Founder effects and the evolution of asymmetrical sexual isolation in a rapidly-speciating clade

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Abstract   Sexual isolation resulting from differences in mate choice behaviors is a hallmark of rapidly-speciating lineages. When present, asymmetrical sexual isolation may provide insights into the mechanisms responsible for the evolutionary change in mate signaling traits. In particular, Kaneshiro's hypothesis suggests that divergence in sexual characters between populations may arise in allopatry when 'derived' founding populations are subject to severe population bottlenecks, accompanied by a relaxation of sexual selection relative to 'ancestral' source populations. In the present study, we tested predictions of asymmetrical sexual isolation between two allopatric species of Hawaiian *Laupala* crickets, representing 'ancestral' (*L. makaio*) and 'derived' (*L. nigra*) taxa. While crickets in this genus are notable for rapid divergence of male courtship songs, these species share similar song types, thus suggesting that patterns of sexual isolation are likely due to other mating cues. Analysis of behavioral responses in conspecific and heterospecific 'no-choice' mating trials revealed pronounced asymmetrical isolation in the direction predicted by Kaneshiro's hypothesis, wherein we observed a significant reduction in mating success for crosses involving 'derived' males paired with 'ancestral' females, compared to the reciprocal heterospecific and both conspecific pairings. Further dissection of courtship behaviors suggested this difference did not reflect male mate choice, but rather, marked reduced spermatophore acceptance rates by 'ancestral' females paired with 'derived' males. The results are discussed with respect to founder effect models of speciation and the potential role of chemosensory signals in mate choice in these species [*Current Zoology* 59 (2): − ,].

Keywords Prezygotic isolation, Kaneshiro's hypothesis, Founder flush models*, Laupala*

Rapidly diversifying clades are commonly characterized by a striking variety of sexual displays and associated mating preferences (e.g., Boake, 2005; Seehausen et al., 2008; Arnegard et al., 2010), thus supporting the hypothesis that the evolution of such traits plays an important role in the origin of reproductive isolation (Panhuis et al., 2001; Ritchie, 2007; Kraaijeveld et al., 2010). In efforts to understand the mechanisms that facilitate such divergence, there has been considerable interest in the consequences of range expansion (e.g., Yeh, 2004) or colonization of new environments (Price, 2006) that may promote sexual trait evolution by altering the direction or relative importance of natural and sexual selection (Svensson and Gosden, 2007; Cornwallis and Uller, 2009).

If colonizing populations are sufficiently small and isolated (e.g., on oceanic islands) sexual trait evolution may also be profoundly influenced by founder effects (Mayr, 1954; Templeton, 1980). Based on observations of Hawaiian *Drosophila*, Kaneshiro (1976, 1980, 1983) posited that, in systems with female mate choice, elements of male courtship could be lost in founding populations due to genetic drift coupled with relaxed sexual selection, resulting from the

evolution of reduced female choosiness at low population densities. A key prediction of this hypothesis is the evolution of asymmetrical sexual isolation wherein females from the 'ancestral' source population discriminate against males from the 'derived' population (due to missing courtship elements) to a greater degree than 'derived' females discriminate against 'ancestral' males.

Kaneshiro's hypothesis has garnered considerable attention due to the frequency with which asymmetrical sexual isolation is observed among recently diverged lineages (Arnold et al., 1996), and the potential to provide inferences regarding the direction of evolution. And while numerous empirical examples have accumulated across diverse taxa (e.g., Ohta, 1978; McLain et al., 1985; Bradley et al., 1991; Fraser and Boake, 1997; Shaw and Lugo, 2001; Tinghitella and Zuk, 2009), Kaneshiro's hypothesis has also been the subject of controversy (e.g., Barton and Charlesworth, 1984), due in part to the failure of early studies to disentangle the effects of mating asymmetry with potential interspecific differences in mating rates. Others have expressed skepticism regarding the generality of the model (Arnold et al., 1996), which assumes (1) a demographic scenario in which a 'derived' lineage is established through a founder event by an 'ancestral' taxon, (2) a cessation of any subsequent gene flow, and (3) that the ancestral lineage has not been subject to severe bottlenecks since the founder event (Giddings and Templeton, 1983). If, however, a study system did meet such requirements, an assessment of mating asymmetry could provide insight into the role of founder effects in the evolution of sexual traits and preferences (Wasserman and Koepfer, 1980; Markow, 1981; Shaw and Lugo, 2001).

Due to the severe bottlenecks that often accompany colonization, island biota have long presented ideal opportunities to test predictions of founder effects speciation models (Carson and Templeton, 1984). In addition to the involvement of small founding populations that can amplify the influence of genetic drift, founder events may sometimes be sufficiently rare that colonization is followed by a cessation of gene flow, and in many cases, knowledge concerning the geochronological patterns of island formation can provide confidence in corroborating the phyletic relationships among clades.

