

Reproductive Costs for Hybridizing Female *Anasa tristis* (Hemiptera: Coreidae), but No Evidence of Selection Against Interspecific Mating

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Abstract

Individuals of different species sometimes mate in nature, and such behavior often carries costs, such as wasted gametes and inviable offspring. One context in which interspecific mating commonly occurs is when closely related species come into secondary contact. Here, we tested whether reproductive isolation is greater in an area of recent secondary contact than in allopatry for two closely related insect species, and we examined whether mating between individuals of these two species constitutes reproductive interference. In Florida, two species of squash bugs (Hemiptera: Coreidae: *Anasa tristis* DeGeer and *Anasa andresii* Guérin-Méneville) have been secondarily sympatric for ≥ 80 generations, and male *A. andresii* copulate with female *A. tristis*. Because hybridization is often costly for females, we predicted that secondarily sympatric females would be less likely to mate with heterospecifics than would allopatric females. We found no evidence of recent selection on reproductive isolation: females from both populations were equally likely to mate with heterospecifics, and heterospecific males did not make more mating attempts than conspecifics to achieve copulations. However, female *A. tristis* paired with heterospecifics produced many fewer eggs and offspring than females paired with conspecifics, and this did not differ according to whether females were from allopatric or sympatric populations. Our findings show that reproductive barriers between these species existed before secondary contact. We suggest that habitat use may limit encounter frequency, and that female choice, multiple mating, and postcopulatory processes may reduce costs for females. Consequently, we suggest that mating systems and ecological factors mediate the effects of reproductive interference.

Key words: Hybridization, reproductive interference, mating behavior

Mating, misdirected courtship, signal jamming, and competition for mates between individuals of different species occur in a great variety of taxa (reviewed in Gröning and Hochkirch 2008, Kyogoku 2015). Any one of this suite of reproductive behaviors is considered ‘reproductive interference’ (RI) when it negatively impacts the fitness of individuals of at least one of the species involved (Gröning and Hochkirch 2008). RI can be a useful lens for examining how organisms make decisions that lead to interspecific mating (Burdfield-Steel and Shuker 2011, Hamel et al. 2015) and the consequences of such matings, including decreased population growth for one or both species (Rhymer and Simberloff 1996, Hettyey et al. 2014), habitat partitioning (Mecham 1960, Höbel and Gerhardt 2003, Gordon et al. 2017), reproductive exclusion (reviewed in Gröning and Hochkirch 2008), and reproductive character displacement (Gabor and Ryan 2001, Höbel and Gerhardt 2003), which can occur rapidly as a result of RI in zones of secondary contact (Bargielowski et al. 2013, Gordon et al. 2017). Despite a surge of recent interest in RI, its

consequences are not easily predicted, in part because a wide range of factors can influence whether and how individual-level effects translate to population-level consequences (Kyogoku 2015, Shuker and Burdfield-Steel 2017).

Such factors include the frequency of RI occurrence (Coyne and Orr 2004, Gröning and Hochkirch 2008), the nature and severity of associated costs (Kitano et al. 2007), and variables likely to influence RI frequency and costs, such as niche overlap of focal species (Gröning et al. 2007), whether costs are environmentally mediated (Pfennig 2007), and whether post-zygotic isolation is complete (Liou and Price 1994). Moreover, fitness losses incurred by individual females in a natural population may be mitigated by female learning (Svensson et al. 2010), or abolished by multiple mating and post-copulatory processes, through a variety of mechanisms (Wade et al. 1994, Howard et al. 1998, Reinhardt 2006, Larson et al. 2012). Focusing more effort in contexts where RI most commonly occurs and on species for which these factors can be defined will improve

our ability to predict ecological and evolutionary outcomes, such as whether selection via interspecific mating reduces the receptivity of females to heterospecific males (Howard 1993).

