Lack of evidence that the false head of male *Callophrys xami* (Reakirt, 1867) (Lepidoptera, Lycaenidae) is a sexually selected trait

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Abstract. Colourful and morphologically complex traits can be used to deflect predators' attacks to a part of a prey's body that is less vulnerable. This is the case of the so-called false head (FH) of some butterfly species, in which the posterior end of the hindwings of individuals perching with closed wings resembles a butterfly's head. Recently, it was shown that the FH of *Callophrys xami* (Reakirt, 1867) females also plays a role in sexual selection. Specifically, females with an experimentally ablated FH received larger ejaculates, suggesting that males exert postcopulatory (cryptic) choice in favour of these females, perhaps because they have shown to be able to deflect predator attacks. Here, we tested experimentally whether *C. xami* females also show a preference for males with ablated FH. In our paired experiment, we presented virgin females either with a male with the FH ablated or with a male with the FH intact. We compared the probability of mating, copula duration and the number of eggs laid by the females two days after mating. We did not find evidence of female choice in relation to the presence/absence of FH in the males. Males with ablated FH were as likely to mate as control males. Copula duration and number of eggs laid were also very similar in females mated with either males with the FH ablated or intact. We suggest that the structure of the courtship behaviour of this butterfly could explain why males use the FH in their choice of mate, while females apparently do not.

Introduction

Darwinian adaptations of prey animals in response to predation are widespread in nature (Ruxton et al. 2004). Sometimes, these adaptations adopt additional functions related to other aspects of an organism's social life. For example, in the poison frog *Oophaga pumilio* (Schmidt 1857), the aposematic coloration deters predators (Saporito et al. 2007) and is also used by females in mate choice (Maan and Cummings 2009). In several butterflies, mainly within the Lycaenidae family, the posterior end of the hindwing of individuals perching with their wings closed resembles the head of a resting butterfly

(Poulton 1890; Robbins 1980; Cordero 2001). This 'false head' (FH hereafter) is thought to deflect predators into attacking its false rather than its real head (Robbins 1980; Cordero 2001). There is observational (Van Someren 1922), experimental (Wourms and Wasserman 1985; Sourakov 2013) and comparative (Robbins 1981; López-Palafox et al. 2015; Novelo-Galicia et al. 2019) evidence supporting this function. On the other hand, recent experimental evidence in *Callophrys xami* (Reakirt, 1867) (Lycaenidae) suggests that the FH also plays a role in sexual selection (Medina and Cordero 2021). In *C. xami*, virgin females with an experimentally ablated FH received larger ejaculates than virgin females with the FH intact (Medina and Cordero 2021). Since in many lepidopterans the ejaculate contains nutritious, hormone-like and protective components that benefit the female (i.e. they are nuptial gifts; see references in Xochipiltecatl et al. 2021), larger ejaculates could provide more benefits to females, thus suggesting that male *C. xami* exert "cryptic" (sensu Eberhard 1996) male choice in favour of females with the FH damaged. Why males favour females with the FH "damaged" is unknown, but Medina and Cordero (2021) proposed the hypothesis that a damaged FH is preferred by the male because it indicates a failed predator attack, and thus reveals the butterfly's ability to deflect a predator attack, a trait that, if inherited, would be useful for the male's offspring.

Whether the FH also influences female mate choice in this species remains unknown. Here, we tested experimentally if *C. xami* females also favours males without an FH, by evaluating if they mate more frequently with males with the FH ablated than with males with the FH intact, and if after copulating with males with the FH ablated the females lay more eggs than after mating with males with the false head intact.

Materials and methods

The butterflies used in the experiment were the offspring of females collected in the Botanical Garden of the National Autonomous University of Mexico (UNAM). *C. xami* is a multivoltine species whose larvae feed mainly on *Echeveria gibbiflora* DC (Crassulaceae). We followed the laboratory rearing method described by Jiménez and Soberón (1989). Briefly, we reared all the butterflies individually from egg to adult in plastic Petri dishes (10 cm diameter, 4 cm height) with pieces of leaf of the food plant; the cages were cleaned and the pieces of leaf changed approximately every other day. When each adult emerged, we recorded the date, sex and the length of its right forewing (a good predictor of body weight; Cordero et al. 2000) from the most anterior point of junction with the thorax to the more distal point using callipers. We kept records of the identity of the mothers to prevent mating of siblings.