Swordtail crickets of the genus *Laupala* have undergone an explosive species radiation across the Hawaiian archipelago (Fig. 1, Otte, 1994), marked by patterns of successive colonization from older to younger islands, followed by subsequent diversification within islands (Shaw, 2002; Mendelson and Shaw, 2005). All species are both morphologically and ecologically similar, inhabiting the understory of mid-elevation rainforests. Individual species are, however, endemic to only a single island and often a single volcano within an island (Otte, 1994). Prior work has pointed to a significant role of sexual selection via female choice in the rapid evolution of reproductive barriers across the genus (Mendelson and Shaw, 2002; Mendelson and Shaw, 2005). In particular, behavioral studies have demonstrated assortative female acoustic preferences based on the pulse rate of male calling song both within (e.g., Grace and Shaw, 2011) and among species (Shaw and Parsons, 2002). Yet other work suggests that, at close ranges, mate choice in *Laupala* is likely to involve the evaluation of additional phenotypic cues, such as tactile and/or chemical signals (Mendelson and Shaw, 2006).

In a previous test of Kaneshiro's hypothesis, Shaw and Lugo (2001) reported asymmetrical sexual isolation between *L. makaio*, an ancestral species that is endemic to the geologically older Maui Island, and *L. paranigra*, a derived species endemic to the younger Hawaii Island. Specifically, mating success between *L. paranigra* males and *L. makaio* females was significantly lower than the reciprocal interspecific cross and both intraspecific pairings, thus suggesting an evolutionary shift in mating preferences that coincided with the colonization of Hawaii Island by an *L. makaio*-like ancestor. However, these species also differ markedly in acoustic behavior, with *L. paranigra* males singing at approximately twice the rate of *L. makaio* (~1.0 pulse per second versus ~0.5 pulses per second, Otte, 1994; Shaw, 2000b), thus presenting the possibility that mating asymmetry is due to female acoustic biases in either species, as opposed to a loss of courtship elements and reduced female choosiness in the derived lineage. Additionally, more recent

phylogenetic evidence (Mendelson and Shaw, 2005) has provided greater resolution concerning the evolutionary relationships in this clade, supporting the hypothesis that *L. makaio* is likely ancestral to the Hawaii Island species radiation (Fig. 1). This suggests that, if the observed *L. makaio* × *L. paranigra* mating asymmetry is a consequence of founder effects from interisland colonization, similar patterns are expected between *L. makaio* and other Hawaii Island endemics, though this prediction has yet to be examined empirically.

In this study we test for asymmetrical sexual isolation between 'ancestral' *L. makaio* and 'derived' *L. nigra*, the latter of which is sister to *L. paranigra* (Fig. 1, Mendelson and Shaw, 2005) and endemic to Mauna Kea volcano on Hawaii Island. The pulse rate of *L. nigra* male song is similar to *L. makaio*, thus allowing us to control for any potential mating biases due to acoustic preferences alone. Using the same experimental approach employed by Shaw and Lugo (2001), we first test for asymmetrical isolation by comparing inter- and intraspecific mating rates in controlled mating trials. Second, we quantify and compare specific courtship behaviors between matings to make inferences regarding potential mechanisms underlying sexual isolation. The results are discussed in relation to general patterns of sexual trait and preference evolution in this clade.

1 Materials and Methods

1.1 Animal rearing

All individuals used in mating trials were drawn from captive breeding populations produced from wild-caught parents: *L. makaio* were collected at Ginger Camp (20˚ 41' 60.0"N, 156˚ 5' 18"W) in Haleakala National Park on Maui Island, *L. nigra* were collected from Eucalyptus Toe (19˚ 47' 60.0"N, 155˚ 8' 60.0"W) on the lower southeastern slopes of Mauna Kea on Hawaii Island. In the laboratory, animals were kept under a 12:12hr light-dark cycle at 20 ˚C ambient temperature, housed in plastic specimen cups with moistened tissues to maintain appropriate humidity and fed cricket chow (Fluker Farms, LA, USA) ad libitum. Species were not acoustically isolated from one another, and thus all individuals were exposed to both conspecific and heterospecific songs during development. Juvenile males and females were separated into individual cages prior to sexual maturity to ensure virginity.

1.2 Mating behavior

Across *Laupala* species, mating in the field typically occurs between 1000 and 1800 hours and courtship is highly protracted, with single bouts lasting 4 to 7 hours. Courtship is remarkably complex (Fig. 1), with progression between steps contingent upon a series of stereotyped behavioral signals and responses between partners (Shaw and Khine, 2004; DeCarvalho and Shaw, 2005; Mendelson and Shaw, 2006). Acoustic attraction brings about initial contact and antennation. Subsequent courtship is then marked by close-range male singing. Following antennal contact, the latency to sing can be interpreted as an indicator of male willingness to mate (Shaw and Lugo, 2001). If females are receptive to a singing male, courtship proceeds through a stereotyped behavioral sequence that is repeated a variable number of times, each culminating with the production (externally visible on the male genetalia) and transfer of a spermless 'micro' spermatophore (Shaw and Khine, 2004; DeCarvalho and Shaw, 2005) by males to their mates, which is subsequently removed and consumed by the female as a nuptial gift. In the final stage of courtship, males transfer a single sperm-containing 'macro' spermatophore to the female. Macrospermatophores are conspicuously large (~3×) compared to microspermatophores and thus are readily identified during behavioral observations.

