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Oviposition site selection and learning in a butterfly under niche expansion: an experimental test

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Organisms that colonize new habitats may gain fitness advantages from changes in habitat selection including oviposition behaviour. Variation in oviposition site selection may contribute to ecological differentiation in response to novel ecological conditions. Both inherited and environmentally induced or learned differences may contribute to variation in preoviposition search behaviour and oviposition site selection. We addressed this issue with the speckled wood butterfly, *Pararge aegeria*. Over recent decades, this species has expanded its habitat use by colonizing anthropogenic environments with populations that show differences in several phenotypic traits (i.e. ecotypes). Here we tested whether preoviposition search behaviour and oviposition site selection differ between field-derived females from forested and agricultural ecotypes, and whether prior experience would change their behaviour differently. We used F_1-offspring reared under common garden laboratory conditions and observed individuals of both ecotypes repeatedly in an indoor experimental arena. The arena offered compartments with host plants under simulated open and woody landscape conditions, as well as control compartments. In contrast with predictions, ecotypic differences in preference were found not for a particular microhabitat, but rather for oviposition site versus control compartment. Nevertheless, exploration and microhabitat use differed between females of agricultural and forest ecotypes. Furthermore, we provide evidence of short-term (i.e. a few hours after initial exposure) learning in the context of preoviposition search behaviour, pointing at the often ignored role of habitat familiarization. Our study sheds new light on behavioural changes that relate to habitat use in novel environments for a species that thrives successfully in landscapes under rapid human-induced environmental change.

Habitat selection is the process whereby individuals preferentially use a nonrandom set of available habitats or habitat conditions (Morris, 2003) and most variation in habitat selection coincides with the environmental conditions to which a species is adapted (Futuyma & Moreno, 1988). In oviparous animals, these adaptations relate to the selection of an oviposition site, which strongly affects the environmental conditions the offspring will experience (Doligez & Boulinier, 2008; Resetarits, 1996). In plant-feeding insects, the introduction of a new plant can lead to a change in host plant preference for oviposition (Lechner et al. 2015; Nosil et al. 2006). In another example, some lizards select oviposition sites based on the microclimatic buffering effect of the surrounding vegetation; in cooler habitats, more open nest sites are selected as they are more heated by solar radiation (Doody et al., 2006). Hence, populations that deal with environments consisting of different resources may consequently differ in oviposition site selection.

Change in oviposition site selection is hypothesized to act as a precursor for rapid ecotypic differentiation among populations that deal with different environments (Hendry et al. 2007). Therefore, intraspecific studies with ecotypes (i.e. populations that have evolved a consistent suite of [adaptive] traits in response to local environmental conditions; Lowry & Hopkins, 2014) that occur in contrasting environments are of particular interest to understand the diversity in oviposition site selection (Eroukhmanoff et al., 2009; Mikheyev et al., 2013; Sandoval & Nosil, 2005). Such studies should deal with both innate preference and induced preference by behavioural plasticity, including learning (Hendry et al., 2007). However, few studies have compared oviposition site selection and learning among different ecotypes. The ecotype-specific oviposition preferences of the stick insect *Timema cristinae* is, for example, assumed to be genetically determined, independent

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of the developmental environment (Nosil et al., 2006). In contrast, in the wasp *Lariophagus distinguendus*, new host plants have been shown to alter naïve adult preference through larval learning. Here, early learning was observed only in the ecotype that had colonized the novel host species, but not in the ancestral ecotype (König et al., 2014).

Whereas these studies tested for learning by rewarding and conditioning insects for relevant cues, fewer have studied learning by focusing on individuals' anticipatory behaviour through repeated exploration of an environment without explicit rewarding (Collett & Zeil, 2018). For example, learning during oviposition search behaviour in butterflies can be inferred through changes in behaviour due to previous experience with the oviposition site (Parmesan et al. 1995; Stanton, 1984; Papaj, 1986; Steward & Boggs, 2020). This type of learning, by gaining familiarity, has often been ignored in habitat selection theory (Piper, 2011).

From an evolutionary point of view, learning in oviposition site selection can be studied as a driver of ecotypic differentiation (Beltman & Haccou, 2005; König et al., 2014), but, more fundamentally, it can also be interesting to focus on the adaptive consequences of learning. Behavioural plasticity of oviposition behaviour comes with several costs and learning is predicted to be selected only under specific circumstances (Mery & Kawecki, 2004; Snell-Rood 2013; Snell-Rood et al. 2013). Some studies have suggested that organisms of more heterogeneous environments are generally more likely to benefit from learning (Tuomainen & Candolin, 2011; Brown, 2013). Indeed, in butterflies, learning in oviposition site selection has been shown to be beneficial when resources are dispersed in a more complex environment (Snell-Rood et al., 2013; Stanton, 1984).

We addressed the issue of oviposition site selection, microhabitat exploration and related experience-induced changes in the speckled wood butterfly, *Pararge aegeria*. This is usually considered a forest (edge) species (Schneider et al. 2021), but in northwestern Europe its ecological niche has expanded from forest to anthropogenic environments over the last few decades (Vandewoestijne & Van Dyck, 2010). This expansion is assumed to explain its regional increase in distribution and abundance under rapid human-induced environmental change (Van Dyck et al. 2009). Currently, it is frequently observed in city parks, gardens, dunes, hedgerows, woodlots and meadows in agricultural landscapes (Berwaerts et al. 1998; Pateman et al. 2016). Since earlier work on population genetics suggested adaptive differentiation between these two ecotypes (i.e. from the ancestral habitat type, forest, and the recently colonized anthropogenic landscapes, Vandewoestijne & Van Dyck, 2010), we may expect inherited differences in traits that relate to colonizing new environments, including oviposition site selection. By using individuals that were reared under common garden conditions, this study system allowed us to test for inherited differences in oviposition site preference and related exploration behaviour between females originating from populations that represent forest and agricultural ecotypes.

