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Cite this article: König K *et al.* 2015 Does early learning drive ecological divergence during speciation processes in parasitoid wasps? *Proc. R. Soc. B* **282**: 20141850. http://dx.doi.org/10.1098/rspb.2014.1850

Received: 24 July 2014
Accepted: 13 November 2014

Subject Areas:
behaviour, ecology, evolution

Keywords:
host preference, early learning, early adult experience, host switch, ecological speciation, *Lariophagus distinguendus*

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2014.1850 or via http://rspb.royalsocietypublishing.org.

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Does early learning drive ecological divergence during speciation processes in parasitoid wasps?

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Central to the concept of ecological speciation is the evolution of ecotypes, i.e. groups of individuals occupying different ecological niches. However, the mechanisms behind the first step of separation, the switch of individuals into new niches, are unclear. One long-standing hypothesis, which was proposed for insects but never tested, is that early learning causes new ecological preferences, leading to a switch into a new niche within one generation. Here, we show that a host switch occurred within a parasitoid wasp, which is associated with the ability for early learning and the splitting into separate lineages during speciation. *Lariophagus distinguendus* consists of two genetically distinct lineages, most likely representing different species. One attacks drugstore beetle larvae (*Stegobium paniceum* (L.)), which were probably the ancestral host of both lineages. The drugstore beetle lineage has an innate host preference that cannot be altered by experience. In contrast, the second lineage is found on *Sitophilus* weevils as hosts and changes its preference by early learning. We conclude that a host switch has occurred in the ancestor of the second lineage, which must have been enabled by early learning. Because early learning is widespread in insects, it might have facilitated ecological divergence and associated speciation in this hyperdiverse group.

1. Introduction

Insects represent the world's largest group of organisms, comprising over 900 000 described species, which is about 70% of all animal species [1]. Their species richness and biological diversity make insects key players in almost every terrestrial ecosystem. Many explanations for this hyperdiversity focus on the ecological niche and on ecological speciation [2]. Central to the concept of ecological speciation is that populations of the same species become genetically isolated because they specialize in different ecological niches [3–5]. This is assumed to result in the evolution of new species owing to restricted gene flow between populations, in allopatry as well as in sympatry [6]. One problem with the concept of ecological speciation is that it must be initiated by the switch of particular individuals of a population to a new ecological niche (e.g. a new host in herbivorous or parasitoid insects) to which they are not yet adapted. It is unclear why the offspring of these pioneers should remain in this niche and how they become genetically isolated from the rest of the population, despite sympatry. A solution could be that in the larval or pupal stage, some insects memorize the host that they consume during development [7]. As adult insects, they afterwards accept or even prefer this host for oviposition. The subsequent genetic isolation of the pioneers could result from mating on or near the host [8]. This mechanism of early learning was first proposed by Thorpe & Jones in 1937 [9] and does not require mutations of genetically fixed preferences. Therefore, early learning has been repeatedly cited as a potential initial mechanism for the separation of subpopulations and

the emergence of new ecological species [7,10,11]. But up to now, there are no studies of speciation in insects combining phylogenetic data with results from behavioural studies on innate and learned host preferences and performance, i.e. the fecundity on different hosts. The role of early learning in speciation thus remains unclear.

One group of insects highly suitable to study mechanisms of speciation are hymenopteran parasitoids [12], which are among the most speciose groups in the animal kingdom [13]. For oviposition, females of these insects choose specific hosts, which are usually immature stages of other insects. The offspring develop in or on the host, thereby killing it [14]. There are a number of well-studied species, mostly parasitoids of agricultural pests. Within some parasitoid species, there are different populations that differ considerably in their reaction to cues from specific hosts, suggesting that they represent host races or even different (cryptic) species [15–18]. As early as 1968, Askew [12] considered chalcidoid parasitoid wasps to be especially susceptible to ecological speciation because of their prevalence of inbreeding, caused by sib-mating in close proximity to the host in combination with monandry. These characteristics help to overcome one of the main obstacles for ecological speciation, that is, a close association between ecological niche and mate choice. In addition, many parasitoid wasp species modify their behaviour through early learning, i.e. learning during development or during and directly after emergence from the host. In some cases, this causes parasitoid females to prefer the host on which they developed for oviposition [19–22]. Early learning could thus lead to the ecological and spatial separation of individuals from different hosts already within one generation.

The cosmopolitan parasitoid wasp *Lariophagus distinguendus* (Förster) (Hymenoptera: Chalcidoidea: Pteromalidae) is reported to attack the larvae and pupae of 15 different beetle species from seven families [23]. However, parasitization of several of these hosts has been demonstrated only in the laboratory [24–27] or is highly doubtful owing to the non-endophytic biology of reported hosts (e.g. *Tribolium* sp.), whereas *L. distinguendus* females clearly prefer endophytic hosts for oviposition. Of 23 samples of *L. distinguendus* collected worldwide, 10 were collected on larvae of the drugstore beetle *Stegobium paniceum* (L.) (Ptinidae), 12 were collected on larvae of the weevil genus *Sitophilus* (Dryophthoridae: Curculionoidea) and one was collected at a location where both hosts occurred (see the electronic supplementary material, table S1). Obviously, drugstore beetles and *Sitophilus* weevils are the main hosts. However, the latter are not suitable as hosts for all *L. distinguendus*. A previous study revealed that certain strains of *L. distinguendus* that were collected from drugstore beetles had a very low fecundity on granary weevils (*Sitophilus granarius* (L.)) compared with strains collected from this host [28]. This indicates that some host specialization has occurred. Host finding in *L. distinguendus* was intensively studied [29–32] and is strongly influenced by adult learning [33]. Mating occurs mostly on the natal host patch, and the protandrous males can perceive the presence of conspecific pupae within the grain kernels and wait for emerging females [34,35]. These features make *L. distinguendus* a suitable candidate species for studying the potential role of early learning in ecological speciation.

Here, we compare different strains of *L. distinguendus* collected from drugstore beetles and granary weevils as hosts. We examine their innate and learned host preferences, their fecundity on the two main hosts, and their genetic divergence, to study the hypothesis that early learning has been involved in the ecological divergence of subpopulations, as a pre-condition for the emergence of new species.

