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Differences in mating strategies in two closely related small ermine moth species (Lepidoptera: Yponomeutidae)

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Abstract. The degree of polyandry in a species is linked to other life history traits such as egg maturation, life span, and male ejaculate size and quality. The study of differences in mating strategies between closely related species can provide a better understanding of the evolution of these strategies and of sperm competition. Mating patterns of two closely related species of small ermine moths (*Yponomeuta*) were investigated in the laboratory. The average female age at first mating was higher in *Y. cagnagellus* than in *Y. padellus*. Both species mated more than once; however, *Y. cagnagellus* females were more likely to remate in a short time frame. Moreover, *Y. cagnagellus* had higher life time female mating frequencies than *Y. padellus* (viz., 3.0 versus 2.0). These differences in mating frequency were confirmed in the field by examining the presence of spermatophores (or their remains) in the bursa as well as sperm in the spermatheca of field-caught females.

INTRODUCTION

Theory predicts that male reproductive success should increase steadily with mating frequency, whereas females should increase their success by maximizing the number of viable eggs laid, for which usually 1–2 matings suffice (Bateman, 1948; Parker, 1970; Arnqvist & Nilsson, 2000). Nevertheless, females of most species mate more than once (i.e., are polyandrous). Apparently, the increased costs of multiple mating, such as energy and time expenditure, vulnerability to predation, male-borne diseases and parasites, and possible life span reduction due to male manipulation are outweighed by an increase in fecundity, fertility, and offspring fitness (Arnqvist & Nilsson, 2000; Morrow et al., 2003). An alternative explanation for polyandry is suggested by systems where the costs of avoiding haressing males are apparantly larger than that associated with mating multiple times, so-called convenience polyandry (see e.g., Rowe, 1994). In any case, mating frequencies of female Lepidoptera show large variation between (Drummond, 1984) and even within species (Bergström et al., 2002; Wedell et al., 2002). Few species mate only once or nearly only once, such as *Erebia ligea* (Nymphalidae) and *Aphantopus hyperanthus* (Nymphalidae; Wiklund, 1982; Svärd & Wiklund, 1989), and many mate 1–2 times (Drummond, 1984; Svärd & Wiklund, 1989). Others, such as *Euxoa perolivalis* (Noctuidae), mate more than ten times (Byers, 1978). Furthermore, within species, individual female mating frequencies can differ widely; for example, in laboratory experiments with *Pieris napi* (Pieridae) females, which had mating opportunities with non-virgin males throughout their life span (about 15 days), females mated between two and more than ten times (see Bergström et al., 2002).

The study of mating strategies, such as degree of polyandry, is important, as mating strategies are an essential part of sexual selection, and are linked to other life history traits such as egg maturation, life span (Wiklund et al., 2003; Jervis et al., 2005) and to patterns of egg-laying and juvenile development (Välimäki et al., 2006; Välimäki & Kaitala, 2007). Jervis et al. (2005) found evidence that in Lepidoptera the maturation of the eggs is negatively correlated with the degree of polyandry. Furthermore, Wiklund et al. (2003) hypothesized that males of monandrous species (species of which females mate only once) have shorter lives than females, whereas in polyandrous species males and females have similar life spans. Although this prediction from life history theory might not hold in cases where males invest by providing nuptial gifts (Wedell et al., 2002) and therefore live shorter, it is nevertheless informative to compare in different species the correlation between mating strategies and aspects of sperm competition such as male ejaculate mass (Svärd & Wiklund, 1989), male protein transfer (Bissoondath & Wiklund, 1995), and sperm length (Morrow & Gage, 2000).

In Lepidoptera, reliable estimates of natural mating frequencies can be made from field-caught females, because remains of spermatophores can be found in the bursa copulatrix, the genital chamber in which the male deposits its spermatophore during mating; this eliminates the need for direct observations of mating (Drummond, 1984). The spermatophore is a balloon-like capsule with a stalk (collum), which contains sperm and accessory sub-

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stances. Sperm migrates out of the spermatophore in the bursa to the spermatheca, the sperm storage pouch of the female. In many species of Lepidoptera, the collum of the spermatophore remains visible in the bursa for an extended period of time, even until after death, so that natural mating frequencies can be easily estimated from analysis of the bursal contents of females (Drummond, 1984).

