Make it simple: mating behaviour of *Euphydryas aurinia provincialis* (Lepidoptera: Nymphalidae)

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Abstract
Courtship in butterflies can vary among species, from simple to elaborate displays, which are thought to promote conspecific mating and drive a female, at first unreceptive, to eventually mate. In this paper, we studied the courtship behaviour of a population of *E. aurinia provincialis* (Nymphalidae) of the Italian region Latium, finding clear differences in acceptance/refusal of females and in consequent departure/persistence of males. Despite the presence of a period of female unreceptivity after eclosion, virgin females usually do not show resistance, and accept to mate after a simple courtship. Once males meet a female, they immediately try to copulate clasping female genitalia, and proceed in mating performing long copulations. They also secrete a mating plug to prevent subsequent matings. On the contrary, already-mated females can strongly reject any approaching male and they always display Wide Wing fluttering, which is a behavioural pattern usually followed by male departure. In case of persistent males, prolonged and energy demanding sexual interactions, as Carrousels, can occur, with rare final copulation. Here, we analyse the effects of female refusal on male sexual behaviour and discuss potential selective pressures that might be acting on such behavioural traits.

Keywords: Butterflies, courtship, monandry, male persistence, female resistance

Introduction
Sexual selection is a strong pressure promoting traits that confer a competitive advantage in reproduction, where success increases through competition for access to mating partners and choice of a sexual partner (Darwin 1871; for butterflies, Rutowski 1984). Both males and females can behave as the competing or the choosy sex; nevertheless, competing males and choosy females are typically found in nature (Andersson 1994).

In butterflies, a large amount of behavioural patterns and cues involved in sexual interactions have been studied (i.e., visual, acoustic, tactile and chemical stimuli), all likely providing information on species identity and individual vigour (Scott 1973; Andersson 1994; Sbordoni & Forestiero 1998). Nymphalids of the genus *Euphydryas* Scudder, 1872 have been deeply investigated by population biologists (Ehrlich & Hanski 2004); however, only few studies focused on courtship behaviour and female responses in these butterflies. Notes on mate-locating behaviour and sexual interactions were briefly reported for some American *Euphydryas* species (*E. editha*, Scott 1986; *E. chalcedona*, Rutowski & Gilchrist 1987; Rutowski et al. 1988, 1989; *E. anicia*, Odendaal et al. 1990) and the English Marsh Fritillary *E. aurinia* sp. *aurinia* (Porter 1981).

*Euphydryas aurinia* (Rottemburg, 1775) is listed in Annex II of the European Community Habitats and Species Directive (92/43/EEC) and suffered a severe decline in most European countries during the twentieth century (Van Swaay & Warren 1999; Van Swaay et al. 2010). In Italy, according to the most recent assessment (under HD art.17), the conservation status of the Continental *E. aurinia* populations is reported as “bad” as in central and northern Europe. At least 12 populations of *E. aurinia* have become extinct due to habitat destruction (Bonelli et al. 2011). On the contrary, the conservation status of the Mediterranean

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and Alpine populations is “favourable” (Balletto et al. 2014a; Casacci et al. 2015).

In Italy, *E. aurinia* is considered as a species complex of three taxa: i) *aurinia* (Rottemburg, 1775), ii) *glaciegenita* (Verity, 1928) and iii) *provincialis* (Boisduval, 1828), alternatively considered as species or subspecies by different authors (Verity 1950; Hartig 1968; Prola et al. 1978; Parenzan & Porcelli 2006; Balletto et al. 2014b; Casacci et al. 2015; Korb et al. 2016).

*E. a. provincialis* is limited to the Maritime Alps and the Apennine Mountains (Balletto et al. 2014a). It is widespread on the Central Apennines, mostly on Monti Reatini (data from Osservatorio per la Biodiversità del Lazio, http://www.leps.it/obl/), and it has been categorised as “Least Concern” in the recent Red List of Italian Butterflies (Balletto et al. 2015; Bonelli et al. 2018).

In previous studies, we provided important information for the conservation of the populations living in Central Italy, where this species is in a better state than elsewhere. For several years we studied a population of *E. a. provincialis* (Boisduval, 1828) focusing on several aspects of the species biology and in particular on larval host plants preference (Pinzari et al. 2016), predators (Pinzari 2016) and parasitoids (Pinzari et al. 2017), adult population dynamics and, finally, on courtship and mating behaviour.

In this paper, we show the results from field and captive observations on the mating behaviour and reproductive biology of *E. aurinia provincialis* and discuss on which potential selective pressures have been acting for maintenance of such sex traits. In particular, we studied a) the behaviour that males adopt to localize the females in the wild; b) the male courtship; c) the female behavioural response; d) copulation and mating frequency.

