Strain differences rather than species differences contribute to variation in associative learning ability in *Nasonia*

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Insect species display a large range of inter- and intraspecific variation in learning and memory retention. Variation in associative learning ability has also been reported for three species in the parasitoid wasp genus *Nasonia*, most notably between *Nasonia vitripennis* and *Nasonia giraulti*, for which inbred isogenic strains have been established and studied intensively. We addressed the question how learning and memory of such isogenic strains compare to the phenotypes found in genetically diverse strains of these species. We recorded memory retention of both isogenic and genetically diverse strains of two species at 4–120 h after either olfactory or visual conditioning. Memory retention typically declined over time, but the pattern of decline differed consistently between strains. The isogenic *N. vitripennis* strain formed long-lasting (>5 days) memory, whereas the isogenic *N. giraulti* strain lost its memory after 48 h. Yet, genetically diverse strains of both species formed long-lasting memory. Memory retention patterns of strains were independent of sensory modality of the conditioned stimulus for all strains. These results show that there is variation for associative learning and memory within the two species, but not clear interspecies differences in memory retention. Without a better overview of the natural variation in learning abilities within a species, individual strains, especially isogenic strains with low genetic variability, are not necessarily representative of the species in general.

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The ability to adjust behaviour based on previous experience has vast adaptive potential, which explains why the ability to learn and memorize is such a common feature of life (Hollis & Guillette, 2015; Johnston, 1982). Yet, we observe huge natural variation in learning ability and memory dynamics, even between closely related species or populations of the same species (Healy, Bacon, Haggis, Harris, & Kelley, 2009; Mery, 2013). This variation has been hypothesized to reflect adaptation to different environments that vary in reliability of information (Dukas, 1998; Dunlap & Stephens, 2016; Smid & Vet, 2016). Insect species, in particular parasitic wasps, display a large range of variation in learning and memory retention, and have therefore been instrumental for studying ultimate and proximate factors involved in the evolution of learning abilities (Dukas, 2004; Mery, 2013). Over the last few decades of research in this field, a strong focus has been placed on identifying species level cognitive abilities, particularly in birds and mammals. This approach tends to ignore possible within-species variation, especially when observations are restricted to a few individuals or laboratory strains with little genetic variation (Boogert, Madden, Morand-Ferron, & Thornton, 2018; Thornton & Lukas, 2012).

Parasitic wasps of the genus *Nasonia* (Chalcidoidea: Pteromalidae) have become an evolutionary model system, providing opportunities to study both ecological and genetic factors involved in natural variation in learning and memory (Hoedjes, Smid, Vet, & Werren, 2014; Kraaijeveld et al., 2018; Lynch, 2015; Werren et al., 2010). The genus encompasses four species of small gregarious wasps that parasitize fly pupae, with partly overlapping distributions and host ranges (Darling & Werren, 1990; Raychoudhury et al., 2010; Whiting, 1967). Both olfactory and visual learning have been

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demonstrated in *Nasonia vitripennis* (Liefting, Hoedjes, Le Lann, Smid, & Ellers, 2018; Oliai & King, 2000), whereas only olfactory learning has been studied in the other species (Hoedjes, Steidle, Werren, Vet, & Smid, 2012; Hoedjes & Smid, 2014). These studies revealed distinct interspecific variation in olfactory learning: *N. vitripennis* and *N. longicornis* formed transcription-dependent long-term memory that lasted at least 5 days after single conditioning, whereas memory of *N. giraulti* females declined sharply within 2 days (Hoedjes et al., 2012; Hoedjes & Smid, 2014). Apparently, a single conditioning trial is not enough to form long-lasting memory in these *N. giraulti* wasps. These differences in memory retention have been tentatively linked to ecological differences between the species (Hoedjes et al., 2012; Smid & Vet, 2016).