Here we examine two small ermine moth species (Lepidoptera: Yponomeutidae), viz., the oligophagous *Yponomeuta padellus* (L.) and the monophagous *Yponomeuta cagnagellus* (Hübner), that are closely related (Menken et al., 1992; H. Turner, N. Lieshout, W. van Ginkel & S.B.J. Menken, unpubl.). These two species are morphologically nearly identical as adults, but their larvae feed on different host plants [respectively, *Crataegus*, *Prunus*, and *Sorbus* species (Rosaceae) and *Euonymus europaeus* (Celastraceae)]. The species also differ in a number of life history traits such as sexual maturation time (Hendrikse, 1979), pupal weight and life span (A. Bakker et al., in prep.). *Yponomeuta cagnagellus* has a higher pupal weight, takes longer to mature, and has a longer life span than *Y. padellus*. A pilot study in the laboratory revealed that only some 20% of once mated females of *Y. padellus* remated in a 4-day trial compared to some 60% of *Y. cagnagellus*. Therefore, in this study we investigated female mating frequencies and the differences therein in *Y. padellus* and *Y. cagnagellus* both in the field and in the laboratory. In the laboratory two experiments were performed. In a short experiment of 1 week we assessed differences in readiness to remate of once-mated females, and in an experiment which lasted throughout most of an individual's life span we assessed female age at first mating and female mating frequencies. Subsequently, natural mating patterns were investigated by analysing bursa contents and spermatheca fillings of field-collected females.

MATERIAL AND METHODS

Mating under laboratory conditions

Yponomeuta cagnagellus and *Y. padellus* caterpillars, whose parents had been collected in the field as fifth instars during the previous summer, were reared from eggs. *Yponomeuta cagnagellus* caterpillars were reared on *Euonymus europaeus* leaves and *Y. padellus* caterpillars on *Prunus spinosa* leaves. The caterpillars were kept in Petri dishes (10 cm Ø) at 19°C, 60% R.H., and 17L : 7D until they pupated. After their pupal cases had hardened, the pupae were weighed individually on an OHAUS Analytical Standard Scale (d = 0.0001 g) (OHAUS, Viroflay, France). They were placed in individual glass vials (8 cm high, Ø 2 cm), which were closed with cotton wool and stored in a climate box at 18–22°C, 65–85% R.H., and 17L : 7D.

In 2004, an experiment was set up in the laboratory to investigate the likelihood of once mated females of both species to remate. Experiments were conducted inside a climate room kept at 20–21°C, 60–85% R.H., and 17L : 7D (of which the photo phase contained 2 h of twilight, 1 h at dawn and 1 h at dusk, provided by a 40 W screened lamp). A red dark room lamp (Philips PF712E, 15 W) was used to check the moths during the scothophase without disturbing them. Twenty-three female moths of *Y. padellus* (8 days old and a minimum pupal weight of 20 mg) and 23 female moths of *Y. cagnagellus* (18 days old and a minimum pupal weight of 25 mg) were each mated with a virgin male (of about the same age as its partner) in a Petri dish on the first day of the experiment. To test their remating frequency, mated females were subsequently presented daily with a new virgin male on days 4 to 7, and thus every female had five mating opportunities. At mating, males of *Y. padellus* were 8–14 days old, and had pupal weights of at least 20 mg. *Yponomeuta cagnagellus* males were 18–26 days old at mating, with a minimum weight of 25 mg. On days 4 to 7 and at least 2 h before the peak of mating activity at twilight ("dawn"), a male partner was introduced into a Petri dish containing a female. At the end of each day the males were removed. We checked whether the moths were in copula about every 30 min until the last pair had separated. Since copulation with virgin males generally lasts some 3–6 h (A.C. Bakker, unpubl. data), a mating duration of less than 1.5 h was not considered as a successful mating. In addition to mating, male sexual activity was also observed and all males were seen wing-fanning and approaching their females at least once during the experiment. All moths were provided with 1% agar (w/v; type 1-D LEEO, Sphaero-Q, Leiden, The Netherlands) cubes of 10% honey once a week before the experiment and on the day before the first mating.

