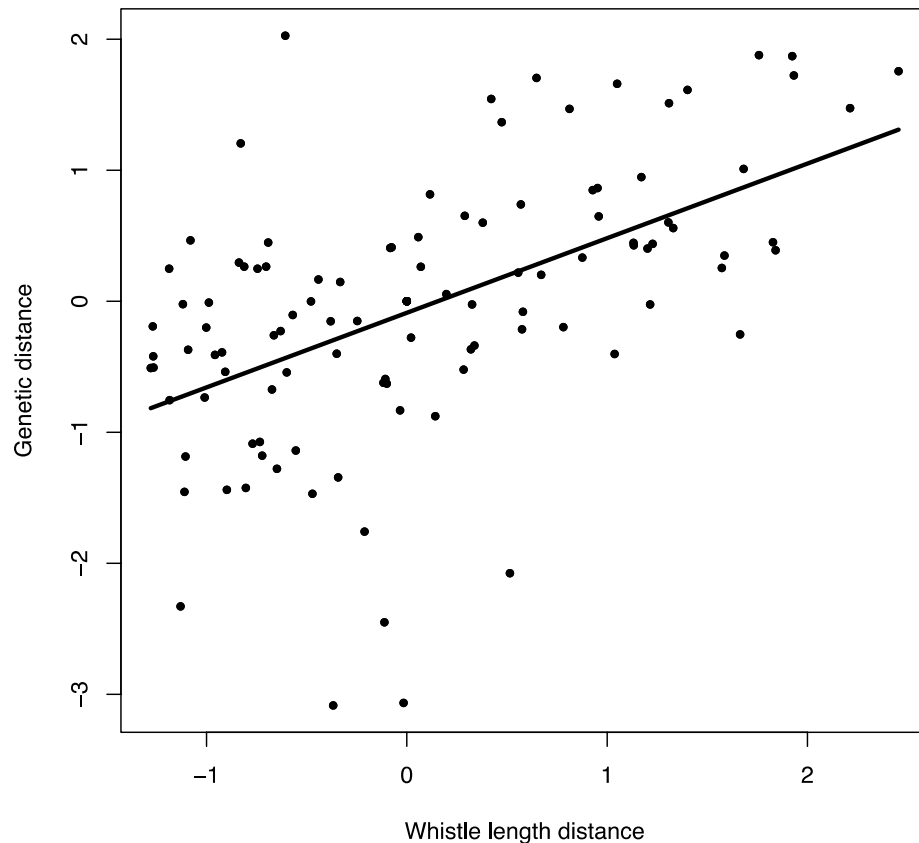


Figure 12. Multiple matrix regression with randomization (MMRR) analysis performed on genetic and song distance.

Isolation by Song

Whistle length and song PC1, which included whistle length and average note length as significant loadings, were both stronger predictors of genetic distance than geography across localities. The regression coefficient for song PC1 distance ($\beta_S = 0.51$, $3 p = 0.0001$) was over twice as large as the regression coefficient for geographic distance ($\beta_D = 0.2$, $p = 0.12$), and the regression coefficient for whistle length distance ($\beta_S = 0.53$, $p = 0.0002$) was over seven times as large as the regression coefficient for geographic distance ($\beta_D = 0.075$, $p = 0.63$), suggesting that isolation by song explained genetic distance more strongly than isolation by distance for these parameters (Figure 12)

Discussion

Overall we found acoustic, behavioral, and genetic evidence that *Z. l. nuttalli* and *Z. l. pugetensis* are distinct evolutionary units and that song is acting as a barrier to gene flow between them. Historical demographic inference suggests that the subspecies diverged relatively recently, and subspecific differences in song have been maintained in the face of hybridization. Putative parental individuals discriminate between the two subspecies based on song, as do putative admixed individuals. We found strong evidence that as songs become more dissimilar, males respond less to these songs in an important functional context: territory defense. There was some evidence that song is a weaker barrier in

the hybrid zone, as putative admixed individuals tended to discriminate less strongly between songs of the two subspecies than did putative parental individuals, though the effect was not statistically significant. On further examination of song divergence, we found that whistle length – an important species recognition cue in song learning in this species – may also function in subspecies recognition.

Our genetic clustering analyses found that *Z. l. nuttalli* and *Z. l. pugetensis* are two distinct genetic populations. This contrasts with previous attempts that could not distinguish the subspecies using allozyme or mitochondrial loci (Corbin 1981; Weckstein & Zink 2001). In both our study and others, pairwise genetic distances among localities both within and between the subspecies were low (Corbin 1981; Zink & Barrowclough 1984). While these two subspecies are genetically distinct in allopatry, population assignment tests revealed genetically admixed individuals at three localities in the hybrid zone. Our simulation-based demographic analyses suggest that this admixture could be explained by secondary contact, with glacial refugia during the last glacial maximum a plausible mechanism for allopatric differentiation. However, further testing of more complex models with selection is warranted in future studies (Roux *et al.* 2016). While many studies of individuals with intermediate admixture proportions assume that taxa are exchanging genes in secondary contact, few explicitly test this model against other evolutionary scenarios (Payseur & Rieseberg 2016, but see Nadachowska-Brzyska *et al.* 2013). Coalescent simulations are a powerful tool for investigating the history of populations, but there are several drawbacks. For one, it can be computationally demanding for genome-scale data, especially as sample size and model complexity increase. Additionally, the stochastic nature of the coalescence process introduces some uncertainty into the estimated demographic parameters (Terhorst & Song 2015). Finally, we interpret these analyses with the caveat that although our results supported secondary contact, it is impossible to evaluate all possible historical scenarios of differentiation.

Songs were divergent between *Z. l. nuttalli* and *Z. l. pugetensis*. The best parameters to distinguish between the subspecies were whistle length and average note duration, and *Z. l. nuttalli* had longer whistles and shorter durations of other notes. Putative admixed individuals from Manchester (6) and MacKerricher (7) had songs more similar to *Z. l. nuttalli*, and individuals from Manchester (6) were also more genetically similar to this subspecies. The pattern of song divergence may provide insight into the features of song that individuals use to discriminate between subspecies. We found that whistle length was the best song parameter to distinguish between the subspecies. The whistle is a likely candidate as a conspecific marker for recognition, as it is universally present across song dialects for both subspecies and is the introductory component of their song. Although song is culturally inherited, song learning is directed by a genetic template (Nelson *et al.* 1995; Soha & Marler 2001a; b). Several song learning experiments with white-crowned sparrow nestlings indicate that the whistle is innate rather than learned (Whaling *et al.* 1997), important for acoustic imprinting (Margoliash 1983), and may reflect an innate template in conspecific song memorization, production and recognition (Whaling *et al.* 1997; Soha & Marler 2000). Thus, the whistle's importance in song learning and recognition for nestlings may also influence adult recognition of potential competitors and mates. Innate recognition of conspecifics may be especially important given that in the non-breeding season, southern localities contain a mixture of overwintering *Z. l. pugetensis* and resident *Z. l. nuttalli* (Blanchard 1941). A rich area of future study lies in exploring regions of the genome responsible for divergence in song between the subspecies, potentially related to innate aspects of song learning.

Males responded less strongly to heterotypic song in parental populations. Signals that elicit a stronger territorial response from males are typically interpreted as signals more effective at maintaining a territory and competing for mates (Searcy & Nowicki 2005), (but see Baker & Mewaldt 1978; Baker *et al.* 1981 for the argument that a stronger response to heterotypic song can facilitate reproductive isolation). Therefore, we interpret a lower response to heterotypic song as evidence of a behavioral barrier between *Z. l. nuttalli* and *Z. l. pugetensis*, such that individuals of one subspecies would not be as effective in territory defense and mate acquisition in a population of the other subspecies. These results are consistent with previous studies that found males in parental populations are more responsive to their own subspecies song, both in white-crowned sparrows (Lampe & Baker 1994) and in other taxa (Turčoková *et al.* 2011; Greig & Webster 2013). Discrimination was not explained solely by familiarity with the local song type, as putative admixed individuals did not discriminate between their own songs and non-local songs *Z. l. nuttalli*, which more closely matched their genotype.

Male territorial responses to playbacks of bird songs are less logistically challenging than

measuring female response and are therefore more typically used to test signal discrimination as an indirect measure of reproductive isolation (e.g. Irwin *et al.* 2001; Dingle *et al.* 2010; Podos 2010; Derryberry 2011; Turčoková *et al.* 2011), although theory also suggests that male-male competition can contribute directly to isolation (Ellers & Slabbekoorn 2003). A useful follow-up experiment could involve testing admixed and parental female preferences for admixed and parental songs, as testing females would provide more direct evidence of whether song is acting as a behavioral barrier between the subspecies. A study in the *Z. l. oriantha* subspecies indicated that females prefer their natal-dialect song over a foreign-dialect or heterospecific song (MacDougall-Shackleton *et al.* 2001), so we predict that females will discriminate between subspecies songs in the parental populations.

We not only found that individuals discriminated between homotypic and heterotypic song, but also that the strength of discrimination between songs was predicted by similarity of the stimulus song to the receiver's song. Finding this association supports the hypothesis that divergence in the signal itself is driving behavioral discrimination between the signals. Co-variation between signal and response has been found in some playback studies (e.g. Sosa-López *et al.* 2016), but not others (e.g. den Hartog *et al.* 2008). However, these studies focus on the strength of behavioral response to a stimulus (e.g. height of behavioral response PC1) rather than the strength of discrimination between stimuli (e.g. slope of behavioral response PC1). Our pairwise design enabled us to relate the difference in song stimuli directly to the difference in behavioral responses to those stimuli. The strength of discrimination between stimulus songs was also positively correlated with pairwise genetic distances for those playback localities. These associations among genetic distance, song divergence, and behavioral response support the role of song as a behavioral isolating mechanism in the hybrid zone.

We also attempted to infer what processes are driving patterns of genetic, acoustic, and behavioral divergence between subspecies. Multiple forces of selection may act on song, causing divergence among populations. Acoustic signals may diverge and converge via sexual and social selection (Fisher 1930; West-Eberhard 1983), acoustic adaptation to environmental conditions affecting sound transmission (Morton 1975; Wiley & Richards 1982; Derryberry 2009), morphological divergence in shape and size that constrains signal production (Podos 1996; Podos *et al.* 2004), genetic or cultural drift (Lemon 1975; Lynch 1996; Irwin *et al.* 2008) or a combination of these social, ecological, and stochastic factors (Mundinger 1982; Price 1998; Wilkins *et al.* 2013). Divergent migratory behavior and/or allopatric temporal isolation between the subspecies could also play a role in genetic differentiation (e.g. Ruegg *et al.* 2012; Delmore & Irwin 2014), given that *Z. l. pugetensis* is migratory and *Z. l. nuttalli* is a year-round resident. However, migratory behavior as an isolating mechanism is not mutually exclusive with our hypothesis that song is a behavioral barrier between the subspecies. After controlling for geographic distance, we still found a significant association between whistle length and genetic differentiation, which suggests that this song feature could be driving genetic divergence between the subspecies. Many studies have looked for an association between song distance and genetic variation in white-crowned sparrows (Baker 1975; Baker *et al.* 1982; MacDougall-Shackleton & MacDougall-Shackleton 2001; Soha *et al.* 2004) and other taxa (Wright & Wilkinson 2001; Nicholls *et al.* 2006; Alstrom *et al.* 2007; Irwin *et al.* 2008; Kenyon *et al.* 2011; Sosa-López *et al.* 2016), but few other than our study have found this association independent of geographic variation (but see MacDougall-Shackleton & MacDougall-Shackleton 2001; Rendell *et al.* 2012).

Conclusions

Our aim in this study was to evaluate when and how song acts as an isolating mechanism in order to gain insight into the evolution and maintenance of behavioral barriers. Our findings provide strong support to the hypothesis that song is a behavioral barrier to gene flow between *Z. l. nuttalli* and *Z. l. pugetensis*, although clearly other barriers to mating are incomplete as introgression is ongoing. We investigated both the signal – bird song – and receiver response to that signal, and found that both songs and behavioral responses to song are divergent between the subspecies, although these differences may be weakening in the contact zone and facilitating hybridization. Our finding that song and genetic distance predict the strength of behavioral discrimination between songs gives insight into the processes driving the evolution of this behavioral barrier.

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CHAPTER II
DIVERGENT COMPETITIVE PHENOTYPES BETWEEN FEMALES OF
TWO SEX-ROLE REVERSED SPECIES

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Abstract

Divergent phenotypes between lineages in the early stages of speciation can promote or impede reproductive isolation. Although divergence in male competitive morphology and behavior has been explored for many hybridizing lineages, it is less known how divergence between females influences hybridization. Here, I compare competitive phenotypes between females and males of two hybridizing, sex-role reversed jacana species in Panama. Previous work suggests *Jacana spinosa* females monopolize mating in the hybrid zone, potentially through a competitive advantage. I tested whether *J. spinosa* females have a more competitive phenotype than *J. jacana* females. I compared morphological traits related to territoriality and measured aggressive behavior using territorial intrusion simulations: the first aggression assay in a shorebird. I also quantified these traits in males, to confirm previous studies reporting males as smaller and less aggressive than females in both species. As predicted, *J. spinosa* females had larger body mass and longer wing spurs than *J. jacana* females. *J. spinosa* females were also more aggressive than *J. jacana* females. Male *J. spinosa* had longer wing spurs than male *J. jacana* but there was no difference in male body mass between the species, and *J. spinosa* males were more aggressive than *J. jacana* males. Additionally, male *J. spinosa* were more aggressive than female *J. spinosa*, suggesting mixed support for females as competitively dominant to males and indicating the need for additional experimental work on sex differences in *Jacana*.

Introduction

For closely related lineages with similar life histories, divergent phenotypes may either promote or hinder reproductive isolation (Safran et al. 2013). Recently diverged species provide good opportunities to investigate the function of divergent phenotypes in reproductive isolation, especially in the context of hybridization. The impact of divergent, sexually selected traits and preferences on reproductive isolation has been studied extensively in the context of mate choice between heterospecifics (Sætre et al. 1997; Wirtz 1999; Stein and Uy 2006; Baldassarre et al. 2013), and is considered a signature of speciation by sexual selection (Safran et al. 2013). In addition to mate choice, divergent phenotypes are also important in mediating interspecific competition over territories, mates and resources (Andersson 1994; Irwin and Price 1999; Grether et al. 2013). For example, differential aggression between hybridizing lineages can lead to displacement of the less aggressive species from breeding territories (Pearson and Rohwer 2000; Jankowski et al. 2010). This competitive exclusion can reduce gene flow, particularly when compounded with reproductive interference (Kishi et al. 2009; Drury and Grether 2014; but see Vallin et al. 2012). Alternatively, differential aggression can facilitate hybridization, for example by driving genetic and/or phenotypic introgression into the less aggressive lineage (e.g. McDonald et al. 2001; Rosenfield and Kodric-Brown 2003; Grava et al. 2012; Robbins et al. 2014; While et al. 2015). Differential aggression between hybridizing species has been typically examined in males, leaving open the question of whether differential aggression in females may also affect hybridization.