According to previous observations on a forest population, *P. aegeria* lays eggs on several grass species with no apparent preference for a single host grass species, but with a preference for tufts of grass in a humid, canopy-shaded microclimate (Shreeve, 1986). A humid, buffered environment during larval development benefits this drought-sensitive species, as it reduces desiccation risks (Oliver et al., 2015; Pateman et al., 2016; Schweiger et al. 2006). Therefore, our study primarily focused on differences in microclimate and vegetation structure surrounding the host plant, rather than differences in the host grass species per se. We observed behaviour in a cross-shaped experimental arena, where females were offered potted host grasses under two simulated microhabitat conditions: open oviposition sites (sun exposed, dry) and woody oviposition sites (canopy-shaded, humid). Both microhabitats can be found in both agricultural and forest landscapes, albeit at different frequencies and configurations.

Because oviposition sites with canopy-shaded microclimates are scarce in agricultural landscapes, one may expect divergence in oviposition site preference between ecotypes. However, even in the absence of differential preference, ecotypes may differ in the ability to accept different oviposition sites. Under the latter assumption, we can expect relaxed selection on allocating time and energy to oviposition site selection in females of the agricultural ecotype. Therefore, we tested whether females of this ecotype are less selective in their oviposition site choice compared to females of the forest ecotype. The latter are predicted to be more frequently drawn to the canopy-shaded oviposition site (prediction 1).

Second, in line with the above-mentioned theoretical framework, we performed three observation trials per female to analyse changes in habitat exploration and oviposition behaviour based on prior experience. We expected a stronger plastic behavioural response due to experience in the agricultural ecotype (prediction 2). From the perspective of an egg-laying *P. aegeria*, agricultural environments are regarded as heterogeneous and microclimatically less buffered; suitable oviposition sites are more likely to be dispersed and fragmented by large areas of crop field and pasture (Schweiger et al., 2006). Therefore, we predicted that learning would be more favoured in agricultural than forest populations.

METHODS

Study Species and Sampling

Twenty-two gravid females were captured in six Belgian regions (Aalst, Tienen, Leuven, Chaumont-Gistoux, Charleroi and Namur; see Table 1) and used to breed an F1-offspring stock of 200–300 individuals. In each region both an agricultural and a forested study area (ca. 500 m^2) were selected based on overall land cover (land cover of interest > 60%) and the presence of *P. aegeria* during the last 4 years (based on our own records; Kaiser et al. 2016).

Ethical Note

Pararge aegeria is a nonthreatened species and not legally protected in Europe. No licences or institutional ethical approval were required for the study. Low numbers of butterflies were caught from populations that show a confirmed stable local presence over the last 4 years (based on previous experience of colleagues). Individuals were transported in small plastic cups kept in a fridge box to keep them cool and inactive. Potential harmful manipulations were restricted to manual marking with a permanent marker and small cage conditions which may potentially induce oviposition. After the study, the laboratory F1-generation animals were killed by freezing for morphological measurements.

Table 1
Number of wild-caught individuals per region and per landscape type

Region	Agriculture	Forest
Aalst	1 (3)	1 (1)
Chaumont-Gistoux	3 (9)	5 (16)
Charleroi	2 (2)	1 (1)
Leuven	2 (4)	1 (1)
Namur	2 (6)	2 (5)
Tienen	2 (6)	1 (4)
Total	12 (30)	11 (28)

The number of F1-lab-reared female offspring used for behavioural tests is shown in parentheses.

Breeding and Mating

Each female was kept separately in a cage (0.3 × 0.3 × 0.3 m) under laboratory conditions (temperature day/night: 23/16 °C; photoperiod light/dark: 16/8 h; solar light spectrum-simulating lamps: Philips HPI-T Plus 400W/645). Females had access to artificial nectar (i.e. 20% honey solution) on a piece of cotton and a tuft of potted host grass reared in our greenhouse (i.e. 50% *Lolium perenne*, 40% *Festuca rubra*, 10% *Poa pratensis*; these species are abundant in the sampled regions and suitable for oviposition and larval feeding). A maximum of 20 randomly selected second instar larvae per wild-caught female (i.e. brood) were transferred to four new grass pots (i.e. five larvae per pot). A single grass pot provided all the food for at least five larvae until pupation. Larvae were checked frequently, and pupae were collected and placed individually in plastic cups until adult emergence. Two to three days after emergence, one F1-female and five F1-males were introduced together into a small cage (0.3 × 0.3 × 0.3 m) for 24 h to ensure mating. Within the cage, males and females were taken from different broods but from the same maternal ecotype. After mating, females were kept at lower temperature (temperature day/night: 14/12 °C) in a Sanyo incubator to reduce activity and energy expenditure. They stayed there for at least 3 days allowing egg maturation (Karlsson, 1987).

Experimental Arena Design

The experimental arena consisted of a central part (1.5 × 1.5 × 1.5 m) connected to four tunnels (growing tunnels for gardening, GardenSkill Ltd, Redditch, U.K.; 3 × 1.5 m and 1.5 m high), made of polypropylene 4 mm insect netting on a frame of carbon fibre rods (Fig. 1). One tunnel end simulated an open oviposition site, one a woody oviposition site and two ends were empty controls. The open compartment contained a 2.4 m × 1.2 m grassland background picture printed on a screen and a single small tree (*Acer platanoides*). The woody compartment was created with a similarly sized woodland background picture on a screen, a few small trees inside the tunnel (*A. platanoides, Crataegus laevigata*), a few larger trees outside the tunnel (*Fagus sylvatica, Quercus robur, Carpinus betullus*, *Acer pseudoplatanus*), a military camouflage netting to simulate canopy cover conditions and a humidifier to increase relative humidity by 10–20%. Both open and woody ends were provided with potted host grasses (*L. perenne* and *P. pratensis*). The remaining two tunnel ends were empty and had a white screen of similar size as the other screens. This setting allowed the butterflies to choose compartments with or without host plants and surrounding simulated microhabitat. If simulated oviposition sites prove attractive for egg laying, gravid butterflies will spend less time in the control compartments than in the oviposition site compartments.