### Material and methods

#### Study site and species

Our field-work was carried out on a population of *E. a. provincialis* discovered during a previous work on Lepidoptera around Vallemare (RI, Lazio) (Pinzari et al. 2010). Here, the species occupies montane grasslands and sometimes steep and rocky slopes, within a mosaic of different habitats including wooded areas, hedgerows, fields and less steep areas occasionally ploughed and cultivated (Pinzari et al. 2016).

*E. a. provincialis* is an univoltine species and, in our study area, the adults fly from May to July, depending on altitude and microclimate. Like in other European sites (e.g., Fric et al. 2010), males start flying 3–6 days before females and largely outnumber them.

#### Behavioural observations

Observations on sexual behaviour were carried out during five consecutive reproductive seasons (2010–2015), both in the wild (on free-flying individuals) and in captivity (on butterflies caught in the wild and on reared individuals) (Table I). When in captivity, individuals were kept in flight cages and provided with food and water. Reared individuals were collected from the host plant *Gentiana cruciata* and bred from the fourth and fifth larval instar.

Male and female sexual behaviour was video recorded for a total of 24 h 32’ 32” of which 7 h 10’ 57” (mainly on captive individuals) were useful for behavioural analysis. Each observational period started when a male-female interaction started, and all behavioural patterns under study (Table II, where all acronyms are specified) were recorded in order of occurrence. Similarly, the observational period ended either with the male-female copulation or with the interruption by one of the two actors.

| Item               | 2010 W | 2011 W | 2012 W | 2014 W | 2015 W | Subtotal W | Subtotal R | Total |
|--------------------|--------|--------|--------|--------|--------|------------|------------|-------|
| **Females tested** |        |        |        |        |        |            |            |       |
| w                  | 5      | 2      | 2      | 2      | 3      | 14         | 0          | 14    |
| c                  | 5      | 2      | 2      | 19     | 1      | 14         | 20         | 48    |
| **TOTAL**          | 10     | 4      | 4      | 34     | 14     | 34         | 20         | 55    |
| **Males tested**   |        |        |        |        |        |            |            |       |
| w                  | 1      | 1      | 1      | 2      | 4      | 9          | 9          | 9     |
| c                  | 1      | 1      | 1      | 21     | 1      | 12         | 34         | 46    |
| **TOTAL**          | 2      | 2      | 2      | 23     | 5      | 12         | 43         | 55    |
| **Copulating pairs** |      |        |        |        |        |            |            |       |
| w                  | 1      | 1      | 1      | 3      | 3      |            |            | 3     |
| c                  | 4      | 4      | 4      | 4      | 4      |            |            | 8     |
| **TOTAL**          | 5      | 5      | 5      | 7      | 7      |            | 4          | 11    |

Setup: w, in the wild; c, in captivity; individuals: W, wild; R, reared.
Depending on whether copulation occurred, courtship was defined as successful or unsuccessful. In successful courtships, we recorded copulation duration (i.e., the coupled pair was placed in a small cage and regularly checked until they separated).

**Observations in the wild**

In the wild, to ensure the encounter of partners, we captured and throw a female towards one or more males to prompt their flight (Rutowski & Gilchrist 1987; Pinzari 2009). This practice encouraged male flight pursuit and allowed us recording some complete courtship sequences and copulations (Table I).

The mating status of wild females (M, mated or V, virgin) was identified in the field (as stated above) or by spermatophore counts in the laboratory (Drummond 1984; Lederhouse et al. 1989).

**Observations in captivity**

Wild-caught and reared individuals were randomly selected from the flight cages and positioned under Plexiglas hemispheres (Figure 1, diameter 10 cm, thickness 1.5 mm) and their behaviour recorded (Table I). Here, their thermoregulation was guaranteed by an incandescent bulb (160 W; distance, 4–30 cm).

The mating status (M or V) of wild-caught females was identified in the field (as stated above) or by spermatophore counts in the laboratory (Drummond 1984; Lederhouse et al. 1989).