Recent empirical and theoretical studies suggest that female-female competition is more widespread than previously thought (Rosvall 2011; Stockley and Bro-Jørgensen 2011; Tobias et al. 2012; Stockley and Campbell 2013). Intraspecific resource and mate defense by females has been demonstrated in numerous taxa (e.g. antelopes, Roberts and Dunbar 2000; birds, Rosvall 2008; lizards, While et al. 2009; and frogs, Meuche et al. 2011). However, a strong understanding of the ecological and evolutionary mechanisms that drive variation in female-female competition across closely related species is still lacking (Cain and Rosvall 2014). Excellent systems in which to examine this question are species for which traditional sex roles are reversed. Differences in female competitive traits have known fitness consequences in sex-role reversed species (Andersson 1995; Butchart 2000; Goymann et al. 2008), in

contrast to species with traditional sex roles, in which female competition is less well-studied. In comparison to males, females in role reversed species are larger, often more brightly colored, and show higher levels of resource defense aggression (Cockburn 2006) – attributes typical of males in other species. In understanding the role that interspecific female competition plays in promoting or impeding speciation, a first step is to understand how female competitive traits vary among closely related species.

Several species of tropical shorebirds in the family *Jacanidae* exhibit classic examples of sex-role reversal, including female-biased size dimorphism (Jenni and Collier 1972; Butchart et al. 1999; Emlen and Wrege 2004a). Wattled Jacana (*Jacana jacana*) females have a more competitive morphological phenotype than males – they are heavier and show greater proportionate development of weaponry relative to body size (Emlen and Wrege 2004b). In *J. jacana* and a closely related species, the Northern Jacana (*Jacana spinosa*) both sexes have keratinized spurs used as weapons in aggressive interactions (Osborne and Bourne 1977; Emlen and Wrege 2004b). Body mass and tarsus length are strong predictors of female mating success in *J. jacana*, and only the largest females control access to mates by defending territories (Emlen and Wrege 2004a, b). Aggressive behavior in female jacanas has been observed in the context of acquisition and maintenance of male territories – males compete over territorial boundaries first, and female territories then encompass male territories (Emlen et al. 1989). Although some observational studies suggest that female jacanas are more aggressive than males because of their competitive dominance over territories, (Jenni and Collier 1972; Stephens 1984), this has not been tested experimentally.

As female competition has consequences for reproductive success within jacana species, variation in competitive traits between females of sympatric jacana species may have implications for reproductive isolation between them. Two closely related jacana species, the Northern Jacana (*Jacana spinosa*) and the Wattled Jacana (*J. jacana*) are known to hybridize in Panama (Miller et al. 2014). There is some evidence of asymmetrical introgression – hybrids shared mitochondrial DNA (mtDNA) haplotypes with *J. spinosa*, but not *J. jacana* (Miller et al. 2014). Because mtDNA is inherited maternally, one hypothesis to explain this asymmetrical introgression is that *J. spinosa* females monopolize mating in the hybrid zone (Miller et al. 2014). A behavioral mechanism by which *J. spinosa* females could monopolize successful matings is by having a more competitive phenotype that allows them to outcompete *J. jacana* females for territories. Here I define the competitive phenotype as the covariance of competitive traits, in this case morphological and behavioral traits involved in competition over mates and territories (West-Eberhard 1983; Andersson 1994; Cain and Ketterson 2012). Although a direct comparison of competitive morphology has not been made between the two species, sexual dimorphism is greater in *J. spinosa*, (female:male mass ratio 1.67:1, Jenni and Collier 1972), than in *J. jacana* (mass ratio 1.48:1, Emlen and Wrege 2004a). It is unknown whether females of the two species are also different in behavioral characteristics of the competitive phenotype, for instance their aggression levels.

In this study, I test the hypothesis that competitive morphological traits and aggressive behaviors are divergent between *J. spinosa* and *J. jacana* females and males. I predict that *J. spinosa* females have more competitive morphological traits than *J. jacana* females, and will be more aggressive to territorial intruders. I also describe the first aggression assay using simulated territorial intrusion in shorebirds. I quantify the same morphological traits and aggressive behaviors in females and males of both species, to place the female competitive phenotype in context and to examine a long-standing assumption that female jacanas are generally more aggressive than males in territory defense (e.g. Stephens 1984; Betts and Jenni 1991). Comparing competitive morphological traits and aggressive behaviors between the two species and sexes expands our knowledge of variation in the *Jacana* competitive phenotype as well as provides a first step towards understanding the potential role of female competition in hybridization.

Methods

Morphological measurements

Birds were captured with mist-nets from April – May in 2012, June – September 2014, and May – August 2015 from Costa Rica to Panama (Figure 13; Table 9). I measured morphological traits from 165 individual adult jacanas (80 *J. jacana* and 85 *J. spinosa*). Individuals were aged based on plumage (Jenni 1996). I measured left and right keratinous wing spurs and tarsi to the nearest tenth of a millimeter with

Avinet dial plastic calipers and then averaged for each individual. To obtain a combined metric of the competitive morphological phenotype for jacanas, I summarized body mass, tarsus length, and spur length using a principal components analysis (PCA) in R 3.2.2 (R Core Team 2015). Prior to the PCA, I log transformed behavioral responses to fulfill assumptions of multi-normality. I retained 1 PC score with an eigenvalue greater than 1 (hereafter Morphological PC1), which explained 79.1% of the variation in competitive morphology (Table 10). I also calculated female:male mass ratios to compare measurements with other published studies (Jenni and Collier 1972; Emlen and Wrege 2004a).

Sex determination

Individuals were sexed based on mass (Wrege and Emlen 2005) and the presence of brood patches underneath the wings, in the case of males. I measured body mass to the nearest tenth of a gram with a Pesola® spring scale. While Wrege and Emlen (2005) identify a 100 – 108g range where male and female mass may overlap, there exists a female *J. jacana* museum specimen with a body mass of 106.7g (LSU 164012) and a male *J. spinosa* specimen in my dataset with mass of 117g (SL 188), suggesting the range of mass overlap may be greater between the sexes. To confirm the sex of 46 jacanas with body masses ranging from 100 – 130g and no brood patches, I used molecular techniques. I collected blood samples with brachial venipuncture and stored them in Queen's lysis buffer (Seutin et al. 1991). I extracted genomic DNA with a DNeasy extraction kit (Qiagen) following the manufacturer's protocol. I amplified the CHD1Z gene using the primers 2550F/2718R (Fridolfsson and Ellegren 1999) in a polymerase chain reaction (PCR). For each 10 µL reaction, I used 5µL of Qiagen Multiplex PCR Master Mix containing 3 mM MgCl₂ (Valencia, CA), 2 µL of molecular grade water, and 1 µL of a 2µM primer mix. I used the following cycling parameters: one cycle of 15 minutes at 95°C, 35 cycles of 30 seconds at 94°C, 90 seconds at 52°C, and 60 seconds at 72°C, followed by one cycle of 30 minutes and 60°C and one cycle of 1 minute at 25°C. I ran the PCR products for 60 minutes on a 2% agarose gel stained with SYBR™ Safe (Invitrogen) and assigned sex based on differences in banding patterns between males and females. There were 16 males sampled from across both species with a body mass higher than 108g, including two males at 124g, which I would have misidentified if sexed based on mass alone.

Aggression Assay Experimental Design

I measured aggression experimentally by simulating territorial intrusion with a taxidermic mount and auditory stimulus. Similar assays of aggression have been conducted on both males and females in a number of avian taxa, primarily in songbirds (Aves: Passeriformes) (Pearson and Rohwer 2000; Uy et al. 2009; Greig et al. 2015). To assay aggression in jacanas, I modified these standard methods using a moving visual stimulus to help the territory holder locate the simulated intruder (Figure 14). Four female taxidermic mounts were prepared per species (8 total), collected outside of the hybrid zone. Mounts were positioned in an identical aggressive stance, with their wings raised upwards, spurs exposed, and necks forward. To facilitate movement, I fixed mounts to a rotating wheel with strings and pulled them from 20m away behind a blind, where myself and another observer conducted behavioral observations. Because the natural coloration of the facial shield and wattles fades when dried, I painted the fleshy bare red ornaments of *J. jacana* with "Deep Red" paint and the bare yellow facial ornament of *J. spinosa* with "Brilliant Yellow" paint from a Crayola 6-color acrylic paint set (Manufacturer number 201997). There are no known visual signals in jacana facial ornaments that are not visible to the human eye, such as UV signaling. No changes were made to the green wing primary feathers of either species, which contain turacoverdin pigment (Bleiweiss 2015).

I used two types of auditory stimuli in the aggression assay – a lure to attract a territorial pair to the mount and a vocal stimulus played during the assay. The lure was a 10 second recording of a pair raucously calling in unison (e.g. Amy et al. 2010; Anderson et al. 2013). Vocal stimuli consisted of repeated-note calls (Mace 1981) of single females responding to conspecific playback. Vocalizations were recorded using a Sennheiser ME67 shotgun microphone and a Marantz PMD-661 MKII compact flash recorder (Saul Mineroff Electronics). Recordings were made at a 44.1kHz sampling rate with 16-bit precision as .wav files. I also used two recordings of *J. spinosa* calls from Costa Rica (XC140613, XC72325) and one of *J. jacana* from Peru (XC47715) downloaded from Xeno Canto (www.xeno-canto.com), for which sex was unknown. I chose five seconds of high-quality calls from each recording, a typical duration for a repeated-note call bout (Jenni et al. 1974; Mace 1981; SEL unpublished data).

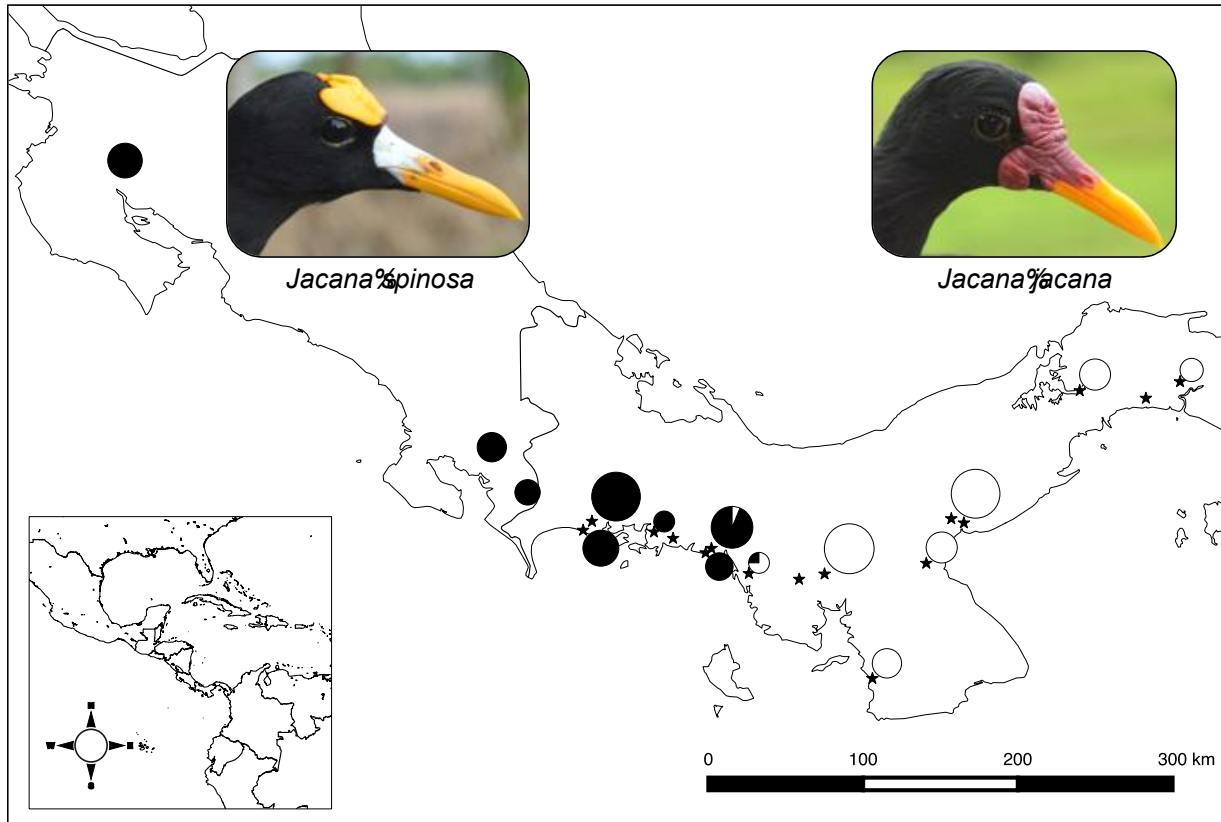


Figure 13. Sampling map of Costa Rica and Panama.

Circle size refers to number of individuals with morphological measurements, ranging from four to 24 individuals, and fill represents *J. spinosa* (black) and *J. jacana* (white). Stars represent sites that were locations of aggression assays

Table 9. Sampling information for morphological and behavioral data.