Two common contexts in which RI occurs include when species that have been historically separated come into secondary contact (Coyer et al. 2002, Servedio and Noor 2003, Coyne and Orr 2004, Lipshutz 2018), and when closely related species are sympatric (Hochkirch et al. 2008, Hochkirch and Lemke 2011). Agricultural settings may provide a particularly rich trove of information about RI in these contexts, because accidental movement and introduction of plant-living insect pests by humans are ubiquitous (Pimentel et al. 2000, Duyck et al. 2004, Chen et al. 2006). The potential for ecologically realistic experimentation is great: for example, field densities of such species may be manipulated by controlling resource abundances (i.e., crops). Moreover, findings from studies testing theory about mating behavior in agricultural contexts can lead to beneficial downstream applications (Boake et al. 1996).

Here, we tested whether interspecific mating has resulted in selection on female receptivity toward heterospecific males, and we examined whether interspecific mating constitutes RI for two closely related insect species that have been in secondary contact for ~40 years (~80 generations). The squash bug (Hemiptera: Coreidae: *Anasa tristis* DeGeer) is an agricultural pest of plants in the cucurbit family that occurs throughout the Americas (Beard 1940). A congener, *Anasa andresii* Guérin-Méneville (Hemiptera: Coreidae), has a largely Neotropical distribution (Jones 1916) and appears to have been introduced to north central Florida in the early 1970s (Baranowski and Slater 1986; J.A.H., personal observations from specimens at Florida State Collection of Arthropods). In the field in north central Florida, we observed mating between individuals of these two species primarily in one direction: the smaller male *A. andresii* copulate with the larger female *A. tristis* (J.A.H., unpublished data). Such matings are driven at least in part by male *A. andresii* preference for larger female body size (Hamel et al. 2015). During laboratory choice tests, most male *A. andresii* directed their first mating attempt toward a female *A. tristis* over a conspecific female, suggesting that if encounter frequency is high in the field, such mating attempts are also common.

We tested the hypothesis that behavioral reproductive isolation between species should be greater when species occur in secondary sympatry (Brown and Wilson 1956, Coyne and Orr 1989, Howard 1993). We predicted that female *A. tristis* from the secondarily sympatric population would be less likely to accept mating attempts from male *A. andresii* than would female *A. tristis* from a historically allopatric population. An assumption of this hypothesis is that interspecific mating is costly (and constitutes RI) for female *A. tristis*. Therefore, we also predicted that females paired with heterospecific males would produce fewer eggs and offspring than would females paired with conspecifics.

Materials and Methods

Insect Collection and Rearing

In June 2015, we collected late-stage (third to fifth instar) juvenile *A. tristis* and *A. andresii* from three community gardens in the zone of secondary contact in Alachua Co., FL. We also collected late-stage juvenile *A. tristis* from three organic farms in a historically allopatric population, in Alamance and Guilford Co., NC. We transported all insects to Elon, NC, where we reared juveniles communally in mesh insect cages separated by both population (NC and FL) and species (*A. tristis* and *A. andresii*). Mesh cages were kept in environmental

chambers (Enconair, GC8-2H; Winnipeg, MB, Canada), and insects were reared under a summer photoperiod (14:10 h light:dark) at 26°C. Insects in each mesh cage were fed with ad libitum potted cucurbits (*Cucurbita pepo*, Linnaeus (Cucurbitales: Cucurbitaceae)).

To ensure that experimental insects were previously unmated, cages of juveniles were checked every 48 h for newly eclosed adults. Adults were removed and maintained together with cucurbit seedlings in individual 32 oz. clear plastic containers with mesh lids. All adults were kept in a climate-controlled greenhouse with seasonal variation in photoperiod. During all insect rearing and maintenance, we monitored temperature and photoperiod with data loggers (HOBO Pendant temp/light UA-002-64; Onset, Bourne, MA).

Do Female *A. tristis* From the Sympatric Population Show Evidence of Selection Against Interspecific Mating?

