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Generation-dependent female choice: behavioral polyphenism in a bivoltine butterfly

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Climatic and biotic circumstances vary as seasons shift, and different cohorts of multivoltine species are likely subjected to different selection regimes. The bivoltine butterfly *Leptidea reali* (Réal's wood white; Lepidoptera: Pieridae) appears during May and June in central Sweden and has a partial second generation in late July. We manipulated both generations to appear simultaneously and performed laboratory mating experiments that showed the presence of a behavioral polyphenism in mating propensity, which is induced during the developmental stages. Females of the summer generation expressed higher mating propensities than spring generation females. Spring females showed an increase in mating propensity with increasing age, whereas summer females accepted most matings already when they were only 1 or 2 days old. It is likely that larval time constraints, a lower abundance of males and a lower risk of accepting a male of their univoltine sister species *Leptidea sinapis* (wood white), have relaxed selection on mate discrimination among summer generation females. A major challenge for future research is to further investigate the developmental pathways causing the polyphenism and the adaptive implications of cohort-dependent behaviors. *Key words:* developmental plasticity, Lepidoptera: Pieridae, mating propensity, population density, sexual selection, time constraints. *[Behav Ecol 18:758–763 (2007)]*

In temperate regions, the environment changes as seasons shift, and animals have evolved several adaptations that allow them to cope with the varying conditions. Multivoltine insects appear in 2 or more discrete generations per year, and the different cohorts are likely subjected to varying selection regimes dependent on the season in which they appear. Indeed, many multivoltine species show seasonal polyphenisms that can influence a variety of character traits (Shapiro 1976; Kingsolver 1995; Lyytinen et al. 2003; Wiklund and Tullberg 2004).

Many studies of seasonal polyphenism have been conducted on butterflies, and most bivoltine butterflies actually exhibit polyphenism in wing coloration, which makes butterflies of different generations easily distinguishable (Tolman and Lewington 1997). In some species, such as the comma butterfly *Polygonia c-album* and the map butterfly *Araschnia levana*, the 2 generations are so different that spring and summer generations have been assigned different names. In the speckled wood, *Pararge aegeria*, seasonal polyphenism is shown not only in wing pattern but also in life-history characters, such as adult weight and larval development time (Nylin et al. 1989), and also body design such as the ratio between wing length and wing loading (Van Dyck and Wiklund 2002). Seasonal polyphenism has been reported also from many other bi- or multivoltine systems, such as leafhoppers (Larsen and Nault 1994; Moya-Raygoza et al. 2005), beetles (Plaistow et al. 2005), and pentatomids (Niva and Takeda 2003).

The same differences in selection regimes that cause seasonal polyphenisms in morphology or color pattern are likely to also cause different cohort-specific behavioral polyphenisms between seasonally distributed generations. In northern temperate areas, many species change from bivoltinism to univoltinism with increasing latitude, and in populations that exhibit partial bivoltinism, the second generation is typically smaller and less dense (Eliasson et al. 2005). Moreover, offspring that are produced close to the onset of winter are more time stressed to reach the hibernation stage (Abrams et al. 1996; Gotthard et al. 1999b; Johansson and Rowe 1999) than offspring produced earlier in the season. Recent theoretical models have predicted a context-dependent female mate choice behavior and a relaxed selection on female choosiness when females are time constrained (Johnstone 1997) or when males are appearing in low densities, leaving females with a sparse supply of potential males (Kokko and Mappes 2005; Kokko and Rankin 2006).

In general, female reproductive output is limited compared with the almost infinite reproductive possibilities of males, and females have therefore much to gain by choosing between potential mates. Benefits resulting from female mate choice might be direct, such as nuptial gifts as food or nutrients transferred along with the male sperm (Vahed 1998), but also indirect if a female can distinguish high-quality males and thereby increase the fitness of her future offspring, or if she, by mating with a high-status male ensures that her male offspring will be favored by future females (Neff and Pitcher 2005).

There are also costs involved with matings. Depending on mating system, a female might face missed opportunity costs (Wickman and Jansson 1997), energetic costs (Arnqvist 1992, 1997; Watson et al. 1998), and an increased risk of predation (Arnqvist 1989; Magnhagen 1991; Fairbairn 1993; Rowe 1994; but see Gwynne 1989) during mating. Moreover, time costs can be profound because recently mated males will make the female remain in copula for a substantial amount of time in many systems (Svärd and Wiklund 1986, 1989; Kaitala and Wiklund 1995). In some organisms, the female even risks male-implemented physical costs during courtship and mating (Daly 1978). Moreover, males also coerce matings on females in many species (Arnqvist and Rowe 2005), and when studying female mating preference it is, hence, important to be aware of the mating system in order to disentangle female mate choice from male mating coercion.