1.3 Sexual receptivity pre-trials

To ensure sexual receptivity, all individuals were subject to 'no choice' mating tests 24 hours prior to experimental mating trials (Shaw and Lugo, 2001). Virgin male and female crickets aged 4−8 weeks (which corresponds to the period

of peak sexual activity) were introduced at 1000 to 1200 hours into an enclosure (two clean 85mm ×25mm plastic Petri dishes) with a virgin conspecific of the opposite sex. Pairs were observed for 3 hours, or until transfer of the first microspermatophore was imminent, at which point mating was interrupted and the partners separated. A male was deemed receptive if he produced a microspermatophore and attempted to back under the female (i.e., spermatophore transfer position), whilst a female was considered receptive if she adopted a hunched posture with her genital opening extended forward toward the advancing male.

We note that this approach may lead to an underestimation of the true degree of sexual isolation if, as a consequence of screening individuals for sexual receptivity using intraspecific pre-trials, we also eliminated females that were unusually choosy and males that were especially unattractive. One might expect that such individuals would be even less likely to mate with heterospecifics, thereby increasing the apparent strength of sexual isolation. However, because of the difficulty in distinguishing such phenotypes from individuals that were simply sexually unreceptive irrespective of the stimulus, we employed the more conservative approach by excluding unresponsive individuals from subsequent tests of sexual isolation.

1.4 Mating trials

No-choice mating trials were carried out with receptive virgin males and females in a procedure similar to the receptivity pre-trials. Individual females were paired with either a single conspecific or heterospecific male between 10: 00 and 12: 00 hours and observed until either mating was successful (i.e., transfer of macrospermatophore), or 18: 30 hours. Individuals were not subjected to more than one trial. While evidence overwhelmingly supports the importance of female mate choice in *Laupala*, the prolonged and energetically costly nature of courtship (Shaw and Khine, 2004) suggests that males might exhibit some discretion in allocating resources to a particular mating attempt. In order to begin parsing the effects of male and female responses, we can reasonably assume that some behavioral transitions are more under the control of the male (e.g., initiation of singing, timing of spermatophore production) whilst others are more directly reflect female decisions. In particular, we assume that spermatophore production is likely costly to males in terms of energy and resources, and thus failure to successfully transfer a spermatophore once produced is likely to reflect rejection by the female. Thus, we recorded and quantified various parameters including number of microspermatophores produced and transferred as well as the latencies to first male song (t_1), first microspermatophore production (t_2) and transfer (t_3), and macrospermatophore transfer (t_4, Fig. 2).

1.5 Statistical analysis

Results from mating trials were first analyzed in JMATING (Carvajal-Rodriguez and Rolán-Alvarez, 2006) to estimate the overall index of pair isolation (I_{PSI}) between species (Rolán-Alvarez and Caballero, 2000). I_{PSI} is calculated using the ratio of observed to expected (under random mating) mating frequencies for each of the four pairing types (e.g., both intraspecific and reciprocal interspecific crosses), and ranges from -1 (interspecific matings only) to 1 (intraspecific matings only). Second, following Shaw and Lugo (2001), we performed a heterogeneity *G*-test (pp. 715–724, Sokal and Rohlf, 1995) to determine if proportions of successful matings were homogeneous among the four pairing types: *L. makaio* male × *L. makaio* female (m × m), *L. makaio* male × *L. nigra* female (m × n), *L. nigra* male × *L. nigra* female (n × n), *L. nigra* male × *L. makaio* female (n × m). This test also partitions the *G*-statistic among each mating type by comparing proportions of successful matings with the mean value for intraspecific matings. The p-values for these tests were subject to Bonferroni correction to account for multiple comparisons.

To further examine the behavioral differences among crosses, the latencies to first singing, microspermatophore production and transfer, and macrospermatophore production were analyzed using one-way ANOVA followed by

Tukey's test for all pairwise contrasts. A heterogeneity *G*-test was also used to compare proportion of microspermatophores produced that were successfully transferred, pooled within each cross type.

