Do juvenile developmental and adult body characteristics differ among genotypes at the *doublesex* locus that controls female-limited Batesian mimicry polymorphism in *Papilio memnon*?: A test for the "cost of mimicry" hypothesis

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ABSTRACT

Female-limited Batesian mimicry may have evolved because of stronger predation pressure on females than on males, but some physiological costs of mimicry may also hinder the evolution of mimicry in males. In *Papilio memnon*, which possesses a female-limited Batesian mimicry polymorphism, two alleles at the *doublesex* (*dsx*) locus strictly control female phenotypes. To examine whether there are physiological costs associated with mimetic genotypes in the juvenile stage, we compered mortality, juvenile growth and development, and the resultant adult characteristics among three *dsx* genotypes (*HH*, *Hh*, *hh*) at a constant temperature (25 °C) and two differing day lengths (LD 14:10 and LD 12:12; the latter might induce pupal diapause) by crossing individuals heterozygous (*Hh*) for the *dsx* allele. All pupae emerged directly without diapause irrespective of day length. The genotype frequencies of the emerged individuals were consistent with the expected 1:2:1 ratio of *HH*:*Hh*:*hh*. The sex ratio was significantly male-biased in one of two families, but not in the other. We found no effect of genotype on any developmental or adult characteristic, although there were sex differences in most traits. The larval development time was longer and growth rate higher in females than in males; pupal weight, forewing length, and total dry mass of the thorax and abdomen were greater in females, whereas the thoracic mass/abdominal mass ratio was greater in males. We also found that the growth rate was higher and pupal period longer with a short day than with a long day. Overall, we found no evidence for physiological costs associated with the mimetic genotypes. However, it is too early to conclude that no physiological cost of mimicry affects the evolution and maintenance of this female-limited Batesian mimicry polymorphism because we have not studied the adults of different genotypes.

1. Introduction

Batesian mimicry is a phenomenon in which palatable mimics avoid predation by resembling unpalatable species (Bates, 1862; Huheey, 1988; Ruxton et al., 2004). One of the most intriguing types of Batesian mimicry in butterflies is female-limited polymorphism, in which females have both mimetic and non-mimetic forms, but males are uniformly non-mimetic (Wallace, 1865; Kunte, 2009). Female-limited mimicry can be attributed to stronger predation pressure on females than on males, resulting in a greater fitness advantage of mimicry only in females (Wallace, 1865; Ohsaki, 1995). It is thought that mimicry is a favorable strategy for females because they are less agile than males due to their larger abdomens and they suffer from predation more

severely (Chai and Srygley, 1990; Srygley and Chai, 1990; Marden and Chai, 1991; Ohsaki, 2005). However, it is unclear why males do not express the mimetic phenotype. One explanation is that female choice for male wing pattern is conservative and makes the mimetic pattern disadvantageous in reproduction (Belt 1874; Müller, 1879; Turner, 1978; Herrel and Hazel, 1995; Lederhouse and Scriber, 1996). In addition, Ohsaki (2005) hypothesized that some physiological costs of mimicry could hinder the evolution of mimicry in males because males could also mimic the models if there were no cost of mimicry. In a greenhouse experiment, Ohsaki (2005) showed that non-mimetic females lived longer than mimetic females of *Papilio polytes* and suggested that producing the mimetic form was costly (e.g., via the production of more carotenoid). However, the physiological cost hypothesis has not

been examined since then, partly because it is difficult to distinguish the genotypes associated with mimetic/non-mimetic forms. In animals, alleles associated with color polymorphism can affect other traits pleiotropically (McKinnon and Pierotti, 2010; Wellenreuther et al., 2014). For example, in the polymorphic damselfly *Ischnura elegans*, larval development time differed between maternal morphs (Abbott and Svensson, 2005), although the genetic locus determining the polymorphism is unknown.

In this study, we examined whether some physiological costs of producing a mimetic form exist in the great Mormon butterfly, *Papilio memnon*, which exhibits female-limited Batesian mimicry polymorphism. *P. memnon* possesses *H* and *h* alleles at the *doublesex* (*dsx*) locus that strictly control female phenotype (Komata et al., 2016), as in the closely related species *P. polytes* (Kunte et al., 2014; Nishikawa et al., 2015). The *dsx* locus corresponds to a hypothesized supergene that controls female phenotype in a Mendelian fashion, in which the mimetic allele *H* is dominant to the non-mimetic allele *h* (Clarke et al., 1968; Clarke and Sheppard, 1971). We developed a method to genotype *dsx* alleles from genomic DNA and found that the frequency of the mimetic allele in Taiwanese *P. memnon* populations has been maintained at intermediate levels spatiotemporally (Komata et al. 2016, 2017). The female-limited polymorphism may be maintained primarily by the action of negative frequency-dependent selection (predation) on the mimetic allele (Poulton, 1908; Barrett, 1976; Kunte, 2009). However, some physiological cost of the mimetic phenotype, if it exists, may have lowered the average level of mimetic allele frequency and hindered the evolution of the mimetic form in males.

