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# Selection for associative learning of color stimuli reveals correlated evolution of this learning ability across multiple stimuli and rewards

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We are only starting to understand how variation in cognitive ability can result from local adaptations to environmental conditions. A major question in this regard is to what extent selection on cognitive ability in a specific context affects that ability in general through correlated evolution. To address this question, we performed artificial selection on visual associative learning in female *Nasonia vitripennis* wasps. Using appetitive conditioning in which a visual stimulus was offered in association with a host reward, the ability to learn visual associations was enhanced within 10 generations of selection. To test for correlated evolution affecting this form of learning, the ability to readily form learned associations in females was also tested using an olfactory instead of a visual stimulus in the appetitive conditioning. Additionally, we assessed whether the improved associative learning ability was expressed across sexes by color-conditioning males with a mating reward. Both females and males from the selected lines consistently demonstrated an increased associative learning ability compared to the control lines, independent of learning context or conditioned stimulus. No difference in relative volume of brain neuropils was detected between the selected and control lines.

**KEY WORDS:** Artificial selection, associative learning, *Nasonia vitripennis*, color, odor, sensory modality.

Animal cognition encompasses the acquisition of sensory information and the storage, retrieval, and use of that information from the environment to modify behavior (Shettleworth 2010). This “processing of information” is specifically important when environments vary within the lifetime of an individual, as it enables an animal to navigate an otherwise unpredictable world by allowing the establishment of predictive relationships (Stephens 1993; Snell-Rood 2013). Animal cognition encompasses a wide range of cognitive abilities like the use of landmarks to navigate (Mather 1991; Steck et al. 2011), memory of locations of high numbers of food caches (Hitchcock and Sherry 1990), formation of positive

and negative associations with a wide range of stimuli (Siwicki and Ladewski 2003), and also more abstract forms of learning like conceptual learning (Murphy et al. 2008; Avarguès-Weber and Giurfa 2013).

After many decades of research on animal cognition it is clear that almost all of the animal species studied, vertebrate as well as invertebrate, are capable of at least simple forms of learning (Papini 2002). It has been hypothesized that the ability to form learned associations might have evolved as far back as the Cambrian explosion (Ginsburg and Jablonka 2010). This is also reflected in the observation that many biochemical, pharmacological, and

behavioral properties of simple forms of learning are conserved throughout evolution (Kandel 2001; Fitzpatrick et al. 2005). However, despite such conserved aspects to cognition, there is much variation observed in the cognitive abilities that underlie specific behaviors both among and within species (Raine et al. 2006; Odling-Smee et al. 2008; Healy et al. 2009). There is a growing understanding of how such natural variation in cognitive ability is caused by different trade-offs between the fitness benefits (Papaj and Vet 1990; Raine and Chittka 2008) and associated costs (Mery and Kawecki 2003; Burns et al. 2011) that are involved in information processing in different environments. Such benefits and costs may also differ between males and females as they face different selective pressures due to fundamentally different reproductive strategies that may require the utilization of different environmental information. Sex-specific correlated evolution can affect behavior in one sex but not the other. For example, selection on paternity assurance behavior in burying beetles resulted in changes in female parental care through correlated evolution, but not in males (Head et al. 2014). Likewise can differences in cognitive ability also arise due to sex-specific genetic correlations with reproductive traits (Hollis and Kawecki 2014; Zwoinska et al. 2016). This means that the fine-tuning of cognitive abilities by selective processes in response to prevailing ecological conditions and reliability of available information may differ between sexes (Healy et al. 2009).

The high level of diversification in cognitive abilities has raised questions on how such fine-tuning is realized through selection (Macphail and Bolhuis 2001; Krause 2015). Consider for example the small, efficient insect brain, where formation of learned associations is governed by specific neurological structures in which multisensory input is integrated and memories are formed (Giurfa 2013; Menzel 2014). How would this associative learning ability be tailored to different environments considering the enormous range in possible relevant cues from different sensory modalities (e.g., audio, visual, chemosensory, shape, pattern) that are encountered during an insect's life? Would an environment with a reliable relationship between, for example flower color and nectar reward select for the ability to specifically associate colors with a reward or rather favor a general appetitive learning ability that transcends sensory modalities? Would this altered associative learning ability involve cross-sex correlations, despite the difference in relevance of certain stimuli between the sexes? Understanding possible correlative evolution is relevant since such correlations may constrain independent evolution of traits (Beldade et al. 2002; Allen et al. 2008; Ellers and Liefting 2015), including behavioral traits (Agnvall et al. 2012; Head et al. 2014), and may therefore also play an important role in the evolution of cognition.

