

A potential role for overdominance in the maintenance of colour variation in the Neotropical tortoise beetle, *Chelymorpha alternans*

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Abstract

The presence of persistent polymorphisms within natural populations elicits the question of how such polymorphisms are maintained. All else equal, genetic drift and natural selection should remove genetic variants from populations. Disassortative mating and overdominance are potential mechanisms for maintaining variation within populations. Here, we consider the potential role of these mechanisms in maintaining variation in colour pattern in the tortoise beetle, *Chelymorpha alternans*. Five colour morphs distinguished by elytral and pronotal coloration are largely determined by a single locus of large effect with four segregating alleles. As many as four morphs co-occur in natural populations. We first assessed whether disassortative mating might maintain this polymorphism. To test for assortative and disassortative mating, we paired females with two males, one with the same colour pattern as the female and one with a different colour pattern and examined the colour patterns of the offspring. We found strong evidence for random mating as a function of colour pattern. We next assessed whether differences in offspring survival among assortative and disassortative male-female pairs maintain colour variation. Crosses involving disassortative pairings had significantly higher offspring survival during development and resulted in more adult progeny. This result is consistent with the effects of overdominance, whereby outcrossed individuals have higher fitness than their homozygous counterparts. Overall, differences in offspring survival appear to play a greater role in maintaining polymorphisms than nonrandom mating in species.

KEYWORDS

colour polymorphisms, differential survival, mate choice, random mating

1 | INTRODUCTION

Persistent variation within natural populations offers an intriguing scenario for evolutionary biologists to explore, because selection and drift are expected to erode intraspecific variation over time (Conner & Hartl, 2004; Hof et al., 2016; O'Neil & Beard, 2010; Stinchcombe & Hoekstra, 2008; Van Belleghem et al., 2017). The

presence of stable, persistent polymorphisms within and across populations suggests the presence of balanced evolutionary mechanisms that maintain alleles at intermediate frequencies (Endler, 1983; Fuller & Johnson, 2009; Lande, 1975; Mitchell-Olds et al., 2007). Several mechanisms have been shown to maintain polymorphisms, including negative-frequency dependence, disassortative mating, spatial or temporal heterogeneity in fitness and

life history trade-offs (Gray & McKinnon, 2007; Jorge et al., 2011; Nachman, 2005; Tuttle,). The mechanisms maintaining polymorphisms have been elucidated in some systems (e.g. disassortative mating for fur colour in North American wolves and negative-frequency dependent predation on colour polymorphisms in some aphid populations). However, for most species with persistent polymorphisms, the mechanisms underlying their maintenance remain unresolved (Hedrick et al., 2016; Losey et al., 1997). In this study, we test the roles of disassortative mating and its consequences for offspring survival in a neotropical polymorphic tortoise beetle.

Disassortative mating occurs when individuals preferentially choose mates with a different phenotype and can facilitate the maintenance of phenotypic variation within populations (Hedrick et al., 2016, 2018; Jiang et al., 2013; Takahashi & Hori, 2008). Disassortative mating for loci of the major histocompatibility complex (MHC) is a well-known example of nonrandom mating and has been shown in primates (Huchard et al., 2013), fish (Landrey et al., 2001), rodents (Penn & Potts, 1998) and reptiles (Strandh et al., 2012). Another classic example of disassortative mating can be found in white-throated sparrows where approximately 98% of observed matings are between two different colour morphs (white and tan) (Hedrick et al., 2018). Moreover, in plants, additional mechanisms can promote disassortative mating, including self-incompatibility where individuals are unable to produce viable zygotes via self-fertilization (reviewed in Delph & Kelly, 2014).

The genetic consequence of disassortative mating is the production of heterozygotes (with respect to the trait in question) and the avoidance of inbreeding. The production of heterozygotes is adaptive when overdominance occurs (i.e. when heterozygotes have increased fitness relative to homozygotes). Both overdominance and the avoidance of inbreeding can favour the evolution of disassortative mating and maintain polymorphisms on their own accord. In the case of MHC loci, disassortative mating is thought to increase heterozygosity at functionally important immune genes, thus increasing the likelihood of preventing disease (Huchard et al., 2013; but see Labkovsky et al., 2019). For decades, overdominance was considered an unlikely explanation for the maintenance of polymorphisms within populations (Endler, 1986; Houle, 1989), but recent molecular studies have increasingly supported a role for this mechanism (reviewed in Liberatore et al., 2013). In addition, a handful of studies have demonstrated that overdominance is involved in the maintenance of genetic polymorphisms in colour patterns such as the butterfly, *Heliconius numata* (Lepidoptera: Nymphalidae; Maisonneuve et al., 2020); the common buzzard, *Buteo buteo* (Falconiformes: Accipitridae; Kruger et al., 2001), and the white-throated sparrow, *Zonotrichia albicollis* (Passeriformes: Passerellidae; Hedrick et al., 2018). In *H. numata* and *Z. albicollis*, a chromosomal inversion associated with deleterious mutations results in a heterozygote advantage and promotes disassortative mating. In *B. buteo*, light and dark homozygous morphs have lower fitness compared to intermediate heterozygous morphs, but maternal imprinting for plumage colour replenishes homozygous morphs.

The best evidence for overdominance requires known genetic markers for quantifiable phenotypes and a demonstration of fitness differences among genotypes, with heterozygotes having higher fitness than either homozygote. Few studies have met this standard (Endler, 1986; Hedrick, 2012). Furthermore, the role of overdominance can be difficult to discern when genotypes differ in fitness in early life stages (Rosenthal, 2017). However, despite these challenges, empirical studies have marshalled evidence for overdominance using classic genetic methods. Comparisons of offspring survival between crosses with parents of similar genotypes and those who differ in genotype have suggested a benefit to outcrossing and a possibility of overdominance in some systems (Caesar et al., 2010; Forsman et al., 2007). In the colour polymorphic pygmy grasshoppers, *Tetrix subulata* and *Tetrix undulata* (Orthoptera: Tetrigidae), evidence suggests that there are benefits to diversely collared offspring, as survival rates were higher for families with higher colour diversity in their offspring when compared to more homogenous families (Forsman et al., 2007). Effects of parental mating dynamics and preferences are highly variable among taxa and can manifest at various developmental stages including adult viability and offspring survival.