	Locality	Province	Country	Latitude	Longitude	Morphology N	Assay N
1	Palo Verde	Guanacaste	Costa Rica	10.3438	-85.33835	11	NA
2	La Gamba	Puntarenas	Costa Rica	8.69993	-83.19388	8	NA
3	Coto 47	Puntarenas	Costa Rica	8.45086	-82.97426	6	NA
4	La Barqueta	Chiriqui	Panama	8.30708	-82.57873	11	13
5	Gaurumal	Chiriqui	Panama	8.35892	-82.52718	22	3
6	Horconcitos	Chiriqui	Panama	8.29655	-82.1673	4	2
8	San Lorenzo	Chiriqui	Panama	8.26013	-82.05614	NA	2
7	Las Lajas	Chiriqui	Panama	8.17595	-81.86723	7	3
9	Remedios	Chiriqui	Panama	8.214	-81.839	16	3
10	Puerto Vidal	Veraguas	Panama	8.05677	-81.6162	4	1
11	Sona	Veraguas	Panama	8.0234	-81.32431	NA	3
12	El Maranon	Veraguas	Panama	8.05352	-81.17674	24	3
13	Quebro	Veraguas	Panama	7.44928	-80.899	8	5
14	El Rincon Cienega de	Veraguas	Panama	8.11416	-80.6095	NA	1
15	las Macanas	Herrera	Panama	8.11542	-80.58603	10	2
16	Las Guabas Puerto El	Cocle	Panama	8.37725	-80.45096	1	NA
17	Gago	Cocle	Panama	8.37363	-80.44085	12	1
18	El Coco	Cocle	Panama	8.34941	-80.36753	7	5
19	Gamboia	Colon	Panama	9.11452	-79.69505	9	6
20	Pacora	Panama	Panama	9.07074	-79.31088	NA	1
21	Chepo	Panama	Panama	9.1664	-79.11244	5	6

Table 10. Mean and standard error for morphological traits related to aggression of adult male and female *Jacana spinosa* and *J. jacana*

Trait	PC1	<i>Jacana spinosa</i>		<i>Jacana jacana</i>	
Eigenvalue	1.78	F	M	F	M
Percent variation	79.2				
Body Mass (g)	0.61	172.7 ± 2.7	103.2 ± 1.0	159.0 ± 2.7	105.9 ± 1.2
Average Spur (mm)	0.56	14.1 ± 0.5	9.6 ± 0.2	11.2 ± 0.4	8.3 ± 0.2
Tarsus Length (mm)	0.56	61.8 ± 0.4	56.3 ± 0.2	60.9 ± 0.5	55.3 ± 0.4
Sample Size		35	50	28	52

The natural rate of call bouts for both species ranges from two to nine bouts per minute (SEL unpublished data). Background noise was minimized using a band filter in Raven Pro 1.4 (Bioacoustics Research Program 2011) and noise reduction in Audacity® 2.1.0. Call bouts were alternated with five seconds of silence to create 9-minute tracks of six call bouts/minute, with four different tracks per species (8 total). Vocal tracks were played at 83-85 dB SPL 1m from a Bluetooth speaker (Bose Mini SoundLink). I used both conspecific lures and vocal stimuli for each species, and all vocalizations were recorded outside of the hybrid zone.

Aggression Assay Protocol

From June – August 2015, I conducted aggression assays on 33 mated pairs of *J. jacana* and 28 mated pairs of *J. spinosa* (Figure 13). These assayed individuals were different from those for which I took morphological measurements – thus, I do not have a direct comparison of aggressive responses and morphological measurements for each individual. I observed focal pairs over a two day period prior to the trial, to determine territorial status, pair status, reproductive status, and territory boundaries (Emlen and Wrege 2004a). Because I identified distinct pairs by their territory locations, I avoided testing adjacent territories for which I could not distinguish the territory-holders. This ensured I did not assay the same individuals twice. In the case of a female with multiple male mates, I tested only the male whose territory was occupied by the female at the time of the assay. I also avoided testing pairs for which a male was incubating a nest or there were chicks present, as that could influence aggression levels. I tested pairs using a randomized combination of four conspecific taxidermic female mounts and four conspecific vocalizations per species. Mount and stimulus combinations were presented in a randomized order across pairs.

Prior to beginning each trial, I placed a mount and speaker in the center of each territory and green flags at distances of 2m and 8m from the mount. I positioned the mount on a 15 cm high platform and elevated the speaker at 1m to project above tall vegetation. At the start of an assay, I played the lure until both focal individuals approached within 20m of the mount. All pairs responded within eight plays of the lure. Once both individuals were within 20m of the mount, I waited 1 min and then started the trial with the 9-min recording of conspecific vocalizations. For the first 30-sec of vocalizations, I rotated the mount (Figure 14).

Myself and an assistant observed each focal individual and recorded behavioral observations using handheld recorders. It was not possible to record data blind because this study involved focal animals in the field. Observers continuously recorded aggressive behaviors towards the mount, including pecks, flyovers, wing-raises, threats, and hoverflights according to ethograms developed for *J. jacana* (Altmann 1974; Emlen and Wrege 2004b) and *J. spinosa* (Jenni and Betts 1978; Stephens 1984) (all responses listed in Table 11). We split each trial into 10-second intervals based on the start of the repeated vocal stimulus. For each 10-sec interval, we scored a focal individual as vocalizing if it called any number of times within interval. We also measured distance to the mount at each 10-sec interval in three distance categories: 0-2m, 2-8m, or 8-20m. To calculate average distance to the mount, I counted the number of instances an individual was in each distance category for all 10-sec intervals, multiplied this count by the midpoint of these distance categories, and divided by the number of 10-sec intervals for the entire trial. We also recorded latency to approach within 0-2m, latency to approach within 2-8m, and the proportion of time spent within 0-2m. I excluded from analyses the 10-sec intervals for which an individual was not observed, including when it was greater than 20m from the mount, because visibility was sometimes reduced in tall grass. For this reason, continuous behaviors and vocalizations are presented as proportions of the total 10-sec intervals for which individual location was known. I calculated this as frequency divided by the number of 10-sec intervals, and the rate of vocalization as the number of 10-sec intervals for which an individual was calling.

To compare aggression between the species I summarized the behavioral responses using a PCA in R. Prior to the PCA, I log transformed behavioral responses to fulfill assumptions of multi-normality. I retained 3 PC scores (eigenvalues > 1) that explained 61.4% of the variation among territory-holders in their response to a simulated intruder (Table 11). I used each PC score (hereafter 'aggression PC1, PC2 and PC3') as a dependent variable in subsequent comparisons of sex and species.



Figure 14. Taxidermic mount used in simulated territorial intrusion.

Table 11. Mean $\pm$ SE behavioral responses to a conspecific, simulated territorial intruder and their principal component score loadings

Sex	PC1	PC2	PC3	<i>Jacana spinosa</i>		<i>Jacana jacana</i>	
				F	M	F	M
Eigenvalue	1.84	1.21	1.14				
Percent variation	33.8	14.6	13				
Cumulative proportion	33.8	48.4	61.4				
Latency 0 - 2 m (sec)	-0.37	0.21	-0.05	422 $\pm$ 35.43	343.6 $\pm$ 41.13	502.86 $\pm$ 22.31	474.33 $\pm$ 31.27
Latency 2 - 8 m (sec)	-0.4	0.02	-0.17	210.4 $\pm$ 41.64	190.4 $\pm$ 41.43	417.86 $\pm$ 38.83	375 $\pm$ 43.58
Proportion time 0 - 2 m	0.44	-0.28	0.18	0.10 $\pm$ 0.038	0.19 $\pm$ 0.058	0.0053 $\pm$ 0.0033	0.014 $\pm$ 0.008
Mean distance	-0.46	0.17	-0.24	10.81 $\pm$ 0.64	9.15 $\pm$ 0.89	13.10 $\pm$ 0.39	12.95 $\pm$ 0.38
Proportion Hoverflights	0.26	0.49	-0.14	0.014 $\pm$ 0.01	0.036 $\pm$ 0.01	0.013 $\pm$ 0.003	0.038 $\pm$ 0.01
Proportion Wing Spreads	0.29	0.39	-0.08	0.031 $\pm$ 0.01	0.13 $\pm$ 0.03	0.018 $\pm$ 0.004	0.020 $\pm$ 0.01
Proportion Threats	0.05	-0.13	-0.75	0	0.010 $\pm$ 0.01	0.0007 $\pm$ 0.001	0.0006 $\pm$ 0.001
Proportion Pecks	0.18	-0.46	-0.45	0	0.026 $\pm$ 0.02	0.0027 $\pm$ 0.002	0.0019 $\pm$ 0.002
Proportion Flyovers	0.21	0.04	-0.24	0.026 $\pm$ 0.01	0.035 $\pm$ 0.08	0.014 $\pm$ 0.004	0.0093 $\pm$ 0.003
Proportion Vocalizations	0.27	0.48	-0.19	0.21 $\pm$ 0.04	0.40 $\pm$ 0.06	0.17 $\pm$ 0.04	0.25 $\pm$ 0.05
Sample Size				25	25	28	30

Model formation and selection

I ran linear mixed effects models using the *lme4* package (Bates et al. 2015) in R. I visually inspected residual plots to ensure they did not deviate from normality, and used the *varIdent* function to account for heteroscedasticity in both species and sex to meet model assumptions. I compared models using a type III ANOVA and AIC_c to account for low sample sizes using the *AICcmodavg* package in R (Mazerolle 2016) (Table 12,13). I used a type III ANOVA to determine which fixed effects were significant predictors of response variables and a Tukey's post-hoc test to compare within and between the sexes and species using the *multcomp* package in R (Hothorn et al. 2016).

To compare morphological traits used in competitive interactions between both species and sexes, I used the morphological PC1 as the response variable, specified species, sex, and the interaction between species and sex as fixed effects, and included site and year as random effects.

To compare aggression between both species and sexes I used aggression PC1, PC2, and PC3 as separate response variables and species, sex, and the interaction between species and sex as fixed effects. To account for mount/vocal stimulus exemplar effects, I included mount ID and vocal stimulus as random effects nested within species. I also included pair as a random effect nested within site, because within a pair, male and female distances from the mount were positively correlated (Pearson correlation: $r = 0.655$).

Results

Competitive morphology varies with species and sex

Morphological PC1 was positively associated with body mass (0.61), tarsus length (0.56), spur length (0.56) (Table 10). The best-supported model for morphological PC1 included all fixed effects and site as the sole random effect (Table 12). Species ($F_{1,145} = 14.6$, $P = 0.0002$), sex ($F_{1,145} = 207.16$, $P < 0.0001$), and their interaction ($F_{1,145} = 6.93$, $P = 0.0094$) were all significant predictors of morphological PC1. Female *J. spinosa* have larger values of morphological PC1 than female *J. jacana* ($z = 3.82$, $P < 0.001$), indicating overall larger competitive traits (Table 10, Figure 15). Comparison of individual morphological traits (Table 10) indicates that *J. spinosa* females have a larger body mass, longer keratinous wing spurs, and longer tarsi than *J. jacana* females. In contrast, males of both species did not have significantly different values of morphological PC1 ($z = 1.96$, $P = 0.194$). *J. spinosa* males have longer average wing spurs, however ($t = -5.01$, $P < 0.001$).

For both species, females had significantly larger values of morphological PC1 than males (*J. spinosa*: $z = 17.1$, $P < 0.001$; *J. jacana*: $z = -14.4$, $P < 0.001$). The differences in body mass were more extreme in *J. spinosa*, which had a female:male mass ratio of 1.67, than in *J. jacana*, which had a ratio of 1.5.

Table 12. Rank of models that describe morphological PC1

Fixed Effects	Random Effects	K	AICc	$\Delta AICc$
Species + Sex + Species x Sex	Site	6	333.89	0
Species + Sex + Species x Sex	Site, Year	7	336.07	2.18
Species + Sex	Site	5	340.46	6.58
Species + Sex + Species x Sex	Year	6	341.35	7.47
Sex	Site	4	354.09	11.93
Species	Site	4	607.09	273.2

Table 13. Rank of models that describe aggression PC1, PC2, and PC3

Fixed Effects	Random Effects	Df	AICc	Δ AICc
PC1				
Species + Sex + Species*Sex	Site/Pair	7	393.07	0
Species + Sex + Species*Sex	Site/Pair, Species/Vocal	9	397.79	4.72
Species + Sex + Species*Sex	Site/Pair, Species/Mount ID	9	397.79	4.72
Species + Sex	Site/Pair	6	398.15	5.08
Species + Sex + Species*Sex	Site/Pair, Species/Mount ID, Species/Vocal	11	402.7	9.63
Sex	Site/Pair	5	406.29	13.22
Species	Site/Pair	9	411.58	18.51
Species + Sex + Species*Sex	Species/Mount ID, Species/Vocal	9	425.56	32.49
PC2				
Species + Sex		4	329.53	0
Sex		3	349.65	20.12
Species		3	352.35	22.82
Species + Sex + Species*Sex		5	353.96	24.43
Species + Sex + Species*Sex	Site/Pair, Species/Mount ID	9	357.96	28.43
Species + Sex + Species*Sex	Site/Pair, Species/Vocal	9	357.99	28.46
Species + Sex + Species*Sex	Species/Mount ID, Species/Vocal	9	362.86	33.33
Species + Sex + Species*Sex	Site/Pair, Species/Mount ID, Species/Vocal	11	362.88	33.35
PC3				
Species		3	338.95	0
Sex		3	339.11	0.16
Species + Sex		4	340.24	1.29
Species + Sex + Species*Sex		5	341.18	2.23
Species + Sex + Species*Sex	Site/Pair, Species/Vocal	9	348.41	9.46
Species + Sex + Species*Sex	Site/Pair, Species/Mount ID	9	348.41	9.46
Species + Sex + Species*Sex	Species/Mount ID, Species/Vocal	9	350.43	11.48
Species + Sex + Species*Sex	Site/Pair, Species/Mount ID, Species/Vocal	11	353.32	14.37