Several studies have described correlations in cognitive abilities, for example capacities for odor and color learning are

positively correlated in Cape honey bee lineages (Brandes and Menzel 1990) and individual bumblebees (Muller and Chittka 2012). The influential work of Mery and coauthors showed that selection for an improved ability of fruit flies to associate the odor or taste of an oviposition medium with an aversive chemical cue (quinine), also led to improved associative learning in a new context where an odor was paired with an aversive mechanical shock (Mery and Kawecki 2002; Mery et al. 2007). However, in these experiments the learned stimulus (odor or taste) was of a single sensory modality (i.e., chemosensory) and does not answer the question if improved associative learning ability extends to stimuli from different sensory modalities that are unrelated to the initial learning context. This is ecologically relevant, since different types of environmental information can signal a consequence or reward. For example, the range of potentially informative, flower-related stimuli that can be relevant to bumblebees during foraging is enormous and can include aspects like color, odor, texture, and pattern (Muller and Chittka 2012).

In this study, we set out to answer two questions; whether selection for enhanced associative learning of one stimulus also leads to improved learning of an unrelated stimulus of a different modality, and whether the evolutionary response in associative learning ability is restricted to the sex under selection. We used the parasitoid wasp *Nasonia vitripennis* of which both males and females can quickly learn to associate stimuli with rewards (Oliai and King 2000; Baeder and King 2004). Female wasps were selected for improved learning of an association between a visual stimulus and a host reward. To detect correlated evolution of learning, the ability to form learned associations in females was subsequently assessed using an olfactory instead of a visual stimulus. To test whether the effect of selection was sex-specific, associative learning ability was also assessed in males of the selected lines, using visual stimuli with a different reward, that is mating opportunity. We additionally compared brain morphology of selected and control lines for volumetric changes in different brain sections because some parts of the insect brain are typically associated with cognitive abilities like associative learning and multisensory convergence (Gronenberg and López-Riquelme 2004; Vogt et al. 2014) and volumetric changes in brains have previously been associated with changes in cognitive ability (Fahrbach et al. 2003; Snell-Rood et al. 2009).

## Material and Methods

### WASP STRAINS AND MAINTENANCE

We used a strain of *N. vitripennis* with relatively high genetic variance (HVRx) as described in Van de Zande et al. (2014), kept at a constant temperature of 25°C (70% RH, 16/8 L/D). At this temperature *N. vitripennis* has a generation time of two weeks. At the start of the selection experiment females were randomly

assigned to four selected and four control lines, and the baseline visual associative learning performance of the starting population was determined. Each selected line was coupled with a fixed control line to assure simultaneous conditioning and assessment of learned preferences. Only the wasps that demonstrated a learned color preference were allowed to reproduce in the selected lines (25 per color, 50 in total). For the control lines, 50 females were selected randomly from the tested wasps. From generation 10 onwards the selection was performed every five generations using exactly the same methodology as described above. The males used to test the cross-sex effects of selection on associative learning ability (the post-selection experiments, see below) were from generation 38, and hence originated from selected lines that went through 14 cycles of selection on female associative learning ability.

### CONDITIONING ASSAYS AND TEST FOR LEARNED PREFERENCES

During classical conditioning, a neutral (conditioned) stimulus is associated with an unconditioned stimulus that naturally elicits a specific behavior. After this process of associative learning, the conditioned stimulus elicits the same specific behavior as the unconditioned stimulus. During the selection experiment, the neutral, conditioned stimulus (color) was associated with the unconditioned stimulus of host finding and feeding. In the post-selection experiments, two other conditioning assays were performed; one with a novel conditioned stimulus (odor) and one with a novel unconditioned stimulus (mating opportunity).

#### *Selection experiment*

Male and female wasps were collected together on the day of emergence to ensure mating and provided with honey and water at a constant temperature of 20°C (70% RH, 16/8 L/D) for 2–3 days. Wasps were conditioned in groups of 40 females by introducing the females into a petri dish (55 mm, Gosselin—Corning Life Sciences B.V., Amsterdam, the Netherlands) containing 30 host pupae. The petri dish was placed on either a sheet of blue or yellow paper (Fastprint color paper, respectively “diepblauw” 746113 and “zwavelgeel” 120858) and the females were allowed to locate a host and probe it, thereby also feeding off the host, for one hour. Females that were not actively drilling or feeding off a host within 15 minutes were removed. A total of eight petri dishes were used per line (four on each color). After the 1 h conditioning session the wasps were separated from the hosts and left in a petri dish on a white sheet of paper for 15 minutes after which they were placed on the other color without a reward for another 15 minutes (i.e., if the wasps were conditioned on blue they would experience yellow without a reward during this phase of conditioning and vice versa). This way, wasps are already familiarized with the nonreinforced color and this procedure also

helps to consolidate the memory of the conditioned stimulus (Hoedjes et al. 2012). After the conditioning session the wasps were provided with honey and water until testing.