2. Material and methods

(a) Insects

In total, nine strains of *L. distinguendus* were studied (electronic supplementary material, table S2). These were collected by us or sent to us by colleagues. Four strains were collected on drugstore beetles (db) as hosts in pantries of private homes: RAVdb (collected 2008 in Ravensburg, Germany), STUdb (2007, Stuttgart, Germany), BIRdb (2011, Stuttgart-Birkach, Germany) and WAGdb (2011, Wageningen, The Netherlands). Five strains were collected on granary weevils (gw) as hosts in grain stores: PFOgw (2005, Pforzheim, Germany), SLOgw (1996, Slough, Great Britain), SATgw (2010, Satrup, Germany), SACgw (2010, Sachsen, Germany) and BYGgw (2005, Bygholm, Denmark). Host preference experiments could not be performed with strains WAGdb, SACgw and BYGgw, and fecundity data are not yet available for BIRdb, because these strains were only recently obtained.

For standard rearing, all strains were reared on their original host, and all insect cultures were kept under constant conditions of 26°C and 45% r.h. and 16 L : 8 D photoperiod, unless stated otherwise. For the experiments, all strains were reared on both hosts. In addition, the granary weevil strains SLOgw and SATgw were reared on bean weevils *Acanthoscelides obtectus* (Say; Bruchinae) and on cowpeas *Vigna unguiculata unguiculata* (L.) Walpers, to exclude potential learning of host cues from drugstore beetles or granary weevils during development, thus elucidating their innate host preference for one of the two hosts.

To rear drugstore beetles, about 1 g of newly emerged unsexed adult drugstore beetles (about 700 beetles) was placed in a Petri dish on 80 g food pellets for koi fish (Hikari Friend, Kamihata Fish Industry Group, Kyorin Corporation, Japan) or on 40 g wheat grain (cultivar: Batis; Saaten-Union GmbH, Hannover, Germany) moistened with 1 ml H_2O for oviposition and left there until they died. After six weeks, *L. distinguendus* were placed on the infested koi pellets or the infested wheat grain, depending on the experiment. To rear granary weevils, 2.7 g of unsexed adult granary weevils (about 600 beetles) were placed in jars (diameter 12 cm, 16 cm height) with a ventilated lid on 40 g of moistened wheat grain (40 ml H_2O on 1 kg grain) for oviposition. After one week, adult beetles were removed. After three weeks, *L. distinguendus* were placed on the infested grain. To rear *A. obtectus*, 6.7 g of unsexed adults (about 1600 beetles) were placed in jars (1 l capacity, 15.5 cm height) on 500 g of cowpeas for oviposition and left there until they died. After three weeks, *L. distinguendus* were placed on the infested cowpeas. Cultures were kept at 27 ± 1°C, 65 ± 5% r.h. and natural daylight conditions.

(b) Host preference experiments

To study host preferences of wasps with different experience from strains RAVdb, STUdb, BIRdb, PFOgw, SLOgw and SATgw, experiments were performed in a small Petri dish (5.5 cm diameter). Wasps were released in the centre of the dish. One grain kernel infested by a drugstore beetle larva, and one grain kernel infested by a granary weevil larva were placed on opposite sides at a distance of 2 cm from the centre. The number of times a wasp inserted her ovipositor into the infested grains (frequency of drilling) [29] was observed for 600 s using a stereo-microscope, and registered using the software 'The Observer v. 5.0' (Noldus, Wageningen, The Netherlands). Frequency of drilling was used to quantify the preference of the wasps, as it is stimulated by external chemical cues [29].

(i) Innate host preference and host preference after pre-imaginal experience

To study innate host preference, and/or host preference after pre-imaginal experience, wasps were reared on drugstore beetles and on granary weevils. Developing wasps were dissected out of the grain in the late pupal stage, when the wasp pupae had turned black [35], and cleaned with wet paint brushes. Then, they were kept singly in Eppendorf tubes under standard rearing conditions until emergence from the pupal case. Wasps were then transferred to small Petri dishes (5.5 cm diameter) and kept under the same conditions for another two days until the preference test (see §2b) was performed. Because these wasps only had pre-imaginal host contact and moulted in the absence of any host-associated chemical cues, their preference must have been innate or owing to pre-imaginal learning. To exclude pre-imaginal experience, and to identify the innate preference for either drugstore beetles or granary weevils in wasps from strains SLOgw and SATgw, they were additionally reared on bean weevils and treated as described above.

(ii) Host preference after early imaginal experience

To study host preference after early imaginal experience, wasps were reared on their original host, i.e. strains RAVdb, STUdb, BIRdb were reared on drugstore beetles, and strains PFOgw, SLOgw and SATgw were reared on granary weevils. To enable early imaginal experience with chemical cues from their original host, wasps were allowed to emerge normally. Directly after emergence from the grains, females were collected from the cultures and kept in a Petri dish for 24 h on grain material infested by their original host from which host beetles had already emerged. The wasps thus received experience with chemical host cues but were not able to associatively learn these cues in direct host encounters by oviposition or host feeding [33,36]. To enable early imaginal experience with chemical cues from the alternative host, wasps were dissected out of the grains in the pupal stage and treated as described above until emergence from the pupal case. Then, they were placed for 24 h on grain material infested by the alternative host from which the beetles had already emerged. The preference test (see §2b) was performed after 24 h. Thus, wasps obtained experience with chemical host cues as early adults in the first 24 h after emergence from the grain. Any difference in host preference between these wasps and those that had pre-imaginal experience on their original hosts must be owing to early imaginal learning.

(c) Fecundity on different hosts

The fecundity of the strains RAVdb, STUdb, PFOgw, SLOgw and SATgw was studied on both hosts. One female and two males were placed in a Petri dish with 15 g koi pellets infested by drugstore beetles (1085 ± 197 beetles per 15 g, $n = 6$) or 12 g wheat grain infested by granary weevils (213 ± 8 beetles per 12 g, $n = 14$) and kept under standard culture conditions. From exploratory work, it was known that this amount of hosts is sufficient to study the weekly fecundity of *L. distinguendus* females. The survival of wasps was checked daily. Wasps were placed on new hosts after one week, and for a maximum of 18 days. Afterwards, the emerging offspring were counted.