Much of our knowledge about differences in memory formation in these parasitoid species comes from highly inbred strains with little or no genetic variation. The use of (near-)isogenic strains to study complex phenotypes is advantageous in genetic analyses because phenotypic variation is reduced (see e.g. Velthuis, Yang, Van Opijnen, & Werren, 2005), but a drawback is that each individual isogenic strain represents only a very limited subset of the existing phenotypes in the natural population (Harvey, Malcicka, & Ellers, 2015; Pischedda, Shahandeh, Cochrane, Cochrane, & Turner, 2014; van Grunsven & Liefting, 2015). Therefore, caution is needed in using isogenic strains to study a trait for which substantial variation is expected to be present in a population, as for example for memory formation in *N. vitripennis*. In this species, the existence of genetic variation for associative learning ability was revealed through artificial selection (Liefting et al., 2018). Also, in an isogenic strain of this species stronger olfactory memory retention was demonstrated compared to two genetically diverse strains (Hoedjes et al., 2012; Hoedjes & Smid, 2014; Koppik, Hoffmeister, Brunkhorst, Kieß, & Thiel, 2015). To maximize insights gained from studies using isogenic or genetically diverse strains, it is important to better understand how their phenotypes compare.

In this study we explored variation in associative learning ability of visual and olfactory cues between isogenic and genetically diverse strains of *N. vitripennis* and *N. giraulti*. We addressed the question how the memory dynamics of the widely used isogenic strains of these species compare to the phenotypes found in genetically diverse strains. Also, by comparing memory formation of all strains after conditioning on olfactory and visual stimuli, we gained insight into correlative patterns of learning and memory formation across stimulus modalities. We included the results from a previous study on the memory formation of two isogenic strains, and collected new data on the memory formation after visual learning in the same isogenic strains, as well as on memory formation of genetically diverse strains after both visual and olfactory learning. This way, memory formation over time could be compared for all strains per conditioned stimulus. The 24–48 h time frame was considered in more detail because this is where the previously described difference in long-term memory formation between two isogenic strains of *N. vitripennis* and *N. giraulti* are evident. This time frame also coincides with a transition period between two intermediate transient memory phases (Schurmann et al., 2012).

**METHODS**

Wasps of the genus *Nasonia* are gregarious and lay eggs in dipteran pupae (Whiting, 1967). We compared four different laboratory strains of two species: for *N. vitripennis* the isogenic AsymCX strain and the genetically diverse HVRx strain, and for *N. giraulti* the isogenic RV2x(U) strain and the genetically diverse NGmix strain. AsymCX (hereafter *Ng iso*) originated from a laboratory strain (Labil) which was further inbred resulting in very low genetic variability (Werren et al., 2010), RV2x(U) (hereafter *Ng iso*) originated from the RV2 isofemale strain and is considered to be effectively isogenic after further inbreeding (Werren et al., 2010). HVRx (hereafter *Ng div*) is an outbred strain from five different *N. vitripennis* outbred lines that each originated from multiple wild foundresses, kept under conditions to maintain a high genetic variability (van de Zande et al., 2014). NGmix (hereafter *Ng div*) is a genetically diverse, outbred laboratory strain from five *N. giraulti* strains (Giesbers, 2016). All strains were reared on host pupae of the blowfly *Calliphora vomitoria* (acquired as maggots from Kreikamp B.V., Hoevelaken, The Netherlands). Mated female wasps were collected on the day of emergence and were provided with honey and water ad libitum until conditioning.

**Conditioning**

During conditioning, 1-day-old female wasps associated either an odour or a colour (conditioned stimulus, CS+) with a host reward (*C. vomitoria* pupae). See Fig. 1 for a schematic overview of the procedure and an overview of the factorial design. For colour conditioning, the wasps were conditioned in groups by introducing 40 females to 40 host pupae in a Petri dish placed on either blue or yellow paper. The females were allowed to probe and host-feed for 1 h; inactive females were removed within the first 15 min. After 1 h of conditioning, the females were transferred to a clean Petri dish for 15 min of rest without any hosts or stimuli present and then another 15 min exposure to the nonrewarding, reciprocal colour (CS−). For odour conditioning (1 h) a 5 µl drop of chocolate or vanilla extract on filter paper was used, followed by the same rest period and exposure to the reciprocal odour (see Hoedjes et al., 2014 for further details). Olfactory memory retention of the isogenic strains has been recorded previously (Hoedjes et al., 2012) and these data were reanalysed here for comparative purposes. The conditioning procedures were highly comparable except that wasps were conditioned individually instead of multiple wasps per Petri dish. This change in conditioning protocol has been thoroughly assessed and no difference in the responses of wasps conditioned individually or in groups could be detected (see Supplementary material of Hoedjes et al., 2014).