To test which wasps had successfully learned to associate a color with a reward, the color preference of the wasps was recorded after 24 h in a T-maze, which consisted of a 35 cm long, closed Plexiglas tube with either color covering the bottom half of each of the distal ends leaving the top half transparent. A release hole was located in the middle of the tube in the section of the T-maze that was left transparent. For each trial, 10 wasps were introduced simultaneously into the T-maze and allowed to move freely for 3 minutes, after which their choice would be recorded. The duration of the test was chosen based on pilot tests showing that 3 minutes sufficed for the wasps to make a choice and initiate searching behavior. Females that remained in the transparent center of the T-maze were recorded as indecisive. Per trial, we calculated the percentage of wasps that chose correctly, that is the color they were conditioned on, ignoring indecisive wasps that remain in the middle of the T-maze. The minimum number of trials was 8 per color. To estimate the innate color preference of the wasps the distribution of naive wasps in the T-maze from generations 0, 3, 6, and 9 was assessed.

#### *Post-selection experiments*

To test for correlated improvement of associative learning ability of stimuli not used during the selection experiment, we conditioned the selected and control lines of generation 11 to an odor (novel conditioned stimulus) instead of a color. The conditioning protocol for associative odor learning followed the same procedure as the protocol for associative color learning, but instead of placing the petri dishes on a sheet of colored paper, a small piece of filtration paper with a droplet of odor (either Natural Chocolate extract or Royal Brand Bourbon Vanilla extract; Nielsen-Massey Vanillas Intl., Leeuwarden, the Netherlands) was placed in the petri dish with the host pupae (see also Hoedjes et al. (2012) for further details). The preference of wasps for the learned odor was recorded in an olfactory T-maze as described in Hoedjes et al. (2012). A minimum of 10 trials per conditioned odor were done.

We assessed the cross-sex effects of selection on associative learning ability by comparing the males of selected and control lines for their ability to form associations. We obtained virgin males by letting unmated females reproduce. As *N. vitripennis* is a haplodiploid species, unmated females only produce males (Werren and Loehlin 2009). The conditioning procedure consisted of associating a color (conditioned stimulus) with mating opportunity (unconditioned stimulus). Mating opportunity in this conditioning assay consisted of a combination of female presence and mating opportunity, as the males were given access to a mixture of both virgin and nonvirgin females. Virgin females were acquired by dissecting parasitized host pupae a few days before

*Nasonia* wasp emergence and selecting the female *Nasonia* pupae. Although virgin females are a stronger reward as females usually only mate once (Boulton and Shuker 2015), the presence of mated females is also a positive stimulus (Baeder and King 2004) and their presence resulted in more male–female interactions than with virgins alone (personal observation). During the 30-min conditioning session a group of 10 males would be introduced to 10 virgin females and 20 mated females in a polystyrene tube with a white foam stopper placed in a small container with colored paper on all sides (blue or yellow). After 30 minutes the males were transferred to a clean tube with honey and water. The learned color preference of the males was assessed 24 h later in the color T-maze as described above. A total of 14 up to 16 trials per line per color were performed.

### PERFORMANCE INDEX

The use of a performance index (PI) as a measure for learned associations is common in *Drosophila* studies (Pitman et al. 2009) and we used an adaptation to the original method of Tully and colleagues (Tully and Quinn 1985; Tully et al. 1994). We calculated PI as follows; the percentage of wasps choosing correctly for yellow-trained wasps minus 50% (i.e., the expected percentage in the absence of learned associations), plus the percentage of wasps choosing correctly for blue-trained wasps minus 50%. Hence, the PI-value can range from –100 to +100. A PI of 0 indicates no learned associations, 100 indicates perfect preference and –100 indicates perfect avoidance of the conditioned color.

For the calculation of the PI-values in the selection experiment, the percentage of wasps choosing correctly was averaged over the total of 8–10 trials per line per color per generation, giving a single PI-value for each line at each generation. We weighted each trial (10 wasps in the T-maze) by the number of wasps that made a choice to value each decisive wasp equally.

For the subsequent post-selection testing of the learned odor associations of females and the learned color associations of males we adopted a method of running two trials with wasps conditioned on either stimulus at the same time. This allowed for the calculation of a PI over those two simultaneous trials instead of the mean response over all trials per color, giving replicate PI values for each set of trials.

### POST-SELECTION EXPERIMENT: BRAIN MORPHOLOGY

After the 10th cycle of selection, adult wasps of the selected and control lines of generation 12 were analyzed for variation in brain morphology. We compared relative volumes of different neuropils of female wasp brains of three selected and three control lines (the samples of one selected line and its control were lost) with five samples per line per treatment.

