The interplay between genetic and learned components of behavioural traits: olfactory responses of predatory mites to signals contained in a herbivore-induced plant volatile

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Genetic variation in learning abilities may mask population-level effects of experience

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Abstract – Effects of learning on individual phenotypes are common, but their role in evolutionary ecology is only starting to be appreciated. In this study we address the question whether there is genetic variation in learning ability and how this affects population-level responses, using iso-female lines of the predatory mite *Phytoseiulus persimilis*. We tested the responses of female predators to methyl salicylate (MeSa) – a volatile compound produced by plants if infested by phytophagous spider mites, the prey of *P. persimilis*. Responses were measured before and after being exposed to MeSa in the presence or absence of food and any change in response to MeSa was taken as an indicator of learning ability. We found variation among isofemale lines in their change in response to MeSa, thus showing genetic variation for learning ability. However, using the base population the same experiments did not show any effect of experience on the response to MeSa. Together, our findings imply that the magnitude of learning as studied in a population as a whole may be underestimated due to genetically determined variation in learning abilities of the individuals constituting the population.

Key words: methyl salicylate, I-tube olfactometer, phenotypic plasticity, *Phytoseiulus persimilis*, preference

In ecological studies it is common practice to study learning behavior by assessing the mean change in response of a group of individuals as a consequence of experience (e.g., Lee and Bernays 1990; Raubenheimer and Tucker 1997; Dukas 1998; Dukas and Bernays 2000; Egas and Sabelis 2001; Mery and Kawecki 2002; Weiss and Papaj 2003). Such groups represent samples from a field population and may therefore harbor genetic variation for the trait under study. In physiological – and sometimes also ecological – studies, learning assays are done using one particular genotype to standardize the genetic background (Dubnau and Tully 1998). This prompts the question whether there is genetic variation in learning behavior and how this affects population-level responses.
If so, it is dangerous to infer learning abilities from experiments on genetically heterogeneous groups from a species. Knowledge on genetic variation for learning is of vital importance to understand population behavior on a longer time scale, because the genetic make-up of the population with respect to this trait may change due to natural selection.

In this article we present results of a study on learning in a predatory mite \textit{(Phytoseiulus persimilis} Athias-Henriot). Its haplodiploid reproductive system allows creating inbred iso-female lines by repeated mother-son or brother-sister-mating. In haplodiploids negative impacts of inbreeding are reduced because deleterious alleles are immediately expressed in the haploid males and therefore these are directly selected against (Crozier 1985; Werren 1993; Henter 2003; Charlesworth and Willies 2009; unless these alleles have female-limited effect; Saito 2001; Tien et al., submitted). In particular, we study variation between such lines in their behavioral response to a relevant odor, as well as the change in response using a learning assay involving either a reward or a penalty. The between-line variation in these responses is then compared with a separate series of responses at the level of the population from which the iso-female lines were obtained. In this way we assessed the consequences of averaging over genetically diverse individuals.

The odor selected for testing behavioral responses of the iso-female lines of \textit{P. persimilis} is methyl salicylate (MeSa). This odor compound is particularly relevant to the foraging behavior of this predatory mite because (1) it is released by many different species of plants in response to the herbivorous spider mite \textit{Tetranychus urticae}, the main prey of this predatory mite (van den Boom et al. 2004), and (2) it is used by the predatory mite as a cue to forage for this prey (de Boer and Dicke 2004). Moreover (3), there is genetic variation in the innate response to MeSa (Sznajder et al. 2010), as well as to the full blend of plant odors containing MeSa (Margolies et al. 1997; Jia et al. 2002).