It has been shown that female mate preferences sometimes vary between different locally adapted populations within species and that female choice (McKinnon et al. 2004; Wong et al.

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2004) and propensity to mate (Gotthard et al. 1999a; Svensson et al. 2006) at these different sites are highly affected by different environmental and ecological factors. A seasonal ecological or environmental variation in selection regimes is likely to affect the female mating propensity similarly, but so far there are no empirical examples of either sexual or other behavioral polyphenisms reported. One circumstance that might explain the lack of evidence of behavioral polyphenism is the difficulty to disentangle cohort-specific behaviors from, more directly induced, adult phenotypic plasticity acting within cohorts.

Here we use a novel approach and demonstrate the presence of a behavioral polyphenism in female mating propensity in the partially bivoltine butterfly *Leptidea reali*. By rearing spring and summer generation butterflies simultaneously, we exclude effects of phenotypic plasticity induced during the adult stage and conclude that females of the summer morph that are subjected to more severe time constrains and less dense populations show a reduced choosiness and a higher mating propensity than females of the spring generation.

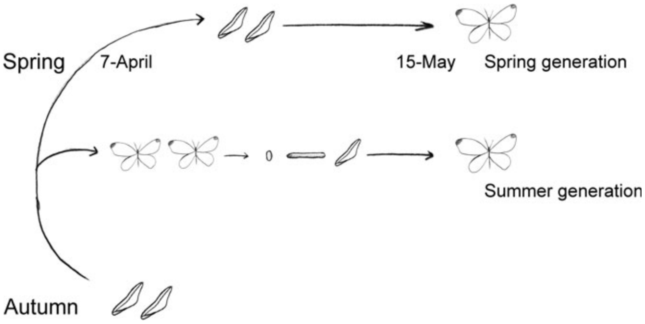

Figure 1
Butterflies of the spring and summer generations were manipulated to emerge simultaneously in spring. On the 7th of April, a fraction of the hibernating pupae was moved into a constant room (22 h light, 23 °C) to induce eclosion into adults. These butterflies were then mated and the larvae that were produced were reared in a direct-development inducing environment (22 h light, 23 °C) to produce a summer generation already in mid-May. On the 15th of May, a new cohort of hibernating pupae were moved into the constant room to produce a spring generation that would emerge simultaneously as the summer generation butterflies. Illustration: Moa Lönn.

MATERIALS AND METHODS

Study species

In 1988, it was found that the wood white, *Leptidea sinapis*, actually consists of 2 species: *L. sinapis* and *L. reali*. The 2 sister species (Martin et al. 2003) are morphologically virtually identical but differ in the size of their genitalia (Lorkovicć 1993) and in DNA sequences (Martin et al. 2003).

Leptidea sinapis is univoltine in south central Sweden (Friberg et al. 2007), whereas *L. reali* is partially bivoltine in the same area with a spring population eclosing from overwintered pupae in mid-May that gives rise to a summer generation in late July and August (Eliasson et al. 2005). The summer generation is constituted of only a fraction of the pupae produced by the spring generation, which leads to lower population sizes and densities during late summer (Eliasson et al. 2005; Friberg et al. 2007). Butterflies of the 2 generations have similar morphology, but the summer generation individuals have less wing melanization and are thus brighter white (Eliasson et al. 2005).

Courtship is usually preceded by a male pursuing a female until she lands in the vegetation. Thereafter, the male places himself opposite to the female and starts oscillating his head and proboscis sideways. Meanwhile, the tips of the antennae move at the same pace as the proboscis. The male courtship can last up to 30 min (Wiklund 1977) and ends either when the female lowers her abdomen and allows the male to mate or when the male gives up and flies away. In many butterfly species, males try to force copulations on females (Wiklund 2003), whereas *Leptidea* males always await an acceptance signal from the female before they try to initiate mating (Wiklund 1977). Males of both *L. reali* and *L. sinapis* cannot distinguish between con- and heterospecific females and are as eager to court females of both species, whereas females exclusively accept conspecifics as mates (Freese and Fiedler 2002).