The procedure to analyze neuropil volumes was taken from Van der Woude and Smid (2015) with some adaptations. Brains were dissected from female wasps, fed ad libitum on honey and water, 3–5 days after emergence. Wasps were anesthetized with CO<sub>2</sub>, and decapitated. Brains were dissected with fine tweezers (Dumont no. 5, Sigma-Aldrich) in ice-cold phosphate-buffered saline (PBS, Oxoid, Dulbecco “A” tablets) and transferred to ice-cold 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), freshly prepared from paraformaldehyde (Merck, Darmstadt, Germany). Brains were further processed for immune labeling of neuropil structures using monoclonal antibody NC82, and nuclei marker propidium iodide as described in Van der Woude and Smid (2015). Brains were dehydrated in graded series of ethanol and mounted in DePeX (Fluka) under cover glass using spacers to prevent deformation of the brains, and dried at room temperature for 3 days. Brains were scanned with a confocal laser scanning microscope using 25x NA 0.8 oil immersion objective with a voxel dimensions (x, y, z) of 0.5089860 × 0.5089860 × 2 μm. We measured the volume of the brain neuropils by image segmentation, using the TrakEM2 plugin (Cardona et al. 2012) in the Fiji package of ImageJ 1.48 s (NIH, Bethesda, MD; RRID: SciRes\_000137) (Schindelin et al. 2012). The following neuropils were analyzed: calyx (Calyx) and lobes of the mushroom bodies (Lobes), together forming the mushroom body (MB); the fan shaped body (FSB) and ellipsoid body (EB) of the central body complex (CBC); medulla (MED) and lobula (LOB) of the optic lobes (OL), the antennal lobes (AL), and the combined protocerebrum and subesophageal ganglion (PC). See Haverkamp and Smid (2014) for schematic representations of the neuropils in *N. vitripennis*.

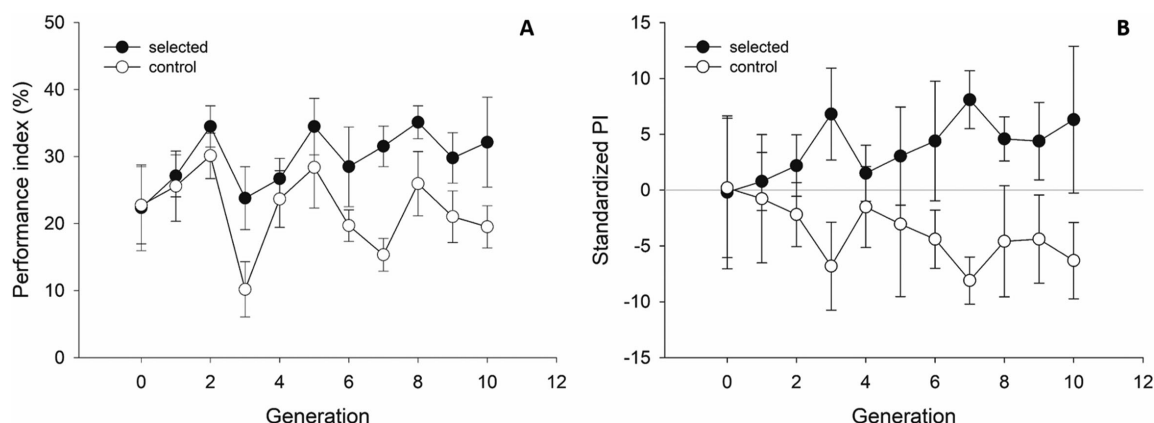
### STATISTICAL ANALYSES

#### Selection experiment

The response to selection over 10 generations was estimated with a linear-mixed model (LMM) with PI as the dependent variable, treatment as fixed factor, generation (continuous) as fixed factor, including a treatment × generation interaction and line as a random factor. Behavior can be affected by subtle changes in environmental conditions like air pressure, despite standardized experimental conditions (Steinberg et al. 1992; Roitberg et al. 1993). To isolate the effect of selection from this type of variation between the generations, we also calculated a standardized PI. This was done by calculating the residuals from a linear model (LM) with PI as dependent variable, generation as categorical fixed factor and line as fixed factor, thus standardizing the PI by removing effects of generation and line.

The color preference of naive wasps from generations 0, 3, 6, and 9 was assessed in the T-maze to check for changes in innate color preferences. Effects were estimated by fitting a linear-mixed model with the percentage of wasps choosing blue





**Figure 1.** Performance index (%) of *Nasonia vitripennis* females averaged over four replicate lines per treatment ( $\pm$ SE) of (A) a visual learning task for each selected generation, (B) the standardized PI corrected for variation over generations ( $\pm$ SE).

as the dependent variable, treatment as fixed factor, generation (continuous) as fixed factor, including a treatment  $\times$  generation interaction and line as a random factor.

### Post-selection experiments

The PI's of females from the selected and control lines of generation 11 in the associative odor learning task were analyzed with a linear-mixed model with PI as the dependent variable and treatment as fixed factor and line as random factor.

