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ORIGINAL ARTICLE

False heads and sexual behaviour in a hairstreak butterfly, *Callophrys xami* (Lepidoptera: Lycaenidae)

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Abstract. In several butterflies, the posterior end of the hindwings resembles a butterfly head when the butterfly is perched with its wings closed. There is evidence that this "false head" (FH) deflects predator attacks towards non-vital parts of the body. If the FH protects from visually oriented predators, its condition in an individual butterfly could provide information about its quality to prospective mates. We tested two hypotheses based on this idea by comparing the probability of mating, duration of copulation and size of the ejaculate received by females of *Callophrys xami* (Lycaenidae) with an intact FH and those with an ablated FH in a paired experiment. The absence of a FH had no effect on the probability of mating, but females with an ablated FH copulated for longer and received larger ejaculates, which supports the hypothesis that males prefer females with damaged FHs because this reveals the female's ability to deflect attacks. Male or female (or both) cryptic choice could also account for our results, but more studies are needed to test this.

INTRODUCTION

Deception is widespread in nature (Wickler, 1968; Ruxton et al., 2004; Howse, 2014; Stevens, 2016). In several species, some individuals provide misleading information that results in fitness benefits for them and fitness costs for the "tricked" organisms (Ruxton et al., 2014; Stevens, 2016). A classic example of deception is the "false head" present in many butterfly species of the family Lycaenidae (Robbins, 1980). In these species, the posterior end of the hindwings resembles the head of a butterfly that is perching with its wings closed. This resemblance is reinforced by specific behaviour, such as the back and forth movement of the hindwings that helps the hindwings tails (the "false antennae") simulate the movements of real antennae (López-Palafox et al., 2015). This false head (FH hereafter) is explained as an adaptation that deflects attacks from predators to non-vital parts of the body (Robbins, 1980; Cordero, 2001). There are only two experimental tests of this idea using species of Lycaenidae (Sourakov, 2013; López Palafox & Cordero, 2017), and in one case the results were inconsistent with the hypothesis (López Palafox & Cordero, 2017). However, comparative data on failed predator attacks on the hindwings support the predictions of the above hypothesis (Robbins, 1981; Novelo Galicia et al., 2020).

If the FH protects them from visually oriented predators, its condition in an individual butterfly could provide valuable information for prospective mates. One hypothesis is that an intact FH could indicate that the individual has been able to avoid attacks from predators and this ability, if it has a genetic base, could be inherited by its offspring. An intact FH could also indicate that the butterfly is young, which could mean a higher probability of being unmated, a desirable state for a male or female mate. An alternative hypothesis is that if the FH shows damage inflicted by a (failed) predator attack, it could reveal the butterfly's ability to deflect attacks. According to the first hypothesis, we would expect a mate preference in favour of mates with an intact FH, and, according to the second, a preference for mates with a FH showing predator inflicted damage. Preference could be expressed as a higher probability of mating, longer copulations or the transfer/reception of a larger ejaculate with respect to FH condition. In this paper, we report an experimental test of these hypotheses in the FH butterfly *Callophrys xami* (Reakirt, 1867).

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We decided to test the effect of ablation of the FH of females on male sexual behaviour because the courtship of this species led us to think that it is more likely that male's assess female's FH rather than the reverse. The rationale is as follows: Males of *C. xami* defend mating territories (Cordero & Soberón, 1990). When a female enters the territory the "male flies following the female … along a route parallel to the ground … the male flies near (<10 cm), slightly behind and a few cm above the female … [t]he female and male alight on vegetation close to each other … the male walks in front of the female until reaching a head to head position, while fluttering vigorously; meanwhile, the female stays motionless with her wings closed … [a] fter a few seconds … the male walks beside the female until reaching a parallel, head to head, tail to tail position" and copulation ensues (Cordero, 1993, p. 100). Thus, in nature, during courtship, males are frequently in positions that allow them to see the rear end of a female, while females, most of the time, presumably see the front part of a male. In addition, there is empirical evidence of male mate choice in other species of butterfly (e.g. Jiggins et al., 2000; Prudic et al., 2011; review in Cannon, 2020) and in *C. xami* it is likely because ejaculate production is costly for males (Cordero, 2000). We compared the probability of mating, duration of copulation and size of the ejaculate received by intact females and females whose FH was experimentally ablated.