(d) Molecular data and phylogenetic analysis

(i) DNA extraction, PCR amplification and sequencing

DNA from two specimens of each *L. distinguendus* strain and one specimen of *Nasonia vitripennis* (Walker, 1836) as an outgroup was extracted following the manufacturer's instructions either using DNeasy Blood & Tissue Kit (Qiagen) for analysis of the partial mitochondrial cytochrome oxidase I (COI) and partial nuclear carbamoylphosphate synthetase (CAD) or using the QiaAmp mini kit (Qiagen) for the three nuclear LOC markers (anonymous markers developed based on genome comparisons in Hymenoptera [37]) LOC100123206 (LOC1), LOC100123909 (LOC2) and LOC100117339 (LOC3) and for the nuclear internal transcribed spacer 2 (ITS2). The LOC markers, which span introns [37], and ITS2 were chosen because of their high proportion of variable regions. For amplification, oligonucleotide primers in electronic supplementary material, table S3 were used. In contrast to Hartig *et al*. [37], the LOC oligonucleotide primers were used without sequencing tails for the sake of simplicity. For studying COI, PCR amplification was performed as follows: denaturation temperature 95°C for 2 min, followed by 40 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min. Final extension was 10 min at 72°C. CAD was amplified by a hot start PCR with denaturation at 95°C for 5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min. Final extension was 10 min at 72°C. Positive PCR products were bidirectionally sequenced by Seqlab (Göttingen, Germany). For studying ITS2 and the LOC markers, PuReTaq PCR beads (GE healthcare) were used according to the manufacturer's protocol, with 1.5 µl of 10 mM primer added to a final volume of 20 µl. A typical PCR cycle started with denaturation 94°C for 4 min followed by 35–38 cycles of 94°C for 1 min, 1 min at the primers' annealing temperature, and elongation at 72°C for 1.5 min, ending with a 5 min elongation period. Touchdown PCRs were chosen when applying some of the more degenerative primers (i.e. for LOC100123206 and LOC100123909), and started with two cycles at annealing temperature + 4°C, two cycles at +2°C and 33–36 cycles at the primers' annealing temperature. PCR products were sequenced on an ABI 377 automated sequencer using Big Dye Terminator technology (Applied Biosystems) at the Swedish Natural History Museum in Stockholm. Chromatograms were checked for ambiguous positions, edited after comparing the forward and reverse sequences of each individual, assembled using the program GENTLE v. 1.9.4 (by Magnus Manske, University of Cologne, released under GPL 2003, v. 1.9.4) and aligned in MAFFT v. 7 [38] with the L-INS-i algorithm [39]. Heterozygous positions were included using IUPAC ambiguity codes. Protein-coding regions of all genes were translated into amino acid sequences using the program 'VIRTUAL RIBOSOME' [40] based on the translation tables of standard genetic code or the code for invertebrate mitochondria in case of COI, and compared with orthologous sequences from *N. vitripennis*. No unexpected stop codons or gaps were observed. Newly obtained sequences were submitted to GenBank (electronic supplementary material, table S4). For the outgroup *N. vitripennis*, we also used the nucleotide sequence EU746610.1 (COI) [41] as well as XM_001607631.2 (LOC100123909) and KC213163 (CAD) [42] from NCBI.

(ii) Data analysis

COI was partitioned in first/second and third codon positions (electronic supplementary material, table S5). DNA sequences of nuclear-encoded genes (nDNA) were manually concatenated in MEGA6 [43] into a single multiple nucleotide sequence alignment owing to the small number of variable sites in each single marker, which does not permit the separate estimation of substitution model parameters for each of them. The nDNA dataset was partitioned into first/second codon positions (p1) and third codon positions (p2) when referring to exonic nucleotides, the intronic nucleotides of CAD and the LOC markers (p3), and ITS2 (p4; electronic supplementary material, table S5).

The models for nucleotide substitutions used in the analyses were selected for each partition by applying the Bayesian information criterion [44] using the program PARTITIONFINDER v. 1.1.1 [45] (electronic supplementary material, table S5). Gaps were treated as missing data.

Phylogenetic relationships were examined using maximum-likelihood (ML) analysis in GARLI v. 2.01 [46] with 100 independent search replicates using the default configuration settings.

Bootstrap replicates (1000 ML) were calculated under the same setting but with only two independent search replicates per bootstrap dataset. Bootstrap values were visualized by PAUP* 4.0b10 [47]. Uncorrected *p*-distances were calculated using MEGA6 [43] (electronic supplementary material, figure S6).

(e) Statistical analysis

We used the software package STATISTICA v. 6.0 (StatSoft Inc. Tulsa, OK) for statistical comparisons. Frequency of drilling on the two grain kernels was compared using the Wilcoxon matched pairs test for paired samples. Outliers were eliminated by using the three-sigma-interval. Statistical differences in fecundity between strains and hosts were calculated using the Mann–Whitney *U*-test followed by sequential Bonferroni correction.

3. Results

(a) Host preference experiments

(i) Innate host preference and host preference after pre-imaginal experience

Regardless of whether females from RAVdb, STUdb, BIRdb and PFOgw developed on drugstore beetles or on granary weevils up to the black pupal stage, and therefore had pre-imaginal experience with these hosts, they all showed a significant preference for grains infested by drugstore beetles over grains infested by granary weevils (figure 1*a*,*b*). Obviously, the host on which the wasps developed did not influence the preference in these strains. This demonstrates that they had an innate preference for drugstore beetles that was not influenced by pre-imaginal learning experience. Remarkably, this was not only true for strains collected on drugstore beetles, but also for PFOgw, which was collected on granary weevils and also, according to the molecular analysis, clearly belongs to the granary weevil group (see below).

In contrast, host experience during development influenced the preference of females from the strains SLOgw and SATgw that were collected on granary weevils. They did not show any preference after development on drugstore beetles (figure 1*a*), but significantly preferred granary weevils when they had developed on this host (figure 1*b*). This demonstrates that pre-imaginal learning takes place in these strains. To exclude the effect of pre-imaginal experience and identify their innate host preference, both these strains were reared on bean weevils. These wasps had no significant preference for drugstore beetles or granary weevils, indicating that they had no innate preference at all (figure 1*c*).