To determine whether female *A. tristis* from the secondarily sympatric population (FL) showed greater discrimination toward heterospecific males than females from the historically allopatric population (NC), we paired female *A. tristis* from each population with a heterospecific male in no-choice trials. We focused our study on responses by female *A. tristis* because interspecific matings observed in the field were overwhelmingly between male *A. andresii* and female *A. tristis* (J.A.H., unpublished data). As a baseline against which to compare female *A. tristis* receptivity, we also paired NC female *A. tristis* with conspecific males. Our pairing combinations were therefore: female FL *A. tristis* × male FL *A. andresii* (hereafter, *sympatry*), female NC *A. tristis* × male FL *A. andresii* (hereafter, *allopatry*), and female NC *A. tristis* × male NC *A. tristis* (hereafter, *conspecifics*).

To ensure that all adults were reproductively mature and to control for unwanted effects of age on female reproductive behavior, individuals were tested 14–21 d after their final molt into adulthood. We chose 14 d post-adulthood as a minimum age because in a laboratory setting, newly eclosed summer adults do not engage in reproductive activity for several days, and because the ovarioles and oviducts of female *Anasa* spp. contain the first clutch of developing eggs at 14–21 d (J.A.H., unpublished data). It is unlikely that any of these females were geriatric, as we have recaptured marked and mating females in the field several weeks after first observing them mating (J.A.H., unpublished data).

Observations were conducted in the same greenhouse where adult insects were maintained. It was not possible to record data blind, because there are observable phenotypic differences between the focal species. Each female was placed in an empty, clear, 32 oz. plastic container with a mesh lid and given 30 min to acclimate before the observation period, which lasted for 2 h. To assess female receptivity to males, we recorded the number of females that copulated during the observation period and, for pairs that mated, the number of male mating attempts before copulation occurred. Copulation, in which male genitalia penetrate female genitalia and the two individuals are attached at distal ends, only occurs in both of these species after a male mating attempt, in which a male approaches and mounts a female, placing all six legs on her dorsal side. Females can reject male mating attempts: they sometimes rapidly jerk or shake when mounted and can thereby dislodge males, and they can move away from and evade pursuing males.

Pairs were included in behavioral analyses if males made mating attempts during the 2-h observation period (sample sizes: conspecific: 23 pairs; allopatric: 21 pairs; and sympatric: 19 pairs). We used logistic regression to test if pairing combination affected whether female *A. tristis* copulated, and we used the Kruskal–Wallis rank

sum test to compare the effect of pairing combination on the number of mating attempts by males before achieving copulation. To assess whether males of the two species demonstrated different levels of mating activity, we also compared the total number of male mating attempts for pairs that did not copulate across pairing combinations using the Kruskal–Wallis rank sum test. All analyses were conducted in R (version 3.3.3).

What Are the Reproductive Costs of Hybridization for Female *A. tristis*?

After each set of behavioral trials ended, pairs were kept together and housed in the same manner as described for adults. To assess the effect of pairing combination on female fecundity, we collected all eggs produced by each female over the 28 ± 2 d after a trial. To increase sample sizes, we included eggs from females regardless of whether or not they had copulated during the 2-h observations. Copulations were anecdotally observed occurring between most pairs of males and females during the 28 ± 2 d timeframe. We excluded data from females who died during the egg collection period. Sample sizes for fecundity and fertility measures were: conspecific: 27 pairs; allopatric: 19 pairs; and sympatric: 20 pairs.

Using a linear model, we fitted total eggs produced to the following fixed effects: pairing combination, female pronotal width, and mean photoperiod experienced by adults before trials, measured over the 14 d after adult molt. We included female body size, here measured as pronotal width, as a covariate because it is typically highly correlated with fecundity (Honěk 1993). We included photoperiod as a covariate because photoperiod can affect egg production for female hemipterans (Dingle 1968, Carvalho et al. 2006).