To test for a change in behavior in response to experience, we associated exposure to MeSa with one day of feeding on eggs of \textit{T. urticae} as a reward, or with one day of food deprivation as a penalty. This protocol was used earlier by Drukker et al. (2000) in experiments to discriminate between different modes of learning (associative learning, sensitization, habituation). Our experiments are not designed to make such distinctions, but merely to detect differences in learning ability among iso-female lines. Any differences in learning ability revealed among the lines indicate the presence of genetic variation (David et al. 2005). We tested five inbred lines established from a single mated female that was selected randomly from the base population (originating from the field thus presumably genetically variable). The genetic variation within the lines was further reduced by repeated brother-sister mating prior to the onset of the experiments.
Therefore, our tests of predator response to experience measured among the lines were conducted against a relatively homogeneous genetic background (as compared to testing iso-female lines each established by different females). This provided a benefit of reducing the confounding effect of variation among the lines in other (genetically determined) traits that may have an influence on animal's behavioural response to experience. The downside of our approach is that the genetic variation in learning ability, from which we aimed to set apart distinct genotypes, might also be reduced (provided genetic variation in learning is present in the studied base population).

**Materials and Methods**

**Predatory mites**

The base population of *P. persimilis* was maintained in our laboratory (25 °C, 70% humidity, and continuous light) on a diet of two-spotted spider mites on detached leaves of lima bean. The population was founded from a sample of predators collected in 2002 at different locations throughout Sicily (Partinico, Scopella, Trappeto, Terrasini, Siculiana, Laghetto, Menfi, Trabia, Alcamo). Thus, it was presumably genetically variable, although local populations of *P. persimilis* are generally considered to be inbred under natural settings (Helle and Overmeer 1973). Inbred iso-female lines used in this experiment were obtained by randomly selecting a mated female from the base population. Subsequently five new iso-female lines were established from this line by randomly selecting five mated daughters of this female. Typically in haplodiploid arthropods iso-female lines are obtained through mating between the virgin female and her sons. However, *P. persimilis*, just like most species in the family of Phytoseiidae, is pseudo-arrhenotokous, i.e., sons and daughters arise from fertilized eggs and male zygotes become haploid due to paternal genome inactivation and elimination during embryogenesis (Schulten 1985). Hence, mating is a prerequisite for oviposition. For this reason we selected already mated females, and propagation of the lines occurs through brother-sister mating (full sibs). The bottleneck of randomly singling out one female to continue the line was applied in each of four subsequent generations. The lines were subject to these repeated bottlenecks in order to obtain genetically homogeneous strains that could be compared with the genetically variable base population. After this, each line was reared to large numbers for use in experiments. Thus the inbred lines used in the study originated from the same female (i.e., female A), as indicated by the first letter in the coded names of the lines (e.g., AAFA). Each iso-female line is named with one letter per generation in which the population experienced a bottleneck, with the letter indicating the founding mother. For instance, lines AAFA and AAFC share ancestry up to the third generation, and differ in the
fourth generation with respect to the mother who founded these lines (mothers A and C from grandmother F, etc.).

Tests of olfactory preference
Behavioral responses of the predators to MeSa were not tested in the commonly used Y-tube (Sabelis and van de Baan 1983; Janssen et al. 1997), but in a single straight glass tube (length 20 cm, φ 0.5 cm) with a small opening (2 mm) in the middle to introduce the predatory mites (hereafter referred to as the I-tube; Sznajder et al. 2010). Opposite the entrance hole, another opening (φ 0.5 cm) was present which was gauze-covered and served as an air-outlet. At the ends of the I-tube there were plastic trap vials (φ 30 mm, height 55 mm) to collect predators and these were connected to jars that either contained a capillary with MeSa or not. The traps were designed as an ‘easy-to-enter-yet-difficult-to-exit’ vial, and were provided with a water source (wet cotton wool). After purification by activated carbon filters, the air was flowing at 20 l/h through the jars, the vials and then through the arms of the glass tube, finally leaving the system through the opening in the middle of the I-tube. At release in the middle of the I-tube, a mite finds itself in air streams coming from right and left, i.e., one with MeSa and the other without. Subsequently, it can move left or right in the I-tube and ultimately enters one of the trap vials or remain in the I-tube. After release of the test animals, the entrance hole was sealed with Parafilm®. Pilot experiments showed that the I-tube olfactometer produces results consistent with our knowledge of the responses of *P. persimilis* to herbivore-induced plant volatiles (M van Wijk, pers. comm.).