(ii) Host preference after early imaginal experience

When wasps had 24 h experience with chemical cues from grain material infested by drugstore beetles or granary weevils, females from RAVdb, STUdb and BIRdb again showed a significant preference for drugstore beetles, regardless of the host experienced (figure 2*a*,*b*). Thus, early imaginal experience did not influence preference in these wasps either, as we already demonstrated for pre-imaginal experience. In contrast, females from PFOgw had a strong, albeit not significant tendency towards drugstore beetles after experience with cues from this host, but no preference for either host after experience with granary weevil cues (figure 2*a*,*b*). Early imaginal learning with chemical cues from granary weevils obviously changed the innate preference for drugstore beetles in this strain. Conversely, early imaginal experience with drugstore beetle cues in SLOgw and SATgw changed the preference for granary weevils, which was induced in these strains after pre-imaginal learning. This led to an equal acceptance of both hosts in SLOgw and even a slight, non-significant preference for drugstore beetles in SATgw. Thus, early imaginal learning influenced host preference in the strains that were collected on granary weevils, but not in the strains from drugstore beetles.

(b) Fecundity

Analysis of fecundity data from experiments with drugstore beetles as host using Kruskal–Wallis ANOVA revealed significant differences between the strains, caused by strain SATgw having superior fecundity relative to all strains, with the exception of STUdb (figure 3*a*). Apart from that, the number of offspring was similar in all strains. In contrast, the fecundity on granary weevils was very low in RAVdb and STUdb, intermediate in PFOgw and high in SLOgw and SATgw (figure 3*b*). When comparing the fecundity between strains after providing them with different hosts, RAVdb and STUdb had a higher fecundity on drugstore beetles when compared with granary weevils (RAVdb: $U = 19.5$, $p < 0.001$; STUdb: $U = 21.5$, $p < 0.001$). PFOgw and SATgw showed no differences between the two hosts (PFOgw: $U = 169.5$, $p < 0.963$; SATgw: $U = 159.5$, $p < 0.937$), and SLOgw had a higher fecundity on granary weevils when compared with drugstore beetles ($U = 41.0$, $p < 0.002$). Thus, the two strains collected on drugstore beetles performed well only on drugstore beetles, whereas strains from granary weevils were indifferent or even showed a higher fecundity on granary weevils.

(c) Molecular analysis

Phylogenetic analysis of mitochondrial and nuclear DNA revealed a clear separation of the strains into two clades (figure 4), supported by high bootstrap values and corresponding to hosts and habitats in which the strains were collected. RAVdb, STUdb, BIRdb and WAGdb belonged to a drugstore beetle group collected in private homes and PFOgw, SLOgw, SATgw, SACgw and BYGgw belonged to a granary weevil group from grain stores. The molecular divergence between these two groups was remarkably high for COI (median of uncorrected *p*-distances: 0.138; minimum: 0.125; maximum: 0.141; $n = 17$) in contrast to the low genetic differences within both groups (median within drugstore beetle group: 0.006; minimum: 0.000; maximum: 0.028; $n = 7$); median within granary weevil group: 0.022; minimum: 0.000; maximum: 0.028; $n = 10$; electronic supplementary material, figure S6).

4. Discussion

(a) The role of early learning for host differentiation in *Lariophagus distinguendus*

To evaluate the hypothesis that early learning enables the switch between hosts during ecological speciation in insects, we studied the host preference, the fecundity and the relatedness of different strains of *L. distinguendus* from two different hosts and habitats. Molecular analysis revealed that our strains can be divided into two distinct lineages that differ in their ecology, one parasitizing drugstore beetles in households, the other