MATERIALS AND METHODS

The butterflies used in our experiment were the offspring of females collected (and released after laying eggs) in the Pedregal de San Ángel Ecological Reserve (PSAER) located within the Ciudad Universitaria (CU) campus of the Universidad Nacional Autónoma de México (UNAM), in Southern of México City. *C. xami* is a multivoltine species whose main larval food plant in PSAER is *Echeveria gibbiflora* (Crassulaceae). We reared and maintained the butterflies following the methods developed by Jiménez & Soberón (1988–1989). We reared all the butterflies individually from egg to adult in plastic Petri dishes (10 cm diameter, 4 cm height) with pieces of its food plant, keeping a record of the identity of their mothers. The day an adult emerged, we recorded the date, determined its sex and measured, using callipers, the length of its right forewing from the most anterior point of junction with the thorax to the more distal point (a good predictor of body weight in this species; Cordero et al., 1996–2000).

Our experimental design was defined by logistics and by the biology of *C. xami*. Collecting a large sample of females in a short period of time would require many collectors (which we did not have) and poses an ethical challenge because the species is relatively rare. Furthermore, we lacked space to maintain a large culture. In addition, sex ratios at emergence are female biased (C. Cordero, pers. observ.) and the emergence of adult butterflies occurs over a period of several days, even for siblings coming from eggs laid on the same day. In consequence, each day just a few adults emerged and since mating propensity declines with age (C. Cordero, pers. observ.) we were not able to produce a large number of butterflies of similar ages in a short period of time. Thus, we performed a paired experiment in which each replicate consisted of two male-female pairs interacting in two independent cages. We decided to use this experimental design, instead of exposing both females to one male within a single cage, because in more than 300 h observing territorial males in the field we never observed more than one female at the same time in a territory (Cordero et al., 1996–2000), in fact Robert Silberglied mentions that in butterflies in general "males do not encounter more than one female at a time" and thus "opportunities for choice between receptive females rarely occur" (Silberglied, 1984: p. 219–220). In the control pair, the FH of the female was intact, while in the experimental pair the FH of the female was ablated (Table 1). In each replicate, both individuals of the same sex were matched as far as possible for wing length and adult age; all individuals were unmated. Twenty replicates were recorded over several days (on most days only one replicate and on a few days two).

For ablation of the FH, the females were first immobilized by placing them, within their plastic Petri dishes, in a refrigerator (4°C) for 5 min. Then, we gently removed the area of the hindwings comprising the tails ("false antennae") and largest eyespots (Table 1) using micro-scissors (Iris scissors, BioQuip). This involved manipulating each butterfly for about 3 min. The control females were manipulated in a similar way, placing them also in a refrigerator for 5 min and simulating the handling involved in the cutting of the FH for about 3 min.

We put each pair in a cylindrical hanging cage made of mesh cloth and metal wire (~60 cm height and ~25 cm diameter). We avoided pairing sibling males and females. Each pair of mating cages were hung, side by side, on a metal frame in an isolated garden of the Instituto de Ecología, located on the CU campus of the UNAM, mostly between 11:00 AM and 13:00 PM (four pairs between 12:00 and 14:00) on sunny days (all matings observed in the field in previous years occurred between 10:00 and 15:00 h; Cordero et al., 1996–2000). If a pair mated, we recorded the duration of copulation. If the pair did not mate within two hours the trial ended (previous experience indicates that the probability of mating after two hours is extremely low). Males and females were used only once.