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Table II. Ethogram of *Euphydryas a. provincialis*. The behavioural codes used in the text are in brackets.

| I. Both sexes                  |                                                                 |
|-------------------------------|-----------------------------------------------------------------|
| A. Immobility (I)             | Remain in one place                                             |
| B. Step approach (SA)         | Move closer to partner (walking, W)                            |
| C. Escape (E)                 | Move away from partner walking (W)                             |
| D. Orientate (O)              | Orientate itself towards the partner while turning             |
| E. Flight (F)                 | Fly                                                             |

| II. Female behaviour          |                                                                 |
| A. Wide Wing fluttering (WWf)| Open widely and beat the wings very rapidly; opening of the wings could reach a 180-degree angle; females can perform WWf together with the other types of behaviour (III and IV) and in any position (head to head (AP); female head towards male side (VF); female side towards male head (TP); other positions (OP) on ground. |
| B. Small Wing fluttering (SWf)| Vibrate the wings while slightly open (<10-degree angle)       |
| B. Twirling (T)               | Turn on itself repeatedly like pirouette, and open and beat the wings very rapidly (PWF); the rotation can occur clockwise or counterclockwise direction and can also reverse more times. |

| III. Male behaviour           |                                                                 |
| A. Copulation Attempt (CA)    | Walk laterally and move towards the side of the female until their heads face the same direction, and then Cl occurs |
| B. Clasping (Cl)              | Flex the abdomen towards the female and make contact with her genitalia. |

| IV. The couple                |                                                                 |
| A. Fronting up (FU)           | The couple adopts a head-to-head position, it is motionless and does not turn, but female beats her wings (WWf) |
| B. Carrousel (C)              | The couple turns on itself maintaining a “temple-to-temple” position and making many spins; rotation can occur clockwise or counter-clockwise direction and reverse more times. During carrousel, female flutters her wings (WWf) while male performs Copulation attempt and Clasping (Figure 2, see detail in Results) |

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Figure 1. Plexiglas hemispheres (diameter 10 cm, thickness 1.5 mm) where the behavioural observations were carried out.
Beihavioural data analyses

Male and female behaviour was identified and scored on a frame-by-frame basis. A description of each behaviour is in Table II. When possible, definitions from Dickinson and Rutowski (1989) were used; otherwise, new behavioural terms were coined. For the behavioural analysis, each “female × male” interaction was considered as a single trial.

Some behaviours were categorised as events, while others as states (Altman 1974; Martin & Bateson 1990); therefore, frequency and duration were measured, respectively. The behavioural parameters were then compared between wild and captive interactions, and between mated and virgin females, using a Mann–Whitney U test.

For Carousel (C) and Twirling (T) behaviours, we additionally determined the number of rotation directions (NR) and the number of turns for each direction of rotation (NT). For the evaluation of the number of turns, we adopted a quarter-turn approximation with an error that was less than one-eighth of a turn.

To investigate female refusal and its effect on male behaviour, we analysed sexual interactions focusing on those behaviours that cause the male to give up and fly away. We evaluated their efficiency calculating frequency (f_FB) and duration (d_FB), and significant differences between wild and captive pairs, using a Mann–Whitney U test. Stereotyped mate-refusal behaviours like wing fluttering are widely present in various species of butterflies (Labine 1964; Scott 1973). For this reason, we evaluated its potential effect on male response and persistence, taking into consideration all those female behaviours that showed wing fluttering, before male retreat. First, in order to investigate the cumulative effect of the Wide Wing fluttering (WWf) on male response, we calculated the total duration (d_NT) of WWf adding up all female behaviours that showed it, without interruption, before male departure. Second, we considered only the very last female behaviour with WWf before male departure. A Mann–Whitney U Test was used to compare values of d_NT between wild and captive conditions.

Finally, as the position of the interacting female can also have a role as a visual signal for the male, we identified three female positions: aligned (AP), transversal (TP) and oblique (OP) (Table II), and counted how often the male departed after each one. When the female is in TP, she exposes a large part of her body (body plus wings) to the male; in AP, she exposes a small part of body, as the partners are head to head; in OP, she exposes an intermediate portion of her body to the male. We hypothesized that female behaviour in AP could be the less effective as the female wing beating gives to the male an important visual flash effect. Results were compared and tested for significance using Chi-Square test for goodness of fit.

All data analyses were performed in STATISTICA version 7.

Observations on mating frequency and signs in females

To evaluate the frequency of mating and its evidence on females, we examined 52 dead females, which were sampled during the 80s (N = 27) or during the study period of this paper (2012–2014, N = 25) in the Central Apennines, and stored dry. Abdomen and bursal sac were dissected after placing them into a microvial with a cold 10% potassium hydroxide solution for 2–3 h.

Results

Male behaviour

From our observations in the wild, males largely outnumber females and they are mainly found in dry meadows, usually resting on low-growing vegetation in dorsal basking posture (i.e., dorsal body surface exposed to sunshine and wings held down and to the sides, Clench 1966; Bernd 1996) or foraging on flowers. From these positions, they fly to intercept females. After following a female in a nuptial flight, the male alights back to the ground near her. Once the pair has settled, the male stands near the female, moves towards her (Step approach, SA) to start the actual courtship. The courtship is very simple, as it involves Copulation attempt (CA) and Clasping (CI). This behavioural pattern was observed both in the wild and in captivity (Table III). As in captivity, the butterflies were placed under a glass hemisphere (see Methods), the interactions started straight with SA, omitting the nuptial flight.