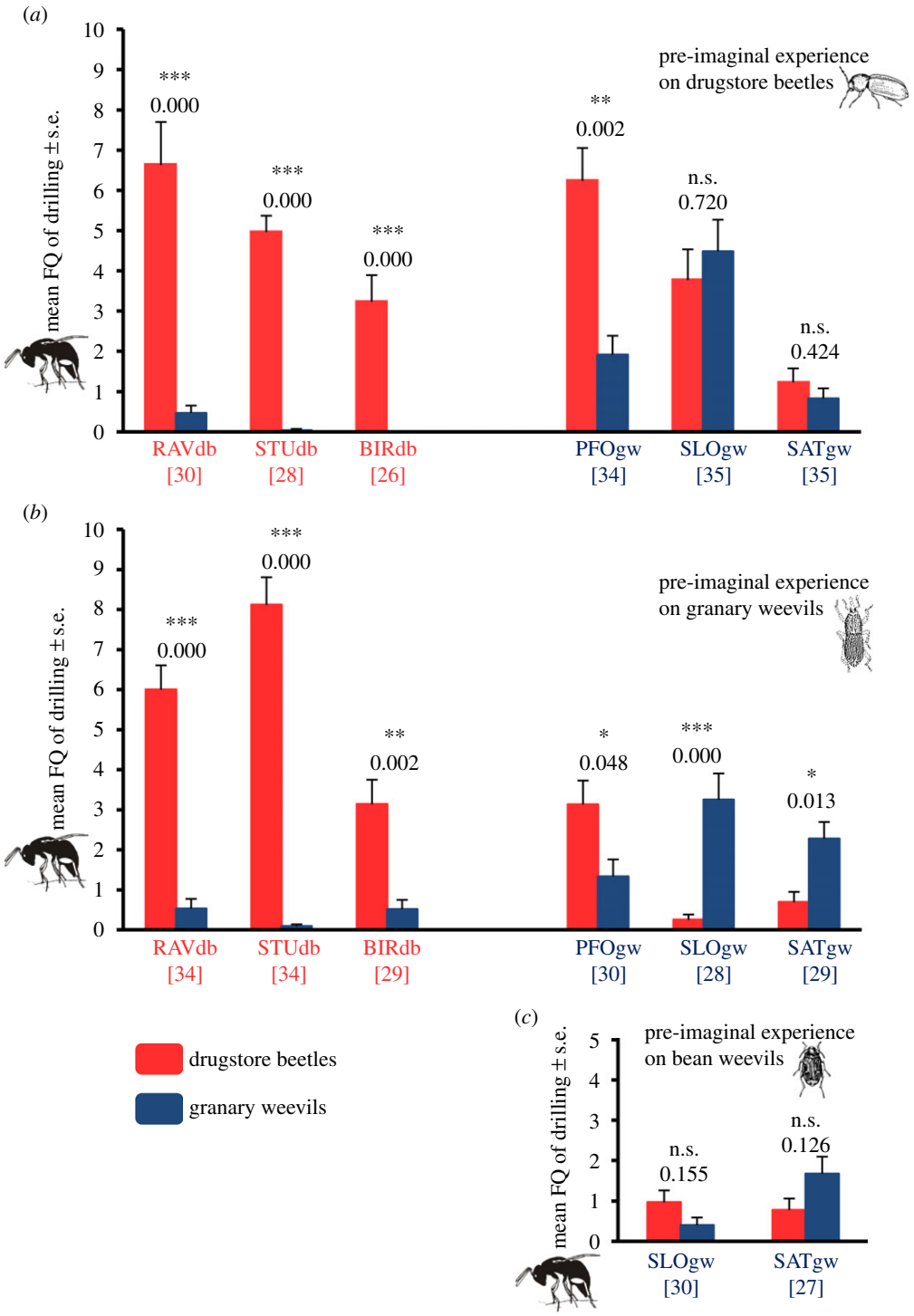

Figure 1. Mean frequency (FQ) of drilling behaviour in 10 min observation time of female wasps from different strains of *L. distinguendus* with different pre-imaginal experience: (*a*) development on drugstore beetles, (*b*) development on granary weevils and (*c*) development on bean weevils. Light grey bars (red online): grains infested by drugstore beetles. Dark grey bars (blue online): grains infested by granary weevils. RAVdb, STUdb, BIRdb, PFOgw, SLOgw, SATgw: different strains of *L. distinguendus*. Numbers in brackets refer to the number of replicates. Means were compared using the Wilcoxon matched pairs test (n.s., not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (Online version in colour.)

one granary weevils in grain stores. Strains from drugstore beetles have a fixed innate preference for this host that cannot be altered by experience. In addition, these strains only perform well on drugstore beetles, but have a very low number of offspring on granary weevils. In contrast, the strains collected from granary weevils change their preference according to pre-imaginal and/or early imaginal experience, by learning to accept or even to prefer one or the other host. Their fecundity on drugstore beetles is comparable to that of the other strains, but they are able to develop on granary weevils as well as or even better than on drugstore beetles. Thus, the ability for early learning in the studied strains of *L. distinguendus* is associated with host and habitat differentiation.

Because all strains have a high fecundity on drugstore beetles and one strain collected from granary weevils has an innate preference for drugstore beetles, the latter were most likely the hosts of the common ancestor of both lineages (figure 5). Therefore, a host switch must have occurred in the ancestor of the strains collected on granary weevils. While such a switch is difficult to conceive in wasps with a fixed,

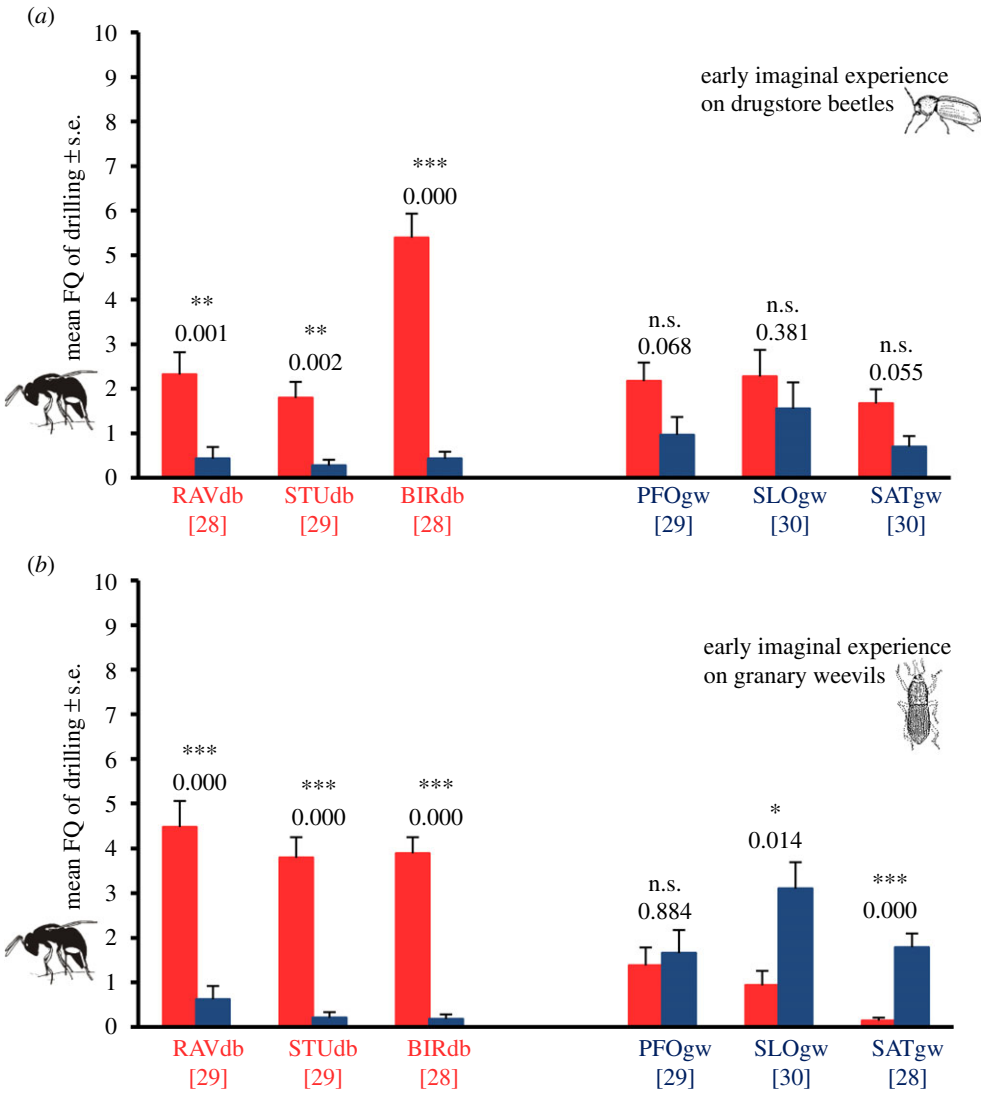

Figure 2. Mean frequency (FQ) of drilling behaviour in 10 min observation time of female wasps from different strains of *L. distinguendus* with different early imaginal experience: (*a*) 24 h experience on grain material formerly infested by drugstore beetles, (*b*) 24 h experience on grain material formerly infested by granary weevils. Light grey bars (red online): grains infested by drugstore beetles. Dark grey bars (blue online): grains infested by granary weevils. RAVdb, STUdb, BIRdb, PFOgw, SLOgw, SATgw: different strains of *L. distinguendus*. Numbers in brackets refer to the number of replicates. Means were compared using the Wilcoxon matched pairs test (n.s., not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (Online version in colour.)