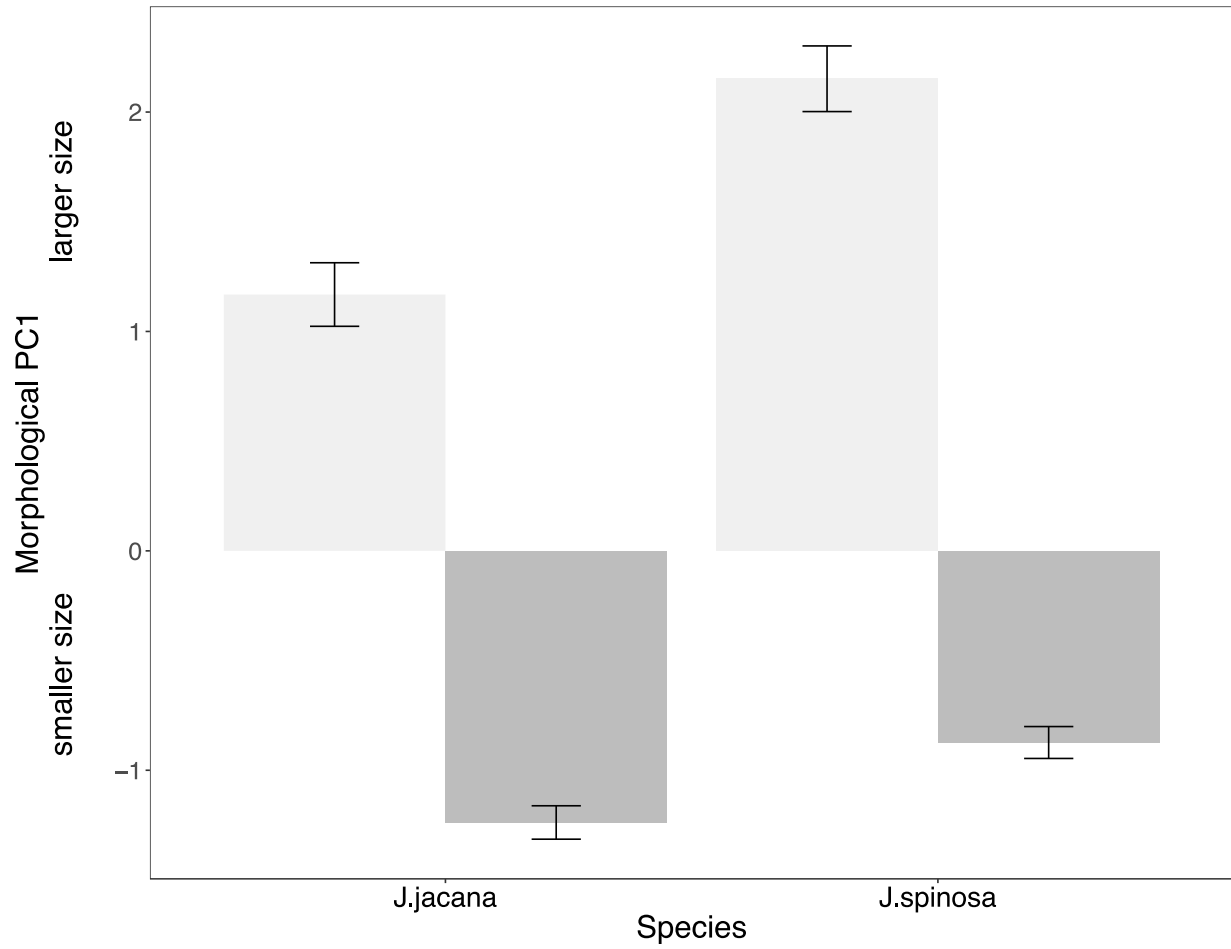


Figure 15. Morphological comparison of competitive phenotype between jacana species and sexes.
Mean ($\pm$ SE) morphological PC1 for female (light grey) and male (dark grey) *J. spinosa* and *J. jacana*

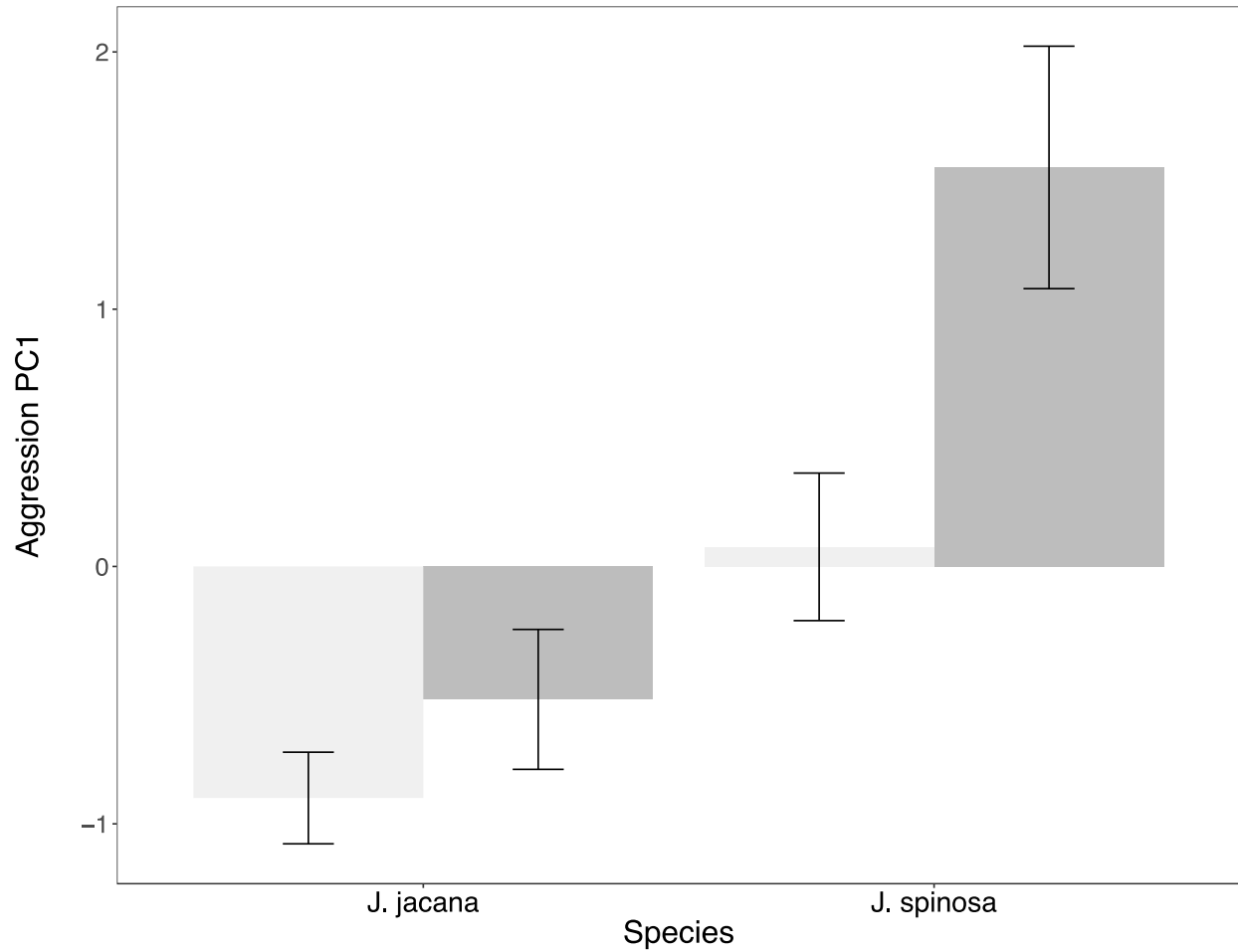


Figure 16. Response by territory holders to simulated territorial intrusion of a conspecific.

Mean ($\pm$ SE) aggression scores (principal component 1) for *J. spinosa* (Females: $N = 25$; Males: $N = 25$) and *J. jacana* (Females: $N = 28$; Males: $N = 30$). *J. spinosa* males (dark grey) and females (light grey) responded more strongly than *J. jacana* males and females

J. spinosa is more aggressive than J. jacana

The aggression score based on PC1, which explained 33.8% of variation in aggressive response, included latency to approach the mount, distance to the mount and time spent close to the mount as important loading variables (Table 11). After model selection, the best-supported model for aggression PC1 included pair nested within site as the sole random effect, and all fixed effects. Species ($F_{1,16} = 9.86$, $P = 0.0063$) and the interaction between species and sex ($F_{1,46} = 8.16$, $P = 0.0064$) were significant predictors of aggression PC1, but sex alone was not significant ($F_{1,46} = 2.32$, $P = 0.134$). *J. spinosa* had higher aggression PC1 score than *J. jacana* for both females and males (female: $z = 3.14$, $P = 0.008$; male: $z = 4.33$, $P < 0.001$) (Figure 16). Male *J. spinosa* had significantly higher aggression PC1 scores than female *J. spinosa* ($z = 4.67$, $P < 0.001$), but there were no differences between male and female *J. jacana* aggression PC1 scores ($z = 1.52$, $P = 0.391$). No fixed effects were significant for either PC2 or PC3 (all $P > 0.09$) (Table 13), which only explained a small proportion of the variation in aggressive response (Table 11).

Discussion

I found support for my prediction that *J. spinosa* females have a more competitive phenotype than *J. jacana* females. *J. spinosa* females on average had a larger body mass and longer wing spurs than *J. spinosa* females, traits involved in competition over territories and mates (Emlen and Wrege 2004b). Consistent with these differences in competitive morphological traits, I found that *J. spinosa* females behaved more aggressively towards a conspecific intruder in measures of distance and time spent near the mount than did *J. jacana* females. Male *J. spinosa* were also more aggressive than male *J. jacana*, and had significantly longer wing spurs. Together, these morphological and behavioral data suggest that *J. spinosa* and *J. jacana* have diverged in their competitive phenotype. Additionally, I found mixed support for a long-standing hypothesis that females have a more competitive phenotype than males in the sex-role reversed *Jacana* genus. As predicted, females were larger than males in both species; however, females were not more aggressive than males in either species. Below, I discuss the implications of these findings for our understanding of these sex-role reversed species.

Differences in the female competitive phenotype between the species may be related to greater sexual selection on secondary sexual traits for *J. spinosa* females. Some evidence suggests stronger competition for mates and territories in *J. spinosa* than in *J. jacana*. For instance, sexual dimorphism in morphological traits is more pronounced in *J. spinosa* than in *J. jacana*, as demonstrated by this and other studies (Jenni and Collier 1972; Emlen and Wrege 2004b). Furthermore, within-population estimates suggest that breeding *J. spinosa* females have an average of 1.8 – 2.5 male mates in their territories (Jenni and Collier 1972; Jenni and Betts 1978), whereas *J. jacana* breeding females have an average of 1.6 – 1.7 mates (Emlen et al. 1998; Emlen and Wrege 2004b). Given that *J. spinosa* has a higher average of male mates in a harem, and therefore higher potential for reproductive skew (Shuster 2009; Kvarnemo and Simmons 2013), the intensity of sexual selection may be stronger in this species. Therefore, I suggest that differences in the female competitive phenotype between *J. spinosa* and *J. jacana* are potentially the result of differences in sexual selection pressures. Future work to determine the ultimate and proximate mechanisms for these differences in competitive phenotype between closely related species could provide valuable insight into how and why young taxa diverge.

This study represents the first experimental measure of aggression in jacanas. Contrary to previous studies, female jacanas were not more aggressive than male jacanas. *J. spinosa* males responded more aggressively than *J. spinosa* females towards a female conspecific intruder, but there were no differences in aggression between male and female *J. jacana*. Observational studies of aggression in *J. spinosa* (Stephens 1984) and in *J. jacana* (Emlen and Wrege 2004a, b) have suggested that female jacanas are dominant to males in territorial interactions because of their larger body size and therefore stronger resource-holding potential. In both species, female territories are larger and may encompass multiple male territories (Jenni and Collier 1972; Emlen et al. 1998). In a related species, the bronze-winged jacana (*Metopidius indicus*), males are less successful at evicting female intruders (Butchart et al. 1999). However, my results are somewhat consistent with the finding from observational studies that male jacanas are more responsive towards territorial intruders than females. For example, *J.*

jacana males are more likely than females to respond first to both male and female conspecific intruders (Emlen and Wrege 2004a), and male *J. spinosa* are more responsive overall to non-jacana heterospecifics than are females (Stephens 1984). Male bronze-winged jacanas were more active than females in multiple measures of territory defense, including time spent alert, frequency of territory patrol and hoverflights (Butchart et al. 1999). Although female jacanas in both species may be physically larger and dominant to males in resource-holding potential, males may be more responsive in aggressive territorial interactions.

Differences in morphology and aggression could allow *J. spinosa* females and males a competitive advantage over *J. jacana* in obtaining and defending territories where they come into contact in the hybrid zone. These morphological and behavioral results can be used to make predictions about the potential introgression of competitive traits and aggression across the hybrid zone. Evidence from other hybrid zones in species with male-dominant mating systems suggests that male aggression drives genetic introgression into the less aggressive species (McDonald et al. 2001; Grava et al. 2012; While et al. 2015). However, these studies did not specifically assay female aggression, and while there may be many systems in which differential female aggression acts in parallel or in opposition to differential male aggression, this has not been empirically investigated. Given that females in a diverse range of mating systems do compete for resources (see Rosvall 2011), this is an important avenue of future research. The current study indicates that both sexes can demonstrate differential aggression in secondary contact. Future work should investigate whether this drives introgression or reduces gene flow in the jacana hybrid zone. As jacanas are role-reversed and polyandrous, we might expect that higher aggression in both sexes of *J. spinosa* has different outcomes for hybrid zone dynamics. For example, *J. spinosa* females could outcompete *J. jacana* females for territories encompassing potential mates irrespective of whether they are conspecific or heterospecific males, which could promote hybridization. In contrast, competition between males of the two species, especially the exclusion of *J. spinosa* by *J. jacana*, could reduce gene flow.

Interspecific divergence in the female competitive phenotype is one behavioral hypothesis that could explain the asymmetrical pattern of *J. spinosa* mtDNA introgression in the hybrid zone. However, divergence in male aggression would not explain this pattern. Future work should examine evidence for Haldane's Rule, another hypothesis regarding asymmetric introgression of mtDNA whereby viability and/or fertility is reduced in the heterogametic sex, which in birds are females (e.g. Carling and Brumfield 2008). Additional work on the *Jacana* hybrid zone should also investigate character displacement in competitive morphology and aggressive behavior, as well as discriminatory behavior between the species. Increased divergence in sympatry could promote reproductive isolation and/or reduce competition between the species (Pfennig and Pfennig 2009). Character displacement could also be asymmetric, whereby they differ in the extent of their divergence in sympatry (Cooley 2007; Dingle et al. 2010; Pfennig and Stewart 2011), which could facilitate asymmetric introgression. The findings presented here provide insight into variation in the competitive phenotype in both sexes and open up new avenues of inquiry about the role that male and female competition play in reproductive isolation between closely related lineages.

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CHAPTER III
DIFFERENTIAL PHENOTYPIC AND GENETIC INTROGRESSION IN A
HYBRID ZONE BETWEEN SEX-ROLE REVERSED SPECIES

Sara Lipshutz and Elizabeth Derryberry conceived the idea and design of the study, Sara Lipshutz and Matthew Miller collected the genetic samples, Sara Lipshutz conducted morphological measurements, Sara Lipshutz and Joana Meier analyzed the data, Sara Lipshutz, Joana Meier, Ole Seehausen and Elizabeth Derryberry contributed to data interpretation, and all authors contributed to writing and revising the manuscript.