The arena was set up within a greenhouse, in which ambient temperature varied between 24 °C and 33 °C, allowing adult female activity (Shreeve, 1986). Ambient relative humidity varied between 20% and 50%.

Oviposition Behaviour Experiment

In total 58 females were individually released in the central part of the arena (see Table 1 for the number of F1-lab-reared females used per wild-caught butterfly of a particular sample location). We did three of these release-and-observe trials for each female: twice on the same day to test for short-term (within 24 h) memory and once more the next day to test for long-term memory. Observation time of each trial was 20 min. Before an observation trial, the female was kept for 10 min in the small cage (0.3 × 0.3 × 0.3 m) in the central part of the arena. The following environmental variables were recorded: temperature, relative humidity, cloudiness (four-level scores: 0: open sky; 1: few clouds; 2: partly clouded; 3: fully clouded), orientation of the open and woody compartments and time of release. The observation started when the lid of the small cage was lifted, and the female was allowed to enter the arena (Fig. 1). Behavioural data (type of behaviour, proportion of time spent for each type of behaviour and proportion of time spent in each compartment of the experimental arena) were recorded using the open-source logging software BORIS (Friard & Gamba, 2016). Types of behaviour included flying (distinguishing three types: bumping against the cage netting, fluttering and flying in a directional fashion), resting (with closed or open wings and type of substrate), walking, arena exploration (frequency of switching between compartments) and ovipositing. If oviposition occurred, the number of eggs was recorded. Recapturing after a release-and-observe trial was done by entering the cage and gently placing the cup over the butterfly on the net surface as soon as it stopped moving. Between observations, we kept the females in a Sanyo incubator (temperature day/night: 14/12 °C). Every two consecutive trials were done with one Forest and one Agricultural female in random order. Compartment orientation remained the same for consecutive trials of the same female, but every few days the

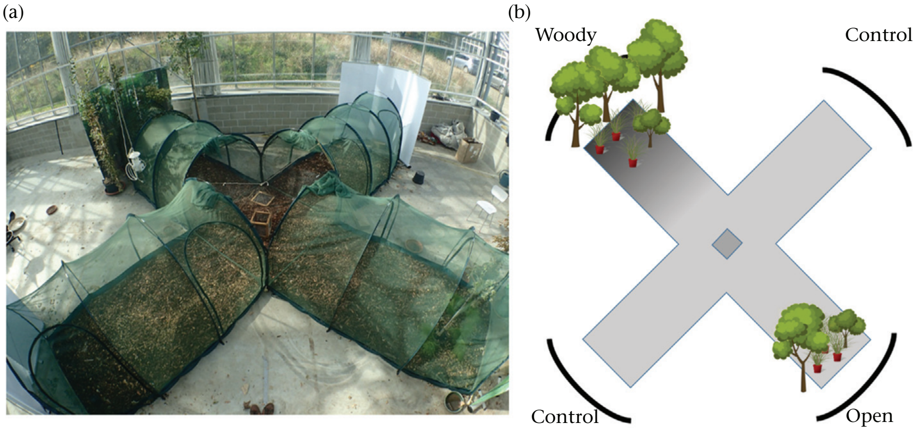

Figure 1. (a) Picture of the experimental arena. (b) Schematic representation of the experimental arena. The two tunnel ends that simulated oviposition sites contained potted host grasses. Curved lines represent the screens showing a picture of the respective conditions of a typical open and woody oviposition site. The control compartments had a white screen. The positions of the compartments were randomized between testing of different females.

orientation of the compartments was altered. A single person (S.B.) made all observations.

Data and Statistical Analysis

All statistical analyses were done in R 3.5.0 (R Core Team, 2018). Prior to analysis, we derived the following variables from the raw observational data. The choice of compartment where butterflies oviposited was our main measure of oviposition site preference. Other preference measures were also considered. The relative use of the compartments was calculated as proportion [open, woody or control compartment]/proportion [all compartments]. We also calculated relative presence for the two oviposition site compartments (open and woody) without the controls, calculated as proportion [open or woody compartment]/proportion [both oviposition site compartments]. The compartment (i.e. open, woody or control) wherein most time was spent and the compartment entered first were recorded as ‘main choice’ and ‘first choice’, respectively. Consistency of compartment choice in the first and second trials was recorded as a binary response (‘yes’ or ‘no’). Presence in a compartment was confirmed when the butterfly occurred at least beyond a third of the tunnel length. ‘Activity’ was measured as the sum of all time spent flying and walking. We defined ‘escape tendency’ as the sum of all active interactions with the netting (e.g. bumping against the netting while flying or moving) divided by the total amount of time spent active (i.e. activity).