A second experiment under the same climatic conditions as the previous experiment was conducted in 2006 to estimate total female mating frequencies for the two species. The experimental conditions differed from the first experiment in that females of both species were given the opportunity to mate with a virgin male every day during day 1–9 days of the experiment. Subsequently, *Y. cagnagellus* females were given access to a virgin male on days 13–16, 20–22, 27–30, and 34–37, and *Y. padellus* on days 13–16 and 20–21. Male partners were replaced after spending 3 days with the same female, and also after mating, or when they failed to be sexually active for 1 day. In this way, *Y. cagnagellus* moths had 24 mating opportunities during 37 days and *Y. padellus* 15 opportunities during 21 days. Females first begin to emit pheromones to call males at 3 days of age for *Y. padellus* and 8 days for *Y. cagnagellus*, Hendrikse, 1979), although many do not start until later. We started the experiment just before the first females became sexually active; the average age at the start of the mating experiment was 6.1 ± 0.2 days (mean ± SE, n = 49) for *Y. cagnagellus* moths and 2.9 ± 0.2 days (n = 49) for *Y. padellus* moths. Since the average adult female life span was 47.0 ± 2.0 (mean ± SE, n = 43) days for *Y. cagnagellus* and 31.1 ± 1.8 (n = 47) days for *Y. padellus*, the species had approximately equal encounter rates of, respectively, 0.59 and 0.53 [calculated as mating opportunities divided by average life span (in days) since the start of the experiment]. Moth pairs were observed at hourly intervals between 9:00 and 18:00 h. Mating occurs around dawn (Hendrikse, 1979) and takes several hours. The light cycle in the climate rooms was offset; lights were on until 7:00 h and the moths dawn started at 14:00 h, so that mating could be detected reliably.

Because of their use in another experiment (K. Parker et al., in prep.), half of the females were once a week fed with honey, and always had access to water (i.e., wet cotton wool). The other half of the females only had access to water. As there was no effect of the presence of honey on the total number of matings (ANOVA, $F = 0.381$ for *Y. cagnagellus* and $F = 0.140$ for *Y. padellus*, both $p > 0.05$) or on the age at first mating (ANOVA, $F = 0.109$ for *Y. cagnagellus* and $F = 0.206$ for *Y. padellus*, both $p > 0.05$), we combined these two treatment groups for this study.

TABLE 1. Mating frequency catagories of field-collected *Yponomeuta padellus* and *Y. cagnagellus* females based on dissection of the bursa copulatrix and the spermatheca. The four mating categories are: "not mated", "1 time mated", "1 or more times mated", and "2 or more times mated". NF means this combination was not found.

	Intact (partially) empty spermatophore	Spermatophore remains	Empty spermatheca	Filled spermatheca	Empty bursa
Intact filled spermatophore	2 or more times mated	2 or more times mated	1 time mated	2 or more times mated	–
Intact (partially) empty spermatophore	–	2 or more times mated	NF	1 or more times mated	–
Spermatophore remains		–	NF	1 or more times mated	–
Empty spermatheca			–	NF	not mated
Filled spermatheca				–	1 or more times mated

Sampling of females in the field

We analysed bursa content and spermatheca filling of female moths collected from their host plant at two field sites in the west of the Netherlands, Amstelveen (52°17′N, 4°51′E) and Kennemerduinen (52°23′N, 4°34′E). Both species pupated in the beginning of July. The majority (60–100%) of *Y. padellus* females are sexually active at 8 days after eclosion, and *Y. cagnagellus* at 13 days after eclosing (Hendrikse, 1979). Focussing on the sexual maturation period, sampling was done around the end of the flight season: females were collected between 16 and 20 July for *Y. padellus* in Amstelveen and Kennemerduinen and between 30 July and 7 August for *Y. cagnagellus* in Kennemerduinen. Samples were frozen at –20°C until dissection. Although the sampling localities were chosen so that in a particular spot only one species was expected to be present, we could not exclude some mixing with the other *Yponomeuta* species. In another study (Bakker et al., in press), it was found that *Y. cagnagellus* adults mate and rest on non-host-plants and could thus also be present on the host plant of *Y. padellus*, and vice versa. Since it is sometimes difficult to distinguish adult moths of the two species in the field, wing tip colour (where wing-wear is least) and a microsatellite locus (see below) were used to assign females to the correct species. Wing colour of approximately 1-week-old *Y. padellus* moths from the laboratory cultures showed that 96.7% of these moths (n = 150) were scored as grey, 2.0% as white, and 1.3% could not be scored. One-week-old *Y. cagnagellus* moths were 100% scored as white (n = 98). Genetic screening with microsatellite loci showed that locus YP09 amplified in only 25% of the *Y. padellus* moths, but in 89% of the *Y. cagnagellus* moths (Voetdijk et al., 2007). If a moth with white wings could be amplified at locus YP09, it was assigned as *Y. cagnagellus*. If a moth had grey wings and did not amplify for locus YP09 (but the positive control was successful, see below), it was assigned as *Y. padellus*. In addition, if a moth was scored for one character as *Y. padellus* and for the other as *Y. cagnagellus*, it was not included in further analyses. If, one of the characters could not be scored, the moth was assigned on a single character.