We used a paired experimental design as in our previous male choice experiment (Medina and Cordero 2021); our sample size was relatively small for several reasons (total replicates: 19 pairs). *C. xami* adults emerge asynchronously throughout several days, even if they emerge from eggs that were laid on the same day, and the female biased sex ratios at emergence observed in previous laboratory cultures reduced the number of potential experimental pairs. Additionally, space limitations in our laboratory and ethical considerations (Crump et al. 2023; Fischer et al. 2023) also influenced our decision to limit the number of replicates in the experiment. Each replicate of the experiment consisted of two experimental mating cages: in each cage there was a virgin female and a virgin male; in one cage the male had his FH ablated and in the other the FH area was intact. For the experimental ablation of the FH area, all males were immobilized for a brief period of time by introducing them in a freezer at -4 °C for about 5 minutes. Males in the 'ablated' treatment had their FH removed with microscissors (Fig. 1), while males in the 'control' treatment received a similar manipulation but without the ablation. We chose this experimental

Figure 1. *Callophrys xami* in the Pedregal de San Ángel (Mexico City). In the experiment, the false head was ablated by cutting the segment to the left of the discontinuous red line with micro scissors. Photo courtesy of Aldair Emmanuel Vergara Paredes.

design instead of exposing the female to the two males simultaneously because males defend mating territories and, thus, females never find more than one male when looking for a mate (Cordero and Soberón 1990). The cylindrical mating cages were made of mesh cloth and metal wire (25 cm diameter, 60 cm height). Each pair of mating cages were hung on a metal frame in a garden of the Instituto de Ecología (UNAM) in Mexico City, which is situated next to the Pedregal de San Ángel, the natural environment where *C. xami* inhabits. Replicates were placed between 11:00 and 14:00 h, and we finished a replicated earlier if the pair mated before the end of this period. Males and females in each replicate were matched, as much as possible, within each sex, for age and wing length. Males and females were never exposed to potential mates more than once, independently of whether they mated or not. When a couple mated, we measured copula duration and the female was transferred to a 1 liter white plastic container with a piece of leaf of the larval foodplant for oviposition. We put the female in the container under a 75W light bulb for two hours over two consecutive days. Under these conditions, females laid most of their eggs in the first two days of egglaying (C. Cordero, personal observation).

We tested whether the age and wing length of males and females was significantly different, as required by the paired design of our experiment, with Wilcoxon Matched Pairs tests. The proportion of females mating in each treatment was compared by means of a Chi-square test. All tests were two-tailed.

We also report the results of a previous pilot experiment with the same paired design, but in which we only recorded if there was a mating or not (copula duration and egg production in the two days following mating were not measured). In this experiment, we were only able to determine age differences between the paired males and females, not absolute ages. The main problem with this experiment (and the reason why we consider it a pilot experiment) was that, due to logistical limitations, most of its replicates used male-female pairs that were siblings (32 out of 40; 80%).

Results

Our effort to match the winglength (our estimate of butterfly size) and age of females and males in each replicate of the paired experiment was successful (full data set in Suppl. material 1: table S1). Wilcoxon Matched-pairs tests did not detect differences in both variables between the treatment and control groups: (1) Female winglength: $W = 21.5$; $p > 0.05$; N = 9 (in the other 10 replicates the winglength of the females was identical). (2) Female age: $Z = 0.82$; $p = 0.41$; N = 15 (in the other four replicates the age of the females was identical). (3) Male winglength: $Z = 1.19$; $p = 0.23$; N = 15 (in the other four replicates the winglength of the males was identical). (4) Male age: Z = 0.65; $p = 0.52$; N = 16 (in the other three replicates the age of the males was identical).

Overall, only 12 out of 38 (31.6%) pairs mated. Five females (26.3%) mated with males with the FH ablated and seven (36.8%) with males with the FH intact; the difference was not statistically significant (Chi-square = 0.49; $p = 0.49$; Fig. 2). Paired comparisons of copula duration and number of eggs laid were not possible because only in two replicates the experimental and control pair mated. The median duration of copulations with males with deleted FH (30 min; minimum-maximum: 29–45; N = 5) was similar to that of males with intact FH (34 min; minimum-maximum: 26–44; N = 7). Also the median number of eggs laid by females mated with males with the FH deleted (23; minimum-maximum: 10–43; N = 5) was very similar to that laid by females mated with males with the FH intact (18; minimum-maximum: 11–45; N = 7).