All mated females were killed by freezing (–4°C) and then dissected to remove and carefully clean with fine forceps their corpus bursae (the bag-shaped organ where the male ejaculate is deposited). Each corpus bursae was placed on a micro meter calibration slide (Walfront™) and photographed under a stereomicroscope (Olympus™ SZH10); since the measurement software (see below) requires a reference scale, in all photographs the scale of the calibration slide was in focus. Since the corpus bursa is distended by the ejaculate, we used the area covered by the bursa as an estimate of ejaculate size (an independent study in progress, David Xochipiltecatl [Instituto de Ecología, UNAM] found that the regression between this area and ejaculate weight is positive, strong and highly significantly in *C. xami*, explaining 77% of the variance). The area was measured in the photographs using the open access software ImageJ (National Institutes of Health USA; http://rsb.info.nih.gov.ij) and the calibration scale as a reference.

After visual examination of the descriptive statistics, the effect size ($Z/\sqrt{N}$) was calculated. Due to small sample size, non-parametric statistics were used. The numbers of matings were compared using a χ^2 test, and duration of copulation and ejaculate area using Wilcoxon Matched Pairs tests. To determine if the ejaculate area was associated with duration of copulation Spearman correlations were used. The effect sizes and Wilcoxon Matched Pairs tests were carried out using www.statskingdom.com and the χ^2 test and Spearman correlations www.socscistatistics.com. The raw data of the experiment is provided in the supplementary Table S1.

Table 1. "False heads" (FH) and sexual behaviour of the butterfly *Callophrys xami*: experimental design and mating results. Highlighted in light green are the replicates in which both pairs mated (data from these replicates were used in the paired comparisons of duration of copulation and spermatophore area), while replicates in which only one pair mated are highlighted in bright green.

Control pairs:
Females with FH intact

Experimental pairs:
Females with ablated FH

Replicate No.	Pair mated?	Pair mated?
1	Yes	Yes
7	Yes	Yes
8	Yes	Yes
13	Yes	Yes
15	Yes	Yes
17	Yes	Yes
18	Yes	Yes
20	Yes	Yes
4	Yes	No
19	Yes	No
3	No	Yes
5	No	Yes
6	No	Yes
9	No	Yes
10	No	Yes
11	No	Yes
2	No	No
12	No	No
14	No	No
16	No	No
Totals	10 Yes / 10 No	14 Yes / 6 No

RESULTS

As expected from the experimental design, there were no differences between treatments in the wing length of females (Wilcoxon matched-pairs test: $Z = -1.25$, $P = 0.21$, $N = 20$) and males ($Z = -1.43.1$, $P = 0.15$, $N = 19$; two males in one replicate had the same wing length and, thus, were not included in this test). With respect to adult age, 16 out of 20 pairs of females and 17 out of 20 pairs of males were the same age when mated, the age difference in the other 7 cases was one day in 5 cases and two days in the other 2.

The descriptive statistics of the response variables (number of matings, duration of copulation and area of the spermatophore) were examined first. Overall, copulations were observed in 24 of the 40 mating cages (60%; Table 1). The number of ablated females mating was 14 out of 20 (70%), while in the control group it was 10 females out of 20 (50%). Only in eight of the 20 replicates (40%) both females mated (Table 1). Considering only these 8 replicates, the values of the median duration of copulation and the median ejaculate area were larger for the ablated females (Table 2). In seven of the eight replicates, the ablated females copulated for longer than those in the control and the difference between medians was 647 s (~11 min; Table 2). Variation in the duration of copulation in matings with females with intact FH was an order of magnitude larger (interquartile range = 705 s ~12 min; Table 2) than that of ablated females (interquartile range = 82.5 s; Table 2). The estimated effect size was large ($Z/\sqrt{N} = 1.92/\sqrt{8} = 0.67$). In 6 of the 8 replicates, the area of the ejaculate in ablated females was larger than that in the control and the difference between medians was 5,063.1 μm^2 (Table 2). In contrast to duration of copulation, variation in the area of the ejaculate was larger in ablated females (interquartile range = 8,373.6 μm^2; Table 2) than in those with an intact FH (interquartile range = 1,174 μm^2; Table 2). The effect size was also large ($Z/\sqrt{N} = 1.76/\sqrt{8} = 0.62$).