DNA isolation and microsatellite amplification

DNA was isolated from a leg of the females collected in the field. DNA was extracted in 100 µl 5% chelex solution in water (Sigma-Aldrich, Zwijndrecht, The Netherlands) with 5 µl 20 mg/ml proteinase K (Sigma). The samples were incubated for 30 min at 56°C followed by 8 min at 95°C. We used 3 µl DNA extract in a volume of 10 µl mix to amplify DNA using PCR [primer description and the PCR and electrophoretic conditions are described in Voetdijk et al. (2007)]. Amplification at locus YP09 was used for species identification; a second locus (YP38) was amplified and run at the same time to check whether DNA isolation had been successful, in case the sample would not amplify at locus YP09. Locus YP38 was chosen because it could be amplified in all 64 *Y. padellus* moths that were tested in the study of Voetdijk et al. (2007).

Mating status of females in the field

Field-collected females were dissected under a binocular microscope. The developmental stage of the eggs (all immature or at least some mature) was scored. Females of *Y. cagnagellus* with immature eggs were excluded from analysis. The bursa copulatrix as well as the spermatheca of a female were placed in Modified Barth Saline buffer (MBS, Gurdon, 1991) on a glass slide. The bursa was ruptured with tweezers and the contents photographed. The number of intact spermatophores, as well as spermatophore remains (collum or parts of the spermatophore wall) in the bursa were scored, and the filling of the spermatheca determined. Based on all possible combinations of filling of the spermatheca (filled or empty) and bursa contents (empty bursa, intact filled spermatophore, intact empty spermatophore, and/or spermatophore remains), females were classified into four mating categories (viz., "not mated", "1 time mated", "1 or more times mated", and "2 or more times mated") (Table 1).

For statistical analysis, we used SPSS (version 12.01, 2003; Copyright SPSS Inc.) for two-sided two-sample Kolmogorov-Smirnov tests to compare female mating patterns of the two species in the laboratory experiments, and R version 2.2.1 (R

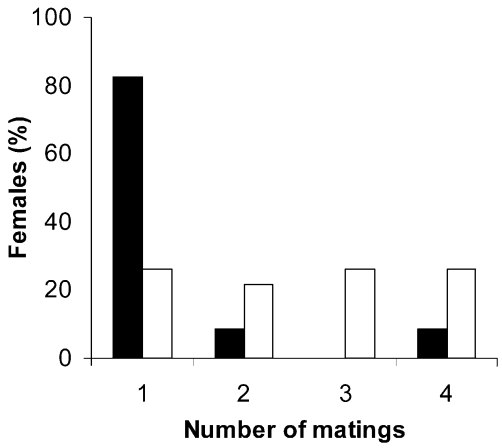

Fig. 1. Percentage of females of *Yponomeuta padellus* (black bars, n = 23) and *Y. cagnagellus* (white bars, n = 23) that mated the indicated number of times out of five mating opportunities.