We compared juvenile growth and development and the resultant adult characteristics among the *dsx* genotypes (*HH*, *Hh*, and *hh*) in *P. memnon*. We reared larvae from an *Hh* × *Hh* cross under two photoperiodic conditions to examine whether (1) the genotypic frequency and sex ratios at adult emergence are consistent with the expected ones under Mendelian segregation and a 1:1 sex ratio and (2) the genotypes differ in juvenile development and adult morphologies. We used two photoperiod conditions (short and long day) because the performance of mimetic genotypes may differ seasonally. In fact, both sexes of *P. memnon* are smaller in early spring than in other seasons in Taiwan (Komata et al., 2017). This seasonal size variation may be related to pupal diapause in winter induced by short-day conditions, as in *Papilio xuthus* (Tanaka and Tsubaki, 1984; Komata and Sota, 2017); short-day conditions do induce pupal diapause of *P. memnon* in Japan (Yoshio and Ishii, 1998).

We determined the mortality, sex ratio, larval development time, pupal weight, growth rate, forewing length, and thoracic mass/abdominal mass ratio of adult butterflies. Of these, examination of the sex ratio at adult emergence was necessary because male *P. memnon* are often captured more frequently than females in the field, as in many other butterflies (Ohsaki, 1995; 2005; Komata et al. 2016, 2017; Jiggins, 2017). This phenomenon can be attributed to differential predation on males and females or behavioral differences between the sexes, but whether the sex ratio at emergence is 1:1 has not been determined. The thoracic mass/abdominal mass ratio was compared among the *dsx* genotypes because the allocation of resources to these two body parts might differ between mimetic and non-mimetic forms. In butterflies, the thoracic mass containing muscles is related to flight ability, whereas the abdominal mass is related to reproductive capacity (Karlsson and Wickman, 1990; Srygley and Chai, 1990; Berwaerts et al., 2002). In *P. polytes*, the mimetic females mimic unpalatable models in both their wing pattern and behavior (Kitamura and Imafuku, 2010, 2015), and mimetic females fly more slowly than non-mimetic females (see also Chai and Srygley, 1990). Such behavioral mimicry may be related to a difference in body mass allocation between the thorax and abdomen.

2. Materials and methods

2.1. Experimental design

We used a cross between females and males that were heterozygous for the *doublesex* allele (i.e., genotype *Hh*) to obtain larval populations consisting of three genotypes: *HH*, *Hh*, and *hh* (expected *HH*:*Hh*:*hh* ratio = 1:2:1). Hatching larvae were reared until adult emergence at 25 °C and under two photoperiods (LD 14:10 or LD 12:12) to compare mortality, larval development time, pupal weight, growth rate, forewing length, and thoracic mass/abdominal mass ratio of adult butterflies among genotypes and between the sexes.

2.2. Rearing and measurements

To obtain male and female butterflies with the *Hh* genotype, we captured one mimetic (M1) and two non-mimetic females (NM1 and NM2) on 6–7 June 2015 at Hualien, eastern Taiwan (23°59′N, 121°32′E). The females laid eggs in the laboratory, and hatching larvae were reared at 25 °C under a LD 14:10 photoperiod to obtain adults. We chose two males and two mimetic females likely of the *Hh* genotype and performed forced copulation by hand pairing for two pairs: female from M1 × male from NM1 (named famA), and female from NM2 × male from NM1 (named famB). The *dsx* genotype of these butterflies was confirmed as *Hh* after copulation and oviposition using the molecular identification method described below. After copulation, the females were reared individually in a cage containing citrus leaves for oviposition; they deposited approximately 300 eggs in total. To study the larval developmental characteristics, the eggs deposited by each female were immediately divided into two groups and reared under LD 14:10 or LD 12:12 at 25 °C. Hatching larvae were reared individually in plastic cups (diameter, 130 mm; height, 100 mm) and fed grapefruit (*Citrus* × *paradisi*) leaves. We measured pupal weight (to 0.1 mg) with an electric balance (Shimadzu LIBROR AEG-220). We defined larval development time as the number of days from hatching to pupation, and growth rate as ln(pupal weight)/larval development time. The pupal period was the number of days from pupation to adult emergence. For adults, we measured forewing length using digital electronic calipers (to 0.01 mm) and determined the dry mass of the thorax (legs and wings removed) and abdomen using an electronic balance (A&D GH-252) after keeping them at 60 °C for 24 h.

2.3. Genotyping

The genotypes of the parental and offspring butterflies were determined following the method described in Komata et al. (2016). Total genomic DNA was extracted from the legs of adults using a Genomic DNA Purification Kit (Promega, Madison, WI, USA). High-resolution melting analysis using a real-time PCR platform was performed to identify *dsx* genotypes (see Komata et al. 2016 for details).

2.4. Statistical analyses

We explored the effect of day length on mortality using a generalized linear mixed model (GLMM) assuming a binomial distribution. The difference in parents (famA vs. famB) was included as a random effect. The observed ratios of the three *dsx* genotypes (*HH*:*Hh*:*hh*) were compared with the 1:2:1 ratio under the null hypothesis of random association of alleles and no mortality difference among genotypes using the chi-square test for the goodness of fit in R (R Core Team, 2016). We explored the effects of sex, *dsx* genotype, and day length on larval development time, pupal weight, growth rate, pupal period, adult forewing length, total mass of the thorax and abdomen, and thoracic mass/abdominal mass ratio using a GLMM assuming a normal distribution. The difference in parents (famA vs. famB) was included as a random effect. In all analyses, all interaction terms with explanatory