Similarly, the PI's of males from the selected and control lines of generation 38 in the associative color learning task were analyzed with a linear-mixed model with PI as the dependent variable and treatment as fixed factor and line as random factor.

The PI's of the four generations that were selected between the end of the selection experiment and generation 38 were analyzed by fitting a linear-mixed model with PI as the dependent variable, generation (continuous) as fixed factor, treatment as fixed factor and line as random factor.

Relative brain volume of neuropils was analyzed with a linear-mixed model with relative volume as the dependent variable, treatment as fixed factor and line as random factor for each of the 11 neuropils with a Benjamini–Hochberg FDR correction over all 11 analyses.

A principal component analysis (PCA) was performed using the neuropil volume data on all independent structures (FSB + EB + Calyx + Lobes + MED + LOB + AL), and the values for the first and second PC were extracted for further analysis. Differences in PC values between females from selected lines and control lines were analyzed using a two sample *t*-test.

All analyses were performed in R 3.4.4. (R Core Team 2018). The function *lmer* in the *lme4* package was used to fit the linear-mixed models and the function *lm* in the base package was used to fit the linear models. For all linear (mixed) model analyses assumptions for homoscedasticity and normality were

met after checking the distributions of predicted and residual values visually.

## Results

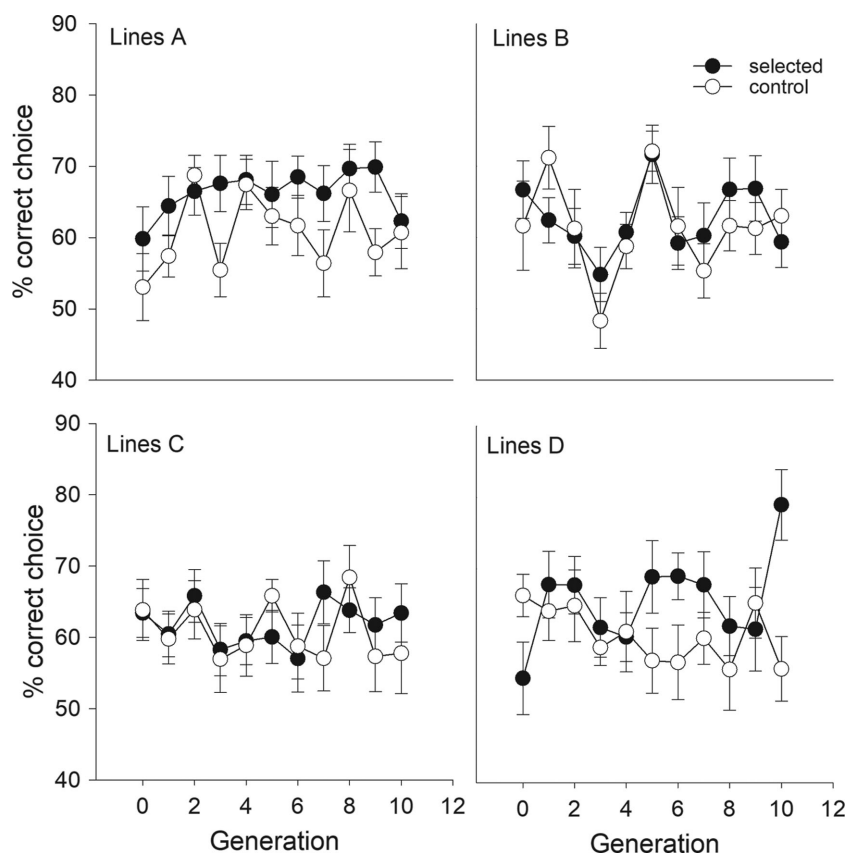
### SELECTION EXPERIMENT: SELECTION ON VISUAL ASSOCIATIVE LEARNING

At the start of the experiment the lines did not differ in their associative learning ability as deduced from their learned preferences, but from the third generation of selection onwards the selected lines consistently had a higher average PI than the control lines (Fig. 1A). The factors generation and treatment did not have a significant main effect, and the generation  $\times$  treatment interaction that indicates an effect of selection is near significant (LMM, Est. = 1.085, S.E. = 0.610,  $P = 0.076$ , see S1 for full model details).

The PI is strongly influenced by environmental effects that differ between the generations, we therefore also calculated a standardized PI that is corrected for such between-generation effects (Fig. 1B). Any effects on this standardized PI are a better indication of a response to selection than the nonstandardized PI. The factors generation and treatment did not have a significant main effect, however there was a significant effect of the generation  $\times$  treatment (LMM, Est. = 1.085, S.E. = 0.533,  $P = 0.042$ , see S2 for full model details).

The percentage of wasps that made the correct choice during the test for learned color preferences in the T-maze is also given for each of the replicate lines separately in Figure 2. These percentages were used to calculate the PI per line and give an indication of the level of variation per generation that is not visible in the PI calculated per generation.