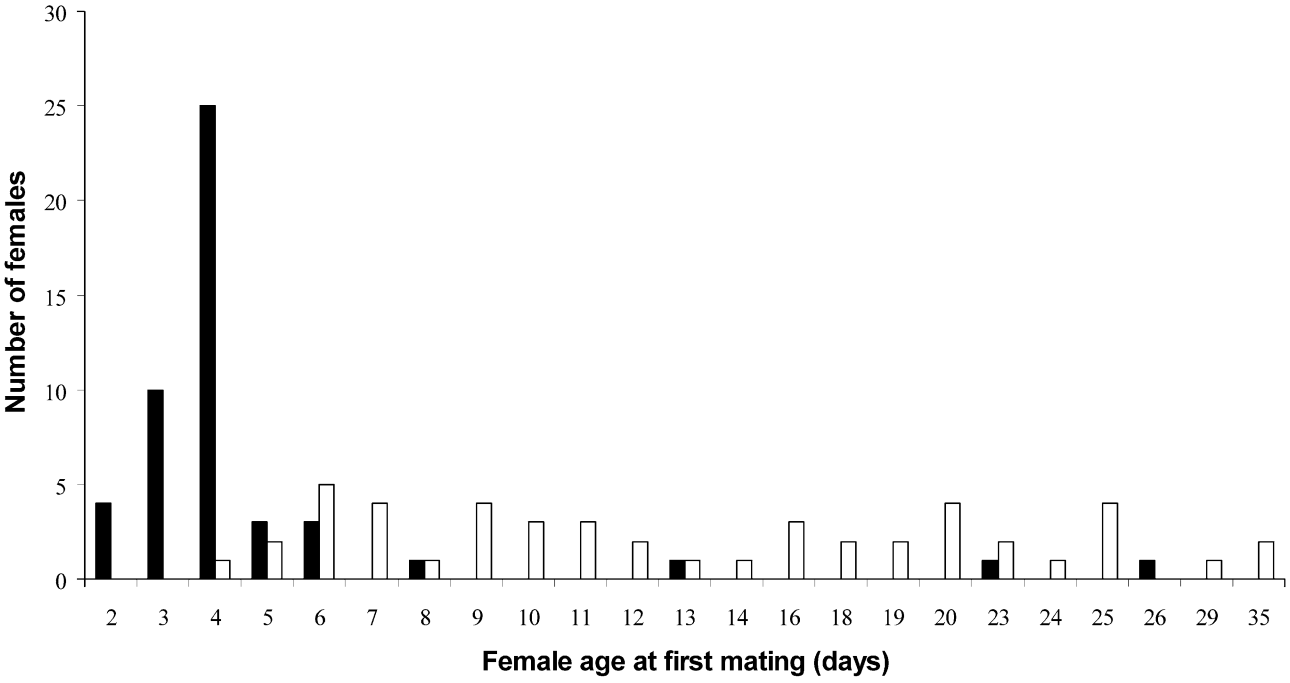

Fig. 2. Age at first mating of *Yponomeuta padellus* (black bars, n = 49) and *Y. cagnagellus* (white bars, n = 48) females.

Development Core Team, 2005) for the one-sided Kolmogorov-Smirnov test used to compare female mating frequency patterns of the two species collected in the field.

RESULTS

Female mating patterns in the laboratory

The laboratory experiment to test the remating frequency of once mated females during a 1-week period showed that 82.6% (i.e., 19 out of 23) of *Y. padellus* females did not remate after the first mating compared to 26.1% (i.e., 6 out of 23) of *Y. cagnagellus*. In *Y. cagnagellus*, almost equal numbers of females mated one, two, three, or four times. In contrast, only two *Y. padellus* females mated twice, and another two mated four times (Fig. 1). The differences in mating frequencies between the two species were significant (two sample Kolmogorov-Smirnov test: Z = 1.917, p = 0.001), with *Y. cagnagellus* females being more likely to remate than *Y. padellus* females.

In the second laboratory experiment, we found that females that had mating opportunities with virgin males throughout most of their life span mated 2.0 ± 0.2 times

TABLE 2. Number of matings (with frequencies in parentheses) of *Yponomeuta padellus* and *Y. cagnagellus* females of the "female mating frequency experiment", and of females collected in the field. The categories "1 or more" and "2 or more" are only found in field-collected females.