Explanatory variables included ecotype (forest or agricultural), trial (1, 2 and 3), sampling location of mating partner, female age (days since adult eclosion), number of days after mating at the time the female was tested, and orientation of the woody compartment. To test trials 1, 2 and 3 pairwise, dependent variables were tested on data sets that included only releases 1 and 2, 1 and 3, and 2 and 3, respectively. Intercorrelated weather and time variables were integrated using a principal component analysis. The first axis (PC1) explained 41% of the total variation. Variables contributing negatively to PC1 loadings were ambient temperature (66%; $F_{1,171} = 334$, $P < 0.001$), hour of the day (11%; $F_{1,171} = 22$, $P < 0.001$) and month (2%; $F_{1,171} = 3.0$, $P = 0.09$). Variables contributing positively to PC1 loadings were relative air humidity (67%; $F_{1,171} = 352$, $P < 0.001$) and cloudiness (56%; $F_{1,171} = 222$, $P < 0.001$), which contributed positively.

The probability of oviposition and the oviposition site compartments used were compared between ecotypes using Fisher's exact tests and one-way goodness-of-fit tests. We tested effects of PC1, ecotype and trial on oviposition probability and choice of oviposition site versus control compartment using a generalized linear mixed model (GLMM) with a binomial distribution and a logit link function (Bates et al. 2015).

We used (generalized) linear mixed models to test the effects of the explanatory variables on the observed behaviours. Effects of ecotype, trial, age and PC1 on egg number were tested with a linear mixed model (LMM; Bates et al., 2015). Effects of ecotype on main choice were tested with multinomial logistic regression (UCLA Statistical Consulting Group, 2006). Effects on proportions (e.g. activity, relative time spent in the control compartments) were first analysed using LMMs. Whenever residuals deviated from normality, a beta regression model was applied as proposed by Ferrari and Cribari-Neto (2004) with the dependent variable y being transformed $((y \times (N - 1) + 0.5)/N$, where N is sample size; Smithson & Verkuilen, 2006). This was done with generalized mixed linear models (R package glmmTMB; Brooks et al., 2017). Random effects in the mixed models included individual, PC1 and hour of the day. For all models, we used backward selection to reduce the full model based on Akaike information criterion (AIC) values in GLMMs or the restricted maximum likelihood (REML) criterion at convergence in LMMs (Zuur et al. 2009). For differences between variances, we applied *F* tests. Differences in consistency of choice were tested by first calculating the expected ratio of individuals that had the same ‘first choice’ or ‘main choice’ assuming random choice, based on the proportions of ‘main choice’ and ‘first choice’ of the first trial. This ratio was tested with one-way chi-square tests against the ratio of individuals choosing consistently. The statistical tests on behaviour were also run with a data set that only included individuals that laid eggs during observation.

RESULTS

Overall Behaviour Within Experimental Arena

Under the experimental conditions of our arena in the greenhouse, 17 of 58 females oviposited. There was no effect of age within the tested range (i.e. 5–18 days) on the tested behaviours. Females became slightly more active with increasing PC1 values. However, when testing all weather variables separately, only cloudiness accounted for an increase in butterfly activity (ANOVA test on ‘cloudiness effect’ of GLMM: $\chi^2_1 = 8.25$, $P = 0.004$).

On average, females bumped against the netting during 37% of the observation time. Average rates of escape tendency were particularly high in the control compartments (90%) and woody compartment (82%) but were substantially lower in the open compartment (67%; GLMM for escape tendency with open compartment compared to woody compartment as main choice: parameter estimate $= -1.023$, $P = 0.014$). During an observation when eggs were laid, females had a 29% lower tendency to escape than during observations when no eggs were laid (59% versus 88%; ANOVA test of escape tendency against the binary variable ‘oviposition during observation’ extracted from GLMM: $\chi^2_1 = 28.51$, $P < 0.0001$).

Ecotype-specific Oviposition and Preoviposition Search Behaviour

No differences in mean activity, escape tendency or mean exploration behaviour were found between ecotypes. However, during the first trial, variation in exploration was higher for Agricultural than Forest females ($F_{1,28} = 3.063$, $P = 0.005$).

Based on our initial three measures of choice (time spent relative to other oviposition site, first choice and main choice), we found no difference in innate preference for either the open or woody compartment. However, when proportion of the ‘woody compartment’ was considered relative to all other compartments, including the control compartments, Forest females initially stayed for 21% less time in the woody compartment than did Agricultural females ($F_{1,53} = 4.29$, $P = 0.040$; Fig. 2a).

In contrast with what we expected, average butterfly presence in the control compartments was relatively high (50%); however, ‘main choice’ as a binary choice between most time spent in simulated oviposition site versus control compartment showed a strong ecotype-dependent effect (Table 2, Fig. 2b, trial 1). Overall, Agricultural females showed a 32% higher occurrence in the oviposition site compartments than Forest females.

Females laid eggs in the open compartment in 15 observations and in the woody compartment in 10. Of the six egg-laying Forest females, five laid eggs in the open compartment and one in the woody compartment. Of the 11 egg-laying Agricultural females, five laid eggs in the open compartment, four in the woody compartment and two in both. Among the females that oviposited, relative proportion of time spent in the woody compartment was 32% lower for Forest than Agricultural females (ANOVA test on GLMM: $I = 9.87$, $P = 0.002$). Hence, the avoidance of woody microhabitat by the forest ecotype was particularly clear during oviposition. For both the oviposited-individuals data set ($F_{32,16} = 6.14$, $P < 0.001$) and the first trial data set ($F_{29,24} = 2.24$, $P = 0.047$), the variance of relative time spent in the woody compartment was higher in Agricultural than Forest females.

Changes in Behaviour Over Trials

When considering 'oviposition site versus control as main choice', we found a marginally significant interaction effect between ecotype and trial: after the first trial, there was a decreased tendency to go to the control compartments in consecutive releases for Forest females, but an increased tendency for Agricultural females. Separate pairwise analyses of trials 1 and 2 and of trials 1 and 3 yielded similar results (Table 2, Fig. 2b). There was no 'trial'*'ecotype' interaction effect between trials 2 and 3 (Table 2). Agricultural females, but not Forest females, differed significantly as they preferred the control compartment more (22% increase; ANOVA test on LMM for control compartment: $\chi^2_1 = 5.97$, $P = 0.015$) and the woody compartment less (18% decrease; ANOVA test on LMM for woody compartment: $\chi^2_1 = 5.14$, $P = 0.023$) in the second trial compared with the first (Fig. 2).