To assess the reproductive costs of hybridization for females, we quantified the proportion of each female's eggs that hatched. To do so, we held eggs in petri dishes in an environmental chamber (Enconair model GC8-2H; Winnipeg, MB, Canada) for 14 d; eggs of *A. tristis* hatch in 9–12 d under similar laboratory conditions (Beard 1940). The effect of pairing combination (i.e., conspecific, allopatric, or sympatric) on the hatching success of each female's eggs (binomial variable: number hatched, number unhatched) was modeled using a generalized linear mixed model (GLMM) fitted to the binomial error structure with a log link, using the lme4 package. This approach allowed us to take variation in total number of eggs per female into account, and also correctly models proportional data (Warton and Hui 2011, Mangiafico 2016). Pairing combination was the fixed effect, and we included pair as a random effect to model overdispersion (Browne et al. 2005).

Results

Do Female *A. tristis* From the Sympatric Population Show Evidence of Selection Against Interspecific Mating?

Pairing combination did not affect the proportion of female *A. tristis* who mated during the 2-h observation periods (logistic regression: $n = 63$, Wald $\chi^2 = 2.06$, $P = 0.357$) (Fig. 1, left panel). Similarly, in pairs that mated, pairing combination did not affect the total number of attempts by males before females copulated (Kruskal–Wallis $\chi^2 = 3.10$, $n = 44$, $df = 2$, $P = 0.212$) (Fig. 1, right panel), and across pairing combinations, most female *A. tristis* who copulated did so after a single male mating attempt (conspecifics: 13/14, allopatry:

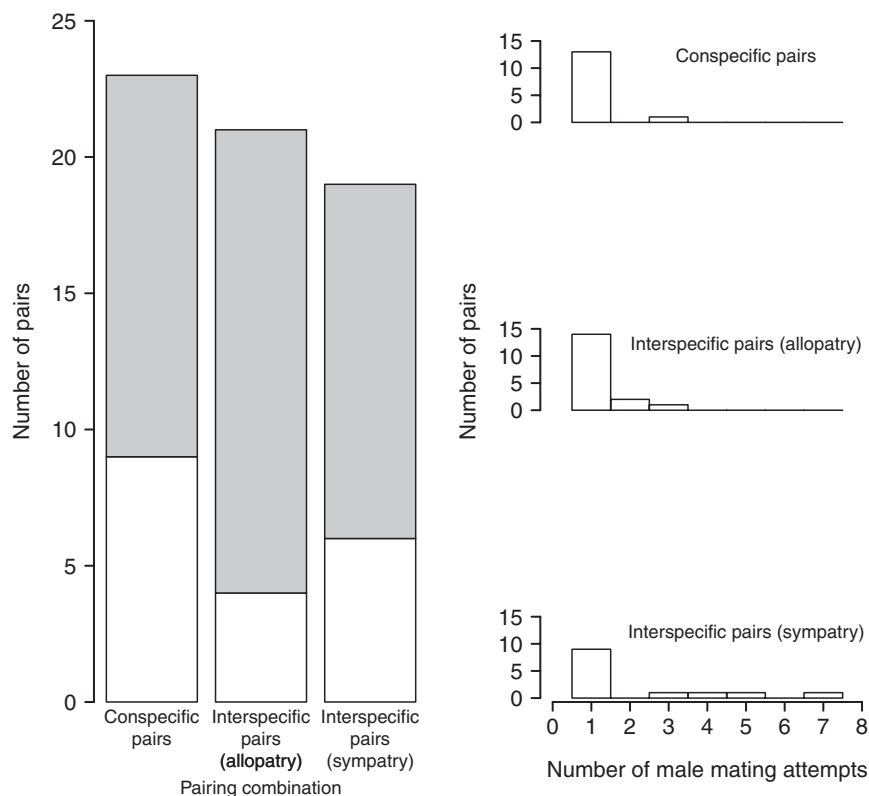


Fig. 1. Left panel: Pairing combination did not affect whether females copulated during a 2-h observation period. Gray portion of each bar indicates pairs that copulated; white portion shows pairs that did not mate. Right panel: Pairing combination did not affect the number of male mating attempts before copulation occurred. Most female *A. tristis* who copulated did so after a single male mating attempt.