Number of matings	*Y. padellus*		*Y. cagnagellus*	
	Laboratory experiment	Field collections	Laboratory experiment	Field collections
	No. (%) of females per mating frequency			
0	0 (0%)	12 (31.6%)	1 (2.0%)	3 (5.6%)
1	20 (40.8%)	11 (28.9%)	10 (20.4%)	16 (29.6%)
1 or more	–	8 (21.1%)	–	26 (48.1%)
2 or more	–	7 (18.4%)	–	9 (16.7%)
2	16 (32.7%)	–	11 (22.4%)	–
3	7 (14.3%)	–	10 (20.4%)	–
4	4 (8.2%)	–	7 (14.3%)	–
5	2 (4.1%)	–	6 (12.2%)	–
6	0 (0%)	–	1 (2.0%)	–
7	0 (0%)	–	2 (4.1%)	–
8	0 (0%)	–	0 (0%)	–
9	0 (0%)	–	1 (2.0%)	–
Total number of females tested	49	38	49	54

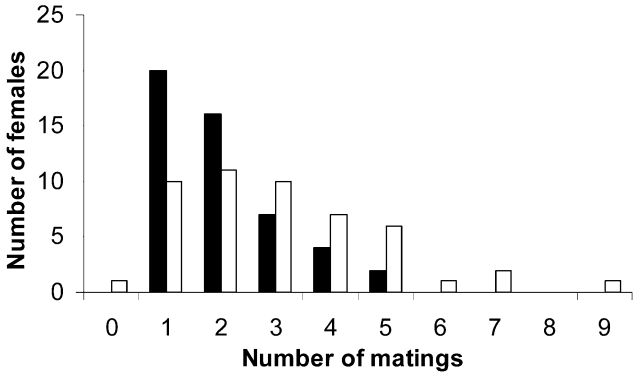

Fig. 3. Number of matings of *Yponomeuta padellus* (black bars, n = 49) and *Y. cagnagellus* (white bars, n = 49) moths with mating opportunities throughout most of their life.

(mean ± standard error, n = 49) in *Y. padellus* and 3.0 ± 0.3 times (n = 49) in *Y. cagnagellus* (Table 2). The youngest female to mate was 2 days old for *Y. padellus* and 4 days old for *Y. cagnagellus*. The average age at first mating was significantly lower for *Y. padellus* than for *Y. cagnagellus* (respectively, 4.9 ± 0.6, mean ± standard error, n = 49 and 14.6 ± 1.2, n = 48; Mann-Whitney U-test: U = 157, p < 0.001). There was considerable variation in the age at first mating (Fig. 2). First mating of *Y. padellus* females peaked at the age of 4 days. Surprisingly, first mating did not peak at a certain age for *Y. cagnagellus* females; their first mating took place anywhere between the age of 4 and 35 days. One *Y. cagnagellus* female did not mate at all (Table 2). In *Y. padellus* most females mated once (40.8%) or twice (32.6%); only 26.5% of the females mated more than twice, and no females were found to mate more than five times (Table 2). In *Y. cagnagellus* females mating once, twice, or three times were found in almost equal frequencies, whereas 55.1% mated more than two times and 8.1% of the females were found to mate between 6 and 9 times (Table 2, Fig. 3). The differences in mating frequencies between the two species were again significant (two sample Kolmogorov-Smirnov test: Z = 1.414, p = 0.037).

Female mating patterns in the field

A total of 109 female moths were collected from the field, 53 during the trip, which was aimed at collecting *Y. padellus*, and 56 during the trip aimed at collecting *Y. cagnagellus*. Thirteen individuals contained immature eggs, of which one was assigned to *Y. padellus*, and 12 to *Y. cagnagellus.* The collection of 12 immature *Y. cagnagellus* moths on 17–20 July at Kennemerduinen indicated that the first collecting (aiming for *Y. padellus* females at the end of their flight season) was indeed too early to catch many mature *Y. cagnagellus*; conversely, we only encountered one *Y. cagnagellus* with immature eggs during the later collection of *Y. cagnagellus.* All immature females were excluded from further analysis. A further four of the 109 individuals collected (3.7%) could not be assigned to a species, as identification through wing color and microsatellite locus gave opposite results. These were also excluded from further analysis. Eighty-six individuals could be readily scored for the two characters; another 24 individuals were assigned on either wing colour or microsatellite locus, because, for example, the wings were too damaged, DNA extraction was not successful, or amplification was doubtful (weak or aberrant bands with many stutter bands). Therefore, 38 *Y. padellus* and 54 *Y. cagnagellus* were used for the estimation of female mating frequencies in the field.