In successful courtships, the male reaches the suitable position in CA, then performs CI and starts copulation. During CI, the male initially bends to the side to approach the female abdomen and then, when male abdomen is under the female abdomen, he flexes upwards to take contact to female genitalia.

In unsuccessful courtships, the male performs the same types of behaviour (SA, CA, CI), but the interaction ends with male immediate departure or after a prolonged interaction with female (Carousel, see below). Of 55 males tested, 54% was involved at least in one Carousel (in the wild, 89%, N = 9; in captivity, 49%, N = 46) (Table I).
Table III. Duration of each behaviour of *Euphydryas a. provincialis*.

| Behaviour | Captivity | Wiki |
|-----------|-----------|------|
|           | N | Mean | SD | Median | N | Min | Max | SD | U | Z | P |
| Male      |   |      |    |        |   |      |     |    |    |   |   |
| SA        | 186.00 | 3.38 | 3.18 | 2.40 | 163.00 | 0.28 | 22.60 | 3.16 | 2.44 | 23.00 | 0.20 | 13.20 | 3.41 | 1733.00 | 0.59 | 0.56 |
| CA        | 328.00 | 2.90 | 4.77 | 1.84 | 301.00 | 0.12 | 54.64 | 4.96 | 1.84 | 27.00 | 0.32 | 6.20 | 1.46 | 3902.00 | 0.34 | 0.73 |
| Cl        | 293.00 | 6.10 | 9.01 | 3.18 | 270.00 | 0.16 | 73.16 | 9.31 | 2.72 | 23.00 | 0.32 | 16.12 | 3.40 | 2585.50 | 1.33 | 0.18 |
| Female    |   |      |    |        |   |      |     |    |    |   |   |   |
| WWf       | 1008.00 | 4.75 | 5.74 | 2.92 | 843.00 | 0.24 | 41.32 | 5.53 | 2.72 | 165.00 | 0.28 | 46.92 | 6.74 | 69,104.50 | 0.13 | 0.90 |
| W         | 14.00 | 10.24 | 8.36 | 11.94 | 12.00 | 1.20 | 24.24 | 8.47 | 3.20 | 2.00 | 1.96 | 4.44 | 1.75 | 6.00 | 1.10 | 0.27 |
| O         | 38.00 | 3.72 | 2.92 | 3.20 | 35.00 | 0.60 | 13.56 | 2.96 | 1.20 | 5.00 | 0.40 | 3.08 | 1.01 | 30.00 | 2.27 | 0.02 |
| I         | 57.00 | 13.20 | 9.63 | 11.28 | 51.00 | 0.84 | 39.00 | 9.97 | 12.56 | 6.00 | 3.44 | 17.80 | 6.27 | 140.00 | 0.34 | 0.74 |
| OP        | 378.00 | 5.28 | 6.55 | 3.20 | 303.00 | 0.32 | 39.00 | 5.93 | 2.20 | 75.00 | 0.32 | 46.92 | 8.66 | 10,030.50 | 1.57 | 0.12 |
| TP        | 120.00 | 7.23 | 7.56 | 4.40 | 111.00 | 0.40 | 33.08 | 7.73 | 1.80 | 9.00 | 0.92 | 8.16 | 2.43 | 353.00 | 1.46 | 0.14 |
| VF        | 37.00 | 7.23 | 7.30 | 4.80 | 35.00 | 0.48 | 24.36 | 7.38 | 1.72 | 2.00 | 1.64 | 1.80 | 0.11 | 10.00 | 1.68 | 0.09 |
| AP        | 44.00 | 5.39 | 6.44 | 3.53 | 37.00 | 0.28 | 29.44 | 6.93 | 3.76 | 7.00 | 0.28 | 4.88 | 1.67 | 125.00 | −0.14 | 0.89 |
| T         | 155.00 | 3.59 | 3.32 | 2.52 | 153.00 | 0.32 | 20.16 | 3.33 | 0.92 | 2.00 | 0.64 | 1.20 | 0.40 | 30.50 | 1.94 | 0.05 |
| The couple|   |      |    |        |   |      |     |    |    |   |   |   |
| FU        | 97.00 | 5.35 | 6.59 | 3.08 | 59.00 | 0.24 | 38.04 | 7.25 | 2.80 | 38.00 | 0.52 | 25.48 | 5.48 | 1089.50 | −0.23 | 0.82 |
| C         | 125.00 | 5.43 | 5.29 | 3.78 | 100.00 | 0.80 | 41.80 | 5.03 | 6.24 | 25.00 | 1.68 | 22.56 | 6.02 | 949.50 | −1.85 | 0.06 |

Duration in seconds, as dB values, is shown. Each type of behaviour was compared between captive and wild observations by a Mann–Whitney U test.