Given that the descriptive statistics of the three response variables pointed in the direction predicted by our second hypothesis (males prefer females with FHs showing predator inflicted damage) and the large effect size recorded in the duration of copulation and ejaculate area, we tested this

Table 2. Summary statistics of the duration of copulation (seconds) and area of the ejaculate (squared microns) in the paired experiment (N = 8 pairs) comparing matings with females with intact "false heads" and those with ablated "false heads". FH – false head.

	Duration of copulation (s)		Area of ejaculate (μm²)	
	Females with intact FH	Females with ablated FH	Females with intact FH	Females with ablated FH
Median	2406	3053	21915.3	26978.4
$Q_{25\%}$–$Q_{75\%}$	2115–2820	3012.5–3095	21550.5–22724.5	20997.3–29370.9
Min–Max	1560–3570	2921–3144	21316.3–24476.7	17500–34357.2

hypothesis using a χ^2 test (number of matings) and one-tailed Wilcoxon Matched Pairs tests (duration of copulation and area of the spermatophore). The number of couples mating was statistically independent of the presence of a FH in the female (Table 1; $\chi^2 = 1.67$, $P = 0.197$, df = 1). Copulations with females with ablated FH were longer than those with females with an intact FH (Wilcoxon Matched Pairs Test, $Z = 1.92$, $P = 0.027$; Table 2). Females with ablated FH received larger ejaculates than females with an intact FH (Wilcoxon Matched Pairs Test, $Z = 1.76$, $P = 0.039$; Table 2).

Ejaculate area was not associated with duration of copulation in either the control (intact FH) or the experimental (FH ablated) treatments. Spearman correlations were not significant whether we considered data for all pairs that mated (Control: $r_S = -0.024$, $P_{2\text{-tailed}} = 0.947$, N = 10; Experimental: $r_S = -0.077$, $P_{2\text{-tailed}} = 0.794$, N = 14) or only the eight trials in which both control and experimental females mated (Control: $r_S = -0.084$, $P_{2\text{-tailed}} = 0.884$; Experimental: $r_S = -0.455$, $P_{2\text{-tailed}} = 0.257$).

DISCUSSION

Our experimental results indicate that in the butterfly *C. xami* ablation of the FH of the female affects duration of copulation and the size of the ejaculate. The median of the duration of copulation of females with ablated FH was ~27% longer than that of females with an intact FH, and the spermatophores transferred were larger when the female had an ablated FH (~23% larger than that in intact females). These results are consistent with our second hypothesis: a larger investment of time and ejaculate materials in females with a damaged FH is favoured because it reflects an ability of the female that the male is looking for because it can be inherited by its offspring. In this case, the damage to the FH implies that when the female was perching (resting, feeding or ovipositing) she deceived a visually oriented predator into attacking a non-vital part of her body (Robbins, 1981; Novelo Galicia et al., 2020).

However, our results are also consistent with a third hypothesis that is based on the idea that females, during copulation, can induce males to modify the duration of copulation and the amount ejaculate transferred. The loss of the FH could favour an increased immediate reproductive effort if it is perceived as evidence of an environment with a high predation risk. One factor that could help increase the immediate reproductive effort is an increase in the amount of ejaculate received, because the ejaculates of lepidopterans commonly contain nutrients and substances that promote egg maturation and oviposition (Cordero, 1995; Eberhard, 1996; Cannon, 2020). Mechanical stimulation by the male via copulatory courtship (for example, with the aedeagus) could also increase female reproductive effort (Eberhard, 1996; Perettti & Aisenberg, 2015) and thus females could also favour longer copulations.