Laboratory experiment

Butterflies used in this experiment descended from wild *L. reali* females collected during 2003 and 2004 from 2 populations: Kronängen, 59°0′ latitude, approximately 100 km south of Stockholm and Riala, 59°30′ latitude, approximately 50 km north of Stockholm. All founders of the laboratory population were allowed to lay eggs and were species determined post mortem using genital preparations and/or DNA sequencing (for rationale, see Friberg et al. 2007). The experimental butterflies were the offspring from butterflies that had been mated in the laboratory in the summer of 2004. The reason for this was to use individuals whose predecessors had functioned well in the laboratory environment and so minimize potential selection effects that might bias the results.

Hibernating *L. reali* pupae were kept outdoors, under natural conditions during winter. On the 7 April, 70 pupae were moved into constant conditions of 23 °C to induce early eclosion of adults. Twenty-six females were then mated with individual males in order to produce a second generation. Mated females were allowed to oviposit on *Lathyrus pratensis,* the most preferred natural host plant (Freese and Fiedler 2002; Amiet 2004), and eggs and larvae were reared in climate cabinets (*Termaks Series KB8000L*) under conditions that induce direct development (22 h day length, 23 °C; Figure 1). We made efforts to prevent selection on early age acceptance of mating by making females of a wide age span (0–11 days at mating) founders of the experimental second generation. The offspring of these females were later randomly chosen to be involved in the experiment (see below).

On 7 May 2005, 100 *L. reali* pupae were moved from the outdoor conditions and were allowed to eclose in an indoor constant room (23 °C). Hence, the individuals that eclosed in this cohort had been hibernating as pupae during winter and belonged to the spring generation phenotype (Figure 1). Their average eclosion date (15 May ± standard deviation [SD] 2.3 days) corresponded to the average eclosion date of the direct development pupae that constituted the summer generation morph in this experiment (16 May ± 1.5 days). Adult males were kept in generation-specific cages (0.8 × 0.8 × 0.5 m) at room temperature (approximately 20–25 °C) and natural day length. All males were fed a solution of sugar and water every afternoon and had additional nectar plants in their cages throughout the experiment period. Females of both generations were kept in individual jars in a cold room, in 8 °C, and 10 h day length until they were used in the experiment.

The experiment was conducted between 0800 and 1230 h between the 17 May and the 22 May 2005. Participating females were collected one at a time from the cold room and released into a cage (0.8 × 0.8 × 0.5 m). Each female was allowed to feed on sugar solution for 10 min before a male was

Table 1
The order of the mating trials during the experiment

Order of presentations	Female generation	Male generation
1	Summer	Spring
2	Spring	Spring
3	Spring	Summer
4	Summer	Summer
5	Summer	Spring
6	—	—

Except for the first presentation, females of the same generation were always used in 2 successive trials, as were males of each generation.

released into the cage. The ages of the participating individuals were noted and used as covariates in the subsequent statistical tests.

During each trial, the male and the female were placed on separate cotton-tipped sticks. Thereafter, the male was moved toward the female until he detected her, climbed over to her cotton stick, and started his courtship behavior. The courtship success was noted, that is, whether the male was accepted or abandoned courtship, as well as the time until the female exhibited mate acceptance by bending her abdomen so that it became visible after having been hidden from view in between her wings. In cases when females did not accept mating, we recorded the male giving up time, that is, the courtship duration until the male abandoned the female and flew away.

Females of both generations were presented to males of both generations in order to detect potential differences in courtship success between spring and summer generation males. All individuals participating in the experiment were individually marked, previously nonmated, and used only once in the experimental setup. In order to avoid potential effects of time of day, and so facilitate the comparisons of mating propensities within and between female generations, we presented 2 females of the same generation to males of different generations subsequently following a fixed schedule (Table 1).

All statistical tests were conducted using Statistica 7.1 (StatSoft 2005). Tests involving mating frequencies were conducted using a generalized linear model (GLZ) with male and female generations as factors, with the response values being binomially distributed (acceptance = 1, reject = 0) and logit as link function. The average time to female acceptance was tested using a general linear model (GLM), again with male and female generations as factors in the model. The time to acceptance was also tested using GLM with female generation as categorical factor and the age of the female as continuous factor. Due to the discrepancy in age distribution between the females of the 2 generations, potential age effects on female propensity were tested within each generation using Mann–Whitney *U*-tests, with female age as response value and mate acceptance and rejection as grouping factors.