One-way chi-square tests on consistency of main choice between oviposition site versus control in the second release (based on main choice of the first release) did not show a significant effect. Of 51 females, 29 showed consistency in their compartment main choice in the second trial, which was not much different from what would be expected from random (one-way chi-square test: $\chi^2_1 = 4.18$, $P = 0.074$). No ecotype effect on consistency was found either.

Trial number did not affect the probability of oviposition, but females that did oviposit laid on average more eggs during the third trial than they did during the first (0.88 eggs on trial 1 versus 3.12 eggs on trial 3) and showed a linear increase over multiple trials (ANOVA test on LMM: $\chi^2_2 = 10.76$, $P = 0.005$). Of nine oviposition events of Forest females, only one oviposited during the first trial, whereas six of 16 Agricultural females did so.

Overall, activity increased on the second trial, compared with the first, but was not different from the third trial (ANOVA test on the 'trial effect' of LMM: $\chi^2_2 = 9.06$, $P = 0.011$; Fig. 3). Despite activity changes being mainly due to changes in the escape tendency

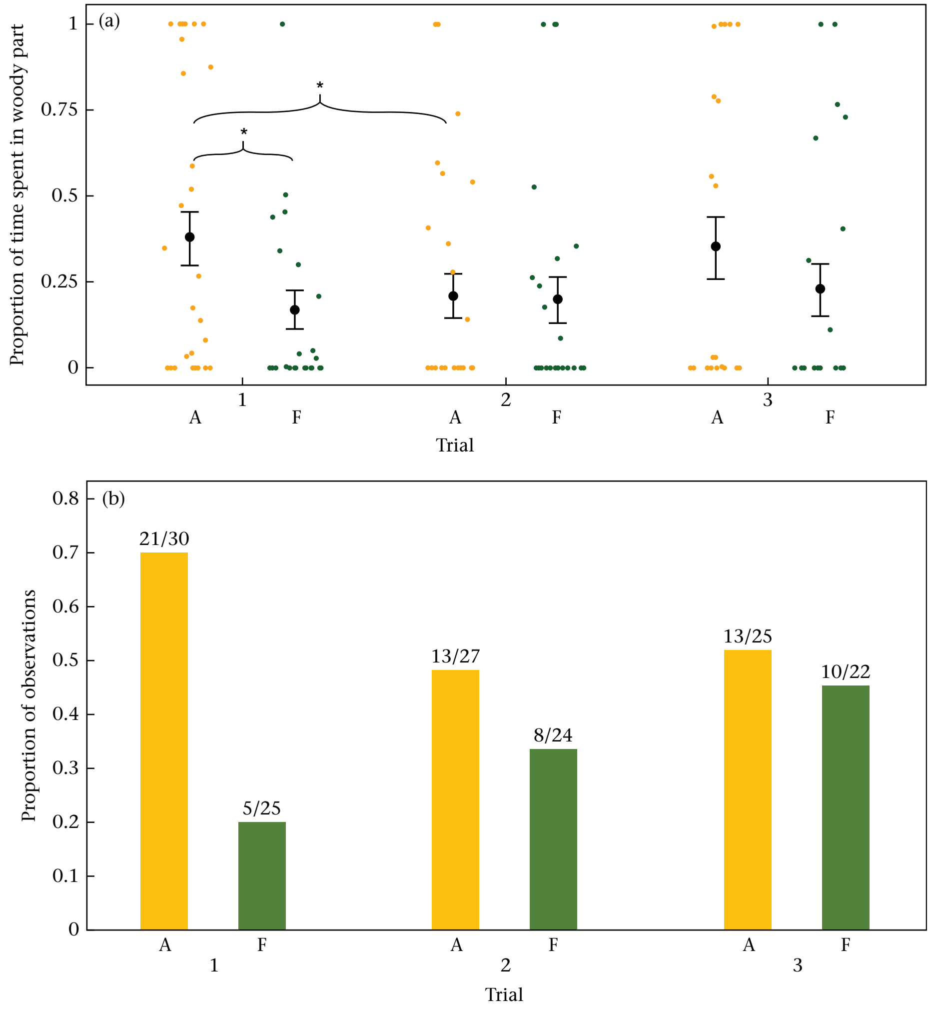

Figure 2. (a) Proportion of time spent in the woody compartment for each of three trials. Means and standard errors are indicated. A: agricultural ecotype; F: forest ecotype. A random choice is expected around a value of 0.25. Braces and an asterisk indicate a difference with $P < 0.05$ according to linear model output. (b) Proportion of observations with oviposition site compartment as main choice as opposed to control compartment as main choice per trial ('1' as a naïve trial, a few hours later '2' and the day after '3') and per ecotype (A: agriculture ecotype; F: forest ecotype). A random choice is expected around a value of 0.5. Above each bar the ratio 'observations with an oviposition site compartment as main choice'/'total amount of observations' is shown.