In this study, we test for (a) the presence of disassortative mating preferences and (b) the presence of increased offspring survival from disassortative versus assortative male-female pairs that would indicate overdominance in the tortoise beetle, *Chelymorpha alternans* (Coleoptera: Chrysomelidae). Across the Isthmus of Panama, *C. alternans* has five genetically determined colour pattern variants distributed nonrandomly (Strickland et al., 2018). These elytral (hardened wings) colour patterns range from entirely red to red with metallic or black stripes. The genetics of this system are detailed in Strickland et al., (2018) and summarized in Figure S1 and Table S1. Briefly, phenotypes are controlled by a single locus or a tight cluster of loci following patterns of Mendelian inheritance. The four alleles follow a strict dominance hierarchy with $R = T > V > r$. Here, "R" and "T" are co-dominant alleles and when inherited together express the *militaris-b* phenotype (bicoloured pronotum and black striped elytra). The dominant "R" allele expresses the *rufipennis* phenotype (black pronotum and red elytra), whereas the dominant "T" allele expresses the *militaris-a* phenotype (black pronotum and black striped elytra). The "V" allele expresses the *veraguensis* phenotype (tan pronotum and red elytra--not used for these studies), whereas the metallic phenotype (tan pronotum and metallic striped elytra) is only expressed in the homozygous recessive state (*rr*). Colour pattern phenotypes are not sexually dimorphic; males and females emerge as adults in roughly 50–50 proportions for each phenotype in polymorphic broods (Strickland et al., 2018). The evolutionary forces maintaining this variation are unknown, but the simple genetic basis of these colour patterns and the relative ease of rearing and maintenance make this a promising system for investigating the mechanisms maintaining polymorphisms within populations.

Here, we tested for the presence of disassortative mating and overdominance via differential larval survival on the maintenance

of colour pattern variation in laboratory-maintained populations. Strong disassortative mating and/or increased survival from offspring of disassortative male–female pairs has the potential to promote diversity and the maintenance of variation within populations. To test for disassortative mating, we first paired females with two males – one male with the same colour pattern as the female and one with a different colour pattern from that female. We allowed these individuals to mate freely and used the colour pattern phenotype of the resulting offspring to assay male mating success. The hypothesis of disassortative mating predicts that females will preferentially mate with males that differ in colour pattern. We also tested for disassortative mating using disassortative and assortative male–female pairs. Here, the hypothesis predicts that females paired with males of a different colour pattern will produce more eggs than females paired with males of the same colour pattern, potentially through differential allocation, which results in the female increasing investment to offspring of preferential matings (Harris & Uller, 2009). Finally, to test for overdominance via differential offspring survival, we compared offspring survival between assortative and disassortative male–female pairs. The hypothesis of overdominance predicts decreased offspring survival in families derived from assortative male–female pairs. We found no strong evidence for disassortative mating, but strong evidence for overdominance where offspring from disassortative pairs had much higher survival than offspring from assortative pairs.

2 | MATERIALS AND METHODS

2.1 | Beetle collection and stock preparation

We used laboratory assays to test for the effects of disassortative mating and overdominance on the maintenance of colour patterns within populations. Our laboratory assays largely eliminated the effects of pathogens, predators and parasitoids.

Laboratory colonies were established by sampling natural populations of *C. alternans* at four locations for the metallic, rufipennis, *militaris-a* and *militaris-b* phenotypes (Figure S1 and Table S1). Egg clutches, larvae, pupae and adults of *C. alternans* were collected and brought to rearing facilities in Gamboa, Panama at the Smithsonian Tropical Research Institute. All wild-caught individuals were reared for at least one generation and mated to metallic individuals to create stocks of known heterozygotes of the *militaris-a* and rufipennis phenotypes for mating triads. The rufipennis phenotype used in assortative pairings (“R-R”) were not all known heterozygotes. Beetles were housed in plastic containers with perforated lids to facilitate airflow and temperature regulation. All containers were given naturally occurring fresh leaves of the preferred host plant *Merremia umbellata* (Solanales: Convolvulaceae) daily. Containers were exposed to natural light, temperature and humidity. Beetles were moved weekly to recycled containers which were washed and bleach sterilized. Coffee filters were added to each new container to absorb excess moisture and collect faeculae.






















An aqueous solution of potassium sorbate was sprayed on the filters to minimize fungal growth.

2.2 | Experiment 1: A test for disassortative mating preferences - Triads

The goal of this experiment was to determine whether females preferred to mate with males whose colour pattern differed from their own. This species is highly promiscuous with males and females mating multiply and no male competition for females. Females can produce and expel spermatozoa droplets from nonpreferred males, and it has been suggested that sexual selection via cryptic female choice is an important evolutionary mechanism in this species (Rodriguez, 1995; Morrison & Windsor, 2018). For this reason, females served as the focal sex for mating triads. Mating preferences were investigated by creating “triads” of beetles. For each triad, we housed a single female with two males: one of her own colour pattern and genotype, and one of another. Phenotypic ratios displayed by the female’s offspring were used to test for the presence of nonrandom mating. Triads were established with nonsibling, virgin beetles of the same age (between 55 and 60 days after oviposition) and given the same daily maintenance. Each triad was checked daily, and new egg clutches were removed, isolated and reared in petri dishes until emergence as adults, 6–8 weeks after oviposition. Offspring phenotypes were assessed at maturity for all egg clutches.

We had seven “triad types” with specific combinations of colour morphs (Table 1). We used the known inheritance pattern of the colour phenotype to infer deviations from random mating. Table 1 shows the colour patterns of the beetles, lists the genotypes of the females and the two males, and gives the expected offspring colour pattern ratios under complete assortative and complete disassortative mating. Table S3 lists the expectations for random mating. Using the genotypes of all three adults and the Mendelian inheritance ratios previously described for this species (Strickland et al., 2018), we determined the paternal contribution of each egg clutch. Deviations from ratios expected under random mating were assessed for each egg clutch. Egg clutches that deviated from random were then assessed to see whether this deviation was assortative or disassortative. These differences were assessed with a series of chi-square tests using the MASS package in R version 3.3.2. Since females of *C. alternans* can store sperm, the first clutch produced was considered separately, as this clutch should most closely resemble the females’ preference if paternity is biased (Morrison & Windsor, 2018). After performing all tests, a Holm’s Bonferroni correction was applied to account for multiple pairwise comparisons. The uncorrected and corrected *p*-values are shown in Table S3. The proportion of the total number of egg clutches which matched a mating strategy (assortative, disassortative, random) was calculated for all females across all triad types (triad types shown in Table 1, averages shown in Table 2).