Types of behaviour: SA, Step approach; CA, Copulation attempt; Cl, Clasping; WWf, Wide Wing fluttering; I, Immobility; O, Orientate; W, walking; and in different positions respect to the male (OP, TP, VF, AP); T, Twirling; FU, Fronting up; C, Carrousel.
Female behaviour

Virgin females. Both in the wild and in captivity, when a male is in close proximity and starts to approach, a virgin female does not adopt opposing behaviours and does not beat her wings; the male carries on with courtship and copulation occurs. Some virgin females might not accept the first male approach and might escape (E) without going too far. This initial mild refusal can be accompanied by small wing fluttering (SWf), during which the female slightly opens and vibrates her wings. This behaviour does not discourage the courting male, which follows the female, starts a second approach and gains a copulation. SWf was observed only 18 times (Mean duration ± St. dev. = 4.76 ± 9.23 s) and only in captive conditions.

Mated females. When males get close to mated females, the females display several types of behaviour that include: wide wing fluttering as immobility (I), walking (E and SA), orientate (O), twirling (T), fronting up (FU) and carousel (C) (Table II).

WWf can start from the first male approach and can be continuously displayed. While the female is displaying WWf, the male can move forwards (SA) but without starting the actual courtship (neither CA nor Cl); he can stop and wait for a female action, or can abandon with flight (F).

In the first case, as soon as the female stops WWf, the male can try to approach her again. A female can perform WWf while adopting different positions (Table II): WWf (TP), the female exposes her side to the male; WWf (VF), the female stands up on the forelegs and points to the partner’s side; WWf (AP), the female orientates her head towards the male’s head (Table II). During T, the female turns on herself and beats her wings, regardless of the activity of the male. T lasts on average 3.59 ± 3.52 s (N = 155, Range: 0.32 to 20.6 s, Table III). The number of rotation directions varied from 1 to 6 (Figure 2a), while the number of turns for each direction of rotation varied from 0.25 to 11 (Figure 2b). During FU (N = 97), the couple adopts a head-to-head position and remains motionless, while the female is displaying WWf. During C (N = 125), the couple turns around together in a “temple to temple” position, making many spins (1 to 9, Figure 3). The female always flutters her wings (WWf), while the male pursues her (W) and tries to attempt copulation (CA), clasping (Cl) the female abdomen. The number of rotations in C varied from 1 to 35 (Figure 3a). The number of turns for each direction of rotation varied from 0.25 to 7.75 (Figure 3b). The female can change the direction of rotation of C by two different actions that are described in (Figure 3d,i). C lasted, on average, 5.45 ± 5.30 s (Range: 0.80 to 41.80 s) (Table III).

Female-female behaviour. In captivity, we observed, therefore, random interactions (N = 8) between females. We did not record any interaction between virgin females: even if a virgin female moved, the others remained motionless without reacting, that is, they adopted the same behaviour of the virgin females during an interaction with the male. On the contrary, the mated females, that have not yet laid their eggs, at the slightest movement of the other females beat their wings and engaged in C (N = 2) and other behaviours (e.g. T, N = 19; FU, N = 3) that were typical of the male-female interactions. Moreover, during the interactions between the females that did not oviposit and those that laid their eggs, we observed that the first displayed the same types of refusal behaviour.

Figure 2. Carousel (C) and Twirling (T): a, number of rotation directions in a single behaviour (NR); b, number of turns for each direction of rotation (NT).
Female behaviour in heterospecific interaction. In the wild, we also recorded a single case in which *E. aurinia provincialis* female displayed WWf in exhibited by the mated females with the male, while the second did not beat its wings and abandon the interaction by flight.

Figure 3. Carrousel (C) and the two ways the couple can reverse the direction of rotation. During C, the female keeps beating her wings. a) the partners (the female is in lower left position) turn together "temple to temple" around the axis orthogonal to the laying surface of the butterflies; each individual rotates towards its free side; b) by moving faster than the female, the male gets close to the female abdomen and attempts copulation (CA); c) the male performs Clasping (Cl), despite the female claps her wings, and both partners rotate; d) the female escapes Cl, by lifting the abdomen and climbing over the male she reverses the sense of C rotation; e–f) the female completes the rotation; g) the partners are again in "temple to temple" position, but in a position opposite to that in (a); h–i) the partners in "temple to temple" position rotate together, each turns towards its free side; l) the female raises her fore side of the body and climbs over the male, head towards head, moving to the opposite side of the male and changing the direction of rotation of the Carrousel; m) the male changes the direction of C rotation; N) C carries on, in the new direction of rotation.
response to a male approach of *Melitaea phoebe* ([Denis & Schiffermüller], 1775). This interaction ended with the female flying away, leaving the male *M. phoebe* alone.