The innate color preference of wasps of all lines was assessed in generation 0, 3, 6, and 9. There was a small consistent preference for blue over yellow (approx. 53–57% of wasps move to



**Figure 2.** Percentage of *Nasonia vitripennis* wasps that made the correct choice in the T-maze after conditioning the prior day (calculated over both colors), per generation given for each of the four coupled selected/control lines A to D ( $\pm$ SE calculated over the number of trials).

blue in each generation), but there was no effect found on this preference of the main effects (generation and treatment) nor of the interaction, see S3 for full model details.

### POST-SELECTION EXPERIMENTS: CORRELATED RESPONSES

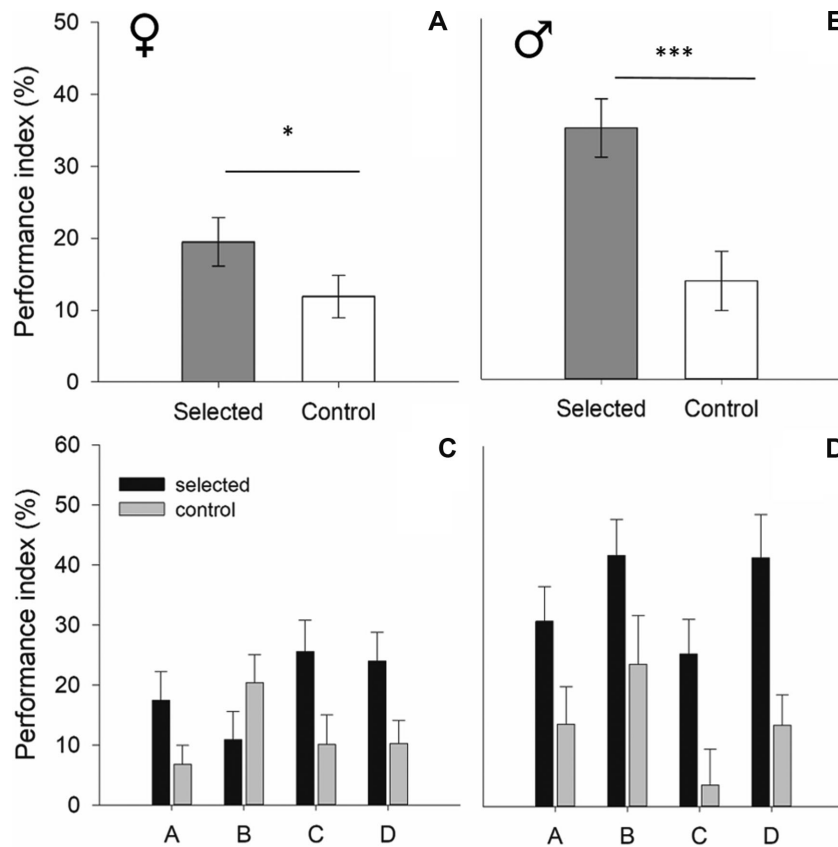
To test for correlated evolution of associative learning ability, females of the selected and control lines also had to form learned associations using an olfactory instead of a visual stimulus. A higher PI was found in the selected lines compared to the control lines, corresponding to what was found for the visual learning stimulus (Fig. 3A, see Fig. 3C for the response per replicate line). There was a significant main effect of treatment (LMM, Est. = 7.238, S.E. = 3.267,  $P = 0.027$ , see S4 for full model details).

The ability to form learned associations was also assessed in males, using visual stimuli with mating opportunity as a reward (see Fig. 3B for the average response and see 3D for the response per replicate line). There was a significant main effect of treatment (LMM, Est. = 21.214, S.E. = 4.389,  $P < 0.001$ , see S5 for full model details) in that the PI of the selected lines was higher than that of the control lines.

There was also a significant effect of treatment on the PI of the four generations in between the end of the selection experiment and the post-selection experiment on the males (LMM, Est. = 10.895, S.E. = 3.134,  $P < 0.001$ , see S6 for full model details) with the PI of the selected lines being on average higher than that of the control lines (data not shown).

### POST-SELECTION EXPERIMENT: BRAIN MORPHOLOGY

No difference was detected between the selected and control lines in the relative volume of any brain neuropil after FDR correction (Fig. 4), see S7 for model details. A principal component analysis (PCA) shows that the first two PCs contain a cumulative proportion of 89.7% of the variation in neuropil volumes. PC1 explains the major part of variation (83.2%), and all neuropils load to a similar extent on PC1. PC2 explains only 6.5%, and two neuropils show strong association with this axis: FSB is positively correlated and EB negatively associated. Neither of the two PCs revealed any divergence in brain composition between selected and control lines (PC1:  $t = -1.06$ ,  $df = 18.87$ ,  $P = 0.30$ ;



**Figure 3.** Performance index (%) averaged over four replicate lines per treatment ( $\pm$ SE) after (A) olfactory conditioning of *Nasonia vitripennis* females of generation 11 and (B) visual conditioning of males of generation 38. The response per replicate line (A–D) is given in panel C after olfactory conditioning of the females and in panel D after the visual conditioning of males ( $\pm$ SE calculated over the number of trials).