Abstract

Mating behavior between recently diverged species in secondary contact can either impede or promote reproductive isolation. Traditionally, researchers focus on the importance of female mate choice and male-male competition in maintaining species barriers. Although *female-female* competition is widespread, little is known about its role in the speciation process. Here, we investigate a case of interspecific female competition and its influence on patterns of introgression between species. We examine a hybrid zone between sex-role reversed, Neotropical shorebird species, the northern jacana (*Jacana spinosa*) and wattled jacana (*J. jacana*), in which female-female competition is a major determinant of reproductive success. Previous work found that females of the more aggressive and larger species, *J. spinosa*, disproportionately mother hybrid offspring, potentially by monopolizing breeding territories in sympatry with *J. jacana*. We find a cline shift of female body mass relative to the genetic center of the hybrid zone, consistent with asymmetric introgression of this competitive trait. We suggest that competition between sex-role reversed females can facilitate hybridization, similar to males in systems with more typical sex roles. We further suggest that the jacana hybrid zone represents one of the few studies in which traits driven by intrasexual competition, not mate choice, influence patterns of hybridization between species.

Introduction

Understanding the factors that determine maintenance or loss of species barriers in sympatry after return from a period of allopatric divergence is a classical goal of speciation research (Coyne and Orr 2004; Mallet 2008). Secondary contact between recently diverged lineages provides a natural laboratory for testing the maintenance of species barriers (Hewitt 1988), particularly for organisms that are not especially fecund or are difficult to breed in captivity. Traditionally, researchers have focused on female mate choice in the evolution of sexually selected male traits important in driving and promoting species barriers (Grant and Grant 1992; Wirtz 1999; Randler 2002). Females are considered the gatekeepers of species through avoiding costly mating with heterospecifics, whereas males are expected to mate indiscriminately to maximize fitness (Darwin 1871; Bateman 1948; Andersson 1994). More recent research has highlighted the role of male-male competition in speciation (Seehausen and Schluter 2004; Tinghitella et al. 2018). However, little is known about the role *female competition* plays in the speciation process (Lipshutz 2017a), despite evidence that female-female competition over mating resources is widespread (Rosvall 2011; Cain and Ketterson 2012; Cain and Rosvall 2014).

Between recently diverged species in secondary contact, mating behavior can influence the likelihood of reproductive isolation (Irwin and Price 1999). For instance, competition over mates can either impede or promote hybridization between species, depending on its interaction with mate choice and the extent to which both forces select for similar or divergent trait optima (Qvarnström et al. 2012; Lipshutz 2017a; Tinghitella et al. 2018). Because sexual traits function both in competition for mating resources as well as mate attraction (Berglund et al. 1996; Hunt et al. 2009), their variation between species can have consequences for reproductive isolation. Sexual characteristics that function in competition include traits important in signaling dominance, such as coloration and vocalizations, morphological traits directly used in fighting, such as body size and weaponry, and behaviors, such as aggression and territoriality (Andersson 1994). Divergence in male traits important for competition can facilitate female-driven assortative mating of conspecifics from heterospecifics (Dijkstra and Groothuis 2011; Okamoto and Grether 2013). Additionally, there is theoretical and empirical evidence that simultaneous sexual selection by male-male competition and female mate choice can facilitate or even cause speciation in primary contact if it is negative frequency dependent (Doorn et al. 2004; Seehausen and Schluter 2004; Dijkstra

et al. 2005). However, negative frequency dependent selection may not be the most common outcome of trait-mediated competition. In fact, signals and traits involved in competitive interactions between sympatric species often converge to facilitate communication over territorial boundaries (Haavie et al. 2004; Tobias and Seddon 2009), which can promote hybridization (Qvarnström et al. 2010). Whether interspecific competition over mating resources erodes or maintains species barriers therefore depends on a variety of factors, including evolutionary history.

Sexual selection can drive the introgression of alleles across a hybrid zone, resulting in the differential exchange of genes across semipermeable species boundaries. This introgression can be asymmetric, whereby traits and their associated genes transition from one population into another more extensively than in the other direction. Many examples of asymmetric introgression are based on introgression of traits involved in local adaptation (Pardo-Díaz et al. 2012; Huerta-Sánchez et al. 2015; Lamichhaney et al. 2015). Traits involved in mate choice are typically thought to promote reproductive isolation, and are thus expected to have restricted introgression compared with ecological traits (Bridle et al. 2002). However, sexual selection has also been implicated in the introgression of traits across species barriers, for instance due to female preference and/or signal dominance of male traits (Parsons et al. 1993; Baldassarre et al. 2014). In one classic example – a hybrid zone between golden and white collared manakins (*Manacus candei* and *M. vitellinus*) in Panama - male differential aggression is hypothesized to explain the pattern of yellow plumage introgression across the hybrid zone relative to neutral genetic markers (Brumfield et al. 2001; McDonald et al. 2001). Morphological or behavioral traits used directly in competition for mates and/or territories can also introgress asymmetrically between lineages. In a hybrid zone between Italian and Western European common wall lizards (*Podarcis muralis nigriventris* and *P. m. bronchialis*), head length along with coloration strongly predict dominance and are asymmetrically introgressed relative to the genetic barrier between the lineages (While et al. 2015). As of yet, there are no studies demonstrating that female competition or male mate choice can facilitate introgression between hybridizing species. To understand the role of female competition in hybridization, a system in which female-female competition is a major determinant of mating success can help to generate predictions.

In the current study, we investigate the role of female competition in hybridization. Two species of Neotropical jacanas, the northern jacana (*Jacana spinosa*) and wattled jacana (*J. jacana*), are allopatric for most of their range but overlap in a narrow hybrid zone in Central America (Miller et al. 2014; Figure 17). Jacanas are a classic example of a polyandrous mating system in that females defend harems containing multiple male mates (Jenni and Collier 1972; Emlen et al. 1998; Emlen and Wrege 2004a). Compared to males, females have larger body mass and show greater development of secondary sexual characters, including keratinous wing spurs (Emlen and Wrege 2004b). Body mass is a strong predictor of female mating success, and only the heaviest females control access to mates by defending breeding territories (Stephens 1984; Emlen and Wrege 2004b). Between the species, *J. spinosa* females have larger body mass, longer wing spurs and are more aggressive than *J. jacana* females; *J. spinosa* males also have longer wing spurs than *J. jacana* males, but they do not differ in body mass (Lipshutz 2017b). Previous work on the jacana hybrid zone found a bias in the introgression of mitochondrial DNA (mtDNA), in which phenotypic hybrids shared mtDNA haplotypes with *J. spinosa* but not *J. jacana* (Miller et al. 2014). One hypothesized explanation for why *J. spinosa* females disproportionately mother of hybrid offspring is that they monopolize breeding territories and mates of both species in sympatry. As the role of female competition in the speciation process has been understudied relative to the role of male competition even for well-studied species in which females compete, (Lipshutz 2017a), the *Jacana* hybrid zone presents an excellent opportunity to examine competition and hybridization from a female perspective.

Here, we test the hypothesis that female competition promotes hybridization between *J. spinosa* and *J. jacana*. We first characterize the population structure of the hybrid zone using thousands of genome wide SNPs to understand the extent of genomic divergence and hybridization between the species. We also compare the introgression of morphological traits associated with competition (body mass and wing spurs) relative to the transition of genome-wide loci and other phenotypic traits that differ between the species (facial ornamentation and dorsal plumage). We previously found that *J. spinosa* has a more competitive phenotype than *J. jacana*, and that morphological differences between females were stronger than between males of the two species (Lipshutz 2017b). We therefore predict that the

geographic cline centers of female competitive traits will be shifted relative to the rest of the genome from the direction of *J. spinosa* into *J. jacana*.

Methods

Genetic sampling

We sampled individuals from 27 localities across Costa Rica and Panama, focusing on the region of geographic overlap between *J. spinosa* and *J. jacana* in western Panama (Figure 17; Table 14). This sampling expands upon Miller et al. (2014) to include more locations in the center of the hybrid zone and parental *J. spinosa* localities outside of the hybrid zone. In 2012 and 2014 we mist-netted and collected blood samples (40 μ L) by brachial venipuncture from 186 individuals; we released birds after banding, ensuring that each sample was unique. We stored blood in Longmire's lysis buffer (Longmire et al. 1997) at ambient temperature. Eighty-five additional tissue samples from across the hybrid zone were provided as loans from the LSU Museum of Natural Science Collection of Genetic Resources (collected 1994 – 2004) and the Smithsonian Tropical Research Institute Bird Collection (collected 2007 – 2012; Supporting Information 1). We classified individuals as adults or immatures based on plumage (Jenni 1996) and sexed individuals in the field based on mass (Emlen and Wrege 2004b) or museum tag information. For ambiguous cases we confirmed sex using PCR of the CHD regions of the W and Z chromosomes (Fridolfsson and Ellegren 1999, see Lipshutz 2017b for protocol details).

Morphological measurements

For both sexes, we measured average left and right wing spur length from the curved edge middle to the pointed tip with dial calipers to the nearest 0.1 mm. We measured body mass to the nearest 0.1 g with a Pesola scale or used information from the museum tag for the specimen associated with the LSU and STRI tissue loans. We included measurements from 51 additional birds sampled in 2015. We only included measurements from live birds to avoid differences due to shrinkage of museum specimens (Winker 1993). We only included adults in our morphological and phenotypic datasets.

Classification of parental and hybrid phenotypes

We calculated a phenotypic hybrid index based on the diagnostic phenotypic characteristics of *J. jacana* and *J. spinosa*. We scored adult phenotypes for each of three phenotypic traits: i) dorsal plumage, ii) facial shield color and shape, and iii) wattle size from live birds ($n = 237$) and previously scored individuals ($n = 67$) from Miller et al. (2014). Two observers (T. Brown, S. Lipshutz) scored each trait independently and then averaged values per trait, with 0 representing a phenotypically un-admixed *Jacana spinosa* and 1 representing a *J. jacana* based on comparisons from birds outside the hybrid zone, following Miller et al.

Sequencing

We extracted genomic DNA using a DNeasy® blood and tissue extraction kit following the manufacturer's recommended instructions (Qiagen, Valencia, CA). We sent DNA extracts to the Cornell University Biotechnology Resource Center in Ithaca, NY, USA, for genotyping-by sequencing (GBS). GBS reduced representation libraries were prepared and analyzed according to the methods of Elshire et al. (2011) using the restriction enzyme *Pst*I (CTGCAG) for digestion and creating three libraries with 96 unique barcodes, one for each individual per plate. We sequenced 285 individuals on three lanes of the Illumina HiSeq 2500 platform, generating 4,281,246 merged 64bp single-end sequence reads. Reads were mapped to a reference genome from *J. jacana* (E. Jarvis, unpublished data) using BWA version 0.7.8-r455 (Li and Durbin 2009). Single nucleotide polymorphisms (SNPs) were called using the Java program TASSEL-GBS 5.0 (Glaubitz et al. 2014). Filtering in the pipeline included minor allele frequency > 0.01 and missing data per site $< 10\%$, resulting in 360,689 variants for 271 individuals. Mean individual depth was 8.2 and mean site depth was 6.4. For population structure analyses we further filtered our SNP dataset in VCFtools v.4.2 (Danecek et al. 2011) and using custom scripts (<https://github.com/joanam/scripts>) to include minor allele frequency > 0.05 and linkage disequilibrium < 0.1 , resulting in 13,339 genome-wide bi-allelic SNPs for the 271 individuals.

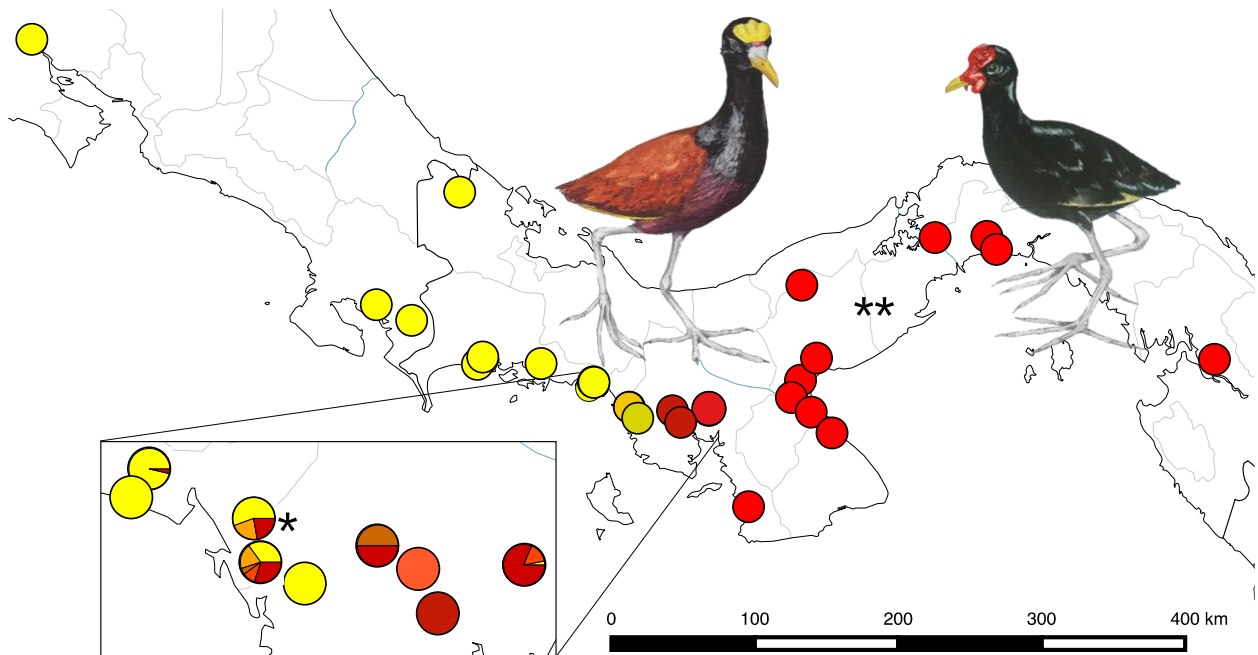


Figure 17. Genetic sampling map.

Pie color represents proportion of *J. spinosa* parental (yellow), *J. spinosa* backcross (yellow-orange), F1 hybrid (orange), *J. jacana* backcross (red-orange), and *J. jacana* parental (red) for each locality. * in inset indicates the genetic center of the hybrid zone. ** indicates the female body mass center of the hybrid zone.