2 Results

A total of 93 trials were staged using sexually receptive virgin males and females: m × m, $N = 21$ pairs; m × n, $N = 23$; n × n, $N = 25$; n × m, $N = 24$. Mating success rates were generally high in both intraspecific pairings, with 81% and 80% of trials culminating in macrospermatophore transfer in *L. makaio* and *L. nigra*, respectively. In contrast, for interspecific crosses, only 61% of pairings between 'ancestral' *L. makaio* males with 'derived' *L. nigra* females, and 29% of the reciprocal cross were successful. I_{PSI} for the two species was estimated to be 0.305 ± 0.1278 SD ($P = 0.024$, 10,000 bootstrap iterations), thus suggesting significant sexual isolation. Furthermore, results of the heterogeneity *G*-test on mating success (Table 1) indicated significant heterogeneity among groups in the proportion of successful matings ($G_H = 17.94$, $df = 3$, $P < 0.001$). After partitioning total *G* into contributions from each pairing type, only 'derived' *L. nigra* male × 'ancestral' *L. makaio* female pairings showed a significant departure from expectations ($G = 29.61$, $df = 1$, $P < 0.001$ following Bonferroni correction), with macrospermatophore transfer occurring nearly one-third as frequently as observed in intraspecific pairings (average = 80.5%).

Analysis of courtship behaviors revealed similar latencies to sing for males in both intraspecific pairings, as well as 'ancestral' *L. makaio* males paired with 'derived' *L. nigra* females, whilst the latency to sing for 'derived' *L. nigra* males paired with 'ancestral' *L. makaio* females was significantly longer compared to both types of pairings involving *L. makio* males ($P < 0.05$, pairwise Tukey's tests; Fig. 3a). There was also variation among pairing types in latency to first microspermatophore production with a tendency for longer delays in interspecific pairings relative to intraspecific (Fig. 3b), though pairwise tests suggest that this was largely due to relatively short latencies in *L. makaio* × *L. makaio* pairings relative to the other three types. Latencies to first microspermatophore transfer were similar among groups, with the exception being 'derived' *L. nigra* male × 'ancestral' *L. makaio* female pairings which exhibited longer latencies compared to intraspecific *L. makaio* matings (Fig. 3c). Furthermore, while the mean number of microspermatophores produced in each trial did not differ among groups ($\chi^2 = 0.575$, $df = 3$, $P = 0.90$), there was significant heterogeneity among mating types in the proportion of these microspermatophores that were subsequently transferred ($G_H = 27.33$, $df = 3$, $P < 0.001$; Table 2). Inspection of the individual *G*-tests indicate that this heterogeneity was largely due to a conspicuous reduction in the proportion of successful spermatophore transfers in 'derived' *L. nigra* male × 'ancestral' *L. makaio* female pairings relative to expected values ($G = 36.85$, $df = 1$, $P < 0.001$ following Bonferroni correction). Finally, for those pairs that were successful, latencies to macrospermatophore transfer was similar across types ($F_{3,57} = 1.62$, $P = 0.19$; Fig. 3d).

3 Discussion

The evolution of behavioral isolation is a hallmark of many rapidly-speciating clades. When present, asymmetry in the direction of behavioral isolation may provide insight into the mechanisms underlying divergence of mate signaling systems and possibly even the direction of evolution (Kaneshiro, 1976; Ohta, 1978; Giddings and Templeton, 1983). Yet, as some authors have observed (Arnold et al., 1996), mating asymmetry can result from a variety of processes (e.g., Markow, 1981), and the significance of models such as Kaneshiro's (1980) may be limited if few empirical systems are likely to satisfy the numerous assumptions. Nevertheless, in cases where these assumptions are met, patterns of mating asymmetries may permit the testing of particular evolutionary scenarios.

Laupala crickets provide a striking example of a rapid species radiation, characterized by the repeated evolution of premating reproductive isolation (Mendelson and Shaw, 2005) and serial colonization events across the geologically young Hawaiian archipelago (Fig. 1). In the present study, we tested for asymmetrical isolation between two species for which both molecular phylogenetic (Mendelson and Shaw, 2005) and biogeographic (Otte, 1994) evidence have strongly supported an 'ancestral' (*L. makaio*) – 'derived' (*L. nigra*) relationship. In comparing mating success between intra- and interspecific crosses, we found that 'ancestral' *L. makaio* males paired with 'derived' *L. nigra* females mated at a similar frequency to either intraspecific pairings (Table 1). In contrast, mating success between 'derived' *L. nigra* males and 'ancestral' *L. makaio* females was significantly reduced relative to expectations, thus demonstrating asymmetrical sexual isolation in a direction consistent with predictions of Kaneshiro's hypothesis (1976, 1980, 1983). The observed similarity in mating frequency between *L. makaio* and *L. nigra* homotypic pairings suggest that this asymmetry was not simply the result of differential mating propensities, thus refuting a central critique (Barton and Charlesworth, 1984) of previous empirical examples.