PC2:  $t = -0.54$ ,  $df = 24.25$ ,  $P = 0.59$ ; Fig. 5, see S8 for model details).

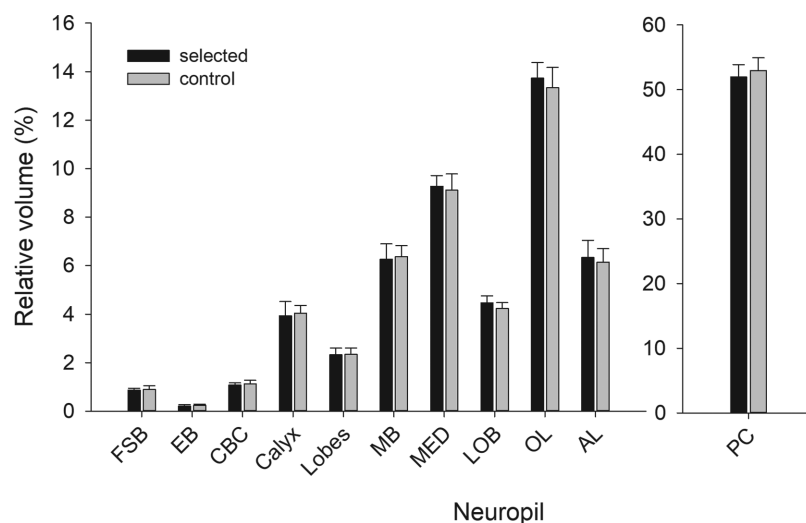
## Discussion

Our results demonstrate that *N. vitripennis* responds rapidly to selection for improved associative visual learning, which indicates that there is genetic variation for this trait. In fact, selection on visual associative learning resulted in a general improvement of associative appetitive learning, which was independent of sensory modality of the stimuli used during selection. In females, selection for associative learning with color as a conditioned stimulus also improved the ability to learn associations with an olfactory stimulus, even though females were never selected in this context (Fig. 3A). Likewise, although selection was designed to enhance female visual associative learning, it also affected the ability in males to form learned visual associations. Males showed an increased PI in a radically different learning context with a different reward, that is mate finding and mating (Fig. 3B). We conclude from these findings that selection for improved visual associative learning ability probably acts

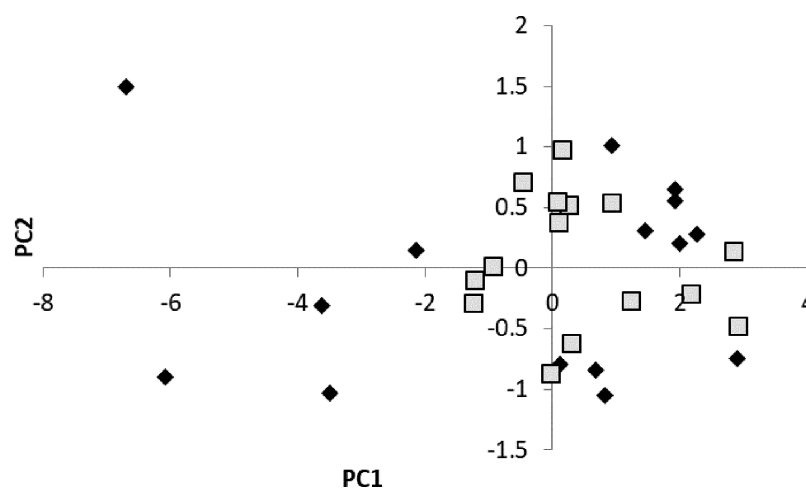
on genes with pleiotropic effects in the different associative learning contexts or affects an underlying process of associative learning.

It is important to carefully define the cognitive ability that underlies specific behavior (Rowe and Healy 2014). In this study, we focus on how the ability to form learned associations responds to selection. We must however assume that to successfully direct searching behavior in the T-maze, different aspects underlying the formation of associations can be fine-tuned. Besides the acquisition of information and formation of associations during the conditioning phase, also aspects of memory formation and retention are expected to act in concordance. This was nicely demonstrated in the *Drosophila* lines selected for an improved aversive associative ability in which both the speed at which associations were formed as well as the rate of memory formation were affected (Mery et al. 2007). We cannot make that distinction in this study, as we focus on the overall effect of changes in associative learning ability on behavior. However, it is important to recognize the limits of what we know as this also generates interesting questions for future research on the evolution of cognitive ability.