Classification of parental and hybrid genotypes

To visualize clustering of parental and hybrid genotypes, we conducted a genetic principal components analysis (PCA) using the 13,339-SNP dataset for all individuals with the package SNPRelate (Zheng et al. 2012) in R (R-Core-Team 2015). We also inferred individual assignment to species using the Bayesian clustering program STRUCTURE (Pritchard et al. 2000). We limited our estimation of assignment to two populations ($K=2$) for *J. spinosa* and *J. jacana*. For 10 replicates of $K = 2$ we used a burn-in period of 100,000 followed by 500,000 Monte Carlo iterations, and an admixture model with correlated allele frequencies. We used CLUMPP (Jakobsson and Rosenberg 2007) to account for potential multi-modality and label switching among replicates and Distruct2.py (Rosenberg 2004; Raj et al. 2014) to visualize admixture proportions (Q). We classified individuals as hybrids if $0.1 < Q < 0.9$. We further classified genotypes as parental *J. spinosa* or *J. jacana*, F1 hybrid, or backcross in the package Hleest in R, which estimates genotypes based on heterozygosity and the proportion of alleles from parental populations (Fitzpatrick 2012). For this dataset we identified 344 SNPs that were fixed between allopatric populations of *J. spinosa* and *J. jacana* and had no missing data.

To estimate genome-wide differentiation between the species, we used VCFtools to calculate Weir and Cockerham weighted F_{ST} between allopatric parental individuals, excluding localities with admixed individuals based on STRUCTURE. We also examined patterns of isolation by distance within each species using a Mantel test in the R package vegan (Oksanen et al. 2017), again excluding localities with admixed individuals. We calculated pairwise F_{ST} within each species across sampling localities with 6 individuals or more (Table 1) in VCFtools and calculated pairwise geographic distances from GPS coordinates using the 'earthdist' function in the R package fossil (Vavrek 2011).

Geographic cline analyses

Examining clinal transitions (i.e., gradual changes in traits or allele frequencies across a geographic range between populations) for traits under selection relative to neutral genetic transitions between species can provide insight into their role in maintaining species barriers (Harrison and Larson 2014). Clines for traits that transition narrowly relative to dispersal ability are expected to contribute to reproductive isolation, whereas genetic loci that move freely between species indicate selection favoring the introgression of these traits in heterospecifics (Barton 1979; Barton and Hewitt 1985).

To estimate the extent of introgression between *J. spinosa* and *J. jacana* across the hybrid zone, we fit genomic and morphological data to geographic cline models (Barton and Hewitt 1985) using the Metropolis-Hastings Markov chain Monte Carlo algorithm in the R package HZAR (Derryberry et al. 2014). To evaluate the genetic center and width ($1/\text{maximum slope}$) of the hybrid zone, we estimated clinal transitions for Q from STRUCTURE for $K = 2$ for all genetic samples collected from 2010 - 2014. For the Q cline we fixed the variance and the mean at either end of the cline to 0 (eastern-most *J. jacana*) or 1 (western-most *J. spinosa*) and evaluated three cline models with none (model 1), mirrored (model 4), or both tails separately (model 5). These tails are exponential decay curves with the parameters delta, indicating distance from the cline center to the tail, and tau, tail slope. Additionally, we estimated clines for genetic loci that were diagnostic between the species, meaning they had allele frequencies >0.9 and <0.1 at each parental locality. We filtered this dataset for loci that did not deviate from Hardy-Weinberg equilibrium using VCFtools.

We also estimated clines for phenotypic traits that were diagnostic between the species and/or involved in competition, including phenotypic hybrid index as well as its individual components: dorsal plumage, facial shield color/shape, and wattle size/shape. We combined data from both sexes for these traits, since they were not sexually dimorphic, and included all live and museum samples collected from 2010 - 2014. In contrast, all competitive traits, including body mass and average spur length, were sexually dimorphic (Lipshutz 2017b), so we also estimated clines separately for males and females for these traits. We fit phenotypic traits to three standard cline models: one with fixed scaling and no tails (model 1), one with free scaling and no tails (model 2), and one with free scaling and both tails (model 3) (Derryberry et al. 2014). Patterns of asymmetric introgression are often visualized by cline centers for traits under directional selection shifted relative to the neutral genetic transition between species (Brumfield et al. 2001). To assess whether any two given clines were coincident (i.e. overlap in their centers), we compared their range of two-log likelihood support for estimated center values (hereafter CIs). We followed the same procedure to assess concordance, i.e. whether cline widths were equal.

Table 14. Genetic and morphological sampling scheme and sizes for each locality.

Q values, calculated from STRUCTURE for K=2, indicate 1 for *J. spinosa* and 0 for *J. jacana*. Geographic distance from western-most site, Palo Verde. Genetic N includes live and museum samples as well as adults and juveniles. Juveniles were excluded from morphological samples. Population genomic summary statistics including fixation index (F_{is}), Tajima's D, and nucleotide diversity (Π) $\pm$ standard deviation also included.

Site	Locality	Latitude	Longitude	Distance	Q	Genetic N	Morph N Live	Morph N Museum	F_{is}	Tajima's D	Π
1	Palo Verde	10.344	-85.338	0	1	11	11	NA	0.09 $\pm$ 0.09	0.38 $\pm$ 1	5.17 ⁻⁵ $\pm$ 5.09 ⁻⁵
2	La Gamba	8.7	-83.194	298.15	0.995	8	8	NA	0.11 $\pm$ 0.07	0.17 $\pm$ 1.03	5.33 ⁻⁵ $\pm$ 5.25 ⁻⁵
3	Changuinola	9.4443	-82.6879	307.39	1	2	NA	1	NA	NA	NA
4	Coto 47	8.6	-82.967	324.75	1	10	6	NA	0.05 $\pm$ 0.1	0.25 $\pm$ 1.04	5.31 ⁻⁵ $\pm$ 5.23 ⁻⁵
5	La Barqueta	8.3061	-82.5746	378.5	0.997	10	12	NA	0.11 $\pm$ 0.06	0.17 $\pm$ 1.04	5.18 ⁻⁵ $\pm$ 5.2 ⁻⁵
6	Orillas del Rio	8.359	-82.527	379.67	0.997	23	22	6	0.15 $\pm$ 0.47	-0.34 $\pm$ 1.03	3.71 ⁻⁵ $\pm$ 4.64 ⁻⁵
7	Horconcos	8.297	-82.167	416.23	0.998	7	3	2	0.09 $\pm$ 0.07	0.15 $\pm$ 1.02	5.7 ⁻⁵ $\pm$ 5.7 ⁻⁵
8	Las Lajas	8.176	-81.867	450.95	0.993	19	8	10	0.15 $\pm$ 0.62	-0.4 $\pm$ 0.9	4.09 ⁻⁵ $\pm$ 4.71 ⁻⁵
9	Remedios	8.214	-81.839	451.63	0.957	28	17	NA	0.23 $\pm$ 0.24	-0.39 $\pm$ 0.95	3.69 ⁻⁵ $\pm$ 4.62 ⁻⁵
10	Rio Tabasara	8.06	-81.64	479.32	0.673	11	NA	7	0.4 $\pm$ 0.32	0.17 $\pm$ 0.89	7.08 ⁻⁵ $\pm$ 6.69 ⁻⁵
11	Puerto Vidal	8.057	-81.617	481.64	0.602	21	5	6	0.37 $\pm$ 0.27	0.52 $\pm$ 0.91	7.57 ⁻⁵ $\pm$ 7.16 ⁻⁵
12	Jorones	8.0199	-81.5537	489.75	0.98	1	NA	1	NA	NA	NA
13	El Espino	8.0568	-81.375	504.42	0.081	1	NA	1	NA	NA	NA
14	Sona	8.02	-81.332	510.59	0.272	2	2	NA	NA	NA	NA
15	Tolica	7.9712	-81.2942	516.95	0.154	1	NA	1	NA	NA	NA
16	El Maranon	8.049	-81.115	523.47	0.09	32	24	NA	0.22 $\pm$ 0.09	0.46 $\pm$ 0.91	8.26 ⁻⁵ $\pm$ 7.78 ⁻⁵
17	La Corocita	8.0804	-81.0661	532.73	0.026	2	NA	2	NA	NA	NA
18	Coclecito	8.813	-80.5482	552.67	0	2	NA	2	NA	NA	NA
19	Las Macanas	8.115	-80.586	578.07	0.026	14	11	NA	0.18 $\pm$ 0.05	0.55 $\pm$ 0.9	8.2 ⁻⁵ $\pm$ 7.69 ⁻⁵
20	El Salado	8.2153	-80.5133	580.58	0.014	6	NA	5	0.23 $\pm$ 0.08	0.41 $\pm$ 0.91	8.5 ⁻⁵ $\pm$ 7.6 ⁻⁵
21	Puerto El Gago	8.3736	-80.4409	581.14	0.029	12	12	1	0.18 $\pm$ 0.05	0.51 $\pm$ 0.9	8.3 ⁻⁵ $\pm$ 7.75 ⁻⁵
22	Quebro	7.444	-80.882	584.93	0.049	12	8	NA	0.22 $\pm$ 0.12	0.5 $\pm$ 0.9	7.87 ⁻⁵ $\pm$ 7.32 ⁻⁵
23	Cenegon del Mangle	8.0502	-80.5001	589.73	0.015	2	7	2	NA	NA	NA
24	Gamboa	9.117	-79.693	634.22	0	17	11	5	0.21 $\pm$ 0.14	0.72 $\pm$ 0.89	7.58 ⁻⁵ $\pm$ 6.97 ⁻⁵
25	Tocumen	9.075	-79.3482	672.22	0	2	NA	1	NA	NA	NA
26	Pacora	9.0322	-79.3036	676.33	0	1	NA	NA	NA	NA	NA
27	Chepo	9.1664	-79.1124	695.45	NA	NA	7	NA	NA	NA	NA
28	Aruza Abajo	8.362	-77.9502	840.88	0	14	NA	9	0.22 $\pm$ 0.22	0.64 $\pm$ 0.88	7.88 ⁻⁵ $\pm$ 7.22 ⁻⁵

Results

J. spinosa and *J. jacana* are associated with distinct genomic clusters

In the genetic PCA of 13,339 SNPs, the first eigenvalue (hereafter genetic PC1) explained 51.8% of variation in the data (Figure 2). Subsequent eigenvalues explained much smaller percentages of variation (PC2 = 2.3, PC3 = 1.7, PC4 = 1.7, etc.). Both genetic PC1 and admixture proportions (Q) for K=2 distinguished the parental species and hybrids across the hybrid zone (Figure 17, 18). STRUCTURE analyses demonstrated that all individuals sampled from localities 1–8 had a high probability of belonging to *J. spinosa* ($Q > 0.9$, mean = $0.985 \pm \text{SD } 0.11$), and all individuals from localities 17–20 and 23–28 had a high probability of belonging to *J. jacana* ($Q < 0.1$, mean = 0.001 ± 0.003) (Figure 17, Table 14). Eighteen hybrid individuals ($0.1 < Q < 0.9$) were sampled at 7 different localities, spanning 105 km in the direction of the major geographical axis of the hybrid zone. The presences of individuals with $Q > 0.9$ and $Q < 0.1$ were found in sympatry at 5 localities, spanning 72 km (Figure 18, Table 14).

Genome-wide differentiation between *J. spinosa* and *J. jacana* in their allopatric populations, estimated using Weir and Cockerham weighted F_{ST} , was 0.52. Within *J. spinosa*, pairwise weighted F_{ST} ranged from 0 to 0.03 between sampling localities, with the exception of the comparison between Palo Verde and Coto 47, which was 0.05. For *J. jacana*, pairwise weighted F_{ST} ranged from 0.01 to 0.03.

We found that *J. jacana* had a pattern of isolation by distance ($r = 0.78$, $p = 0.01$), but *J. spinosa* did not ($r = 0.49$, $p = 0.099$) (Figure 19). Classification of parental and hybrid genotypes based on heterozygosity and the proportion of alleles from parental populations indicated both F1 hybrids and backcrosses with *J. spinosa* and *J. jacana*, but no F2 hybrids (Figure 20). Heterozygosity peaked in the localities in the center of the hybrid zone (Table 14). Both Tajima's D and nucleotide diversity (π) were higher in *J. jacana* than *J. spinosa* (Table 14).

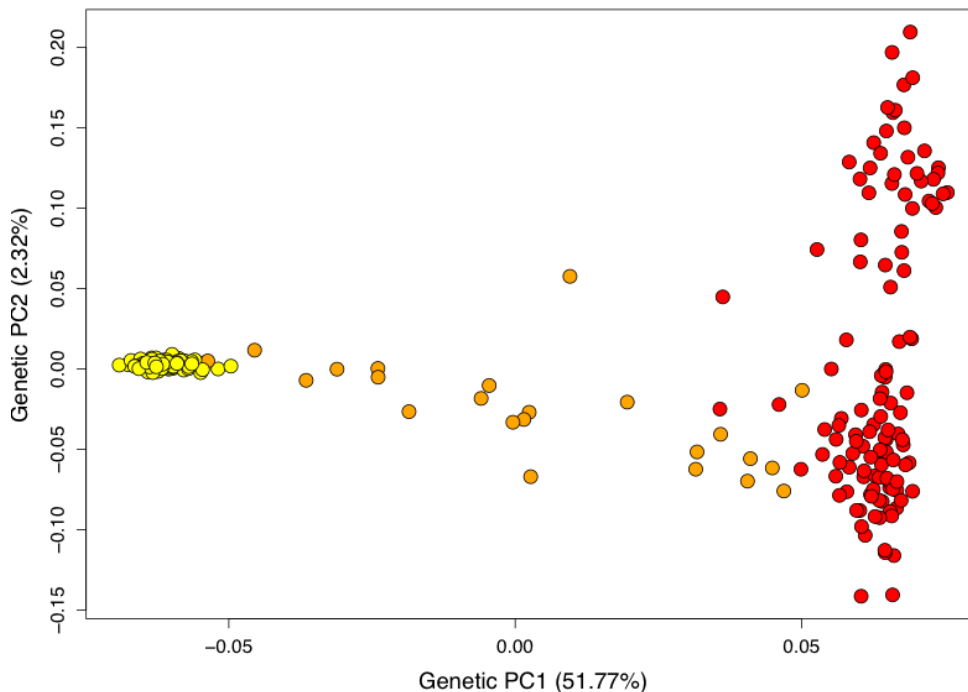


Figure 18. Genetic PC1 and PC2 of 13,339 genome-wide SNPs.

Color codes indicate phenotypic classification based on STRUCTURE admixture proportions for K=2: yellow = *J. spinosa* ($Q > 0.9$), orange = hybrid ($0.1 < Q < 0.9$), red = *J. jacana* ($Q < 0.1$).