Yponomeuta cagnagellus and *Y. padellus* differed significantly in their mating patterns (one sided, two sample Kolmogorov-Smirnov test: Z = 0.2656, p = 0.044). The two species showed differences in two of the four mating categories, viz., "not mated" and "1 or more times mated". In total, 5.6% of the *Y. cagnagellus* females had not mated compared to 31.6% of *Y. padellus* females (Table 2). Although many *Y. padellus* females had not mated, they all contained mature eggs and were found on different sampling dates. This means that the finding was unlikely to be a consequence of sampling too early in the mating season. 48.1% of *Y. cagnagellus* fell in the catagory "1 or more times mated" versus 21.1% of *Y. padellus* (Table 2). This was mainly because 13 of the 51 mated (25.5%) *Y. cagnagellus* females showed no (remains of) spermatophores but had clearly mated, as sperm was found in their spermatheca. In *Y. padellus* this number was lower, namely three of the 26 mated females (11.5%).

DISCUSSION

Differences in female mating strategies

We investigated possible differences between the mating strategies of *Y. padellus* and *Y. cagnagellus* and assessed female mating frequencies for both species. The short term remating experiment showed that *Y. cagnagellus* was more likely to remate than *Y. padellus*. In the long term mating experiment we measured female mating frequencies during most of their life span and found that the number of times a female mated was higher for *Y. cagnagellus* than *Y. padellus*. Furthermore, the results of field collected females showed a significant difference in mating pattern in the expected direction.

The short term remating experiment showed that 80% of the *Y. padellus* females did not remate during the first week after mating. In contrast, 75% of *Y. cagnagellus* females remated during this week. The long term mating frequency experiment showed that 59% of *Y. padellus* females mated more than once, and the average mating frequency was 2.0 ± 0.2 times (mean ± standard error, n = 49). Thus *Y. padellus* can be considered a polyandrous species. In the same experiment, it appeared that *Y. cagnagellus* exhibited a significantly higher mating frequency than *Y. padellus*: 78% of *Y. cagnagellus* mated more than once and the average mating frequency was 3.0 ± 0.3 times (n = 49).

It should be noted that although the difference in mating frequencies between the two species is clear, it is as yet unknown if this difference is a consequence of a higher willingness of *Y. cagnagellus* females to remate, or

the result of a reduced female ability to withstand male copulation attempts (Rowe, 1994).

Variation in mating pattern among individuals within species

We found in the long term mating frequency experiment that mating frequencies varied from 1 to 5 times for *Y. padellus* and 0 to 9 times for *Y. cagnagellus*. Torres-Vila et al. (2001, 2002) and Wedell et al. (2002) showed that female mating rate is a heritable trait in several Lepidoptera species. Furthermore, female mating frequency can vary between females of the same species (Wedell et al., 2002), or due to non-genetic individual differences in resources (such as nutrient intake in the larval and adult stage, including nuptial gifts), age at first mating (Kaitala & Wiklund, 1994; Torres-Vila et al., 1997; Bergström et al., 2002), or conditions influencing individual mating opportunities such as encounter rates and operational sex ratio, which are themselves influenced by local population density (Drummond, 1984, and references therein; Arnqvist & Nilsson, 2000). No correlation was found between female pupal weight and the number of times females mated divided by their life span (K. Parker et al., unpubl. results). In addition, in our experiment all females had the same mating opportunities and therefore differences in encounter rate fail to explain the within-species differences in numbers of times that females mated.

Variation in mating pattern between species

Between species differences in mating strategies may depend on distribution patterns, which influence encounter rates (Arnqvist & Nilsson, 2000, and references therein). In this study, the duration of the experiments led to *Y. cagnagellus* and *Y. padellus* having nearly-equal encounter rates and thus nearly-equal mating opportunities (respectively, 0.59 and 0.53 encounters per day of their adult life span, which was considered to begin on the first day of the experiment).