Within a single replicate experiment a group of females was released sequentially into the I-tube, i.e., the individuals were released in the olfactometer one after the other. Visual cues play no role in predator orientation as the predator is blind and orients itself by means of chemical cues. Previous studies using a Y-tube set-up showed that there is no effect of possible residues deposited along the path taken by an individual on the choices of subsequent individuals (Sabelis and van de Baan 1983; van Wijk et al. 2010). Hence we considered the choices of individual mites in this set-up as independent measurements.

In total, 20-30 adult females were released per replicate experiment. After 25 minutes, the number of mites in each of the two trap vials was counted. The number that remained in the I-tube, was scored as ‘no choice’. For each consecutive replicate of a line, the arm containing air with MeSa was changed to exclude any unforeseen asymmetries in the experimental set-up. Per line per treatment, roughly 120 mites were tested in 4-8 replicate experiments (except for one case, where three replicates were performed).
Synthetic MeSa (Sigma-Aldrich Fluka, pure; assay ≥ 99%) was offered undiluted in a small capillary (9 μl, Ø 0.60 mm, Omnilabo) placed in one of the jars connected to the I-tube. The air flow was led through the set-up at least 2 h before the start of the test. The MeSa evaporation rate was c. 30 μg/h. This evaporation rate was chosen based on preliminary experiments to find a concentration to which the base population exhibited a neutral (50:50) unconditioned response. Unfortunately, this evaporation rate is hard to compare with previous studies attempting to measure the dose-response relation, because these assessments were based on filter paper as a substrate (de Boer and Dicke 2004). Unlike filter paper, capillaries generate a constant evaporation rate.

**Experimental design**

We tested for change in response to MeSa in the base population as well as five randomly chosen iso-female lines. Adult females from the base population and the lines were subjected to either of two treatments determining their experience with a reward or a penalty: 24 h feeding on eggs of *T. urticae* (hereafter called FED) in the presence of MeSa and 24 h food deprivation (STARVED) in the presence of MeSa. Shorter exposures are ineffective, according to Drukker et al. (2000). The experiment involved first a test to establish the innate response of well-fed females, and then a conditioned response test with starved or fed females. The conditioned response was tested immediately after the conditioning treatment. Under the FED regime the mites were kept on a wet piece of filter paper containing eggs of *T. urticae* (see below), whereas under the STARVED regime they were kept in the presence of MeSa on wet filter paper without prey eggs. All conditioning took place in vials identical in design to the trap vials of the I-tube using the exact same concentration of MeSa as in the tests.

Adult females used in all experiments were collected directly from the populations. Such samples contain adult individuals of various ages (i.e., very young ovipositing females as well as old females). We tested mixed-age groups of individuals to avoid any confounding effect of age on predator response to the volatile. To minimize any potential effects of the time of day on predator responses, replicates of all tests were randomized with respect to the time of day.

To obtain eggs for conditioning, spider-mite infested bean leaves were put in a 3-l jar with water provided with a droplet of mild detergent. The content of the jar was gently stirred, and then deposited on a pyramid of fine sieves. The eggs were collected on the finest sieve, where they were thoroughly rinsed to remove the detergent and the plant-derived odors. Finally, the eggs were washed off the sieve onto a piece of filter paper. This method of obtaining eggs has been used successfully by Drukker et al. (2000) to obtain neutral innate responses of the predatory mites in a Y-tube olfactometer.
Chapter 4

The set-up of these experiments allows us to compare the preference for MeSa before and after experiencing this odor in the presence or absence of food. This set-up does not allow us to discriminate between different kinds of learning, but this is not the aim of our study. Our actual aim is to measure whether there is genetic variation in effects of experience on preference for MeSa and how that affects the response at the population level. In fact, food deprivation alone could cause preference for MeSa to change, and hunger in general is known to be an important motivational factor in animal learning (Dethier 1976). Because our set-up does not control for effects of food deprivation alone, it is possible that changes in preference for MeSa in the STARVED regime are modulated by a hunger effect. This argument does not hold for the FED regime, since all measurements on innate preference are performed on well-fed mites.