RESULTS

Females of the spring generation were more reluctant to mate than females of the summer generation regardless of the generation affiliation of the courting male. There was, however, no difference in mating success between males of the spring and summer generations (GLZ [binominal, logit]: male generation Wald $\text{stat}_1 = 1.58$, $P = 0.21$; female generation Wald $\text{stat}_1 = 8.51$, $P = 0.004$; male generation × female generation Wald $\text{stat}_1 = 2.05$, $P = 0.15$; Table 2). In the spring generation, female age affected her propensity to mate (Mann–Whitney *U*-test: $U = 2.138$, $n_{\text{acceptance}} = 17$, $n_{\text{rejection}} = 22$, median $\text{age}_{\text{acceptance}} = 4$, median $\text{age}_{\text{rejection}} = 3$, $P = 0.03$; Figure 2a) but not in the summer generation (Mann–Whitney *U*-test: $U = 0.0600$, $n_{\text{acceptance}} = 23$, $n_{\text{rejection}} = 5$, median $\text{age}_{\text{acceptance}} = 2$, median $\text{age}_{\text{rejection}} = 2$, $P = 0.95$; Figure 2b).

Table 2
Number of spring and summer generation females *L. reali* that accepted or rejected courting males from both generations

	Spring males		Summer males		Total	
Generation	Accept	Reject	Accept	Reject	Accept	Reject
Spring females	9	11	8	11	17	22
Summer females	9	4	14	1	23	5

Males and females were used in one trial only.

The females of the summer generation took less time to decide whether to accept the male or not, regardless of whether the male belonged to the spring or summer generation. The average duration of male courtship to female acceptance was 179 s (±SD 169 s; $n = 17$) for females of the spring generation and 64 s (±140 s; $n = 23$) for summer generation females. In the statistical analysis, we used the natural logarithm of the female acceptance time as dependent factor in order to meet the assumption of equal variances (GLM: male generation $F_{1,36} = 1.18$, $P = 0.28$; female generation $F_{1,36} = 13.71$, $P < 0.001$; male generation × female generation $F_{1,36} = 0.64$,

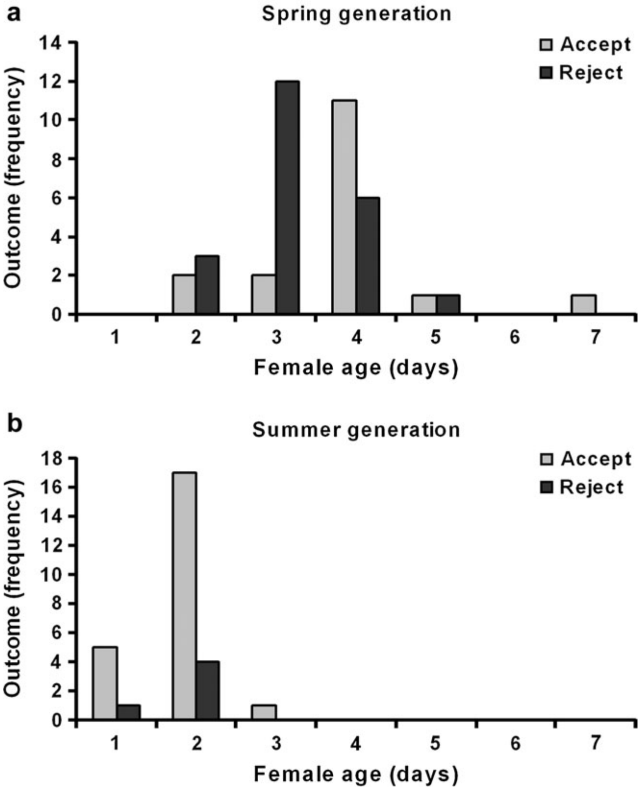

Figure 2
(a) The mating and rejection frequency of females at different ages and generations. The propensity to accept courting males increased with age (Mann–Whitney *U*-test: $Z_1 = 2.138$; $P = 0.03$). (b) Females of the summer generation did not show any age effect in the propensity to accept mates (Mann–Whitney *U*-test: $Z_1 = 0.0600$, $P = 0.95$).