innate preference for drugstore beetles, it can be easily explained by the ability for pre-imaginal and/or early imaginal learning, as demonstrated in the strains collected on granary weevils. It remains unclear whether the ability for early imaginal learning was present in the ancestor of the two lineages and was lost in the drugstore beetle lineage, or if it did emerge only in the ancestor of the granary weevil group. In any case, the ability for early learning would have resulted in an increased exposure to granary weevils, leading to an improved adaptation and increased fecundity on the new host, and to a loss of the innate preference for drugstore beetles.

Although our molecular data clearly show that the PFOgw strain belongs to the granary weevil group, it seems to have a transitional position. It has retained the probably plesiomorphic innate preference for drugstore beetles, but its host preference is influenced by early imaginal learning and it has an improved ability to use granary weevils as host, the species on which it was collected in the field.

Our molecular analysis revealed a very high uncorrected pairwise distance between the two lineages in COI of 0.138. For comparison, the thresholds for the delimitation of putative species in DNA barcoding of insects, which uses the same gene region, are usually set at 0.02 or 0.03 uncorrected *p*-distance [48,49]. Furthermore, we also found consistent differences between the two lineages in all the nuclear markers. Although mating experiments are required to demonstrate reproductive isolation, this suggests that the two lineages are in fact two biological species, with no or only very little genetic exchange.

In agreement with our hypothesis, these data strongly suggest that a host switch from drugstore beetles to granary weevils occurred within *L. distinguendus*, which was enabled by the ability for early learning. Given that *L. distinguendus* is monandrous and females generally mate on the host substrate directly after emergence, often with their own siblings [50], we are tempted to assume that the ecological divergence in two populations with different hosts resulted in increased separation, reproductive isolation and finally speciation within *L. distinguendus*.

We are currently testing alternative mechanisms of reproductive isolation, for example hybrid breakdown based on genetic differences [51], cytoplasmic incompatibility caused by *Wolbachia* infection [52] and sexual isolation owing to different pheromones [53]. Remarkably, a recent study revealed that cuticular hydrocarbons, which are used as mate recognition

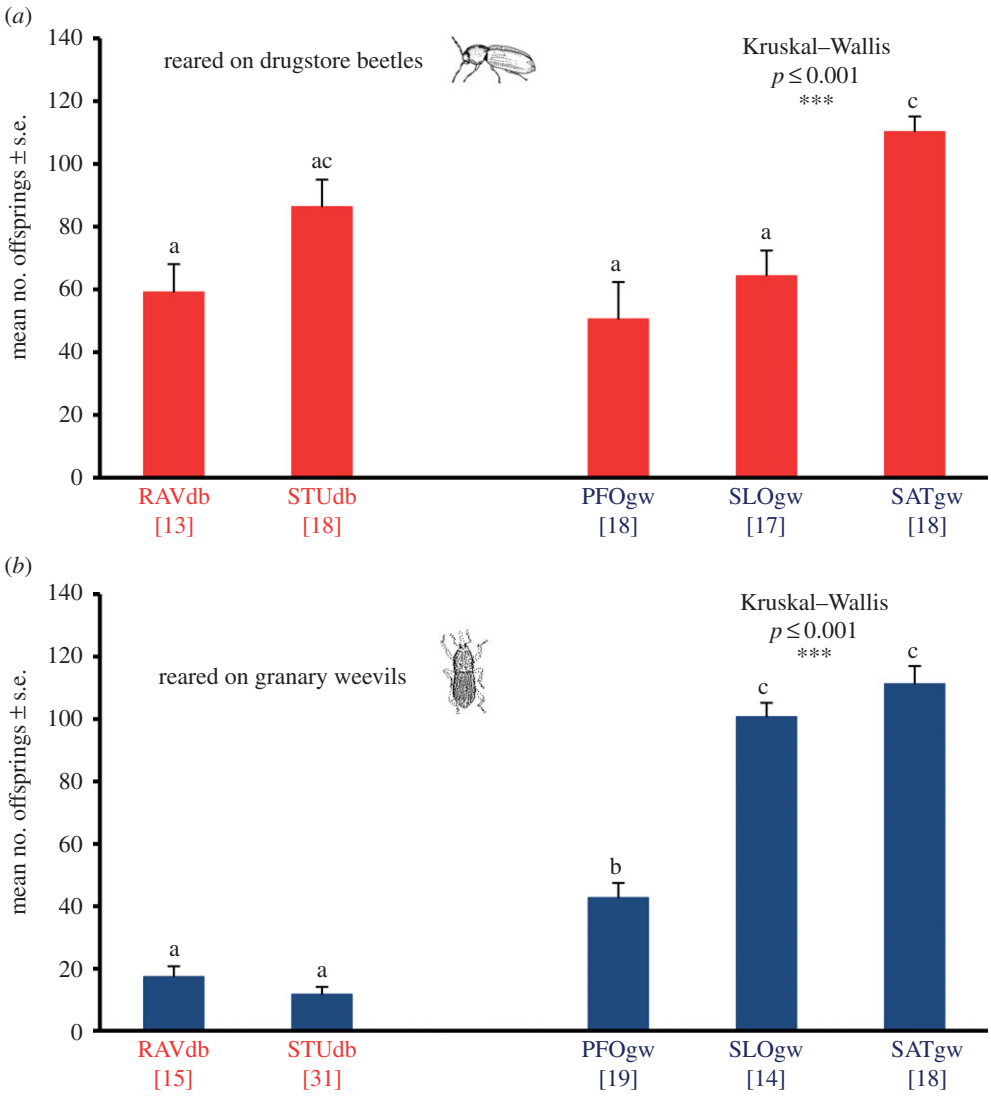

Figure 3. Mean fecundity of female wasps from different strains of *L. distinguendus* on different hosts: (*a*) drugstore beetles as hosts, (*b*) granary weevils as hosts. RAVdb, STUdb, BIRdb, PFOgw, SLOgw, SATgw: different strains of *L. distinguendus*. Numbers in brackets refer to the number of replicates. Different letters indicate a significant difference at the 0.05 level using pairwise Mann–Whitney *U*-tests followed by sequential Bonferroni correction for multiple comparisons. (Online version in colour.)

pheromones in *L. distinguendus*, change within one generation after a host switch [54]. Together with early learning and on-host mating, this could further promote reproductive isolation of wasps from different hosts. In addition, we are studying which of the two species agrees with the type specimen of *L. distinguendus* and which has to be assigned a new name or one of the former synonyms [55].