Statistical analysis
We asked: (A) whether feeding under exposure to MeSa leads to increased preference for MeSa, and starvation under exposure to MeSa leads to avoidance of MeSa; (B) is there a variation in the response to these two types of conditioning among the inbred iso-female lines? and (C) is there a difference between the responses of the inbred iso-female lines and the base population (that is presumably genetically variable)? To address these hypotheses we constructed mixed-effects logistic regression where the data points consist of the choices of individual mites in the test MeSa versus clean air (see Tests of olfactory preference for arguments for treating the responses of individual mites in this experiment as independent). To address the first hypothesis (A) we included two fixed effects: line (six levels - inbred lines1-5 and the base population) and test (three levels - pre-conditioning, post-fed conditioning and post-starved conditioning). The effect of test contains three levels because there is no reason to expect that the predator response measured before starvation under exposure to MeSa is on average different from predator response measured before feeding under exposure to MeSa. The lines are treated as a fixed effect, although they are in fact a subset of genotypes randomly drawn from the base population. Therefore, the correct interpretation of a significant effect of line in this model is that the differences are found within this specific group of lines (but a different subset of iso-female lines may show a different effect). A random intercept was introduced with observations grouped by replicate (i.e., replicate is a random effect). R software was used for these analyses (package lme4).

Results
Prior to the conditioning treatment all lines (and base) exhibited, on average, avoidance to MeSa (Figure 4.1). There was a significant difference in predator responses between tests such that feeding under exposure to MeSa did indeed
increase preference for MeSa after the conditioning treatment \(P<0.001\), estimate (SE) = 0.67 (0.11)]. However we found that starvation in MeSa also increased predator preference for MeSa \(P = 0.002\), estimate (SE) = 0.32 (0.10)].

Exploring these unexpected results further we included an interaction term between line and test. This interaction term was highly significant both including \(P<0.0001\), log-likelihood ratio (df) = 42.5 (10)] and excluding base population as a factor level \(P<0.0001\), log-likelihood ratio (df) = 40.6 (8)]. Therefore this result supports the hypothesis (B) that there is variation in the response to the conditioning treatment among the iso-female lines.

**Figure 4.1** – Mean proportion of individuals choosing MeSa in the innate response test (open bars) and the conditioned response test (closed bars), in five iso-female lines and the base population. The conditioning consisted of feeding in the presence of MeSa (A), and food deprivation in the presence of MeSa (B). Numbers superimposed on the bars above the x-axis show the total numbers of individuals per line per treatment.
Next we examine the pairwise tests of the responses to the conditioning treatment within each line. Here, we found that both for feeding under exposure to MeSa and starvation under exposure to MeSa, there is one line (AAFA for feeding and AAC for starvation) whose response after conditioning was significantly different from its response prior to conditioning (for AAFA $P = 0.002$, estimate (SE) = 1.39 (0.44) and $P = 0.001$, estimate (SE) = 1.71 (0.53) for AAC). In both cases these lines show a strong preference for MeSa after conditioning (i.e., after feeding for AAFA and after starvation for AAC; Figure 4.1). Other lines showed similar responses, albeit not as strong (Figure 4.1). In contrast, the response of the base population after the conditioning was not different from its response prior to conditioning ($P = 0.35$, estimate (SE) = 0.9 (0.31) for feeding in MeSa and $P = 0.42$, estimate (SE) = 0.27 (0.33) for starvation in MeSa). This supports hypothesis (C) that there is a difference in the response to experience measured at the level of the lines versus the level of the base population. In particular, the effect of experience on animal behaviour is revealed when studied at the level of inbred iso-female lines but not at the level of the base population.

We conclude that the interaction between the effect of the test and the iso-female line indicates intraspecific genetic variation in learning abilities in *P. persimilis*.