The if and how males or females induce males to prolong copulation, and if and how females induce males to transfer more ejaculate, remains to be studied. Research on cryptic choice mechanisms is currently of great interest, although its taxonomic coverage is still relatively limited (Eberhard, 1996; Peretti & Aisenberg, 2015). However, if FH damage due to a failed predator attack favours an increase in the immediate reproductive investment of the female (as the third hypothesis proposes), selection on males could also favour an increase in the amount of ejaculate transferred and possibly in the duration of copulation (as the second hypothesis proposes), even in the absence of female traits evolved to induce appropriate male responses. Thus, both hypotheses could account for our results.

Finally, in the Introduction we justified our "male choice" experiment by presenting evidence that, during courtship, only males are in position of visually assessing the FH of their mates. However, a reviewer suggested the interesting possibility that during copulation, particularly during the tail-to-tail position, the butterflies could use tactile information to evaluate the condition of the FH of their mates. Thus it is important to assess the relative importance of visual and tactile information on the duration of copulation and ejaculate transfer and carry out a "female choice" experiment similar to the one reported in this paper.

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Table S1. Complete experimental data. Notes: Replicate number in this table corresponds to that used in Table 1. In Replicate 2 the wing length of both males was the same and, therefore, this replicate was not included in the Wilcoxon Matched Test.

	Females with false head intact									Females with false head ablated								
Replicate number	Female Id	Female wing length (mm)	Female age (d)	Male Id	Male wing length (mm)	Male age (d)	Mated?	Copula duration (s)	Ejaculate area (μm^2)	Female Id	Female wing length (mm)	Female age (d)	Male Id	Male wing length (mm)	Male age (d)	Mated?	Copula duration (s)	Ejaculate area (μm^2)
1	2	17.17	1	8	16.27	1	Yes	3570	21646.7	5	16.8	1	9	16	1	Yes	3090	28059
2	12	16.18	0	7	15	1	No			14	16.9	0	1	15	1	No		
3	18	16.33	1	16	16	1	No			17	16.1	1	15	15	1	Yes	2400	19577.2
4	12A	16.9	5	11	15.46	5	Yes	2896	31068.1	19	16.33	5	20	15.74	5	No		
5	26	15.62	5	32	14.26	5	No			33	16	5	29	14.61	5	Yes	722	16870.4
6	36	16.2	5	31	16.89	6	No			35	14.15	5	28	15.88	6	Yes	800	203054
7	37	15.64	6	27	15.64	7	Yes	1560	24476.7	38	16.2	6	34	16.27	7	Yes	2921	34357.2
8	43	15.37	7	40	15.88	7	Yes	2592	22183.8	48	15.47	6	41	15.68	7	Yes	2957	33306.6
9	52	16.36	3	44	16.1	5	No			50	16.4	3	45	14.75	5	Yes	4140	14405
10	7	16.21	2	13	15.7	2	No			12	17.03	2	5	14.74	2	Yes	3000	21541.6
11	11	14.6	5	6	15.08	5	No			24	15.51	3	2	15.35	6	Yes	1560	20647.7
12	27	15.94	4	28	15.76	4	No			21	15.5	5	16	13.62	5	No		
13	23	15.18	5	25	15.87	5	Yes	1800	21316.3	26	15.55	5	29	14.82	5	Yes	3031	17500
14	33	16.38	4	18	14.57	6	No			36	15.9	4	19	14.85	6	No		
15	24	15.51	7	41	15	5	Yes	2760	21563.6	31	16.33	5	42	14.79	5	Yes	3031	21767.5
16	32	16.27	8	39	15.45	8	No			43	16.17	8	35	14.1	8	No		
17	40	15.32	9	54	14.85	7	Yes	2220	22778.1	34	17.36	9	49	13.02	8	Yes	3075	27079.5
18	51	15.66	9	57	12.1	8	Yes	3000	22706.6	47	16.1	9	59	15.24	8	Yes	3110	18686.5
19	62	15.05	8	73	15.86	7	Yes	2100	23889.2	72	15.76	8	74	15.27	7	No		
20	78	16.95	3	76	15.64	3	Yes	2220	21511.1	85	16.59	3	83	16.31	3	Yes	3144	26877.4