Table 2
Model output on proportional occurrence in tunnels relative to ecotype and trial

Response variable	Statistical model	Independent variable	*df*	$\chi2$	$P\ (>\chi2)$
Trials 1, 2 and 3					
Oviposition site or not	GLMM	Intercept	1	3.52	0.060
		Ecotype	1	11.00	**0.001**
		Trial	2	2.62	0.27
		Ecotype*trial	2	5.67	0.059
Relative time in control	LMM	Intercept	1	20.53	**<0.0001**
		Ecotype	1	0.22	**0.004**
		Trial	2	5.70	0.058
		Ecotype*trial	2	5.73	0.057
Trials 1 and 2					
Oviposion site or not	GLMM	Intercept	1	3.57	0.059
		Ecotype	1	8.97	**0.003**
		Trial	1	2.27	0.13
		Ecotype*trial	1	4.12	**0.042**
Relative time in control	LMM	Intercept	1	24.23	**<0.0001**
		Ecotype	1	9.52	**0.002**
		Trial	1	6.03	**0.014**
		Ecotype*trial	1	4.55	**0.033**
Trials 1 and 3					
Oviposition site or not	GLMM	Intercept	1	3.64	0.056
		Ecotype	1	10.30	**0.001**
		Trial	1	1.77	0.18
		Ecotype*trial	1	4.8723	**0.027**
Relative time in control	LMM	Intercept	1	19.89	**<0.0001**
		Ecotype	1	8.25	0.25
		Trial	1	1.66	0.20
		Ecotype*trial	1	3.81	0.051
Trials 2 and 3					
Oviposition site or not	GLMM	Intercept	1	0	0.99
		Ecotype	1	0.99	0.32
		Trial	1	0.093	0.76
		Ecotype*trial	1	0.0030	0.96
Relative time in control	LMM	Intercept	1	50.37	**<0.0001**
		Ecotype	1	0.14	0.71
		Trial	1	0.57	0.45
		Ecotype*trial	1	0.021	0.89

Mixed ANOVA output based on linear mixed models (LMM) and generalized linear mixed models (GLMM) using both the ‘time spent in control compartment’ proportion’ and ‘oviposition site versus control’ as response variable. Results were from the model with the lowest AIC/REML-score: ecotype + trial + ecotype*trial. PC1 and individual were included as random effects. Values in bold show estimate values with P value < 0.05.

(i.e. spending more time interacting with the netting), escape behaviour alone did not increase over subsequent trials.

From the first to the third trial, average arena exploration rates halved (ANOVA test on GLMM with trial as a numeric variable: $\chi^2_1 = 6.17$, $P = 0.013$; Fig. 3) and variance of exploration rate dropped in the third release (F test; trial 1 compared to trial 3: $F_{54,46} = 3.58$, $P < 0.001$; trial 2 compared to trial 3: $F_{51,46} = 2.56$, $P = 0.002$). Trials with high exploration rates (i.e. switching compartments more than three times; $N = 14$) were only performed once per individual (Fig. 4).

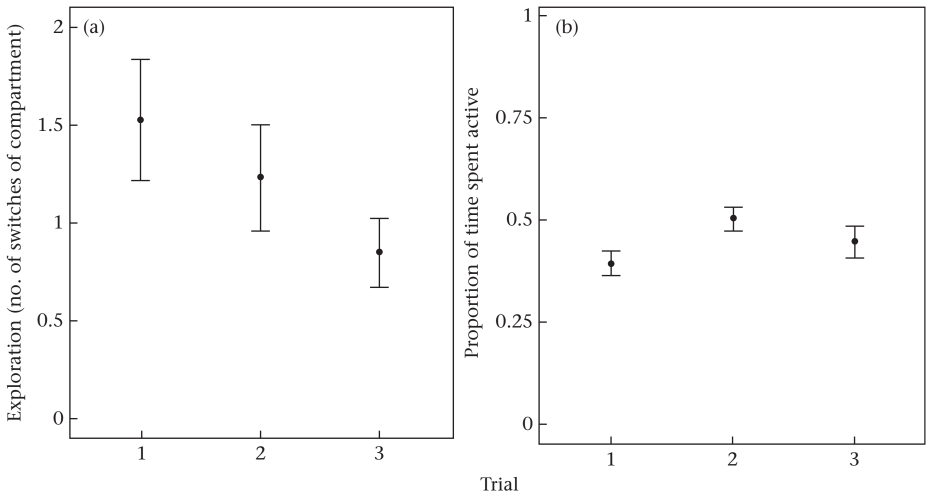

Figure 3. (a) Average number of times a female crossed the central part of the arena to switch compartments during the 20 min observation for each of three trials. (b) Average proportion of time active (i.e. walking and flying) for each of three trials. Bars indicate standard error.

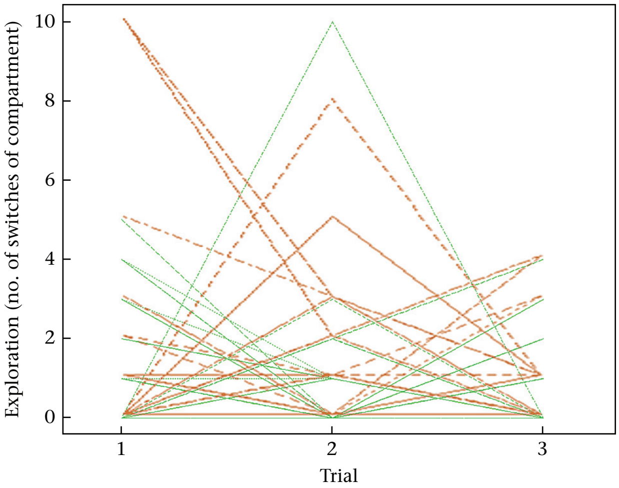

Figure 4. Exploration behaviour (frequency of crossing the central part of the arena) in the three trials. Lines with the same dash type connect the same individual. Brown: agricultural ecotype; green: forest ecotype. Brown lines are moved slightly upwards to make green lines visible.