14/17, sympatry: 9/14; Fisher's exact test: $P = 0.298$). In trials where copulation did not occur and males made at least one attempt, the total number of male mating attempts did not differ according to pairing combination (Kruskal–Wallis $\chi^2 = 2.26$, $n = 19$, $df = 2$, $P = 0.323$).

What Are the Reproductive Costs of Hybridization for Female *A. tristis*?

Pairing combination affected the number of eggs produced by females ($F_{2,61} = 8.489$, $P < 0.001$). Females in conspecific pairs produced more eggs (least squares [LS] mean \pm 1 SE: 75.85 ± 6.79) than did females in interspecific pairs (allopatry: 33.60 ± 8.61 , sympatry: 41.78 ± 7.41) (Fig. 2, left panel; Table 1). The number of eggs produced did not differ between sympatric (FL) and allopatric (NC) females in interspecific pairs (pairwise comparison: $t = -0.705$, $P = 0.762$). Mean photoperiod after adulthood also affected the number of eggs produced ($F_{1,61} = 50.497$, $P < 0.001$). Surprisingly, female body size did not affect the number of eggs produced ($F_{1,61} = 0.018$, $P = 0.892$), although pronotal width varied among females (FL female *A. tristis*: 5.35 ± 0.56 mm, range: 4.75–6.37 mm; NC female *A. tristis*: 5.26 ± 0.38 mm, range: 4.40–6.03 mm; values are means \pm SDs).

Pairing combination also affected hatching success (binomial GLMM, $n = 66$, log-likelihood ratio = 23.615 from comparison against null model, $P < 0.001$). A lower proportion of eggs hatched from interspecific than from conspecific pairs, but the proportion

of eggs that hatched did not differ between the two interspecific pair types (Fig. 2, right panel; Table 2). Compared with conspecific pairs, interspecific pairings reduced egg number (allopatric females, by 55.70%; sympatric females, by 44.92%), and the probability of egg hatching (allopatric females, by 92.50%; sympatric females, by 93.30%). The reproductive value of an interspecific pairing is therefore a small percentage of that of a conspecific pairing (allopatric females 3.32%; sympatric females 3.69%).

Several interspecific pairs produced hybrid offspring. We reared hybrid offspring in 32 oz. clear plastic containers together with seedling squash plants (*C. pepo*). Hybrid nymphs were maintained under the same abiotic conditions as field collected juvenile *A. tristis* and *A. andresii*, as described in Materials and Methods. Of 36 total nymphs produced by eight female FL *A. tristis* paired with male *A. andresii*, 3 individuals (2 males and 1 female) survived to reproductive maturity (≥ 14 d post adult eclosion). Of 69 total nymphs produced by 10 female NC *A. tristis* paired with male *A. andresii*, 4 individuals (3 males and 1 female) survived to reproductive maturity (> 14 d post adult eclosion). We backcrossed all hybrid adults with NC *A. tristis*. None of these F1 adult pairs produced any offspring.

Discussion

In an area of secondary sympatry, individuals of two closely related insect species copulate in the field. Our findings suggest that reproductive isolation mechanisms were present before secondary contact,