TABLE 1 Triad combinations and expected ratios

Triad type	Female phenotype/ genotype	Male 1 phenotype/ genotype	Male 2 phenotype/ genotype	Assortative expected ratio	Disassortative expected ratio
1	 Metallic (rr)	 Metallic (rr)	 Rufipennis (Rr)	1:0 Metallic:Rufipennis	1:1 Metallic:Rufipennis
2	 Metallic (rr)	 Metallic (rr)	 Militaris-a (Tr)	1:0 Metallic:Militaris-a	1:1 Metallic:Militaris-a
3	 Rufipennis (Rr)	 Rufipennis (Rr)	 Metallic (rr)	3:1 Rufipennis:Metallic	1:1 Metallic:Rufipennis
4	 Rufipennis (Rr)	 Rufipennis (Rr)	 Militaris-a (Tr)	3:1 Rufipennis:Metallic	1:1:1:1 Rufipennis:Militaris-a:Militaris-b:Metallic
5	 Militaris-a (Tr)	 Militaris-a (Tr)	 Rufipennis (Rr)	3:1 Militaris-a:Metallic	1:1:1:1 Rufipennis:Militaris-a:Militaris-b:Metallic
6	 Militaris-a (Tr)	 Militaris-a (Tr)	 Metallic (rr)	3:1 Militaris-a:Metallic	1:1 Militaris-a:Metallic
7	 Militaris-b (TR)	 Militaris-b (TR)	 Metallic (rr)	1:2:1 Militaris-a:Militaris-b:Rufipennis	1:1 Militaris-a:Rufipennis

Note: Seven types (column 1) of mating triads were performed to determine whether mating is random or nonrandom. Each virgin female (column 2) was housed with two males, one of her own genotype (column 3) and one of a different phenotype/genotype (column 4). Expected ratios for both assortative mating (column 5) and disassortative (column 6) are based on Mendelian inheritance following the allelic dominance hierarchy previously described for this species (Strickland et al. 2018).

TABLE 2 Resulting offspring of all triad types

	Triad Type 1	Triad Type 2	Triad Type 3	Triad Type 4	Triad Type 5	Triad Type 6	Triad Type 7
# of Females	8	12	12	8	5	16	5
Avg. # of egg clutches/ female	8.125 (0.76)	6.83 (0.6)	7.1 (0.56)	6.34 (0.62)	5.6 (0.51)	7.75 (0.72)	6.4 (1.1)
Avg. # of offspring/ female	107 (20.2)	111.75 (9.9)	103.2 (17.7)	78.75 (11.9)	67 (8.1)	125.75 (13.3)	97.4 (20.8)

Note: The number of females (replicates) for each triad type is shown in the first row. The average number of egg clutches produced by each female and the average number of offspring are shown for all triad types, with the standard error for each shown in parentheses.

2.3 | Experiment 2: A second test for disassortative mating preference and increased offspring survival from disassortative pairs

To assess whether outcrossing for colour pattern affects fitness, we asked how parental fecundity and offspring survival varied as a function of assortative and disassortative matings. We used assortative pairs for both rufipennis and metallic beetles (female–male pairings: R-R and M-M). We then established three sets of disassortative pairs each with reciprocal female–male combinations: rufipennis-metallic (R-M and M-R), rufipennis-militaris-a (R-Ma and Ma-R), and metallic-militaris-a (M-Ma and Ma-M). All pairings were established with non-sibling, virgin individuals of the same age and given the same daily maintenance. Pairs were checked daily for egg clutches, and the first egg clutch was removed and reared in a petri dish until adulthood. For each clutch, we recorded the number of eggs laid, the number

of larvae that emerged, the number of larvae that developed into pupae, and the number of pupae that became adults.

The hypothesis that disassortative mating contributes to the maintenance of colour pattern polymorphism predicts increased fecundity for disassortative male–female pairs. To test this, we compared fecundity (# of eggs laid) between assortative crosses (M-M and R-R) and disassortative crosses (M-R, R-M, Ma-M, M-Ma, R-Ma, Ma-R). We performed an ANOVA where fecundity was the dependent variable and cross type was the independent variable. We used post hoc tests in *emmeans* to examine the pattern in fecundity between assortative and disassortative mating pairs. Finally, we performed a simple *t*-test on fecundity between assortative and disassortative pairs by pooling the observations across all cross types.

The hypothesis of overdominance makes two predictions. First, it predicts increased survival for offspring from disassortative versus

assortative female–male pairs. To test this, we compared overall offspring survival (proportion of offspring surviving to adulthood from the number of eggs laid) as well as survival in each of the life stages (survival to larval stage = #larvae/#eggs, survival to pupal stage = # pupae/# larvae, survival to adulthood = #adults/#pupae). Differences in the proportion of offspring surviving through development between assortative and disassortative pairings were assessed using a nested repeated measures ANOVA with stage and pair type (assortative vs. disassortative) as explanatory variables and cross type (M-M, R-R, M-R, R-M, Ma-M, M-Ma, R-Ma, Ma-R) nested within pair type. All analyses were performed using the *dplyr*, *lme4*, *lmerTest* and *lsmeans* packages in R v. 3.5.1. Due to the excess of zeros produced (half of all egg clutches failed to produce any offspring surviving to adulthood), a zero-inflated negative binomial regression was performed on count data using the *pscl*, *MASS* and *boot* packages in R v. 3.5.1.

Second, overdominance makes the prediction of increased offspring survival from crosses that produce more heterozygotes. Previous work on the inheritance of the colour pattern allowed us to infer the genotype of most parents and to calculate the expected proportion of heterozygotes and homozygotes among the offspring. We tested whether cross types with higher expected proportions of heterozygous offspring had higher survival. There were eight different cross types that varied in the expected proportion of heterozygous versus homozygous offspring (Table S3). One cross (metallic (rr) ♀ × metallic (rr) ♂) was expected to produce 0% heterozygotes and 100% homozygotes. Four crosses were expected to produce 50% homozygotes and heterozygotes, and two crosses were expected to produce 75% heterozygotes and 25% homozygotes (militaris ♂ × rufipennis ♀ and rufipennis ♀ × militaris ♂).

For one of the assortative crosses (rufipennis-rufipennis), it was unknown whether both individuals in a given cross were heterozygous, homozygous or mixed. We performed an analysis where we assumed that rufipennis-rufipennis crosses involved all homozygous parents (RR × RR) and which created an expectation of 100% homozygous offspring. We also performed an analysis where we assumed that rufipennis-rufipennis crosses involved either two heterozygotes (Rr × Rr) or a cross between a homozygote and a heterozygote (RR × Rr). Both of these crosses create clutches where the expected ratios of homozygotes to heterozygotes is 50%. For the first cross (Rr × Rr), the offspring should be 25% RR, 50% Rr, and 25% rr. For the second cross (Rr × RR), the offspring should be 50% RR and 50% Rr.