DISCUSSION

Differences in oviposition site selection between ecotypes have the potential to drive adaptive divergence in species that make use of different habitats (Doligez & Boulinier, 2008). We addressed the question whether speckled woods that originated from populations of recently colonized agricultural landscapes differed in preoviposition search behaviour and oviposition site selection compared to conspecifics from populations of forest landscapes, and whether these differences are largely innate or differently altered by exploration experience. Our common garden approach allowed us to detect differences between the ecotypes under these experimental conditions. Below, we discuss (1) ecotype-related differences in innate oviposition behaviour, (2) the role of environmental experience on preoviposition searching behaviour and the interaction effect between experience (i.e. trial) and ecotype, and (3) the limitations and advantages of our experimental arena approach.

Ecotypes and Innate Behavioural Differences

Contrary to prediction 1 that Forest females would be more frequently drawn to the canopy-shaded oviposition site, (naïve) Forest females did not occur more, nor did they lay eggs more, in the compartment that simulated a woody microhabitat than the one that resembled an open microhabitat. Instead, our findings suggest that Forest females were less attracted to the woody compartment than Agricultural females were. Furthermore, Agricultural females had a higher tendency to visit potential oviposition sites.

However, our hypothesis on ecotypic differences was made under the assumption that simulated oviposition sites were representative of oviposition sites found in the field. Considering that the simulated compartments were not ideal for oviposition (see below), ecotypic differences can be explained by the behaviour-at-boundaries hypothesis tested by Merckx et al. (2003). According to this hypothesis, crossing boundaries between suitable and unsuitable habitat does not signify the same risk of not reaching another suitable habitat patch for individuals adapted to agricultural landscapes and forest landscapes. Agricultural landscapes are characterized by scattered woodlots and hedgerows while large forests have suitable oviposition sites along their edges and in rides that are more easily accessible. Forest females therefore experience lower costs when crossing boundaries and are thus more motivated to do so. Merckx et al. (2003) showed that Forest females were three times more likely to cross a woodland-to-open habitat boundary than Agricultural females. Experiments by Öckinger and Van Dyck (2012) expanded on this hypothesis by showing that agricultural individuals are able to detect woodland from a greater distance than forest individuals when released in an open field.

Furthermore, a study on female fecundity on *P. aegeria* ecotypes showed that eggs laid by females descending from agricultural populations are heavier (Karlsson & Van Dyck, 2005). Assuming investment trade-offs between exploratory flight and fecundity (Tigreros & Davidowitz, 2019), Agricultural females are expected to invest more in initial oviposition bouts, as they face higher risk by exploring other sites. Karlsson and Van Dyck (2005) also found that females lay more eggs on the first day, but only at high temperatures (30–35 °C). Dry and warm periods may limit larval food sources, primarily in agricultural habitats (Schweiger et al., 2006), thus increasing the risks of exploring for other oviposition sites. These and our results support the hypothesis that females of the agricultural populations are both physiologically and behaviourally adapted to a sparser distribution of suitable oviposition sites. Under such strong selective pressure from the environment *P. aegeria* may have undergone ecotypic differentiation, allowing them to thrive in newly colonized environments (Hendry et al., 2007; Richardson et al. 2014; Vandewoestijne & Van Dyck, 2010). Although it is unknown whether these ecotypic differences result from genetic, maternal or epigenetic effects, our experiments demonstrate inherited behavioural differences by using F1-lab-reared females obtained from eggs of field-caught parental females. Future research is required to elucidate whether ecotypic differentiation mainly occurred under processes of (1) local adaptation or matching habitat choice (Hendry et al., 2007; Edelaar et al., 2017) or (2) transgenerational plasticity via epigenetic or maternal changes (Bitume et al. 2014; Jensen, 2013).

Preoviposition Searching Behaviour and the Role of Learning

In our experiment, ecotype-specific differences disappeared after prior experience (i.e. trial 1) as experience, but not age, resulted in an ecotype-specific change in microhabitat preference. Prior experience also resulted in the reduction of the mean and the variation in exploration behaviour and in an overall increase in eggs laid per trial. Our results point to short-term and long-term changes in preoviposition and oviposition behaviour that are unrelated to motivational changes due to age. Behavioural shifts are consistent with butterflies of both ecotypes acclimating to the cage, and differences between ecotypes are consistent with the agricultural population being more tolerant of completely new environments.

Here, we argue that these behavioural shifts may occur because of spatial learning, a cognitive trait that is expected in butterflies that lay eggs selectively over the course of several days (Wiklund & Persson, 1983). Following a broad definition of learning (i.e. an experience-based behavioural change in which conditioned stimuli are not specified; Thorpe, 1963), butterflies may have learned from experience in the experimental set-up. As exploratory behaviour is an important but costly requirement for spatial learning (Brown, 2013), we would expect more exploratory behaviour prior to switches in compartment preference (i.e. high exploration bouts in the first or second trial), which is indeed what our results show: high exploration bouts tended to be followed by lower levels of exploration in the following trial of the same individual (see Fig. 4).

Earlier work on butterfly movements suggested that habitat exploration may be advantageous because it increases familiarity with, and hence optimal use of, the surrounding environment (Conradt et al. 2001), which implies learning and spatial memory. Environmental familiarization is a type of (micro)habitat learning that may be more common than often thought (Collett & Zeil, 2018). Planarians, for example, learn their chemical environment and memorize the information up to 14 days (Shomrat & Levin, 2013). However, the exact mechanism of how *P. aegeria* learns spatially is not clear from our study, as we could not determine whether behavioural changes resulted only from learned spatial cues or also from motivational changes induced by other cues. Suboptimal spaces or recapture after a trial may induce tendencies to flee in subsequent trials. This is in agreement with a decrease in exploration, while no decrease in activity was observed (see Fig. 3). Nevertheless, the proportion of active time spent in interaction with the netting did not increase over trials, suggesting that prior experience in the experimental set-up does not influence tendency to escape.