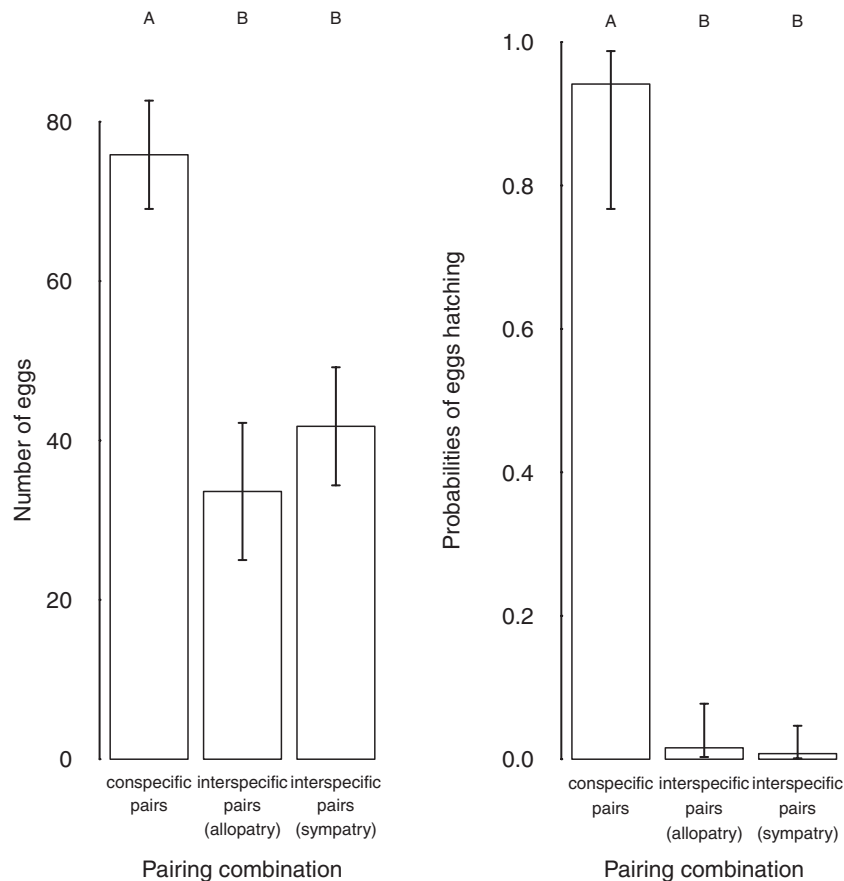


Fig. 2. Left panel: Pairing combination affected the mean number of eggs produced by females. Bars show LS means; error bars show ± 1 SEs. Different letters denote differences at ≤ 0.05 from Tukey pairwise comparisons. Right panel: A lower proportion of eggs hatched from interspecific pairs than from conspecific pairs. Bars show probabilities from GLMM; error bars show 95% CIs back transformed from the logit scale. Different letters denote differences at ≤ 0.05 from Tukey pairwise comparisons.

Table 1. Results of linear model of how pairing combination influenced number of eggs produced ($N = 66$, $F_{4,61} = 14.72$, $R^2_{adj.} = 0.458$, $P < 0.001$)

Fixed effects	Estimate	SE	<i>t</i>	<i>P</i>
Interspecific pairs (allopatry)	-42.252	11.850	-3.566	< 0.001
Interspecific pairs (sympatry)	-34.077	9.925	-3.433	0.001
Female body size	1.314	9.674	0.136	0.892
Mean photoperiod	148.099	20.841	7.106	< 0.001

Interspecific combinations were contrasted against conspecific pairs in the model.

Table 2. Contrasts from binomial GLMM showing how pairing combination influenced proportion of eggs that hatched ($N = 66$, log-likelihood ratio = 23.615 from comparison against null model, $P < 0.001$)

Pairwise comparisons	Estimate	SE	<i>z</i>	<i>P</i>
Conspecific–interspecific (allopatry)	6.904	0.962	7.176	<0.001
Conspecific–interspecific (sympatry)	7.628	1.023	7.457	<0.001
Interspecific, allopatry–sympatry	0.724	0.980	0.739	0.740

Pairwise comparisons were performed on the log-odds ratio scale and corrected using a Tukey adjustment.

and also that interspecific matings constitute RI: if female *A. tristis* mate with only heterospecifics, they produce many fewer offspring than if they mate with conspecifics. We note that we only assayed female fecundity and reproductive success, and RI may also impact female longevity (McLain and Pratt 1999, Kishi et al. 2009, Kyogoku and Sota 2015, Shuker et al. 2015).