**Memory Retention**

Memory retention of groups of wasps was recorded once at either 4, 24, 48, 72, 96 or 120 h after conditioning in either a T-maze with coloured paper on either arm or an olfactometer with an airflow carrying odours down either arm (Hoedjes et al., 2012; Liefting et al., 2018). Groups of 10 wasps were released in the visual T-maze and 10–12 wasps in the larger olfactometer. The distribution of wasps in the two arms was determined after 3 and 10 min, respectively (Fig. 1). Indecisive wasps that remained in the centre of the T-maze were excluded from the analysis. The trial was dismissed if more than four wasps were indecisive, which rarely happened. Distributions were always assessed simultaneously in two T-mazes for the two colours, while response to the odours was recorded in direct succession.

We use a performance index (PI) as a measure of memory retention (Hoedjes et al., 2012; Liefting et al., 2018). A PI is calculated based on the percentage of correct choices in the visual T-maze or olfactometer, minus 50%, which is the expected distribution in the absence of learning. For example, if 85% of the wasps conditioned on blue move to blue, and in a simultaneous recording 65% of wasps conditioned on yellow choose yellow, the PI in this case is (85 − 50) + (65 − 50) = 50%. Hence, the PI value can range from −100 (perfect avoidance) to 100 (perfect preference) with a
value of 0 indicating no learned associations. For odour learning 10 PIs per period per strain were recorded and for visual learning $10^23$ PIs per period per strain.

The absolute PI value, although correcting for innate preferences, can be sensitive to factors that may influence a wasp’s motivation but cannot easily be standardized, such as host quality during conditioning or seasonal variation in for example air pressure. Therefore, comparing absolute PI values obtained at different time points should always be done with caution. Likewise, two types of T-mazes were used to measure memory retention of two very differently perceived stimuli, making the PI in part modality specific. To minimize these effects, measurements in the olfactory and visual T-mazes were always performed in the same laboratories and with the same devices. Also, at least two strains were always measured simultaneously in one series of T-maze tests.

### Statistical Analysis

The memory retention over time for each of the four strains was analysed for olfactory and visual learning separately. These linear models included PI as the dependent variable and strain ($N_v$ iso, $N_v$ div, $N_g$ iso, $N_g$ div) and time ($4$, $24$, $48$, $72$, $120$ h) after conditioning as (continuous) fixed factors, including the interaction. Differences between the strains were analysed in a contrast analysis per time point (using the emmeans package in R), including a Tukey correction for multiple measurements. The $24$–$48$ h time frame is of specific interest because this is where the memory patterns between the two isogenic strains start to deviate and this time period also coincides with a transition between two transient middle-term memory phases. Therefore, the memory response of the four strains within this time frame was also analysed in a separate model that enables the interpretation of the interaction term of that specific time period. The $24$–$48$ h subset was analysed per stimulus modality of the CS$^+$ with a linear model with PI as the dependent variable and strain ($N_v$ iso, $N_v$ div, $N_g$ iso, $N_g$ div) and time ($24$ and $48$ h) after conditioning as (continuous) fixed factors, including the interaction. Estimated effects of all factors included in the model are presented in the Results but note that when an interaction is significant, the estimated effects of the factors included in the interaction cannot be properly interpreted.

To explore correlative patterns in olfactory and visual memory retention we also compared the memory retention of both modalities per strain. Memory retention over time after olfactory and visual learning was analysed per strain with a linear model with PI as the dependent variable and modality of the CS$^+$ and time as (continuous) fixed factors, including the interaction.

All analyses were performed in R 3.5.3 (R Core Team, 2019) and homoscedasticity and normality were visually assessed.

### Ethical Note

All experimental work reported here abides to the principles endorsed by the ASAB/ABS Guidelines for the Use of Animals in Research. After experiments, wasps were directly killed by placing them in a freezer. We took care not to breed exceedingly high numbers of wasps or hosts by default and experiments were designed to meet the desired number of measurements for statistical analyses without requiring a disproportionately large number of wasps.
RESULTS

Strain Differences in Olfactory Memory Retention

There was a significant effect of strain on memory retention after olfactory conditioning ($F_{3, 232} = 11.5, P < 0.0001$; Fig. 2a), an effect of time ($F_{1, 232} = 34.8, P < 0.0001$) and an interaction effect between strain and time ($F_{3, 232} = 3.9, P = 0.01$). A contrast analysis including a Tukey correction revealed how the individual memory retention differed between the strains per time period (see Supplementary Material). The significance groups are indicated with letters in Fig. 2a.