Closer examination of mating behaviors indicated that *L. makaio* males in both conspecific and heterospecific mating commenced singing sooner than did *L. nigra* males that were paired with *L. makaio* females (Fig. 3a). While there was an apparent tendency for *L. nigra* males to delay singing when paired with *L. makaio* females, this latency was accompanied by substantial variation among trials and the mean did not differ significantly from *L. nigra* males in conspecific pairings. With respect to production of the first microspermatophore, *L. makaio* males in homotypic matings produced microspermatophores earlier than any other pairing type (Fig. 3b), pointing to another distinction in male mating behavior compared to *L. nigra*. However, the latency to transfer the first microspermatophore, which is likely dependent to some extent on female acceptance behavior, did not differ between homotypic matings (Fig. 3c). Rather, we observed a significant increase in latency to accept the microspermatophore by *L. makaio* females mated to *L. nigra* males compared to *L. makaio* females paired with conspecifics. This pattern suggests discrimination against 'derived' *L. nigra* males by 'ancestral' *L. makaio* females—a conclusion that is corroborated by comparing the total numbers of microspermatophores produced and then successfully transferred in each type of mating (Table 2). Of particular note, despite all groups producing comparable numbers of microspermatophores, 'derived' *L. nigra* males paired with 'ancestral' *L. makaio* females showed markedly lower success in transferring microspermatophores than males in the three other test groups. Ultimately, however, for those pairs that progressed to the final stages of courtship, the latency to macrospermatophore transfer did not differ among groups (Fig. 3D), suggesting that sexual barriers between these species are likely due to disruption early in the courtship process.

Taken together with similar findings of asymmetrical sexual isolation between *L. makaio* and *L. paranigra* (Shaw and Lugo, 2001), the present study indicates that the evolution of asymmetrical sexual isolation can, at the very latest, be placed at the root of the *L. nigra* – *L. paranigra* clade (Fig. 1), thereby strengthening the case for an origin of behavioral isolation that coincides with the colonization of Hawaii from Maui. Furthermore, because interisland colonization is likely to have involved small and isolated populations, these results suggest that founder effects may have played an important role in the speciation process (Templeton, 2008). While numerous mechanisms for founder effect speciation have been hypothesized (e.g., Mayr, 1954; Carson, 1975; Templeton, 1980), only Kaneshiro (1976, 1980, 1983) focused explicitly on the evolution of behavior isolation via sexual selection as a causal mechanism. Interestingly, in later work, Kaneshiro (1989) postulated that changes in the form of sexual selection, brought on by the evolution of less choosy females in small founding populations, might promote the formation of novel genetic recombinants as a consequence of the destabilization of coadapted genetic systems. In this way, the consequences of founder effects for mate choice phenotypes may ultimately contribute to the adaptability of colonizing populations to new ecological conditions (Kaneshiro, 2006). Determining the degree to which such a process may have accompanied

interisland founding events in the *Laupala* study system will require explicit tests of specific demographic scenarios, particularly those that probe for genetic evidence of bottlenecks in both 'ancestral' and 'derived' taxa (Giddings and Templeton, 1983).

While *Laupala* exhibits a conspicuous diversity of acoustic signals and preferences (Shaw and Parsons, 2002; Mendelson and Shaw, 2005), previous work suggests that male calling song functions primarily in long-distance signaling, whereas other modalities are likely more important at the proximities examined in our study (Mendelson and Shaw, 2006). Moreover, *L. makaio* and *L. nigra* males sing at similar pulse rates, the most diagnostic feature of song in *Laupala*, suggesting that mate choice among these species may be based on other phenotypic cues. While there are no conspicuous differences in body size or other morphology between the species we studied (Otte, 1994; Shaw, 2000a), the extensive and repeated antennation commonly observed between mates during courtship (Shaw and Khine, 2004) suggests that contact pheromones such as cuticular hydrocarbons (CHCs, Singer, 1998) might be particularly important in mediating mating decisions. Extensive research has established mate choice based on CHCs in diverse insect taxa (Howard and Blomquist, 2005), including other orthopterans (e.g., Thomas and Simmons, 2009). In *Laupala*, a survey of CHC variation (Mullen et al., 2007; Mullen et al., 2008) found evidence of substantial reductions in CHC diversity in species occurring on Hawaii Island compared to species endemic to Maui—the number of unique compounds observed in *L. nigra* was approximately half of that found in *L. makaio*. This finding is thus consistent with the mechanism proposed by Kaneshiro (1980), which predicts a loss of courtship elements in males of the 'derived' species as a consequence of extreme population bottlenecks. Whether these 'lost' CHC compounds can explain the rejection of *L. nigra* males by *L. makaio* females will require further ethological study.