When hybridization results in reproductive penalties, we generally expect selection for reproductive isolation (Brown and Wilson 1956, Coyne and Orr 1989, Howard 1993), and we predicted that *A. tristis* from an area of secondary sympatry would be less likely to accept mating attempts from heterospecific males than would females from an allopatric population. Despite the costs of interspecific mating, we found no evidence of selection against such matings. We consider several explanations for our findings. First, females in this study each encountered only one male, and rejecting a prospective mate in this scenario may be equivalent to choosing not to mate. Female *Anasa* that encounter multiple males may express a preference that is undetectable in a no-choice paradigm. Although recent work on an ecologically similar insect species suggests findings from no-choice and choice tests could be similar (Dougherty and Shuker 2014), we note that in the field, female *Anasa* may simultaneously encounter multiple prospective mates, as they can occur at high densities (J.A.H., unpublished data).

Secondly, although models suggest that low levels (e.g., 2–5%) of interspecific mating can drive population dynamics if reproductive costs from reduced female fertility are high (Ribeiro and Spielman 1986; Kishi and Nakazawa 2013), mating system and postcopulatory processes affect the presence and magnitude of such costs (Marshall et al. 2002, Hartman et al. 2012, Shuker and Burdfield-Steel 2017, Lipshutz 2018). Because female (and male) *Anasa* mate multiply, females might abolish the costs of a first or single mating with a heterospecific by also mating with a conspecific (Price 1997, Noriyuki et al. 2012, Burdfield-Steel et al. 2015). Such mitigation can occur through a variety of mechanisms, including males preferentially allocating sperm to females of their own population or species (Reinhardt 2006) or conspecific sperm precedence (Howard et al. 1998, Lorch and Servedio 2007) via cryptic female choice (Tyler et al. 2013, Yeates et al. 2013). The reproductive costs we measured therefore represent maximum potential costs, and we suggest that postcopulatory processes are likely to affect reproductive outcomes. Females that develop in areas of high heterospecific densities may also learn to avoid heterospecifics after an initial encounter, reducing rates of interspecific matings (Svensson et al. 2010).

Recent empirical studies on RI have focused on ecological factors likely to influence its frequency of occurrence and population-level consequences, including relative densities of species (Vallin et al. 2012, Hettyey et al. 2014, Rohde et al. 2015), habitat use (Rohde et al. 2015), and behavior of one or both species involved (Hochkirch and Lemke 2011, Izzo and Gray 2011, Bath et al. 2012). Although reproductively mature adult *A. tristis* and *A. andresii* substantially overlap in the field during a season, their generations are not entirely synchronous, and relative abundances change over 1 y (J.A.H., unpublished data). Moreover, although both species feed, mate, and oviposit on *Cucurbita* spp. (Jones 1916, Beard 1940), *A. tristis* typically occur on substrates and plant stems near the soil surface, whereas *A. andresii* are observed near the substrate but also on vertical surfaces (e.g., vining, trellised plants) ≥ 1 m above the soil. Quantifying *Anasa* spp. habitat use and relative abundances in the area of sympatry would provide necessary information for inferring the frequency of RI.

In summary, although we documented substantive potential costs for mating between male *A. andresii* and female *A. tristis*, we found no evidence of selection against interspecific mating. Female *A. tristis* may choose conspecific males over heterospecifics when males of both species are available for mating. Generations that are not completely synchronous in the field and differences in habitat use may limit encounter rates between species. When interspecific matings occur, female *A. tristis* may mitigate or abolish costs by also mating with conspecifics. We therefore suggest that the level of gene flow from mating between these two species is unlikely to lead to introgression and collapse of species boundaries. Assessing female response to heterospecific males in a choice paradigm, examining whether female learning affects mating decisions, documenting relative species densities and habitat use in the field, and investigating the potential roles of postcopulatory processes are necessary next steps in understanding the consequences of interspecific mating in this study system. More thorough descriptions of male and female mating behavior would also be informative, as mating preferences and other components of mating systems may differ between *A. tristis* and *A. andresii*.

We suggest that generalizations about the consequences of RI will be facilitated by further study of factors such as habitat use and mating systems that affect pre- and postcopulatory processes for focal species.

Data Availability Statement

Data from this study are available from the Dryad Digital Repository: doi:10.5061/dryad.f548pb4 (Hamel et al. 2018).

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