**Female refusal behaviour and its effectiveness on male behaviour**

In unsuccessful courtships, male actions always end with flight (F), both in the wild and in captivity (N\text{wild} = 46, N\text{captive} = 501). In particular, male flight follows female flight (nuptial flight) (N\text{wild} = 10, 8\%; N\text{captive} = 39, 22.7\%) or female WWf behaviours (N\text{wild} = 35, 76\%; N\text{captive} = 371, 74\%) (escaping flight); only rarely (2\% and 18\%, respectively) the male flies away spontaneously (i.e., with females not showing refusal behaviours such as WWf).

We recorded a total of 279 female flights (N\text{wild} = 15, N\text{captive} = 264), which involved 116 mated females (N\text{wild} = 14; N\text{captive} = 152) and 113 virgin females (N\text{wild} = 1; N\text{captive} = 112). Among female flights, 17.5\% (N = 49) caused the male to fly (i.e., a real nuptial flight), and involved 34 mated (N\text{wild} = 10; N\text{captive} = 20) and 19 virgin females (N\text{wild} = 0; N\text{captive} = 19). Males were similarly attracted to virgin (17\%) and mated females (18\%) (Mann–Whitney U test, NS).

When we analysed female WWf, the behaviours I, E, O, T, FU, C, VF and F would be those followed by male flight (Figure 4). No statistical significant differences between wild and captivity were showed, for each behaviour. Among the actions displayed by the female alone (I, W, O and T), Twirling had the highest value of efficiency in rejecting the male (Figure 4). In fact, by adopting this behaviour, a female can literally impede the male approach (therefore avoid CA and CI), beating her wings and turning on herself to the female body. If only actions with the male involvement were considered (FU, C, VF, F), the Flight was more effective than other types of female behaviour, followed by VF, FU and C.

When we analysed the duration efficiency of female behaviour, we found out that, given the same duration (e.g. 5 s), behaviours that involved the male were more effective in him abandoning the interaction (FU, 90\% and C, 80\% vs T, >50\%; O, >70\%; W, 50\%; I, <50\%) (Figure 5a). Finally, the study of the cumulative of WWf showed that the majority of male flights followed female behaviour of low duration (d\text{INT} = 5 s, 50\% wild, 60\%, captive) (Figure 5b).

When we analysed the female position in respect to that of the male, we found out that it could make a difference in prompting male departure; in fact, Wide Wing fluttering (WWf) in transversal position (TP) was more powerful than WWf in aligned (AP) and oblique (OP) position (Figure 6) (Chi-square test: \(\chi^2 = 72.621, df = 2, p < 0.001\)).

**Copulation and mating frequency**

The duration of copulation was documented for 11 pairs of butterflies (wild: 3 pairs; captivity: 8 pairs) and had an average of 4 h 1 min (range: 1 h 45 min–6 h 37 min). At the end of copulation, the male produces a mating plug and leaves visible signs of sexual events on the female bursal plate (Figure 7a, c).

Among the females whose abdomens were examined (N = 52), 10 (19\%) were unmated (they had an empty bursa copulatrix and the bursal plate was partially covered by scales or bristles (Figure 7d); 42 (81\%) were mated (females had visible signs of scales rubbed off the bursal plate and mating plug, Figure 7c). Forty (95\%) of the mated females copulated once and only two (5\%) twice.

The abdomens dissections also revealed that during copulation a single spermatophore is passed from male to female, and it generally lingers in a recognizable shape inside the female bursa copulatrix and occupies the whole volume of the bursa. In those females that copulated twice, each spermatophore occupied half of the bursa copulatrix.