We tested whether the expected proportion of heterozygous offspring was associated with increased survival. We did this in two ways. First, we tested whether there was an association between complete clutch failure (all offspring die before reaching adulthood) and the proportion of expected heterozygous offspring. We did this using a generalized linear model on the binary variable of clutch failure. Our initial analyses indicated that the data were overdispersed, so we used a quasibinomial distribution with a logit link function. We performed the analysis twice: once with the assumption that the rufipennis-rufipennis cross produced 50% homozygous offspring

and once with the assumption that the rufipennis-rufipennis cross produced 0% homozygous offspring.

For the majority of the cross types, the “rr” genotype was the expected homozygous genotype produced. This could potentially create a problem of whether there was genuine evidence for overdominance or whether the “rr” genotype has low survival. Fortunately, the rufipennis-rufipennis cross type was expected to produce either 25% r-r, 50% R-r and 25% R-R (Rr × Rr), 100% R-R offspring (RR × RR) or 50% R-r and 50% R-R (Rr × RR). This allowed us to compare the relationship between the expected proportion of homozygotes and clutch failure with the relationship between the expected proportion of “rr” homozygotes and clutch failure to ask which variable better explains clutch failure. We also examined the relationship between survival and the expected number of homozygotes in clutches that produced offspring surviving to adulthood to determine whether there were effects on survival that manifested in clutches that did not suffer complete clutch failure. Here, we pooled the number of offspring that survived to adulthood and the number of eggs laid for the eight cross types and asked whether the expected proportion of homozygotes predicted offspring survival to adulthood.

3 | RESULTS

3.1 | Experiment 1: A test for disassortative mating preferences - Triads

The hypothesis of disassortative mating predicts that females preferentially mate with males of a different colour pattern. The data did not support this prediction. The vast majority of first clutches (90%) produced offspring in ratios that were consistent with random mating with regard to colour morph (Figure 1a). The remaining 10% of clutches were consistent with disassortative mating, and none indicated assortative mating for first clutches. Deviations from random mating were found in triad types 2, 5, 6 and 7. In triad type 2 (metallic female, metallic male and militaris-a male), 2 of 12 females produced first clutches with phenotypic ratios indicative of disassortative mating, whereas the remaining 10 females produced clutches that did not differ from random. In triad type 5 (militaris-a female, militaris-a male, and rufipennis female), 2 of 5 first egg clutches had offspring ratios which matched disassortative mating, whereas the remaining 3 matched ratios expected from random mating. In triad type 6 (militaris-a female, militaris-a male, and metallic male), 2 of 16 females produced clutches indicative of disassortative mating whereas the remaining 14 females produced clutches that did not differ from random. In triad type 7 (militaris-b female, militaris-b male, and metallic male), two of the five females produced offspring with colour pattern ratios that were consistent with disassortative mating for the first egg clutch. Considering all 488 egg clutches produced, 26% followed ratios of assortative mating, 24% followed ratios of disassortative mating and 50% followed ratios of random mating. For every triad type (with the

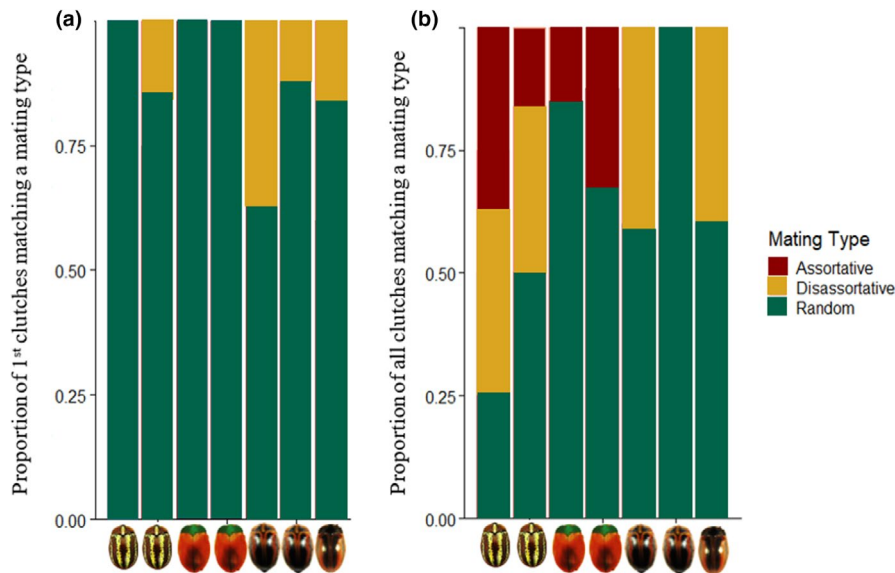


FIGURE 1 The proportion of egg clutches produced by triad types (1–7 in numerical order; female colour morph is shown on the x axis) producing offspring colour morph ratios that aligned with the expectations of assortative (red), disassortative (gold) and random mating (green). To account for multiple pairwise comparisons, a Holm's Bonferroni correction was applied, and the corrected p -values were used for this figure. (a) Only the first egg clutch produced from each female within a triad type to assess initial preferences. (b) All egg clutches produced by females within a triad type

exception of triad type 1), the phenotypic ratios were consistent with the expectations from random mating in $\geq 50\%$ of clutches (Figure 1b, Table S2).

3.2 | Experiment 2: A second test for disassortative mating preference

Disassortative mating preferences predict increased clutch size when females are paired with males whose colour pattern differs from their own. Again, the data did not support this prediction. Instead, the data suggested that there was a $\sim 10\%$ increase in fecundity in assortative pairings (average clutch size \pm SE: 32.3 ± 0.1 eggs) relative to disassortative pairings (29 ± 0.1). An ANOVA indicated a significant effect of pair type (assortative versus disassortative; $F_{1,2,212} = 22.55$, $p = 2.14e-6$) and cross type ($F_{10,822} = 8.5$, $p = .002$). Subsequent post hoc tests indicated that both assortative cross types (M-M and R-R) had higher fecundity than most disassortative cross types (Table 3; Figure 2a).