The data for the 24–48 h period, which is of particular interest (see Methods), were analysed with a similar linear model (see box in Fig. 2a indicating the time frame). Over this period, there was no effect of strain ($F_{3, 72} = 1.5, P = 0.217$) or of time ($F_{1, 72} = 0.4, P = 0.553$), but there was an interaction effect indicating that the strains responded differently over time ($F_{3, 72} = 3.8, P = 0.013$).

Figure 2. Memory retention of the four Nasonia strains expressed as a performance index ($PI ± SE$) at different time points (4–120 h) after single conditioning with either (a) an odour or (b) a colour as the conditioned stimulus (CS+). A PI of 0 indicates an absence of the learned preference; a PI of 100 indicates a perfect learned preference. Different letters indicate differences based on contrast analyses (with Tukey correction, $P < 0.05$) between the four strains at each time point. The 24–48 h time frame (indicated with a grey box) was analysed separately.
Strain Differences in Visual Memory Retention

For the visual memory retention (Fig. 2b), the results of the overall linear model and the linear model on the 24–48 h time frame are similar to the olfactory memory retention. There was a significant effect of strain on memory retention (F3, 298 = 4.2, P = 0.006), an effect of time (F1, 298 = 19.6, P < 0.0001) and an interaction effect between strain and time (F3, 298 = 3.9, P = 0.024). For the 24–48 h time frame, there was no effect of strain (F3, 88 = 2.7, P = 0.051) or of time (F1, 88 = 1.4, P = 0.247), but there was an interaction effect (F3, 88 = 2.9, P = 0.041). The differences between the strains per time period based on a contrast analysis including a Tukey correction (see Supplementary Material) are presented in Fig. 2b with letters.

Correlative Patterns Between Olfactory and Visual Learning

To test whether memory retention over time shows consistent patterns per strain for both olfactory and visual learning, we ran four separate linear models for the four strains (see Fig. 3). As mentioned before, mean PI values based on learned associations of stimuli of different modalities cannot necessarily be directly compared. However, an interaction effect would be a strong indication that the way memory retention decays over time is dependent on the modality of the conditioned stimulus. The results of the four models for each strain are summarized in Table 1. For all strains, time was the most important factor in explaining the observed variation. For two strains, i.e. the *N. vitripennis* isogenic strain and the *N. giraulti* diverse strain, there was also an effect of

![Graphs](image-url)

Figure 3. Memory retention expressed as a performance index (PI ± SE) at different time points (4–120 h) for (a) *Nv iso*, (b) *Ng iso*, (c) *Nv div* and (d) *Ng div*, organized per strain for the response to olfactory and visual learning.
the modality of the Cs+). However, there was no interaction effect for either strain, indicating that memory retention typically deteriorates over time in a similar fashion for olfactory and visual learning within a strain.

**Discussion**

In this study, we investigated differences in olfactory and visual learning between two isogenic and two genetically diverse strains of the parasitoid wasp species *N. vitripennis* and *N. giraulti*. Memory retention declined over time for all strains, but the pattern of decline differed between strains (Fig. 2). The two isogenic strains of both species stand out for different reasons. The isogenic *N. vitripennis* strain appeared to learn olfactory stimuli exceptionally well and the memory retention after a single conditioning remained relatively high over time. Ps measured at different time points should be compared conservatively, but a similar high performance of this particular strain (AsymCx) has been reported in other studies as well (Hoedjes & Smid, 2014; Koppik et al., 2015). In a study that explored differences in learning ability of *N. vitripennis* strains, the AsymCx strain repeatedly demonstrated higher learning performance in comparison to the genetically diverse HVRx and ‘Hamburg’ strain (Koppik et al., 2015). The isogenic *N. giraulti* did not form long-term memory as demonstrated by the lack of memory retention after 48 h and this was independent of the modality of the conditioned stimulus. These striking differences in memory have previously been interpreted as being species specific (Hoedjes & Smid, 2012). However, the two diverse strains (*Nv div* and *Ng div*) both formed memory lasting longer than 48 h (i.e., middle-term to long-term memory; see Schurmann et al., 2012 for an overview of the memory phases in *Nasonia*) after a single olfactory or visual conditioning trial with an intermediate response compared to the two isogenic strains. These differences were particularly apparent in the 24–48 h time frame (highlighted in the grey box in Fig. 2a and b). The contrast analysis clearly separated the two isogenic strains from the diverse strains, particularly when the conditioned stimulus was olfactory. These results, together with an earlier study on strain-specific variation in *N. vitripennis* (Koppik et al., 2015), show that there is considerable variation in learning ability and memory retention between strains and that previously described memory patterns of the isogenic strains are not necessarily representative of the species in general.