**Discussion**

During courtship interactions, different signals can be involved to provide males and females with information regarding species identity and individual quality ([Andersson 1994](#)). Courtship is mostly

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**Figure 4. Efficiency of the last female behaviour in causing male departure: wild vs captive observations. Female behaviour: I, Immobility; W, walking; O, Orientate; T, Twirling; FU, Fronting up; C, Carrousel; VF, Wide Wing fluttering (VF); F, flight. During these behaviours, females always display also Wide Wing fluttering.**
envisaged as promoting conspecific mating (Scott 1973), and should therefore include components that allow males to distinguish conspecific females, and females to distinguish conspecific males from all others. The actual courtship behaviours differ among species, and go from simple to more complicated displays (see Scott 1973; Silberglied 1977; Rutowski 1984). Rather complex sexual patterns have been described in nymphalids (Tinbergen 1941, 1972; Magnus 1958; Scott 1973; Nieberding et al. 2008; Pinzari 2009; Pinzari & Sbordoni 2013), while in other butterflies males perform simple courtship displays comprising a reduced set of behaviours and few events (Crane 1955; Lederer 1960; Brussard & Ehrlich 1970). In this paper we show that *E. a. provincialis* belongs to the latter category, as it shows one of the simplest butterfly courtship behaviour: males immediately try to clasp the female genitalia. This male behaviour is similar to that previously reported in the American Fritillary species, *E. calchedona* (Rutowski & Gilchrist 1987), *E. editha* (Scott 1986) and *E. anicia* (Odendaal et al. 1990), and the English Marsh Fritillary, *E. aurinia aurinia* (Porter 1981), revealing a basic courtship plan throughout the genus *Euphydryas*.

As a novel finding, we showed that the ways males overcome female *Carrousel* and other refusal actions and reach their reproductive success. In our analyses, most of the times males departed after a brief female refusal (*Wide Wing fluttering*) and the interaction ended; in some cases, they stayed and kept courting the female, regardless of her strong opposition, and sometimes obtain copulation. Accordingly, also Labine (1966) reports males of *E. editha* harassing mated females and “capturing” to attempt copulation, not considering their signals of no receptivity.

Virgin females in our study generally did not oppose any reaction to the male approaches. Only
rarely females performed *Small Wing fluttering*, as a first response to male approach, and it was never followed by male departure. SWf better resembles types of behaviour like *Fanning* in *Hipparchia* species (Pinzari 2009; Pinzari & Sbordoni 2013) and *Short wing fluttering* in *E. chalcedona* (Rutowski & Gilchrist 1987).

Females started mating only when they were at least 2–3 days old, and they were therefore displaying SWf in the days before the copulation. In many groups of butterflies, female virginity is associated with egg immaturity (i.e., a period of a few hours or days needed to begin or stimulate egg maturation) and during this period they are unreceptive to courtship and mating (Drummond 1984). Unlike *E. a. provincialis*, in *E. a. aurinia* and *E. editha* the females emerge with a fully developed set of eggs and can lay as soon as mating is finished (Labine 1966; Porter 1981). *Euphydryas a. aurinia* females can mate immediately after the emergence (Porter, personal communication, 11 February 2013).

As regards mated females of *E. a. provincialis*, they always responded to approaching butterflies (i.e., heterospecific or conspecific, both sexes) using behaviours that always included wide and prolonged fluttering (WWf). This strategy could be a consequence of their reproductive status and be adopted as an egg defence: the female aims at keeping the intruder away, defending the product of her one-in-lifetime copulation, which are the eggs not yet laid.

The rate of success of WWf increased with the proportion of female body exposed to the approaching individual. It was minimal (i.e., only few times the approaching individual left) when the two butterflies had their bodies aligned, and it was maximum when the female placed her body sideways (Figure 6). This let us suppose that WWf in transversal position could functions as a warning signal, as it happens in many other animal species during sexual contexts, from mammals (*Cervus elaphus*, Clutton-Brock & Albon 1979) to insects (Ruxton et al. 2004). Here, the warning signal could be the result of a combined effect of visual (wide female surface, flash effect), mechanical (wing stroke) and chemical (wing fanning) stimuli, which are displayed during WWf.

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**Figure 7.** Pictures of *E. aurinia provincialis* females taken in the field (a, b) and from collection (c, d). Arrows show the genitalia structure (*sterigma*), in the mated females, where it is exposed after copulation (a and c), and in the unmated females, where it is not completely visible because it is still covered by the body scales (d).
In general, the female butterflies succeeded in making males fly away after few seconds of WWf. Only the most determined males resisted longer and flew away under the cumulative effect of the different types of WWf (after 40–180 s, Figure 5b). WWf varied from simple behaviour exhibited by the female alone (I, W, O, T) to complex behaviours displayed by the couple (FU, C). In all cases, both performance and duration were the keys to success, as its efficiency increased with the duration of the last female behaviour preceding the male flight (Figure 5a). The effect of WWf on male response gradually increased from Immobility to Orientate (I < W < O < T, see Figure 4).