3.3 | Experiment 2: A test for increased offspring survival from disassortative pairs

Overdominance predicts that the offspring from disassortative male–female pairs should have increased survival relative to assortative pairs. Our data support this hypothesis. Disassortative pairings had much higher survival from the larval to the pupal stage (43.8% disassortative) than did assortative pairings (19.7%, repeated measure ANOVA: $F_{1,2,467} = 344.54$, $p = 9.6e-12$). There were also distinct differences in survival between developmental stages, particularly between the larval and pupal stages with 66% of all offspring dying before pupation (repeated measure ANOVA: $F_{2,2,467} = 741.57$, $p = 0.0009$). Additionally, there was a strong interaction between larval survival during development and parental pairing, with offspring from assortative pairings being less likely to survive to pupation ($F_{2,2,467} = 12.12$, $p = 2.4e-9$; Figure 2b). Moreover, there was variation within assortative and disassortative pairings as different cross types produced different rates of offspring survival ($F_{13,2,467} = 3.78$, $p = 0.008$; Figure 3).

	Assortative	95% CI	Disassortative	95% CI
No. of pairs	316	–	513	–
Avg. # of eggs/pair	32.29	31.2–33.4	29.02	28.2–29.9
Avg. # of larvae/pair	22.38	20.8–23.9	21.36	20.3–22.4
Avg. # of pupae/pair	4.41	3.47–5.37	9.36	8.54–10.2
Avg. # of adults/pair	4.18	3.3–5.2	7.99	7.22–8.78

TABLE 3 Resulting offspring of assortative and disassortative pairs

Note: The average number of eggs produced for each pair and the average number of surviving larvae, pupae and adults for “different” and “same” colour morph pairings. The 95% confidence intervals are also shown for pair types at each stage. The total number of pairings, eggs, larvae, pupae and resulting adults are shown below averages in parenthesis.

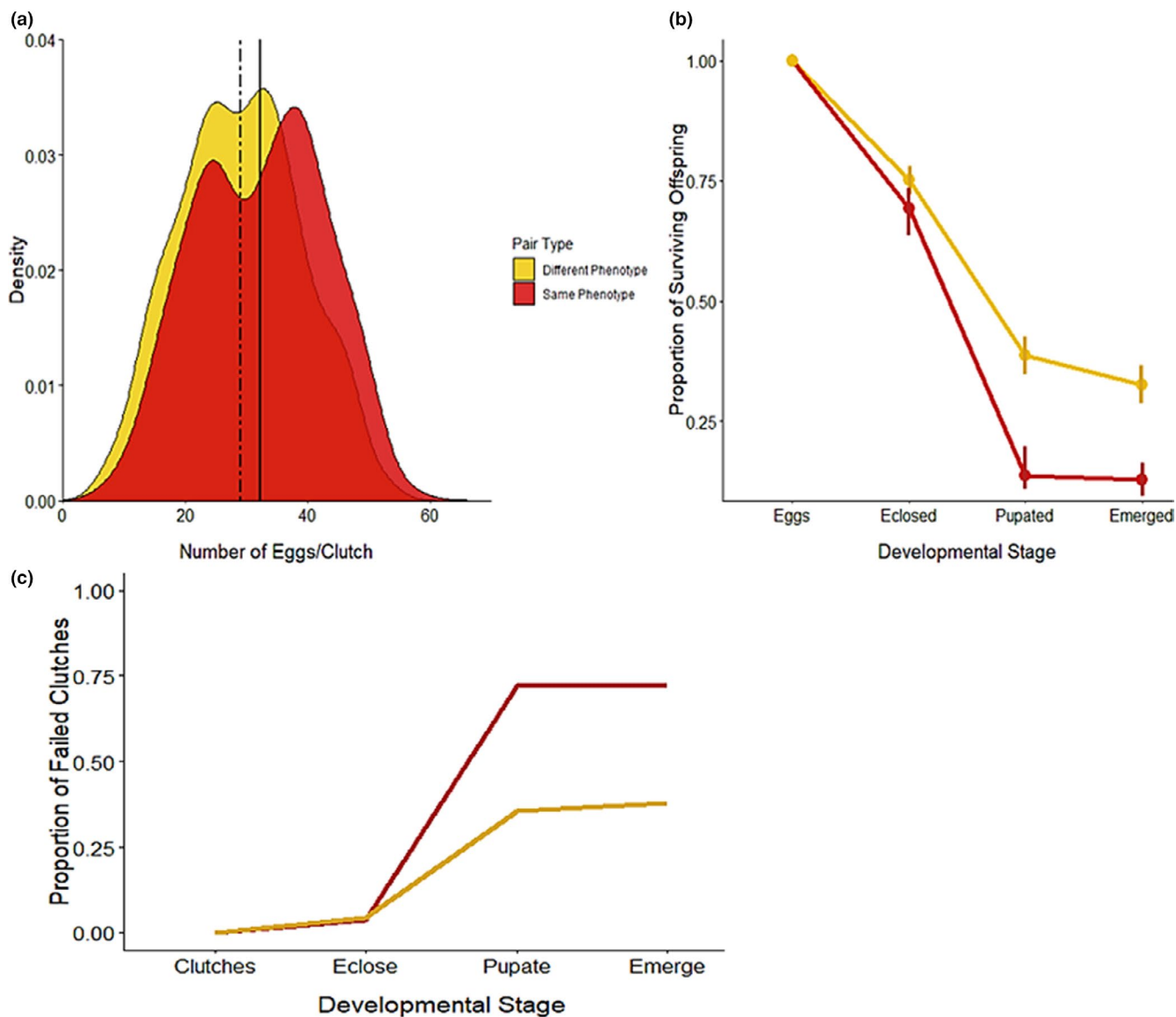


FIGURE 2 Differences in egg production (fecundity) and larval survival through development. Assortative pairings are shown in red and disassortative pairings in gold. (a) Density plot for the number of eggs produced/egg clutch for all females. The solid line shows the average number of eggs produced/clutch for assortative pairs, and the dashed line shows the average for disassortative pairs. (b) The proportion of offspring surviving through development from the total number of offspring produced for assortative (red) and disassortative (gold) colour morph pairings. Eclosed refers to the proportion of larvae that emerged from eggs, pupated is the number of larvae who became pupae, and emerged is the number of individuals who emerged from pupae as adults. The standard error is shown for both assortative (red) and disassortative (gold) pairs at each developmental stage. (c) The proportion of completely failed egg clutches through development for assortative (red) and disassortative (gold) pairings. The larval stage experiences the highest levels of mortality (time between emergence from eggs and pupation). Standard errors are shown for each developmental stage for assortative (red) and disassortative (gold) pairs

The pattern in differential larval survival was partially attributable to the fact that complete clutch failure was 1.9× higher for offspring from assortative pairs when compared to disassortative pairs. Specifically, 72% of clutches from assortative pairs failed to produce any offspring surviving to adulthood compared with 38% of clutches from disassortative pairs ($\chi^2 = 93.06$, $df = 1$, $p = 2.2e-6$; Figure 2c). Analysis of count data with a zero-inflated negative binomial regression showed that there was no significant effect of assortative or disassortative pairings on offspring survival to adulthood, once the excessive number of zeros was taken into account for this

data set (ZINB count model, $Z = 6.655$, $p = 2.83e-11$; zero-inflation, $Z = -0.011$, $p = 0.991$; Figure 3).