In addition to CHCs, sexual isolation in these *Laupala* species might be mediated by gustatory cues contained in the microsphermatophores consumed by females during courtship (Vahed, 1998). Specifically, if speciation has been accompanied by evolutionary change in the quantity or composition of spermatophores produced by males of the 'derived' species, 'ancestral' females may choose to terminate a heterospecific mating after tasting the first microspermatophore transferred. Such a mechanism would be consistent with the behavioral responses observed in our study, in which the proportion of successful microsphermatophore transfers was significantly reduced when *L. nigra* males were paired with *L. makaio* females compared to reciprocal heterospecific crosses. Interestingly, rapid evolutionary divergence of spermatophore seminal proteins has been suggested to play a role in prezygotic barriers in other gryllid lineages (e.g., Andrés et al., 2008), though these typically involve sperm-egg interactions occurring in the female reproductive tract as opposed to mating decisions based on gustatory cues. Thus, understanding variation in the composition of microspermatophores across *Laupala* species, and the degree to which these nuptial gifts might contribute to sexual isolation, represents a promising future research direction.

A third potential mechanism underlying asymmetrical sexual isolation is interspecific differences in temporal components of courtship. In particular, Shaw and Lugo (2001) conjectured that rather than losing components of courtship per se, species might diverge in relation to diel timing or rhythmicity of mating. Divergence in courtship timing has been implicated in other studies of behavioral isolation in *Laupala* (Danley et al., 2007; Fergus et al., 2011), and is likely important due to the protracted nature of courtship. Consequently, if the diel timing of courtship initiation is delayed in the 'derived' species, as was observed in relation to microspermatophore production in *L. nigra* males, then interspecific matings might fail due to the pair falling behind the typical mating schedule of the 'ancestral' *L. makaio* female. In the reciprocal direction, the relatively fast 'ancestral' *L. makaio* males might be less constrained by a fixed temporal window and thus still capable of successfully completing the courtship process with 'derived' *L. nigra* females. Thus, our results are consistent with at least a partial contribution of interspecific differences in behavioral timing to asymmetrical sexual isolation between these species.

In conclusion, this study revealed significant asymmetrical behavioral isolation in a pattern consistent with a founding event that influenced both male secondary sexual traits and female mate choice. Given the numerous interisland colonization events that have likely accompanied the species radiation of *Laupala* crickets (Otte, 1994), we suggest that such demographic events may have repeatedly played key roles in the rapid diversification of this clade.

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Table 1 Heterogeneity *G*-test for proportions of successful intra- and interspecific matings between *L. makaio* (m) and *L. nigra* (n)

Test	Proportion successful	*df*	G
$m_a \times m_a$	0.81	1	0.003
$m_a \times n_d$	0.61	1	4.696
$n_d \times m_a$	0.29	1	29.61[*]
$n_d \times n_d$	0.80	1	0.004
Heterogeneity		3	17.94[**]
Pooled	0.62	1	16.37[**]
Total		4	34.31[**]

In each test, observed proportions of successful matings were compared to the mean value for both intraspecific crosses (0.805). All pairings are designated as male × female; subscripts indicate 'ancestral' (a) and 'derived' (d) species. * *P* < 0.001 after Bonferroni correction for multiple tests (*k* = 4). * *P* < 0.001 after Bonferroni correction for multiple tests (*k* = 4). ** *P* < 0.001.

Table 2 Heterogeneity *G*-test for proportions of microspermatophores successfully transferred in intra- and interspecific matings between *L. makaio* (m) and *L. nigra* (n)

Test	Proportion successful	*df*	G
$m_a \times m_a$	0.74	1	0.843
$m_a \times n_d$	0.67	1	5.970
$n_d \times m_a$	0.54	1	36.85[*]
$n_d \times n_d$	0.81	1	1.043
Heterogeneity		3	27.33[**]
Pooled	0.70	1	17.38[**]
Total		4	44.71[**]

* *P* <0 .001 after Bonferroni correction for multiple tests (*k* = 4). ** *P*<0 .001.   In each test, observed proportions successfully transfered were compared to the mean value for both intraspecific crosses (0.774). All pairings are designated as male × female; subscripts indicate 'ancestral' (a) and 'derived' (d) species.
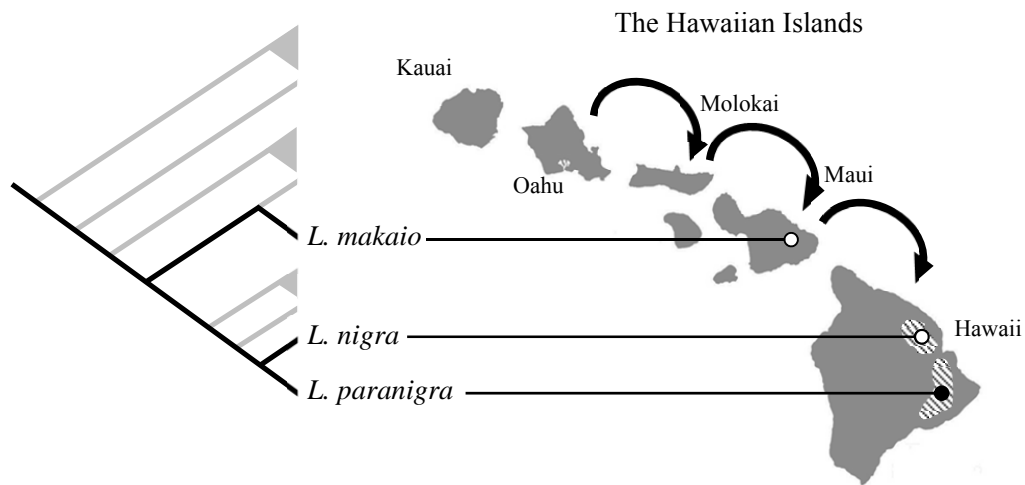
Fig. 1 Cladogram (simplified from AFLP phylogeny of Mendelson and Shaw, 2005) for the 'pacifica' species group and geographic location of *L. makaio*, *L. nigra* and *L. paranigra* within the Hawaiian archipelago