(b) Hopkins' host selection principle and neo-Hopkins host selection principle

Our study sheds new light on the processes of early learning in insects. Based on the observation by Hopkins [56], that adult insects choose for oviposition the host on which they had developed, the 'Hopkins' host selection principle' was established. It states that a memory for chemical cues learned during development can be transferred through the pupal stage, affecting the host choice behaviour of the adult insect [22]. Alternative to this pre-imaginal learning, the phenomenon described by Hopkins can also be explained by early imaginal learning of chemical cues that are present at the emergence site of the adult insect [57]. This process was termed 'neo-Hopkins host selection principle' by Jaenike [58]. In addition, Corbet suggested the 'chemical legacy hypothesis', proposing that traces of chemical cues that have been transferred from the larva could be learned by the early adult [59]. Convincing demonstrations of Hopkins' host selection principle were first presented with odour-shock conditioning in *Drosophila* [60], and by the transfer of neural cells from trained to untrained house fly larvae, which stimulated odour preferences in recipient adult flies that reflected the training of the donor larvae [61]. For parasitoid wasps, the Hopkins' host selection principle was first demonstrated by Gandolfi *et al.* [62]. Our study revealed that pre-imaginal learning according to the Hopkins' host selection principle is present in the strains SLOgw and SATgw of *L. distinguendus*, whereas early imaginal learning according to the neo-Hopkins host selection principle occurs in all three strains from the granary weevil lineage (PFOgw, SLOgw, SATgw). Obviously, both forms of learning represent separate processes that evolve independently, but can also be present in the same species.

(c) Implications of early learning for classical biological control

The finding that a host switch in insects is associated with the ability for early learning is important from an applied point of view. In classical biological control, exotic pests are

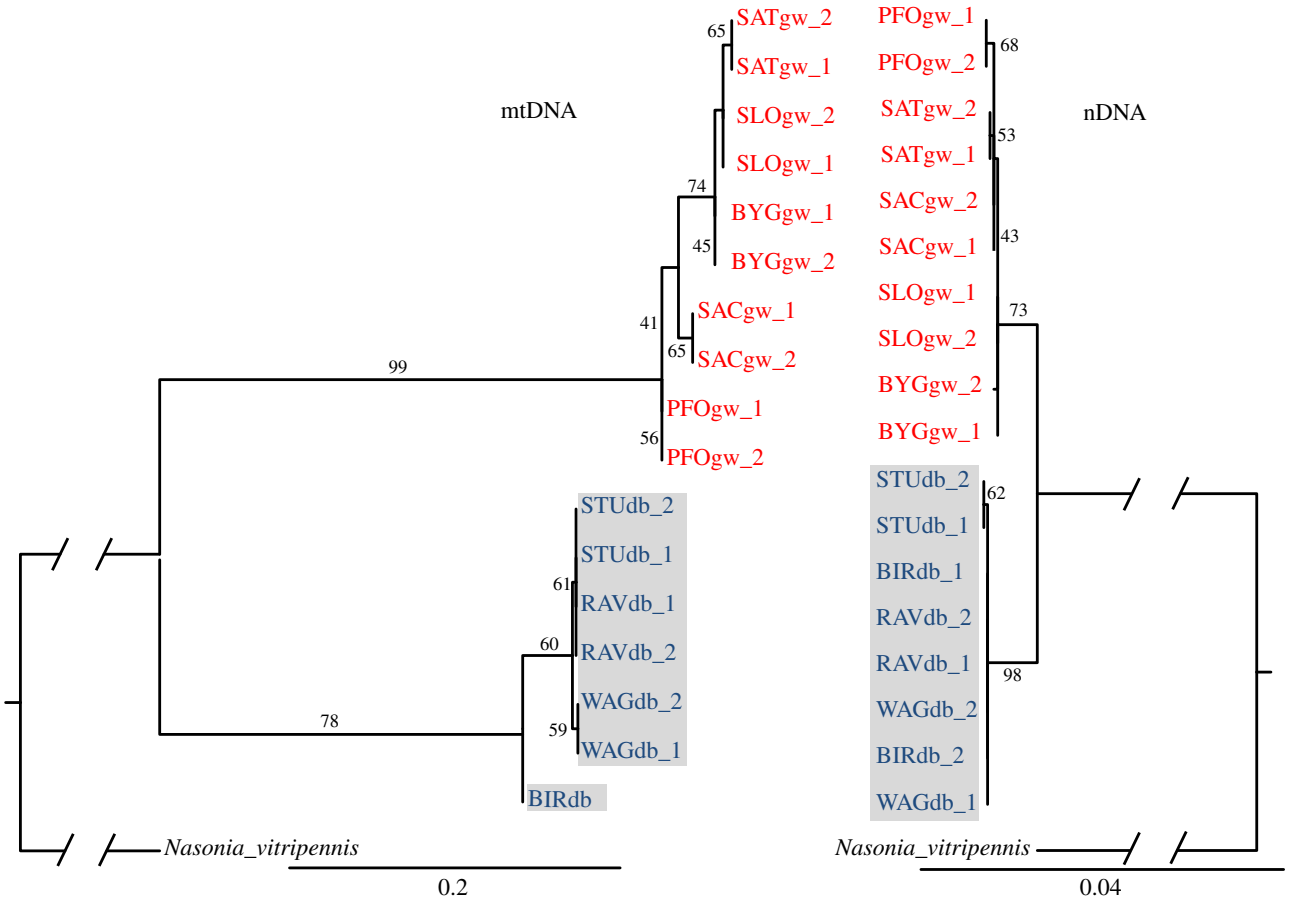

Figure 4. Maximum-likelihood phylogenetic relationships of strains of *Lariophagus distinguendus* inferred from analyses of the partial mitochondrial gene COI (mtDNA) and combined sequences from the nuclear markers CAD, LOC100123206, LOC100123909, LOC100117339 and ITS2 (nDNA). COI was partitioned in first/second and third codon positions (electronic supplementary material, table S5). The nDNA dataset was partitioned into first/second codon positions (p1) and third codon positions (p2) when referring to exonic nucleotides, the intronic nucleotides of CAD and the LOC markers (p3), and ITS2 (p4; electronic supplementary material, table S5). Branch support values are given in percentages for maximum-likelihood bootstrapping (1000 bootstrap replicates). Light grey (red online): strains collected from drugstore beetles in private homes. Dark grey (blue online) with grey shading: strains collected from granary weevils in grain stores. (Online version in colour.)