Overdominance also predicts that cross types that produce more heterozygotes should have higher survival. Our data supported this hypothesis. Figure 4 shows the relationship between complete clutch failure and the expected proportion of heterozygotes. Cross types that were expected to produce more homozygotes had greater complete clutch failure. This result was obtained regardless of whether the rufipennis-rufipennis cross was modelled with an expectation of 50% homozygous offspring ($Rr \times Rr$ or $RR \times Rr$;

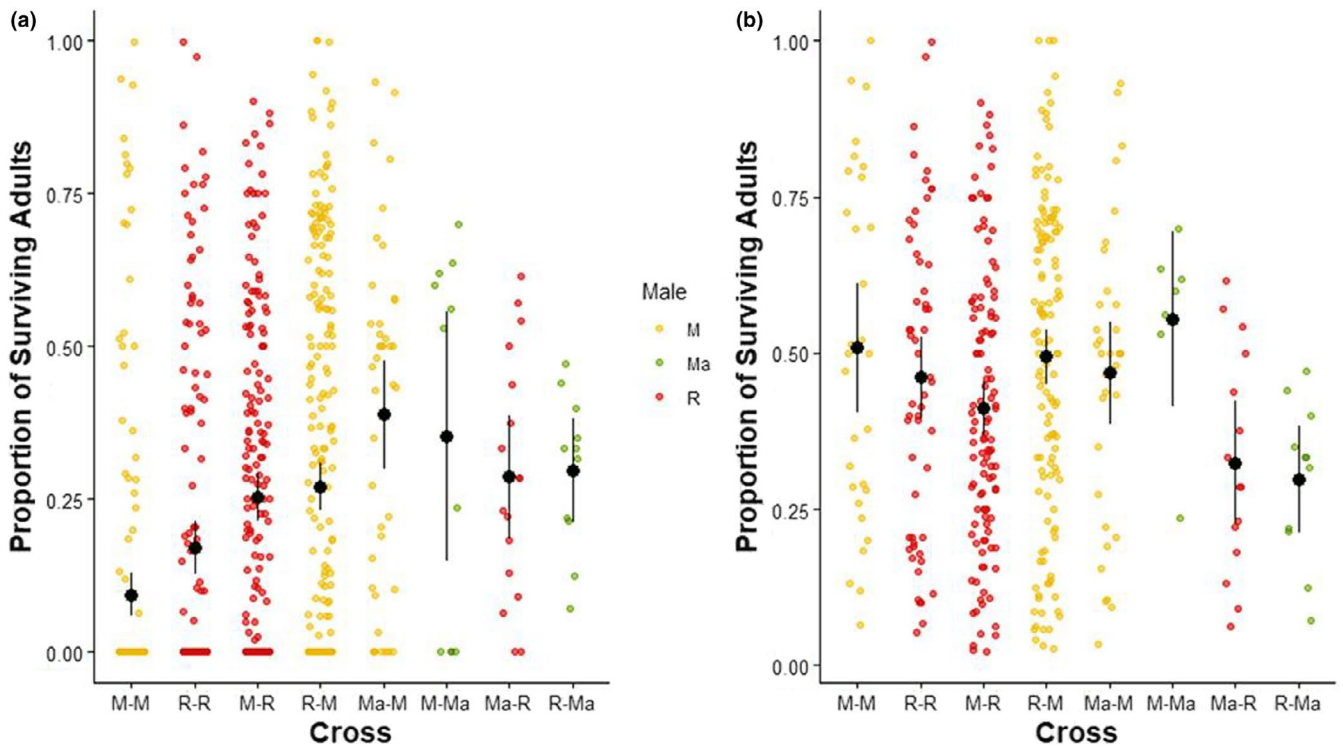


FIGURE 3 The proportion of offspring surviving to adulthood separated by cross types. Each point represents a single egg clutch. The average and SE are shown for each cross type in black. Cross types are colour coded by male colour morph. Crosses refer to phenotypes as metallic = M, rufipennis = R, Ma = *militaris-a*. (a) The proportion of offspring from each egg clutch surviving to adulthood from the entire data set. (b) The proportion of offspring surviving to adulthood from each egg clutch, with those egg clutches that completely failed to produce adult offspring removed from the analysis