**Discussion**

Our study showed that experience with MeSa – a signal of prey presence – does not modify the innate response of *P. persimilis* to this compound if measured as the average response of the base population. Had this been the only experiment to assess effects of experience, we would have concluded that learning plays little role in this behavior. However, we further showed that this apparent lack of an effect of experience can arise when individuals vary in their ability to learn: iso-female lines of *P. persimilis* originating from the base population do show significant effects of experience. In this study we only tested a small subset of all possible iso-female lines (and thus genotypes), which additionally all originated from a single female. This may explain why the innate response tests of the iso-female lines consisted mostly of females avoiding MeSa. Our study demonstrated that genetic variation in learning can be already found within a group of lines established from a single founder sampled from a natural population of *P. persimilis*. A larger subset of iso-female lines from the same population yields innate test responses varying from avoidance to preference (Sznajder et al. 2010). A different (larger) subset of iso-female lines might also show more variable responses to the conditioning treatment used in this experiment. All lines and the base population were reared under identical con-
ditions prior to the experiment and were subsequently subjected to the same treatment, so that the iso-female line is the only factor making a difference. We conclude, therefore, that the observed differences in the response of the iso-female lines to experience have a genetic basis.

Exploring the full array of genotypes determining learning abilities is beyond the scope of this article. In contrast to Drukker et al. (2000), we have not found evidence for associative learning. They tested the same predatory mite species, but from a different origin, and used intact odor blends from infested and uninfested plants, instead of a single odor compound. Such differences in experimental set-up may well explain the contrasting results, but should stimulate more research into mechanisms of learning in this predatory mite.

Although it was not our aim to distinguish different modes of learning, it is relevant to ask which of them may explain the observed changes in behavior. Overall, the change in the behavior of the iso-female lines amounted to an increase in the percentage of individuals choosing MeSa after the predators were fed in MeSa (particularly prominent in line AAFA) as well as starved in MeSa (particularly prominent in line AAC). An associative learning mechanism should result in a decreased preference when starved. Therefore, this mechanism can be ruled out in the tested subset of iso-female lines. This leaves non-associative mechanisms of learning such as, e.g., sensitization or habituation. Sensitization consists of an increased response to a variety of stimuli following an exposure to an intense or noxious, repeated stimulus (Kandel et al., 1993). Therefore this mechanism of learning should result in a significantly increased preference for MeSa when exposed long enough. For instance, the responses of line AAC in the STARVED regime (Figure 4.1B) may indicate sensitization to MeSa because conditioning resulted in significant preference of MeSa in a way that excludes habituation. Habituation result in an indifferent response to MeSa when exposed long enough (Kandel et al. 1993) and it may explain the behavior of the lines AAFC in the STARVED regime and line AACA in the FED regime. For these lines we observed an increase in the percentage of individuals choosing MeSa but of the magnitude that effectively amounts to an indifferent response.

This study provides evidence for the role of genetic factors in learning ability in the predatory mite *P. persimilis*. This in itself may not be surprising: genetic variation in learning and memory is often found when looked for, in many different study organisms (Dukas 2004). However, this finding has two important consequences. First, the ability to learn may simply not be detected if a population of various learning genotypes is examined collectively and their various responses cancel out in the overall effect. This conclusion is relevant for ecological and evolutionary studies of animal behavior that frequently use the effect of experience.
manifested in behavioral change as a measure of learning ability. Second, learning ability has attracted attention in evolutionary studies due to its potential to affect the rate of evolutionary change in phenotypic traits (Robinson and Dukas 1999). However, we caution to take into account the fact that learning ability is a trait in itself, subject to natural selection (see, e.g., Mery and Kawecki 2004). The effect of learning on individual phenotypes, and consequently on selection acting on a population of such phenotypes may depend on the amount of variation in learning ability. It is therefore crucial to control for genetic variation in learning ability when studying the effects of experience at the population level.

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References


Genetic variation in learning abilities