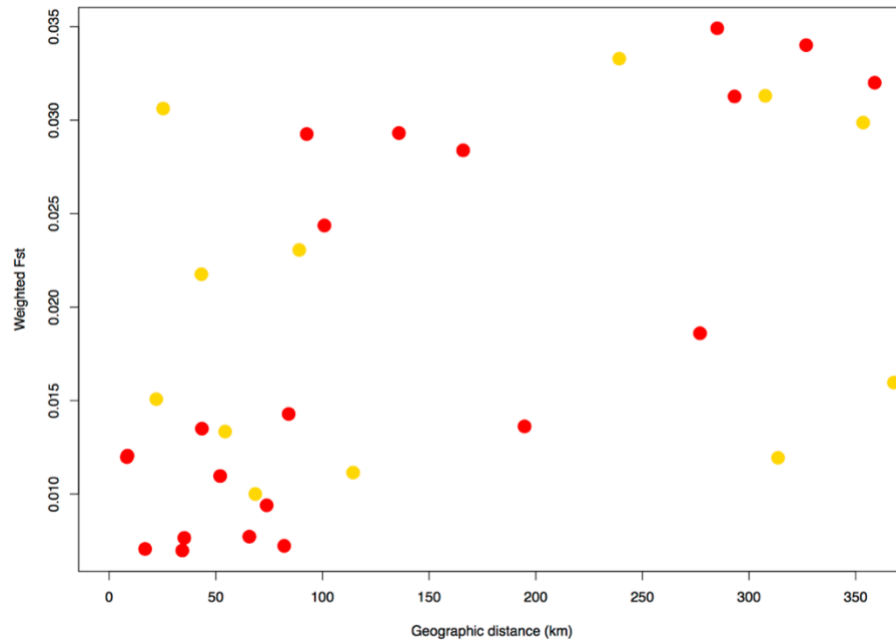


Figure 19. A pattern of isolation by distance was found in *J. jacana* (red) but not *J. spinosa* (yellow).

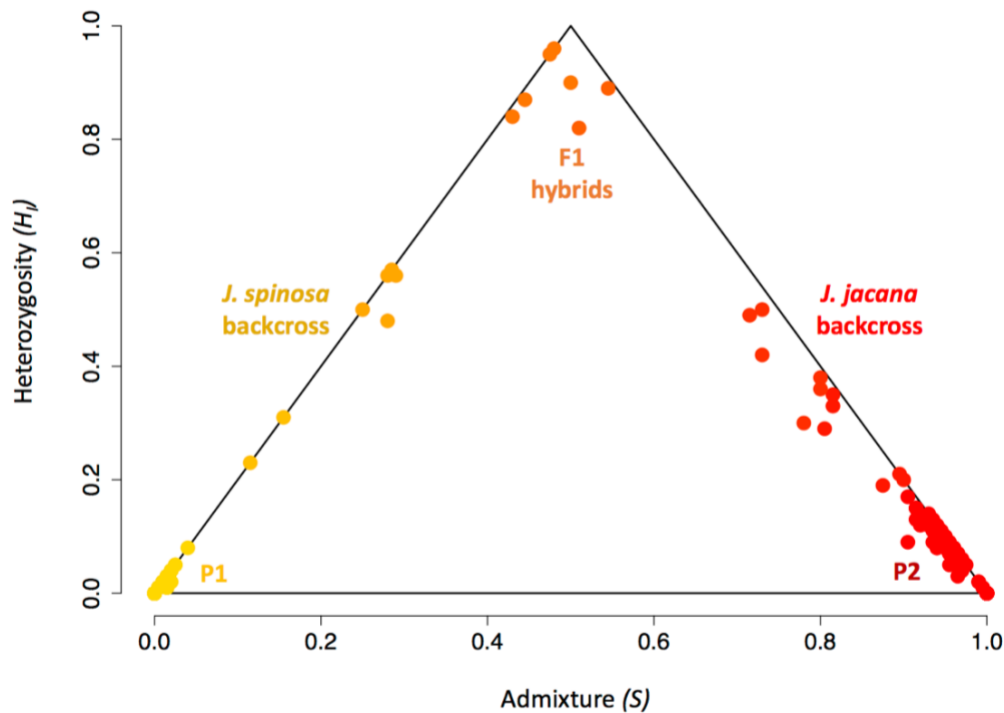


Figure 20. Classification of hybrid and parental genotypes indicated the presence of hybrid F1s (orange) as well as *J. spinosa* backcrosses (left center) and *J. jacana* backcrosses (right center).

Geographic clinal transitions

Female body mass is asymmetrically introgressed relative to Q

The best supported models for each trait are reported in Table 2. For Q (admixture proportions for $K=2$), the cline center was estimated near Puerto Vidal at 483.2 km (CI: 479.2 - 489.3) from the western-most site, with a width of 32.4 km (CI: 27.5 - 41.8). For female body mass, the cline center was estimated farther east at 611.0 km (CI: 550.9 - 672.9), near Gamboa. The log-likelihood values for the female body mass cline center do not overlap with the Q cline center, indicating that larger female body mass associated with *J. spinosa* is asymmetrically introgressed relative to genome-wide markers (Figure 21). Another way to interpret these results is to compare female body mass in sympatry and allopatry. Near the hybrid zone genetic center, females with *J. jacana* genotypes have an average body mass more similar to *J. spinosa* (*J. jacana* = 157.7g, *J. spinosa* = 167.0g), and the species do not significantly differ in female body mass (Welch Two Sample t-test: $t = -1.41$, $df = 35.9$, $p = 0.17$). In allopatry, however, female *J. jacana* body mass (134.3g) is significantly smaller than female *J. spinosa* body mass (169.7g) ($t = -5.7166$, $df = 22.351$, $p = 8.935e-06$). Plotting individual variation in female body mass across the hybrid zone for each species (Figure 22 reveals that *J. jacana* female body mass significantly increases in sympatry ($R^2 = 0.17$, $p = 0.0043$). The cline width of female body mass was estimated at 288.5 km (CI: 140.4 - 485.1). These log likelihood values do not overlap with Q cline width. Male body mass was not clinal, so we did not fit cline models for this trait.

We identified 8479 genome-wide loci in Hardy-Weinberg equilibrium that were diagnostic and clinal between the species (Figure 23). The vast majority aligned with the Q cline.

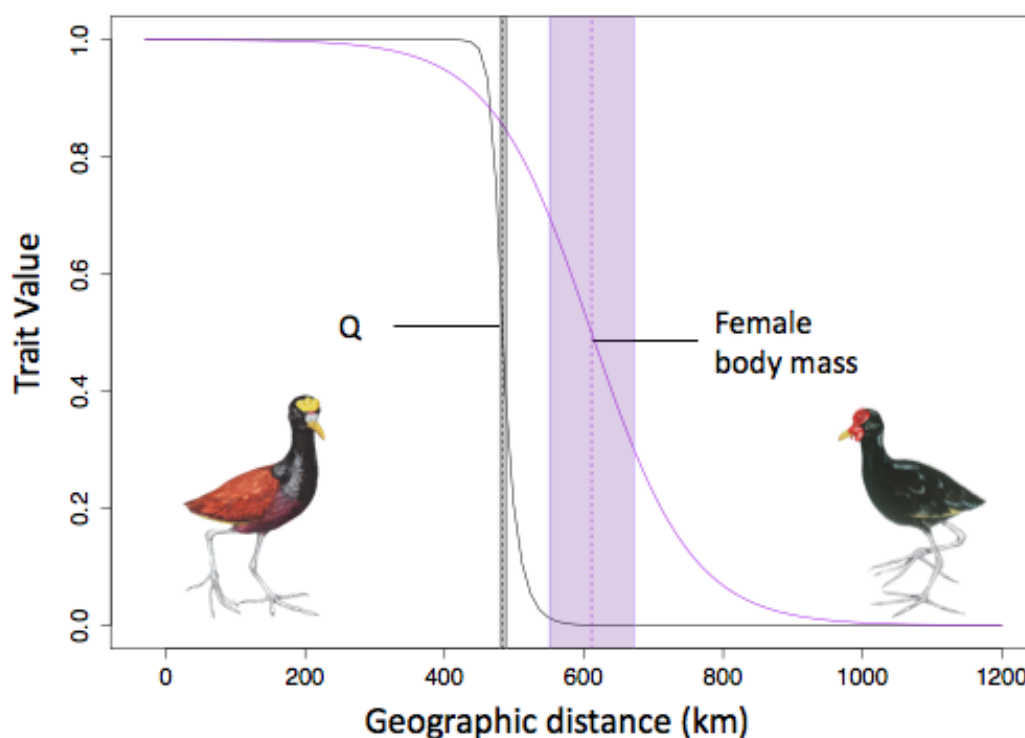


Figure 21. Best supported cline models for Q (based on STRUCTURE admixture proportions for $K=2$ from 13,339 genome-wide SNPs) and female body mass.

Dotted lines indicate cline centers, and colored vertical bars indicate confidence intervals. Distance depicted from western-most *Jacana spinosa* locality in Palo Verde, Costa Rica.

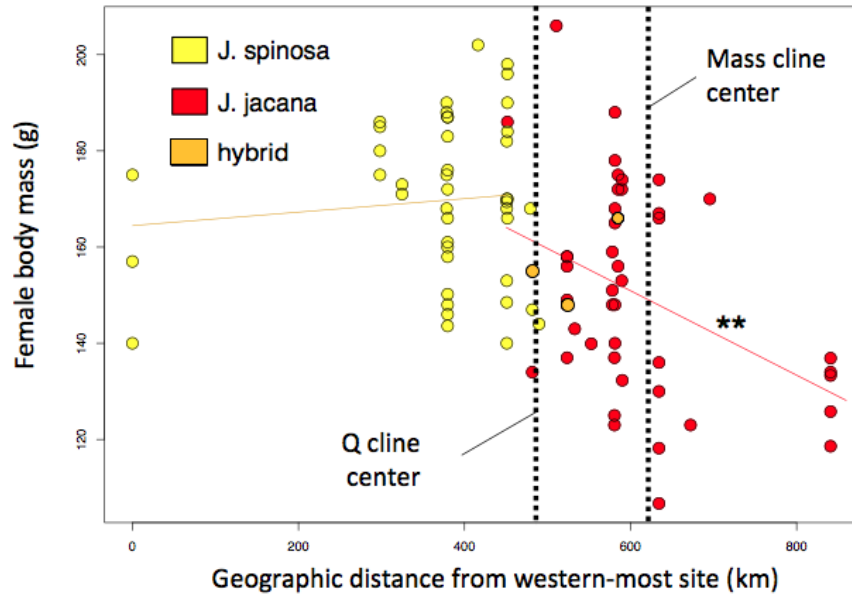


Figure 22. Individual variation in female body mass across the hybrid zone.

Dotted vertical lines indicate cline centers for Q (STRUCTURE admixture proportion for K2) and female body mass. ** Indicates a significant increase in *J. jacana* female body mass in sympatry with *J. spinosa*. ($R^2 = 0.17$, $p = 0.0043$).

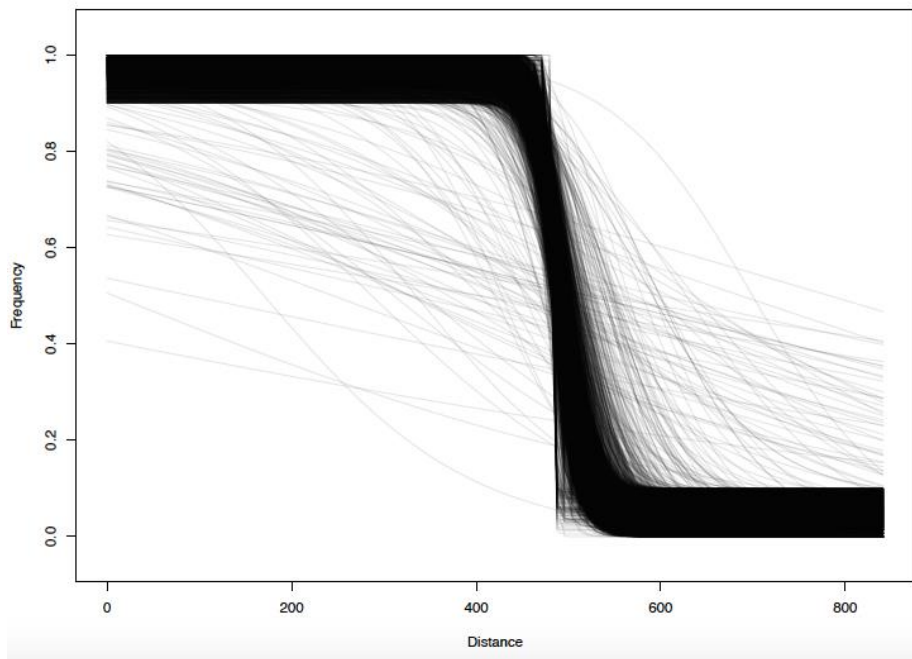


Figure 23: Best supported cline models for diagnostic genomic loci.

Geographic cline models for 8479 diagnostic, genome-wide loci in Hardy-Weinberg equilibrium.

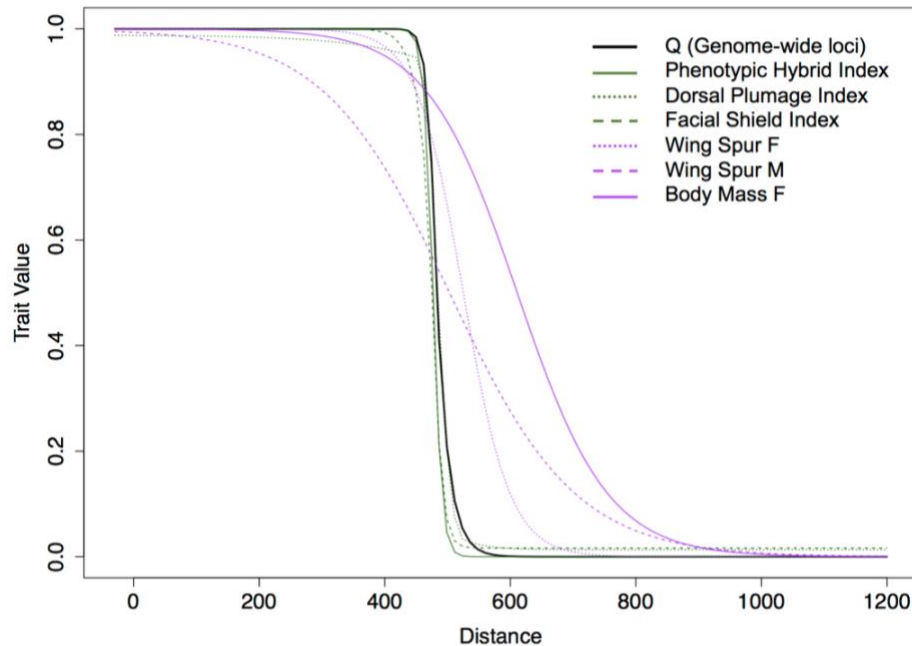


Figure 24. Best supported cline models for Q (black, genome-wide loci from STRUCTURE for K = 2), competitive traits (purple) and putative species recognition traits (green).