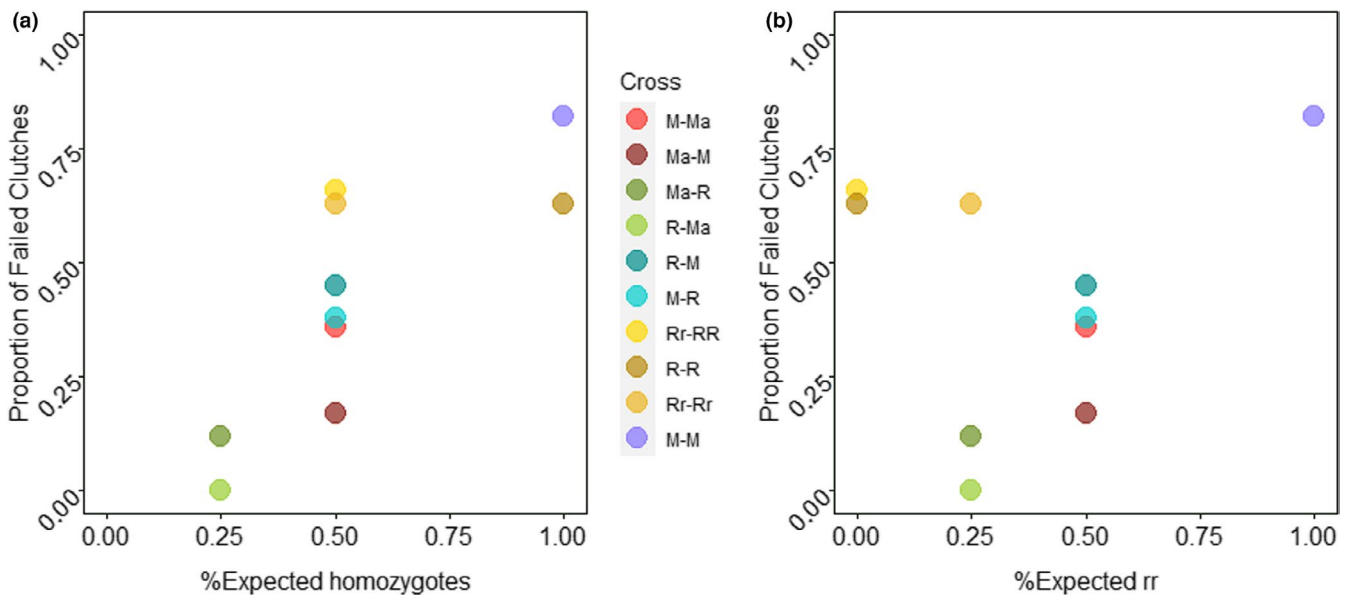


FIGURE 4 The proportion of failed egg clutches as a function of the percentage of expected homozygotes and the percentage of metallic individuals (the recessive genotype *rr*) produced by cross types. For most crosses, the proportion of expected homozygotes is equivalent to the proportion of metallic individuals, with the exception of R-R crosses. In this case, because not all rufipennis individuals were known to be heterozygous, Rr-Rr indicates two heterozygous individuals, R-R is two homozygous individuals, and Rr-R_r indicates crosses where one individual is heterozygous and the other is either heterozygous or homozygous. (a) The proportion of failed clutches as a function of the expected proportion of homozygotes produced by cross types. (b) The proportion of failed clutches as a function of the expected proportion of metallic offspring produced by cross types

generalized linear model, $\chi^2 = 14.2$, $df = 1$, $p = .00016$) or whether the rufipennis-rufipennis cross was modelled with an expectation of 100% homozygous offspring ($RR \times RR$; $\chi^2 = 21.68$, $df = 1$, $p = 3.2e-6$).

An alternative hypothesis is that the decreased survival for assortative mating pairs is driven by the production of metallic "rr" homozygotes. A weaker relationship was found between complete clutch failure and the proportion of "rr" homozygotes. This result was obtained regardless of whether the rufipennis-rufipennis cross was modelled with an expectation of 50% homozygous offspring ($Rr \times Rr$ or $RR \times Rr$; generalized linear model, $\chi^2 = 2.9$, $df = 1$, $p = 0.088$ or $\chi^2 = 0.922$, $df = 1$, $p = 0.337$) or whether it was modelled with an expectation of 100% homozygous offspring ($RR \times RR$; generalized linear model, $\chi^2 = 0.921$, $df = 1$, $p = 0.337$). The proportion of expected homozygotes did not account for a significant amount of variation in survival among the clutches that had at least 1 adult survive. Again, the result was obtained regardless of how the rufipennis-rufipennis cross was modelled (50% homozygous; generalized linear mixed model, $\chi^2 = 0.128$, $df = 1$, $p = 0.721$; 100% homozygous; generalized linear model, $\chi^2 = 0.082$, $df = 1$, $p = 0.774$).

4 | DISCUSSION

We investigated the roles of disassortative mating and differential survival to adulthood as a function of assortative versus disassortative mating in Panamanian populations of *Chelymorpha alternans*. We found little evidence for disassortative mating (or any form of nonrandom mating for colour), suggesting that this mechanism is likely not a selective force maintaining colour variation. Moreover, random mating in this system supports previous reports of high admixture between colour morphs within a single population (Strickland et al., 2018). Instead, we found a strong pattern where survival to adulthood was higher for disassortative than assortative pairs, suggesting a role for overdominance. Hence, although mating appears to be largely random, differential offspring survival as a function of parental colour morph appears to play a role in maintaining this polymorphism.

Offspring from disassortative colour morph pairings had higher rates of offspring survival and produced more offspring than assortative mating pairs, despite the fact that assortative mating pairs had slightly higher fecundity. Moreover, the proportion of failed clutches was highest for assortative pairs, with 72% of egg clutches failing to produce any adult offspring. Heterozygote advantage may explain this pattern. In the morning-glory, *Ipomea purpurea* (Solanales: Convolvulaceae), the locus associated with colour is also associated with offspring production and seedling viability, leading to heterozygotes having an intermediate colour phenotype and an increased seed size (producing greater seed viability) compared with homozygotes. This contributes to the stabilization of this polymorphism throughout its distribution (Mojonnier & Rausher, 1997). How heterozygote advantage influences larval survival and the maintenance of colour polymorphisms in *C. alternans* requires further investigation under field conditions where factors such as parasitism and

predation may also act as selective forces producing this balanced polymorphism.

Previous work with this species has elucidated the geographic distributions of phenotypes across the Isthmus of Panama. In 12 of 28 sampled populations, at least three phenotypes co-occur, and in almost all sampled populations at least two phenotypes are shown to co-occur, indicating that most populations are polymorphic (Strickland et al., 2018). The *militaris-a* and *militaris-b* phenotypes are found from the eastern edge of the Panama Canal and reach greatest frequencies closer to the Colombian border. The metallic and rufipennis phenotypes are the two most widely distributed phenotypes occurring in nearly all collection sites across Panama. There are only two populations found which were shown to be monomorphic (for the metallic phenotype), and these are located on the northern, Caribbean coast of Panama and were not sampled for this study. All four phenotypes used for this study can be found naturally occurring at the experimental site (Gamboa, Panama) suggesting that the differences observed in this study are not the product of local adaptation.

The fact that fecundity was higher in assortative mating pairs is perplexing. A hypothesis of adaptive mate choice would suggest that females should invest more in disassortative matings (Sheldon, 2000). One possibility is that females have limited mating opportunities in nature and engage in reproductive compensation, where females invest more resources in the offspring of suboptimal matings (Bluhm & Gowaty, 2004; Gowaty, 2008). Allocating more resources to offspring of suboptimal matings has been shown to occur in several bird species, including mallards, zebra finches and house finches (Ratikainen & Kokko, 2009). This is expected only under a limited set of conditions, specifically when females are constrained to matings with nonpreferred males, when there are predictable fitness deficits in offspring of suboptimal pairings, and when the associated fitness cost of increased resource allocation to the parent is relatively low (Bluhm & Gowaty, 2004; Gowaty et al., 2007; Gowaty, 2008; Harris & Uller, 2009). In mallards (*Anas platyrhynchos*), females forced to copulate with nonpreferred males produce offspring with lower viability (decreased likelihood of survival to independence) and compensate for this by laying heavier eggs (Bluhm & Gowaty, 2004). It is possible that in this study females from assortative pairs compensated for reduced larval survival with increased fecundity (laying more eggs).