Most of the times, low-energy female behaviours (i.e., wing fluttering in I) were sufficient to cause the male to fly away. Other times, males flew only after more expensive behaviours (wing fluttering in W, O, T) and maybe stressful (C and FU) female behaviours. We could not evaluate the energetic expenditure of the diverse types of female refusal behaviours; however, it is reasonable to think that, for a given duration, a complex behaviour (i.e., combined actions of wing fluttering, walking, rotation, etc.) requires higher energetic cost than a simple behaviour (i.e., wing fluttering, I). During C, male could try several times to attempt mating and clasp the female, which, in turn, would perform all possible actions to prevent copulation: she could beat her wings and disorientate the male turning on herself and continuously changing direction of rotation. Sometimes, C allowed the males to circumvent female premating resistance and gain a copulation. This was the case of a double copulation of a female after a prolonged sexual interaction (171 s) that we filmed in the wild: the male kept courting the female by displaying Cl 18 times, within nine C and six FU, and gained mating at the 19th attempt, when the female became motionless.

Why do the males “waste their time” courting an unreceptive or mated female instead of searching for more available females? Our findings revealed that *E. aurinia provincialis* is a monandrous species and after copulation females display evident signs of their mating on their abdomen and also show a mating plug. Moreover, although thecopulation duration was effectively recorded only for 11 pairs, it was on average longer than in congenic species (105–397 min vs 43–124 min of *E. chalcedona*, Rutowski & Gilchrist 1987 and 109 min in *E. editha*; Labine 1966). Long copulations and mating plugs are likely results of intrasexual selection in males, as they prevent a female second mating, at least until sperm of the first copulation has migrated out of the spermatophore, and therefore reduce last-male sperm precedence (Drummond 1984).

In fact, although a second copulation did not frequently occur, in *E. aurinia provincialis* the females could mate more than once, and, as in other *Euphydryas* butterflies, the mating plug did not always seem to be effective. In *E. editha* males can also try to displace a mating male; if the plug of the first male has not hardened yet, two spermatophores can be introduced in the same female (Labine 1964). In *E. calchedona*, the mating plug neither reduces courtship persistence nor mechanically prevents clasping by males, but with a plug in place, males are less successful in the intromission (Rutowski & Gilchrist 1987; Dickinson & Rutowski 1989). In some congenic species, some authors suggest that double copulations could occur when the first copulation is not satisfactory and the female has small spermatophores from mating with not fully potent males (Labine 1964; Drummond 1984; Rutowski & Gilchrist 1987; Dickinson & Rutowski 1989; Odendaal et al. 1990). This is consistent with the studied dissections data, as the females that mated twice had two small spermatophores in the bursa copulatrix (i.e., the first copulation was not fully adequate).

Following the above reasoning, in our butterflies, male persistence may be an advantageous strategy to achieve copulation. The benefits of multiple mating to males are typically obvious: more mating, more offspring (Andersson 1994). How can males win the female opposition behaviour? Wearing the females weary out until they are motionless. In fact, it was only when the females were motionless as virgin females that the males were able to attempt copulation (CA and Cl). In general, mated females would defend themselves from approaching males using their time and energy to chase them off. At the beginning of the interactions, it would perform without the male involvement, and would therefore be only them spending energy and getting tired: the male if not “spooked” by female behaviour, would stand his ground and successively engage her in Fronting up and Carrousel. All these behaviours are energy demanding and could wear the female out to the point when she allows a male clasping her genitalia and gain copulation (even if it would be her second copulation). In this context, the final response of females to male persistence would simply be to give up and a second mate, and thereby avoid further costly harassment, a behavior that has been referred to as “convenience polyandry” (Thornhill & Alcock 1983).
Concluding remarks

Courtship behaviours of a strictly monandrous butterfly, *E. aurinia*, was described and discussed for the first time. In particular, those that lead to second copulations were discussed as results of “struggles” between persistent males and reluctant females. Although female costs during courtship are unknown, their resistance to mate suggests that mating carries significant costs (e.g., signs of mating, predation risk for long copulation). The fact that resistance itself is often costly reinforces the view that females try to avoid mating when costs overcome advantages. Female resistance has two potential selective results: one is to reduce mating rate, and the second is to bias mating success of males toward certain phenotypes that can overcome resistance. The first seems obvious: every rejected male is potentially one mating less for the female. The second can be a simple side effect of reducing the mating rate and is, by definition, sexual selection by female mate choice. If we consider a scenario where female degree of resistance to mating is independent from the phenotype of the attempting male, we can assume that male phenotypes able to overcome female resistance would be those that achieve higher mating success, and would be therefore favoured by selection. As it is, costly mating, and the resulting female resistance towards second mating, could be a powerful force of sexual selection.

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