Bergström et al. (2002) noted that not only sex ratio and population density during the fertile period of the female, but also the direct costs of mating and male investment influence the number of matings of female Lepidoptera. An example of such male investment is the nuptial gift supplied by the male during copulation. In *Utetheisa ornatrix* moths (Arctiidae), the male provides the female with pyrrolizidine alkaloids that can deter predators and serve to protect both the female and her offspring (Bezzerides & Eisner, 2002). Females of *U. ornatrix* are expected to frequently remate to replenish their supply of pyrrolizidine alkaloids and indeed mate multiple times (Bezzerides & Eisner, 2002). We do not know if mating has positive effects on, or costs for *Yponomeuta* females.

Natural female mating frequencies

We wanted to compare the female mating frequencies from the laboratory experiment, in which females were allowed to mate with virgin males throughout most of their lives, with female mating frequencies in the field. Unfortunately, it was not possible to estimate natural mating frequencies in the field-collected females using the technique of counting spermatophores or colla in the female's bursa. This technique avoids the need for direct field observations of matings, which are difficult to carry out (Drummond, 1984). In *Yponomeuta,* this technique proved to be problematic. Time series of females of both species that were deep-frozen over a period of up to 20 days after mating, showed that spermatophores gradually dissolved and from about 7 days onwards spermatophore remains completely disappeared (data not shown). The presence of sperm in the spermatheca will still indicate whether a female has mated, but this will only reveal that the female mated at least once, i.e., the number of matings tends to be underestimated. The deviation from the real mating frequencies is expected to be greater in *Y. cagnagellus*, as this species lives longer and, at least in the laboratory, mates more, there is a greater chance that spermatophores in the bursa have dissolved in *Y. cagnagellus*, and that its female mating frequencies in the field are underestimated. Indeed, we encountered a greater number of at least once mated (i.e., with filled spermatheca) females without spermatophore remains in their bursae in field-collected *Y. cagnagellus* than we did in *Y. padellus* (25.5% and 11.5%, respectively).

Several studies show that female mating frequencies inferred from laboratory experiments match well with those obtained from counts of spermatophores and colla in field-collected females (Byers, 1978, Torres-Vila et al., 2001; Välimäki & Kaitala, 2006). However, mating frequencies obtained from laboratory tests are expected to be overestimates, as partner encounter rates can be often much higher than in natural situations, and there are few possibilities for females to escape insisting males (Drummond, 1984, and references therein). In our laboratory experiments, males and females were placed within close range of each other (in a Petri dish), mimicking a high encounter rate. *Yponomeuta* are usually found in high densities in the field (20–40 individuals per bush of about 4.5 m^3 is common, pers. observ. A.C. Bakker; Menken et al., 1992), likely resulting in high encounter rates. Furthermore, we do not believe that forced matings frequently occurred in our laboratory set-up, as several females of both species were found to mate only once, while they passed many days together with a willing male (the males would indeed chase the females for hours every day). Females fled when males attempted mating and they were even observed to shake off males. Hence, females seemed very adept at keeping males at bay even in close quarters.

A further overestimation of natural mating frequencies, based only on laboratory experiments, originates from the fact that female and male life span is longer in the laboratory than in the field. This can influence the number of times a female is able to mate (Drummond, 1984). Nonetheless, in *Yponomeuta*, it is clear from laboratory and field results that both species can have multiple matings, as we found several two-times-mated *Y. padellus* and *Y. cagnagellus* females in the field.

From another point of view, natural female mating frequencies can be underestimated if they are based on labo-

ratory experiments that offer only virgin males as partners (as was the case in our laboratory experiments). In lepidopteran species, virgin males generally transfer larger spermatophores than recently mated males (Kaitala & Wiklund, 1994; Cook, 1999; Watanabe & Hirota, 1999; Hughes et al., 2000) and these larger spermatophores can increase female refractory time, thus reducing the likelihood of females remating (Kaitala & Wiklund, 1995, and references therein). Female *Pieris napi,* for example, that were paired in the laboratory with mated males, had higher mating frequencies than females mated with virgin males (Kaitala & Wiklund, 1994). In the wild, females will receive small and possibly sperm-depleted spermatophores from already-mated males and therefore may need to realise a higher mating rate than those in a laboratory set-up with virgin males only.

In summary, we conclude that the two closely related *Yponomeuta* moths show significant differences in matting strategies. The observed changes in timing and frequency of mating provide a basis for further research of the evolution of these traits and their possible role in the radiation of the European *Yponomeuta* species

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