Patterns of memory retention for all strains were very similar between conditioned stimuli of different modalities: for example, the absence of memory after 48 h (most likely a form of mid-term memory; see Schurmann et al., 2012) in *Ng iso* was observed for both colour and odour stimuli (Fig. 3). This pattern is consistent with the findings of a selection experiment in which selection for associative learning of colours in *N. vitripennis* also improved olfactory learning (Liefing et al., 2016). Such correlated evolution in learning has been observed before in fruit flies, *Drosophila melanogaster*, and honey bees, *Apis mellifera* (Brandes & Menzel, 1990; Mery, Pont, Preat, & Kawecki, 2007; Zwoinska, Lind, Cortazar-Chinarro, Ramsden, & Maklakov, 2016), and is expected when traits are dependent on (partly) shared pathways. Therefore, the evolution of one aspect of learning cannot be understood in isolation from other cognitive abilities (Ellers & Liefing, 2015).

There was no sign of a deleterious effect of inbreeding on learning performance as has been reported in inbred *Drosophila* strains (Népoux, Haag, & Kawecki, 2010). Rather, both isogenic strains demonstrated a strong (short-term) memory response compared to the genetically diverse strains. This seems in correspondence with the absence of any deleterious effects on memory retention in *N. vitripennis* strains selected for either relatively large or small brain size (van der Woude, Groothuis, & Smid, 2019). Differences in learning behaviour between the strains are therefore unlikely to be caused by any sensory deficiency or motivational differences. Moreover, the *Ng iso* strain is able to form long-term memory after repeated conditioning (Hoedjes et al., 2012; Hoedjes; Smid, 2014). The memory retention patterns of the isogenic strains should thus not be considered deviant but rather part of the natural variation in learning and memory retention within the two species. This opens exciting opportunities for studies on the evolution of cognitive abilities, as there is likely to be ample natural variation present in wild populations of *Nasonia* given that in this study only four different laboratory strains showed strikingly different patterns of memory retention.

In conclusion, we have shown clear differences in memory retention between strains of *N. vitripennis* and *N. giraulti*. Additionally, the associative learning ability of all strains appeared independent of the sensory modality of the conditioned stimulus, which adds to our knowledge on general learning ability and correlated patterns in the evolution of learning. However, our results suggest that there are no clear interspecific differences in memory retention between the two species, although more strains need to be studied to fully characterize inter- and intraspecific variation. It is precisely these dramatic differences between genotypes that warrants caution in extrapolating characteristics measured on single genotypes to interspecies differences (Harvey et al., 2015; van Grunsven; Liefing, 2015). Recently, the fixation of many studies on identifying species level cognitive abilities has been criticized as it ignores crucial existing variation in such traits that underlies evolutionary processes (see e.g., Boogert et al., 2018; Thornton & Lukas, 2012). Using a panel of isofemale lines from a natural population would be one way to explore variation in, for example, learning ability, which would provide more insight into the distribution of learning phenotypes in said population (David et al., 2005). These findings stress both the need for careful consideration of the suitability of isogenic strains in experimental design and the need to further explore natural variation in cognitive ability to better understand how and why this variation is maintained.

**Data Availability**

The data supporting this article have been uploaded in the Zenodo Data Repository (DOI 10.5281/zenodo.3885652).

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Declaration of Interest

None.

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Supplementary Material

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