Patterns of interisland colonization (curved arrows) within this clade have generally followed a progression from older to younger islands (Mendelson and Shaw, 2005; Shaw, 2002). Species' geographic ranges indicated with diagonal striping and population location for two species used in this study indicated with white circles.
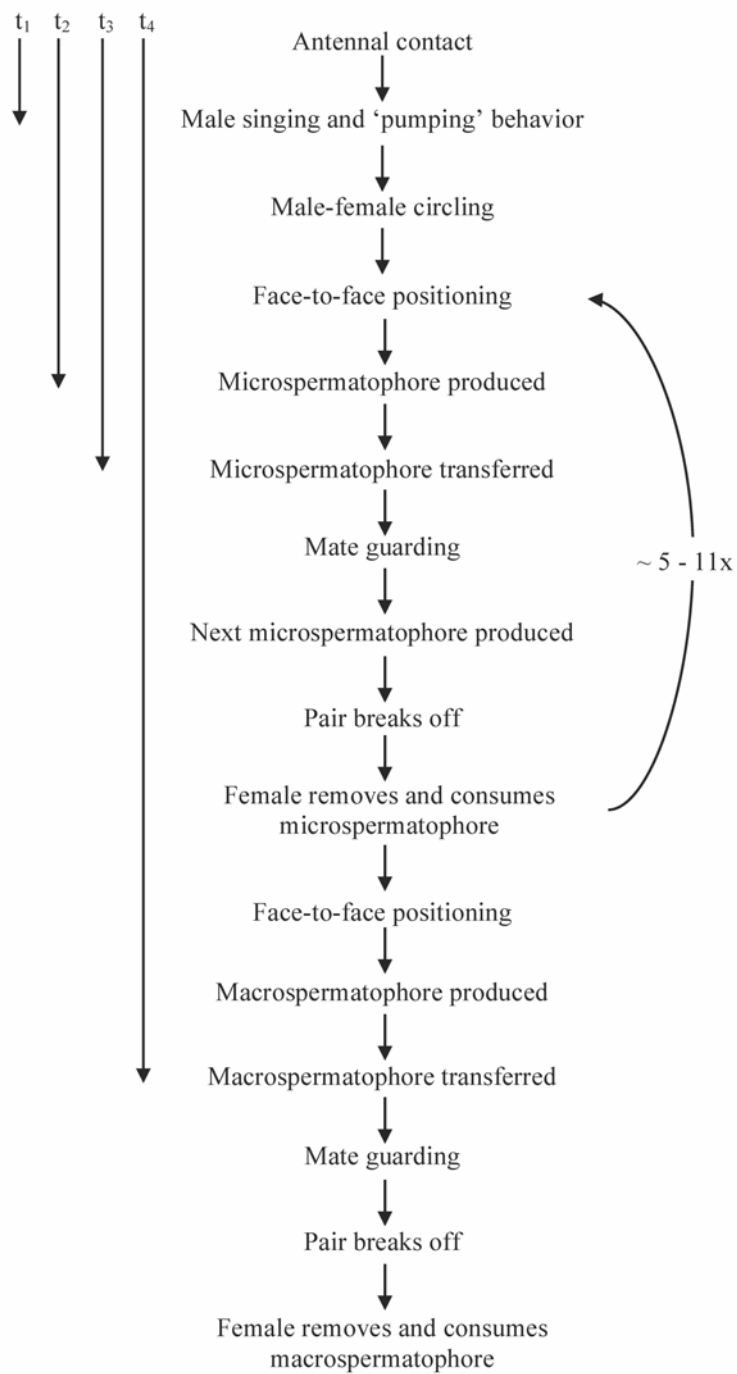
t_1 t_2 t_3 t_4