The results of the pilot experiment (full data set in Suppl. material 1: table S2) were similar to those of the main experiment. There were no significant differences in wing length between experimental and control females ($Z = 0.64$; $p = 0.52$; N = 19), although the difference was close to significance in males ($Z = 1.9$; $p = 0.06$; N = 20), with control males being smaller than males with the FH ablated (mean ± SD: males with FH ablated: 26.7 ± 1.07, males with FH intact: 25.9 ± 1.52). With respect to age, female pairs in 16 out of 20 replicates (80%) and male pairs in 17 out of 20 replicates (85%), emerged the same day. Twelve out of 40 pairs mated (30%); this proportion was almost identical to that of the main experiment (Chi-square = 0.02; $p = 0.88$). In this pilot experiment the proportion of couples mating in the control and in the ablated FH treatments was identical (30%, 6 out of 20, in each case).

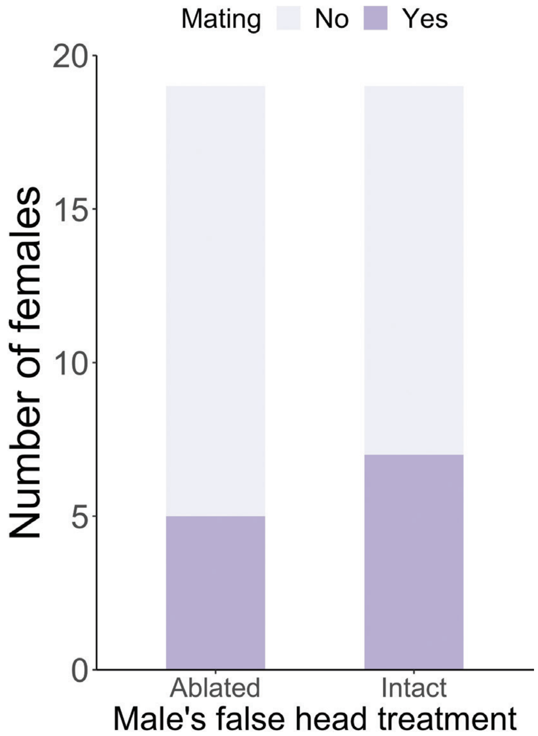

Figure 2. Number of females mating (grey) or not mating (white) with males with the false head intact and with the false head (FH) experimentally ablated. The difference was not significant ($p = 0.49$).

Discussion

According to our results, the lack of an FH on virgin *Callophrys xami* males had no effect on their probability of mating. Although we could not test it statistically, copula duration and the number of eggs laid by their mate did not appear to be affected by the lack of a FH. These results suggest a lack of female choice regarding male absence or presence of FH. This is somewhat unexpected, considering that a previous experiment on male mate choice in the same species detected a positive effect of the experimental ablation of the FH of females on copula duration and on the amount of ejaculate transferred, suggesting cryptic (i.e. copulatory and postcopulatory) male choice in favour of females that apparently survived a predator attack (Medina and Cordero 2021).

It could be argued that our sample size was limited, but it was very similar to that of our previous male mate choice experiment (Medina and Cordero 2021). Another important difference with the male mate choice experiment was the smaller proportion of matings observed (almost half of those observed by Medina and Cordero (2021): 12 out of 38 (31.6%) vs. 24 out of 40 (60%) (Chi-square = 6.33; $p = 0.012$). We do not have an explanation for this difference. Butterfly rearing, experimental design and the facilities in which both experiments were performed were the same in both studies. Furthermore, in the pilot female choice experiment the proportion of matings was very similar to that of the main experiment (30%). An obvious difference is that Medina and Cordero (2021) manipulated female individuals, whereas in the experiments here reported, males were the manipulated sex. If males are more sensible to manipulation (although we consider extremely improbable that we hurt any specimen in the experiment) and more easily disturbed, this could explain the difference observed. Currently, we have no evidence in support of this difference. Alternatively, females could pay more attention to other traits, such as territory size and quality, and may consider the state of the male's FH less important.

The courtship and mating pattern of this species may also explain why males use the ablation of the FH of females on male mate choice, but perhaps not females. Specifically, males of *C. xami* defend mating territories (Cordero and Soberón 1990), and when a female enters the territory the male flies behind the female, staying less than 10 cm behind her. When the female is perched, the male walks beside the female until reaching a parallel, head to head, tail to tail position until copulation takes place. Therefore, in nature, males can see the rear end of a female, while females, most of the time, presumably see the front part of a male. It is therefore possible that females do not manage to evaluate the ablated FH of the males, due to the courtship behaviour of this species.

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Supplementary material 1

Additional information

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Data type: docx

Explanatory note: **table S1.** Complete data set of the main experiment. **table S2.** Dataset of the female choice pilot experiment.

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