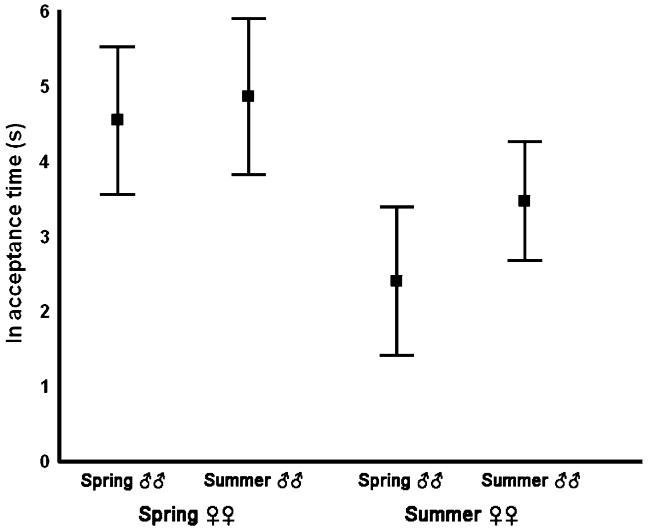

Figure 3
The natural logarithm of the time to female acceptance of courting males (mean + 95% confidence intervals) by females of the spring and summer generations. Summer generation females accepted males after a shorter time than did females of the spring generation. Neither spring nor summer generation females discriminated between summer or spring generation males.

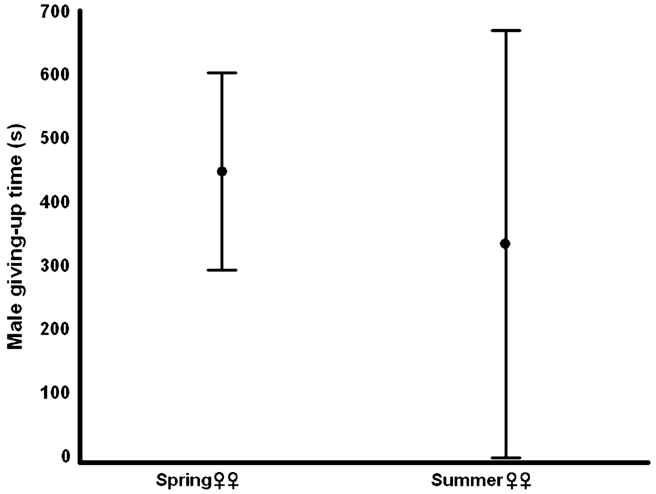

Figure 4
The male giving up time of unsuccessful courtships (mean + 95% confidence intervals) of spring and summer generation females, respectively.

$P = 0.42$; Figure 3). The duration of male courtship until female acceptance decreased with age among spring generation females, but no such pattern was detectable among females of the summer generation (GLM: female generation $F_{1,36}$= 8.83, $P = 0.0053$; female age $F_{1,36} = 0.85$, $P = 0.036$; female generation × female age $F_{1,36} = 5.85$, $P = 0.021$).

There was no significant difference in the average male giving up times of unsuccessful courtships, regardless of both the male and the female generations. The average male courtship of spring females that did not accept mating lasted 447 s (±SD 364 s, $n = 22$), whereas courtships involving summer females that did not accept mating lasted 295 s (±270 s, $n = 5$; Figure 4). Spring males spent on average 353 s (±262 s, $n = 15$) of courtship on an unwilling female, whereas the average giving up time of summer males was 302 s (±432 s, $n = 12$) when courting unwilling females (GLM: male generation $F_{1,24} = 0.830$, $P = 0.371$; female generation $F_{1,24} = 0.407$, $P = 0.529$).

DISCUSSION

Leptidea reali is behaviorally polyphenic. Females of the summer generation were less discriminatory toward courting males than females of the spring generation. Summer females were more prone to accept matings and also were faster to decide whether to accept a male or not. Neither spring nor summer females discriminated between males of the different generations. Likewise, males of the 2 generations did not discriminate between spring and summer generation females because there was no detectable between-generation difference in the duration of unsuccessful courtships, regardless of the generation affiliation of the courted female. It is noteworthy that unsuccessful male courtships of spring generation females lasted more than twice as long as the average time to female acceptance among spring generation females, and unsuccessful male courtships of summer generation females lasted more than 4 times longer than the average time to female acceptance. Hence, the between-generation difference in female propensity to mate cannot be explained by male preference for a certain female phenotype; this conclusion is further corroborated by the difference in female average acceptance time with summer generation females accepting male courtships after shorter time durations than spring generation females.

It is likely that the behavioral polyphenism in female mating propensity is a result of different selection pressures during spring and summer. In central Sweden, the flight period of the summer generation is about 2 months later than that of the spring generation, which means that the offspring of the summer generation have a correspondingly shorter time to develop to the pupal stage before the onset of winter compared with diapause-entering larvae produced by the spring generation. Johnstone (1997) has shown the benefits of a reduced choosiness in mate choice when females are time constrained. *Leptidea reali* females of the summer generation can less afford to delay the onset of egg laying and so may be more prone to accept mates. We contend that this difference in development time available to offspring from the first versus second generations will be valid for virtually all partially bivoltine species, and so we predict that the observed higher willingness for second generation adult females to be more eager to mate quickly will be a common pattern in most bivoltine insects in temperate areas.