Antennal contact

Male singing and 'pumping' behavior

Male-female circling

Face-to-face positioning

Microspermatophore produced

Microspermatophore transferred

Mate guarding

Next microspermatophore produced

Pair breaks off

Female removes and consumes microspermatophore

$\sim 5 - 11x$

Face-to-face positioning

Macrospermatophore produced

Macrospermatophore transferred

Mate guarding

Pair breaks off

Female removes and consumes macrospermatophore

Fig. 2 Diagrammatic representation of the typical courtship process in *Laupala* (see methods for details)

Arrows at left depict time latencies measured for behavioral analyses: t_1, latency to sing; t_2, latency to first microspermatophore production; t_3, latency to first microspermatophore transfer; t_4, latency to macrospermatophore transfer.
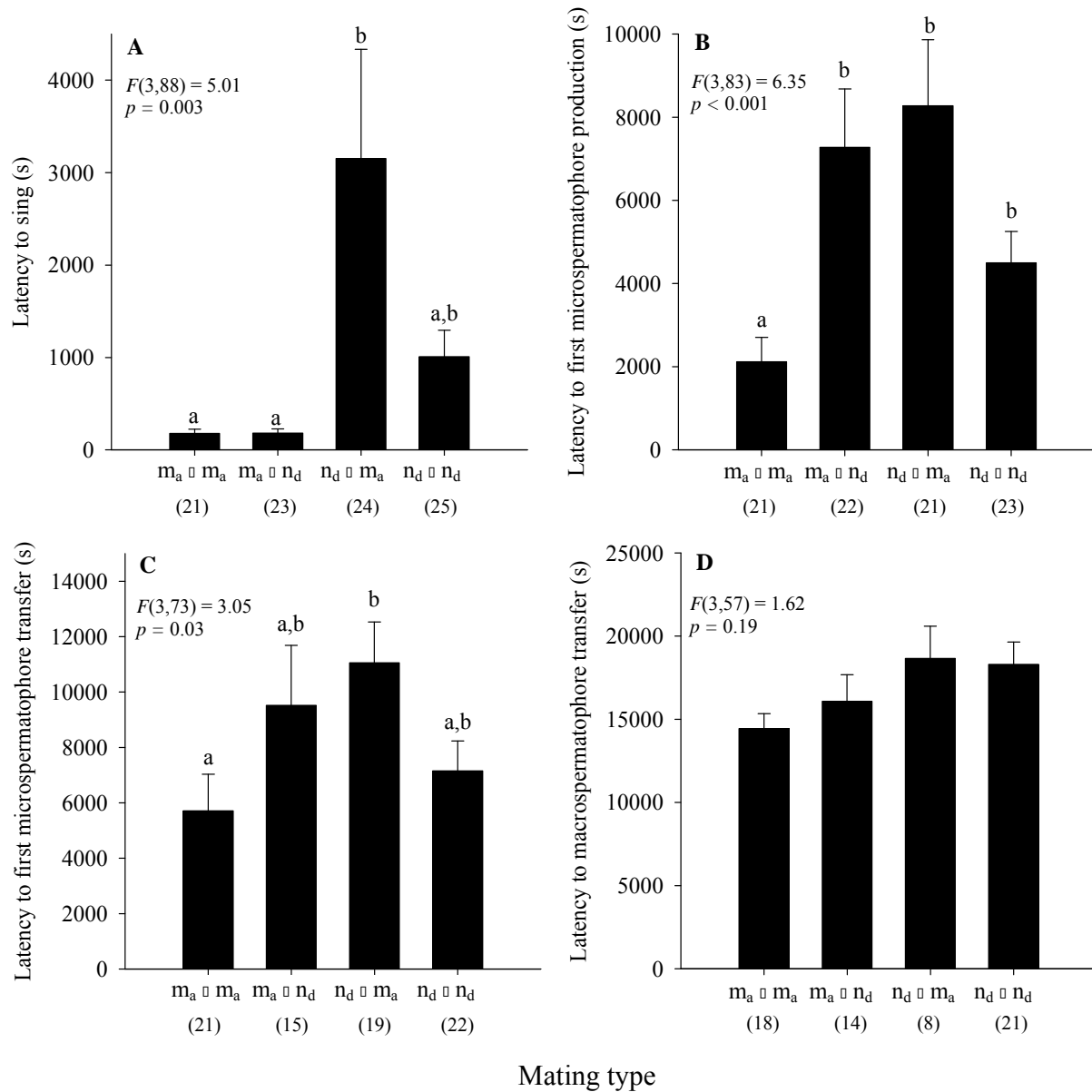
Fig. 3 Latencies (measured in seconds from initial antennal contact) to (A) first male singing, (B) first microspermatophore production and (C) transfer, and (D) macrospermatophore transfer for intra- and interspecific mating trials in *L. nigra* (n) and *L. makaio* (m)

All pair types are designated as male × female; subscripts indicate 'ancestral' (a) and 'derived' (d) species. Lower case letters over bars indicate groups that are not significantly different (pairwise Tukey range test), error bars are standard errors, and numbers below axes are sample sizes.