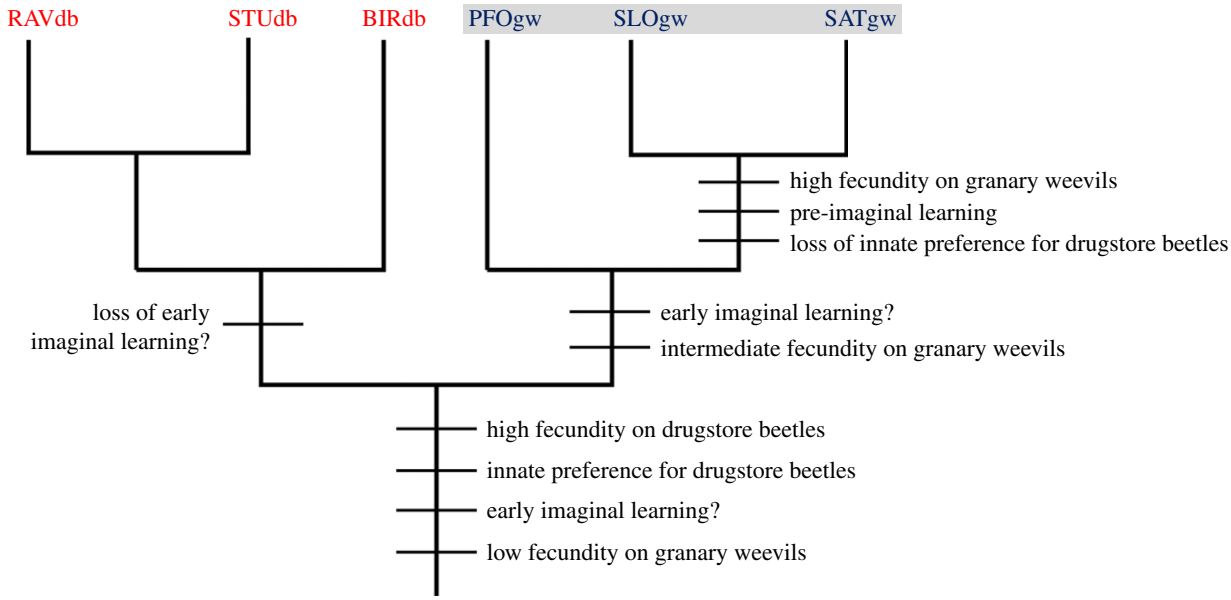

Figure 5. Hypothetical scenario of character evolution in *L. distinguendus*. Light grey (red online): strains collected from drugstore beetles in private homes. Dark grey (blue online) with grey shading: strains collected from granary weevils in grain stores. (Online version in colour.)

controlled by introducing their co-evolved natural antagonists from the same place of origin [63]. It is crucial to introduce only those natural enemies that are not expected to switch to non-target organisms [64]. Our results indicate that studies on early learning ability should be integrated in risk assessments of exotic natural enemies used in classical

biological control, as it could facilitate the switch to non-target, native host species.

5. Conclusion

To the best of our knowledge, our study is the first experimental support in insects for the long-standing hypothesis that early learning can be a first step for ecological divergence as pre-condition for ecological speciation. Using mathematical models, it has been demonstrated that speciation through learning of habitat features is possible in theory [65]. However, empirical data supporting this hypothesis exist only for parasitic African indigobirds. In these finches, nestlings are reared by different host species and acquire the songs of their respective host by imprinting. As a consequence, male indigobirds mimic host songs and females use these songs to choose their mates and the nests they parasitize. This provides a mechanism for reproductive isolation after colonization of a new host [66–68]. Interestingly, the induction of host and habitat preferences seems to be widespread in herbivorous and parasitoid insects [7,69], which represent the most species-rich insect groups. Therefore, the induction of new ecological preferences by early learning, leading to a switch to a new niche within one generation, might well be a general mechanism in speciation, helping to explain the huge diversity of insects. To further address this hypothesis, behavioural and molecular studies with different host races and/or closely related species of herbivorous and parasitoid insects with different ecology are required.

Data accessibility. DNA sequences: Genbank accessions KJ867375–KJ867409 and KJ923849–KJ923923. For details, see the electronic supplementary material, table S4. Original data of experiments on preference and fecundity: see electronic supplementary material, tables S7 and S8.

Acknowledgements. We thank Lise Hansen (Department of IPM, Aarhus University, Danmark), Steffi Niedermayer (Department of Animal Ecology, University of Hohenheim, Germany), Sabine Prozell and Matthias Schöller (BiP GmbH, Berlin, Germany) and Hans Smid (Laboratory of Entomology, Wageningen University, The Netherlands) for providing us with the *L. distinguendus* strains BYGdb, PFOgw, SATgw, WAGdb, and Natalie Dale-Skey Papilloud (Natural History Museum London, UK) for the loan of material. We greatly appreciate the help of Matthias Tisler (Department of Zoology, University of Hohenheim, Germany) in preparing the figures, and of the whole animal ecology working group of the University of Hohenheim for good advice and proofreading. Joachim Ruther (Department of Chemical Ecology, University Regensburg, Germany) kindly improved an earlier version of the manuscript and Elsa Obrecht (Natural History Museum Bern, Switzerland) revised the English of the manuscript. We thank three unknown reviewers for their careful reading as well as their helpful suggestions and comments.

Funding statement. Kerstin König was financially supported by the Landesgraduiertenförderung Baden-Württemberg.

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