Metallic-metallic pairings (M-M) produced the fewest number of offspring surviving to adulthood when compared with all other cross types. Interestingly, the metallic colour morph is the most widely distributed across the Isthmus of Panama, including at least two populations where this allele is fixed (Strickland et al., 2018). Moreover, studies using predator bioassays indicate that the metallic morph also experiences lower levels of predation suggesting that an interplay between morph fecundity, larval survival and adult palatability may work to maintain high levels of phenotypic variation in this species (Strickland, 2020). The melanic colour morph in the colour polymorphic spittlebug, *Philaenus spumarius* (Hemiptera: Aphrophoridae), experiences higher fecundity (higher egg production) and lifetime

longevity, when compared with a more widely distributed recessive nonmelanic phenotype, but it also incurs higher rates of predation than nonmelanic morphs (Silva et al., 2015; Halkka & Halkka, 1990).

Surprisingly, we did not observe strict disassortative mating although there are strong advantages conferred to offspring of disassortative pairings. There might be several reasons that account for the absence of mate preferences in this study. Most sampled populations across the Isthmus of Panama have at least two co-occurring phenotypes with the relative frequencies of these phenotypes fluctuating between populations. Therefore, it may serve as a bet-hedging strategy for females to increase the likelihood of reproductive success as morph ratios change between populations. Additionally, since there is no male competition for females and females can theoretically engage in cryptic female choice by expelling spermatozoa droplets after matings (Rodríguez, 1995), nondiscriminatory mating may serve as a mechanism to avoid male harassment.

The bright and contrasting coloration of *C. alternans* as well as the relatively close association with their various host plants (Morrison & Windsor, 2018) suggests that this species may sequester host plant secondary metabolites as a defense mechanism. The vast majority of egg clutches were viable (>95% of clutches produced at least one larvae), but more than half of all clutches failed to produce larvae surviving until pupation, indicating high larval mortality. In this study, the preferred host plant, *Merremia umbellata* was utilized for all beetles. Previous work has shown that when given a choice, gravid females of *C. alternans* prefer to deposit egg masses on *M. umbellata*, and larvae also show higher rates of survival when feeding on *M. umbellata* compared with other potential hosts (Morrison et al. 2019). This suggests that the mortality rates observed in this study is not a product of utilizing a nonpreferred host plant or one that results in lower fitness. However, a high larval death rate may be the result of larvae incorporating and sequestering secondary metabolites from host plants which can directly reduce survivorship (Bowers, 1992; Harvey et al., 2005; Zvereva & Kozlov, 2016). Although many studies have focused on the effects of host plant variation on the survival and sequestration ability of their insect herbivores (Bowers, 1992; Dimarco et al., 2012; Fordyce & Nice, 2008), few studies have assessed how intraspecific genetic variation of the herbivore can influence larval survival and development. Notable exceptions include two studies on the pipevine swallowtails. As caterpillars, the pipevine swallowtail, *Battus philenor* (Lepidoptera: Papilionidae) sequesters toxic chemicals from its host plants, species of the genus, *Aristolochia* (pipevines). Fordyce and Nice (2008) studied the trade-offs of insect chemical defense and found that variation between families accounted for 40% of the total variation in larval growth and that sequestration ability (measured as the mean alkaloid content sequestered in individuals) was positively correlated with survivorship to the second instar. Furthermore, a follow-up study with the same species found that 44% of larval sequestration ability was due to family identity, suggesting that sequestration ability, and highly complex, is a heritable trait with intraspecific variation (Dimarco et al., 2012).

Investigating whether heterozygote advantage in this study plays a role in conferring a greater ability to sequester noxious plant compounds; thus, increasing larval survival would be a fascinating endeavor. Elucidating how intraspecific variation of sequestration ability relates to differential survival of larvae as well as fecundity and adult palatability would provide insights into community and spatial distributions of toxin-producing plant hosts, sequestering herbivores and their predators.

Two caveats should be addressed. High juvenile mortality rates could have obscured actual parental mating preferences if parents did mate nonrandomly. Although differential larval survival may have skewed adult phenotypic ratios, the expectation is that it would have produced a pattern indicating a preference for disassortative mating. The fact that we mainly found random mating suggests that the mating was either random or assortative. Second, infections of the intracellular bacteria, *Wolbachia*, are known to influence egg production and viability through cytoplasmic incompatibility, whereby females lacking the infection produce no or a highly reduced number of progeny when mated with infected males (Hoffmann et al., 1998). This manifests principally through increases in the number of inviable egg clutches (resulting in zero larvae being produced from a clutch rather than larval death later in development) between incompatible pairings. Testing for the presence of *Wolbachia* infections with PCR primers for the *wsp* gene in the individuals used for these studies might have offered additional evidence regarding the influence of *Wolbachia* (or lack thereof). However, previous studies on *Wolbachia* infections in *C. alternans* have shown easily detected responses to cytoplasmic incompatibility (CI) (Keller et al., 2004). Additionally, in this study we observed hatch rates of 96%, consistent with previous work studying *Wolbachia*-compatible crosses (88.5%–98%; Keller et al., 2004) suggesting that hatch rates were not compromised in this study. Furthermore, CI from *Wolbachia* infections would not produce the pattern observed in differential survival through larval development and emergence as adults between assortative and disassortative pairings (Keller et al., 2004; Werren & Windsor, 2000). Additionally, there were no methodical differences in the population of origin between individuals used for assortative and disassortative pairings; therefore, any differences in *Wolbachia* infection status would remain random throughout the dataset. Finally, crosses between different populations can often show a pattern of heterosis due to the masking of deleterious recessive alleles from each population. Because individuals from various populations were randomly assigned to pair types and multiple phenotypes can be found in a single population, this would not be biased towards disassortative matings in our dataset (i.e. a disassortative pairing is just as likely to consist of individuals who originate from the same geographic population as assortative pairings).

In conclusion, we found no evidence that disassortative mating preferences contributes to the maintenance of polymorphisms in colour pattern within populations. Interestingly, we found a pattern of increased survival during development for crosses

involving parents with different colour patterns in comparisons with crosses involving parents with the same colour pattern. This pattern is consistent with overdominance. The exact mechanism through which overdominance might occur is unknown, but data from other studies suggest a role for differential palatability between the colour morphs, which, combined with the bright coloration present in both sexes, suggests that differential sequestration of secondary chemicals—which often occurs early in development—may play a role.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

LRS conducted all experiments and data collection, participated in data analysis and preparation of the manuscript. RCF contributed to experimental design, data analysis and preparation of the manuscript. DW contributed to experimental design and preparation of the manuscript. CEC contributed to data analysis and preparation of the manuscript.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/jeb.13779>.

DATA AVAILABILITY STATEMENT

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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