Other competitive traits are not asymmetrically introgressed in the hybrid zone

For female wing spur length, the cline center was estimated at 501.9 km (CI: 434.3 – 560.1), slightly shifted to the east of the Q cline center (Figure 24; Table 15). However, the log-likelihood values overlap with Q cline center, suggesting that the female wing spur and Q cline center are coincident. The cline width for female wing spur length was estimated at 359.9 (CI: 0.1 - 580.8). The overlap of these log-likelihood values with Q cline width suggests they are concordant.

For male wing spur length, the cline center was estimated at 525.16 km (CI: 367.3 - 863.6), also shifted east of the Q cline center. However, the log-likelihood values overlap with Q cline center, suggesting that the male wing spur cline and Q cline centers are coincident. The cline width for male wing spur was estimated at 148.9 km (CI: 4.0 - 915.3). This overlaps with the log-likelihood values for Q cline width and spans the entire hybrid zone, suggesting the cline widths are not independent.

Facial ornamentation and plumage are concordant with neutral genetic markers

In contrast with female body mass, the cline widths of species-specific traits including facial shield index (34.3 km, CI: 31.1 - 36.7), dorsal plumage index (39.5 km, CI: 30.3 - 46.6), and phenotypic hybrid index (29.3 km, CI: 19.8 - 36.1), were concordant with Q cline width (Figure 24, Table 15). However, the cline center for facial shield index was slightly west of the Q cline center. The models for wattle index had low likelihoods and were excluded from analyses.

Discussion

Asymmetric introgression of female body mass, a sexually selected competitive trait

We found that a female competitive trait – body mass – had a cline center shifted east relative to genome-wide markers and other species-specific traits tested. This pattern is suggestive of asymmetric introgression from the larger *J. spinosa* into *J. jacana* across the jacana hybrid zone. Considering that female *J. spinosa* are larger in morphological traits used in competition and more aggressive than female *J. jacana* (Lipshutz 2017b), our results are consistent with the hypothesis that female competition facilitates introgression between the jacana species. Our findings align with studies of other hybrid zones (Baldassarre

et al. 2014; While et al. 2015) in which traits under selection by either female choice or male competition introgress asymmetrically relative to the genome-wide cline center between species.

Interspecific competition may select for increased female *J. jacana* body mass in sympatry, though the underlying mechanism has not yet been determined. The cline shift of female body mass across the hybrid zone could be driven by the adaptive introgression of *J. spinosa* alleles for larger body mass into *J. jacana*. Loci controlling variation in avian body mass have been identified on several chromosomes (Henriksen et al. 2016), and given that size is typically a quantitative trait with small effect loci on many different chromosomes, it is likely that introgression has contributed the underlying genetic variation targeted by selection. Future work should identify whether such loci in jacanas have asymmetrically introgressed across the hybrid zone relative to the genome-wide average. An alternative hypothesis is that there is increased selection on existing genetic variation for larger body mass within *J. jacana*, due to aggressive interactions with a heterospecific competitor in sympatry. However, several *J. jacana* females in sympatry with *J. spinosa* exceed the size range observed for *J. jacana* females in allopatry, suggesting that this genetic variation may not exist outside of the hybrid zone. It is also unlikely that this cline shift is due to ecological differences within and outside of the hybrid zone, as female *J. spinosa* body mass did change across geography, whereas female *J. jacana* body mass significantly increased in sympatry.

A number of empirical studies suggest that asymmetry in competitive ability can facilitate asymmetric introgression (reviewed in Lipshutz 2017a; Tinghitella et al. 2018). One case study example is a hybrid zone between subspecies of the common wall lizard (*Podarcis muralis*), in which asymmetries in male dominance leads to directional hybridization (MacGregor et al. 2017) and asymmetric introgression (While et al. 2015). An asymmetry in competitive ability can also facilitate hybrid zone movement via geographical displacement (Grava et al. 2012). Our findings broaden the framework of this literature on male competition and hybridization by presenting the first example of female competition facilitating introgression between hybridizing species. In the context of other hybrid zone studies, we find that intrasexual competition appears to have similar evolutionary outcomes whether the competitors are males or females, such that a female competitive trait is asymmetrically introgressed relative to genome-wide markers, from the direction of the more dominant into the less dominant species. Whether intrasexual competition is similar or different between the sexes has been debated recently (Tobias et al. 2012; Cain and Rosvall 2014). It has been argued that when females compete, they are more driven for access to high quality mates, whereas males are concerned more with quantity than quality of mates (Rosvall 2011). Given that competition is more important than mate choice in determining reproductive success in jacanas (Emlen and Wrege 2004a,b) we hypothesize that interspecific competition between females is more driven by access to mate quantity rather than quality. Future work will test the hypothesis that female competition may also be driving geographic displacement and hybrid zone movement, an empirical question that requires an adequate time series (e.g. Taylor et al. 2014) which is not yet available.

For some hybrid zones in which intrasexual selection is the hypothesized mechanism driving introgression, the trait of interest has not been behaviorally linked to competitive interactions. For example, in a study hypothesizing that differential male aggression causes hybrid zone movement between hermit and Townsend's warblers, introgression of plumage color is interpreted as evidence of competition driving hybridization (Rohwer and Wood 1998; Pearson and Rohwer 2000). Although Townsend's warblers are more aggressive and have higher testosterone than hermit warblers, the plumage trait itself plays no demonstrable competitive function. In another instance, the introgression of yellow plumage from golden-collared into white-collared manakins is explained by differential male aggression (McDonald et al. 2001) but also by female choice (Stein and Uy 2006). Thus, it is not clear whether inter- or intra-sexual selection explains introgression in this system. In jacanas, female body mass is associated with female competition over breeding territories (and by extension, the males contained on those territories), but males do not appear to use female body mass in mate choice (Emlen and Wrege 2004b). Thus, the jacana hybrid zone represents one of the few studies in which there is clear evidence that competition, not mate choice, drives asymmetric introgression of a trait between hybridizing species. Long-term breeding observations of sympatric populations, along with paternity analyses, could test the hypothesis that larger bodied females monopolize harems of males in the hybrid zone.

A recent debate in the sexual selection literature is whether traits that influence female-female competition, such as female body mass in jacanas, are sexually selected (Rosvall 2011). Here, we consider traits that influence competition for mates (either in terms of mate number or quality) as sexually

selected, whereas traits that directly influence female fecundity or offspring survival are naturally selected. From a long-term study of *J. jacana* mating behavior at one site sampled in our transect, we know that females with larger body mass have larger territories, which allows them to include more mates in their harems and increase reproductive success (Emlen and Wrege 2004a; Emlen and Wrege 2004b). Sexual dimorphism is often a key indicator of sexual selection acting on a trait, and females in both species are 48% - 67% larger than their respective males (Emlen and Wrege 2004b; Lipshutz 2017b) – this dimorphism is more pronounced in *J. spinosa*. Female body mass is likely under sexual selection via female competition in these species, making it a strong candidate trait for asymmetric introgression due to interspecific competition for territories. In contrast, neither female nor male wing spur length significantly predicted reproductive success in that study (Emlen and Wrege 2004b). This could explain why these competitive traits were not asymmetrically introgressed relative to genome-wide markers in the hybrid zone.

Selection against hybridization

We suspect intrinsic incompatibility selection against hybridization in the jacana hybrid zone. The presence of F1 hybrids and backcrosses suggests that gene flow is ongoing between the two species. However, species boundaries are also being maintained, as we found no F2 hybrids and mostly early generation backcrosses. To infer selection against hybridization, the width of a cline should be interpreted relative to dispersal ability. A steep cline for genome-wide markers relative to high dispersal ability could suggest selection against hybridization between *J. spinosa* and *J. jacana*. However, dispersal has not been well characterized for the family Jacanidae, although jacanas disperse locally with seasonality (Jenni and Mace 1999). Selection against hybrids could be the result of low hybrid fitness due to intrinsic postzygotic barriers such as Dobzhansky-Muller incompatibilities (Barton and Hewitt 1985), although no study to date has examined postzygotic barriers in jacanas.

Table 15. Best supported model and parameters for cline center, width, and shape of genotypic and phenotypic clinal transitions.

Model 1 has fixed scaling (mean fixed at 0 or 1 and variance fixed at 0 for either end of the cline). Model 2 has free scaling and no tails. Model 3 has free scaling and both tails. Model 4 has fixed scaling and mirrored tails. Model 5 has fixed scaling and both tails. Mu is the mean allele frequency and phenotypic trait value for the left and right cline tails. Delta and tau are exponential decay curve parameters for left and right tails.

Model Information	Q Value	Body Mass	Wing Spur Length	Wing Spur Length	Facial Shield Index	Dorsal Plumage	Phenotypic Hybrid Index
Sex	Both	F	F	M	Both	Both	Both
Best Model	Model 5	Model 2	Model 2	Model 2	Model 3	Model 3	Model 2
Center	483.2 (479.2 - 489.3)	611.0 (550.9 - 672.9)	501.9 (434.3 - 560.1)	525.2 (367.3 - 863.6)	474.8 (473.5 - 476.0)	484.7 (482.0 - 489.5)	476.7 (473.6 - 480.2)
Coincident with Q center?	-	No	Yes	Yes	No	Yes	Yes
Width	32.4 (27.5 - 41.8)	288.5 (140.4 - 485.1)	359.9 (0.1 - 580.8)	148.9 (3.96 - 915.3)	34.3 (31.1 - 36.7)	39.5 (30.3 - 46.6)	29.3 (19.8 - 36.1)
Concordant with Q width?	-	No	Yes	Yes	Yes	Yes	Yes
muL	0	171.5 (164.7 - 178.4)	15.23 (12.96 - 16.91)	9.28 (8.94 - 10.16)	1 (0.98 - 1)	0.99 (0.97 - 1)	0.03 (0.02 - 0.04)
muR	1	128.5 (116.1 - 136.7)	8.69 (7.49 - 11.11)	7.91 (4.5 - 8.61)	0.02 (0.01 - 0.02)	0.01 (0.01 - 0.02)	0.99 (0.98 - 0.99)
varL	0	241.4 (115.1 - 436.9)	6.03 (3.11 - 13.57)	3.14 (1.87 - 4.44)	0	0	0
varR	0	0.11 (0 - 179.0)	0.24 (0 - 7.14)	1.46 (0.08 - 2.63)	0	0	0
varH	0.21 (0.14 - 0.3)	259.4 (34.2 - 606.0)	4.58 (0.1 - 19711.9)	0 (0 - 11.81)	0.41 (0.23 - 0.46)	0.14 (0.09 - 0.22)	0.24 (0.12 - 2.01)
deltaL	69.6 (59.8 - 90.0)	None	None	None	2.7 (2.0 - 6.6)	30.05 (21.4 - 34.2)	None
deltaR	1.41 (0 - 16.2)	None	None	None	128.72 (64.4 - 288.1)	37.5 (64.4 - 288.1)	None
tauL	0.18 (0.14 - 0.25)	None	None	None	0.48 (0.4 - 0.5)	0.09 (0.4 - 0.48)	None
tauR	0.44 (0.37 - 0.59)	None	None	None	0.25 (0.21 - 0.93)	0.28 (0.21 - 0.93)	None

Prezygotic barriers, such as species recognition traits, could also maintain species boundaries between jacanas by promoting reproductive isolation (Hudson and Price 2014), though this would not restrict the rest of the genome from introgressing, given that backcrossing occurs. We found that species-specific phenotypic traits, including facial ornamentation and dorsal plumage, had steep clines that were concordant with genome-wide markers, whereas the widths of clines associated with competitive traits were much wider. Across the Neotropical *J. jacana* species complex, all subspecies have chestnut-brown dorsal plumage except the black Panamanian *J. jacana* subspecies. This plumage difference in sympatry with a heterospecific is suggestive of reproductive character displacement.

Hybridization can promote the exchange of beneficial alleles between species despite the maintenance of species barriers across the majority of their genomes (Grant and Grant 2010), which is becoming known as 'adaptive introgression'. Recent examples include insecticide resistance in mosquitoes (Clarkson et al. 2014), color pattern in *Heliconius* butterflies (Enciso-Romero et al. 2017), and opsin genes and nuptial coloration in Lake Victoria cichlids (Meier et al. 2017; Meier et al. 2018). In jacanas, the adaptive potential of hybridization via introgression of female body mass could potentially outweigh the costs of hybridization between the jacana species, as a larger body mass in females is associated with higher reproductive success (Emlen and Wrege 2004a; Emlen and Wrege 2004b). Comparing the introgression of putative loci for larger body mass, which is likely sexually selected, with loci resistant to introgression and associated with reduced hybrid viability, which indicate natural selection against hybridization, could lend insight into the potential roles of sexual and intrinsic incompatibility selection in the jacana hybrid zone.

Conclusions

In summary, we find the first evidence for asymmetric introgression of a trait important in female-female competition. This trait is associated with reproductive success due to competition for territories and mates, not due to mate choice, which has been inferred to explain asymmetric introgression in other hybrid zone studies. We therefore conclude that competition between sex-role reversed females can facilitate hybridization, similar to males in systems with more typical sex roles.

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CONCLUSION

Historically, research on the role of mating behavior in hybridization has focused on competitive males and choosy females. Although female-female competition is widespread and has important fitness consequences for many species across numerous taxa, little is known about the role of female competition in mediating hybridization between closely related lineages. I compare morphological and behavioral traits related to competition between two hybridizing species of jacanas, tropical shorebirds with sex-role reversal. I find that *Jacana spinosa* females have a more competitive morphological phenotype and higher aggression than *Jacana jacana* females, which may allow them a competitive advantage in obtaining and defending territories and mates in the hybrid zone. These patterns align with a pattern of asymmetrical introgression of *J. spinosa* mtDNA previously described in the hybrid zone, as well as findings from other hybrid zones in which male-male competition can potentially explain asymmetric patterns of gene flow from the more dominant species into the less dominant species.

VITA

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