In line with prediction 2 that there would be a stronger plastic behavioural response due to experience in the agricultural ecotype, the Agricultural, but not Forest, *P. aegeria* females changed their preference from the woody compartment in the first session to the more open control compartments in the second. As Brown (2013) suggested, complex, heterogeneous resource distributions may indeed promote learning towards those resources. However, one needs to be cautious with generalizing this relationship. First, learning is tested on one type of behaviour. A heterogeneous environment refers to a single type of resource distribution (here, suitable oviposition sites) and learning therefore only applies to the behaviour related to this resource (here, oviposition search behaviour). Understanding how learning occurs in the context of oviposition site selection does not necessarily translate to how learning occurs in other contexts (e.g. foraging: Jones & Agrawal, 2017). Second, to understand why heterogeneous environments often promote learning, one needs to look at the more specific environmental conditions under which learning occurs. Theoretical models predict that learning can be selected for under environmental change with high within-generation and low between-generation predictability (Botero et al., 2015; Stephens, 1993). Heterogeneous environments may indirectly elicit such specific levels of environmental change over time. Within a *P. aegeria* generation, the distribution of potential oviposition sites is unlikely to change at high rates and thus is likely to remain predictable. However, between *P. aegeria* generations, the distribution of oviposition sites can change drastically, either because of rapid human-induced environmental change within the existing habitat (Sih, 2013) or via colonization of new, fragmented habitats in the agricultural landscape (Yeh & Price, 2004). In this context, the environmental change is predictable and occurs within a lifetime. Further experiments in related species may help elucidate the relative contribution of environmental heterogeneity and predictability on learning. Our results show that, rather than only assuming innate oviposition site preference, studies on oviposition site selection should explicitly test the additional role of (micro) habitat familiarization.

Note that we did not test the role of early developmental plasticity in this study. In holometabolous insects, behavioural changes based on larval experience is considered less relevant for oviposition site preference than is adult learning (Barron, 2001; Davis, 2008). Nevertheless, host plant preference is affected by larval experience in some species as well (Petit et al., 2017) and larval developmental conditions have been shown to affect the adult *P. aegeria* phenotype (Merckx & Van Dyck, 2006). Therefore, experiments that integrate reciprocal transplants of the larvae in both agricultural and forest rearing sites, will be our next step to test the role of larval learning in shaping preoviposition searching and oviposition behaviour.

Experimental Arena and Perspectives

The approach we adopted to simulate microhabitat conditions in an indoor greenhouse setting has both advantages and disadvantages for the study of oviposition site selection and microhabitat use in general. Arguably, these conditions may have been suboptimal for *P. aegeria* oviposition behaviour. We observed frequent behavioural interactions with the tunnel netting, low oviposition rates (29% of the individuals) and high occurrence in the control compartments. Potential suboptimal conditions include limited cage size and overall low humidity (20–50%); despite artificial humidification inside the greenhouse, atmospheric humidity was low compared to typical field conditions (outside, relative humidity on sunny afternoons fluctuates around 40–60%; Simon Braem & Hans Van Dyck, 2019). Furthermore, although temperatures were sufficient for active flight, cloudiness appears to interfere with the motivation to oviposit (Braem & Van Dyck, 2021).

Cage observations of butterfly behaviour unavoidably have constraints not found in free-roaming behaviour in the field. Nevertheless, our experimental arena provided an interesting opportunity to test several individuals repeatedly for spatial memory used in an oviposition context and to compare ecotypes in a time-efficient manner with minimal interference of weather variables. Under field conditions, individuals are more likely to be lost limiting the possibility of repeated tests, which is essential to study learning processes.

Shreeve (1986) observed that ovipositing *P. aegeria* females showed specific exploratory flight behaviour for both small- and large-scale exploration and laid eggs on sites that are characterized by specific, season-dependent microclimatic conditions. Therefore, outdoor conditions may better reflect ecologically relevant microclimatic fluctuations at the level of the grass layer, where wind, soil and canopy cover play an important role in regulating the buffering capacity of near-ground temperature and humidity (von Arx et al., 2013). Large outdoor cages in real-world habitat settings may provide complementary tools to study exploration and oviposition site preference in butterflies under more ecologically representative conditions (Nieberding et al. 2018). For example, in a Swedish study with large outdoor cage experiments, 70% of *P. aegeria* females oviposited in sessions of 20 min (Bergman et al. 2011). However, although cage size can affect rate of oviposition (see also Braem & Van Dyck, 2021), we need to be prudent when comparing oviposition results of Swedish and Belgian populations. Shady places to oviposit, as in our woody compartment, were highly preferred in Swedish forest populations of *P. aegeria* (90% in shady sites; Bergman et al., 2011; Wickman & Wiklund, 1983), whereas we did not find such a relationship.

In this study, we tracked individuals for a relatively long time and repeatedly in seminatural environments under standard conditions. Although frequently applied in vertebrates (Dawkins, 2007), ethogram-based live observations are rather rare in insect behaviour studies. Moreover, studying the oviposition behaviour of different ecotypes under a common garden approach provides interesting scope for tackling ecological divergence in a species undergoing niche expansion in anthropogenic landscapes.

Author Contributions

All authors codesigned the study. S.B., C.T. and H.V.D. prepared the experiment. S.B. collected and analysed the data in interaction with C.T., C.N. and H.V.D. S.B. wrote the first draft of the paper in close collaboration with H.V.D. All authors discussed the results and contributed critically to the final manuscript. All authors gave final approval for publication.

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