Both female propensity to accept mating and male courtship duration before female acceptance were correlated to female age in the spring generation. This means that the older the females got, the more prone they were to accept male courtships. It is possible that also summer generation females show a similar pattern of age-specific female mating propensity, but any such pattern was undetectable among summer females because they appeared in a narrower age window in the experiments (1–3 days old) than the spring generation females (2–7 days old). Nevertheless, although most of the summer females were between 1 and 2 days old during the trials, that is, experiencing the same ages as the females of the spring generation that were most reluctant to accept the male courtships, 23 out of 28 1- to 3-day old summer generation females tested accepted to mate, whereas only 4 out of 19 spring generation females in the same age category accepted mating (Figure 2a,b). Hence, it is possible that also summer generation females increase their mating propensity with increasing age, but they also show an overall higher propensity to accept male courtships than spring generation females of corresponding age.

Despite their young age a higher proportion of females of the summer generation accepted to mate and did it faster than females of the spring generation, which further emphasizes the differences in mating propensity between the generations. The reluctance to mate early in life among spring generation females corresponds to studies of the speckled wood *P. aegeria*. Female *P. aegeria* from Sweden, where populations are dense during a restricted spring flight period, showed less propensity to mate during the first days as adults than did *P. aegeria* from Madeira where the butterflies are present in more sparse populations all year around (Gotthard et al. 1999a); a pattern predicted from recent theoretical models (Kokko and Mappes 2005; Kokko and Rankin 2006), where a female is expected to show higher mating propensity in situations when it is unpredictable when she will encounter a male the next time (Gotthard et al. 1999a). Such an interpretation is analogous to the differences between Swedish *L. reali* generations because only a fraction of the larvae produced in spring enter direct development (Eliasson et al. 2005). Hence, the risks included in rejecting a male courtship are therefore larger for a summer generation female because the probability of soon meeting a new male is smaller in the less dense summer population than in the larger spring population. With increasing age, the cost of rejecting a male courtship will increase also for females of the spring generation and so female willingness to accept males is likely to increase with time.

Additionally, the cohort-dependent female mating propensity might be explained by differences between the generations in the occurrence of the sister species *L. sinapis*. *Leptidea reali* females do not only have to choose among conspecific males but also have to discriminate between conspecifics and males of *L. sinapis* that readily court females of both *L. sinapis* and *L. reali* (Freese and Fiedler 2002). In Sweden, *L. reali* is specialized on open meadow habitats, and *L. sinapis* dwells both on meadows and in the forest (Friberg et al. 2007). Hence, both species are present on the meadows, and female *L. reali* constantly stand the risk of being courted by heterospecific males and must therefore assess the species affiliation of their mates carefully to avoid heterospecific matings. That risk is, however, larger for spring generation females because the proportion of *L. reali* that enters direct development and appears in a second generation is much larger than the proportion of *L. sinapis* that do so at the locations where the animals participating in this study descend from (Friberg et al. 2007). Therefore, the risk of choosing a heterospecific mate is lower for a *L. reali* female of the summer generation.

The occurrence of diverging female mate preferences between different locally adapted populations due to varying selection on a geographic scale has been shown to be a common pattern in nature (McKinnon et al. 2004; Wong et al. 2004). Likewise, ecologically implemented sexual selection might be variable on the temporal scale, and further attention must be drawn to the possibilities of plastic female mate preferences between temporally distributed cohorts within the same population (Qvarnström 2001; Rodriguez and Greenfield 2003; Greenfield and Rodriguez 2004). Here we show, for the first time, seasonal polyphenism in a behavioral trait, with females of the summer generation being more eager to mate and accepting mates more quickly than spring generation females. Females of both generations belong to the same population and so share the same gene pool, and it seems as if mating propensity is genetically modified in the different generations. In our novel approach, females of both generations were manipulated to emerge simultaneously and so experienced the same environmental cues during the experiment. Therefore, the difference between female generations was not a direct, plastic response caused by environmental factors per se but must have been, more directly, induced during earlier developmental stages. A challenge for future research will be to investigate the developmental pathways that determine the gene expression of female choosiness in the different generations and also to further analyze the evolutionary consequences of cohort-dependent differences in mating propensity.

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