**Figure 4.** Relative volumes for each neuropil in the *Nasonia* brain of the selected and control lines ( $\pm$ SD).



**Figure 5.** Variation in neuropil volumes for *Nasonia vitripennis* females from control (gray squares) and selected lines (solid black diamonds) presented in a biplot between PC1 and PC2 that are both not significantly different between lines.

A possible alternative explanation for the difference in PI between the selected and control lines could be that the selection regime changed the color perception of the wasps, making them more responsive to the color stimuli. Something similar has been described in honeybees where individual heritable responsiveness to sucrose in turn affected the learned response (Scheiner et al. 1999). However, the innate color preference of the wasps remained constant over the whole experiment, invalidating this hypothesis. Moreover, improved ability to form learned associations was found to extend to associative learning tasks involving different rewards in the different sexes and stimuli from different sensory modalities. This is a strong demonstration that the response to selection was not based on for example enhanced salience of a particular visual stimulus used in the selection regime (Rescorla 1988), but affected genes in underlying neurological pathways.

Single genes associated with learning or memory formation (like, e.g., *dunce* or *rutabaga*) have been identified in laboratory-generated mutants of *Drosophila melanogaster* (Tully 1996; Dubnau and Tully 1998; Margulies et al. 2005). It is however very unlikely that such single loci have contributed to the segregating variation in associative learning ability that we found in this study. The gradual and modest increase in PI typically does not support an argument for a single major gene associated with associative learning ability. It rather suggests that selection drew on standing genetic variation for polygenic traits (Barrett and Schluter 2008; Burke et al. 2010), for example in the many genes associated with underlying neural pathways or processes such as the dopaminergic neural circuitry (Waddell 2010) or neural plasticity in brain cells that influence approach or avoidance behavior (Waddell 2016). Also, changes in regulatory regions and

translational repressive mechanisms can contribute significantly to the variation cognitive ability (Costa-Mattioli et al. 2009; Santini et al. 2014; Cho et al. 2015). It would prove highly informative to explore both gene expression differences and changes in gene allele frequency in these selected and control lines and compare these findings with recent genomic studies on learning behavior in parasitoid wasps (van Vugt et al. 2015) and *Nasonia* (Hoedjes et al. 2014; Hoedjes et al. 2015) in particular.

In their influential work on experimental evolution of associative learning in *Drosophila*, Mery and colleagues demonstrated that selection for improved chemosensory avoidance learning (Mery and Kawecki 2002) was correlated across different aversive cues (Mery et al. 2007). These results convincingly demonstrate generalized associative learning across different unconditioned stimuli, using conditioned stimuli of the chemosensory modality (odor and taste). Our findings significantly add to this knowledge by demonstrating the occurrence of correlated associative learning across drastically different conditioned stimuli (color and odor), and across sexes, all within one experimental set-up. Taking the results of this study and earlier work into account creates a strong case for the generality of associative learning ability in both aversive (Mery et al. 2007) and appetitive learning paradigms (this study).

Our results on brain morphology showed no difference in relative volume of neuropils between selected and control lines. Although learning performance has been associated with volumetric changes in brains or neuropils (Fahrbach et al. 2003; Kotrschal et al. 2013), and relative volumes of multiple neuropils in brains of *N. vitripennis* have been found to differ between wasps of different sizes (Groothuis and Smid 2017), this is only one of many possible mechanisms through which changes in cognitive ability can be mediated. Possibly, selection has resulted in subtle changes in sites of synaptic plasticity in underlying pathways, since this does not require large morphological changes of the brain but can influence cognitive ability (Margulies et al. 2005; Waddell 2016). This would be an interesting research line to pursue, especially when considering structures that are important for integrating learned olfactory and optic information, like, for example the mushroom bodies in many Hymenoptera (Menzel 2014). The output neurons of the mushroom bodies play a crucial role in encoding valence and whether a stimulus should be approached or avoided (Aso et al. 2014) and considering variation in synaptic plasticity between the selected and control lines in and around this region would be most interesting.

Another promising avenue for further research would be to explore if the covariance in the ability to form learned associations transcends the appetitive and aversive pathways. These two forms of associative learning are considered to operate independently and to be governed by (partly) different neurological circuitry and neurotransmitters (Honjo and Furukubo-Tokunaga

2009; Nakatani et al. 2009; Mizunami and Matsumoto 2017). Also of great interest from an ecological perspective is to resolve to what extent correlated responses in associative learning ability form an integrated phenotype or how easily they can be uncoupled (Head et al. 2014; Ellers and Liefing 2015). There are many examples of specialized learning in natural populations, such as the ability of some *Polistes* wasps to memorize conspecific faces better than other visual stimuli (Sheehan and Tibbetts 2011) or the cases of prepared learning where some associations are more readily learned than others (Dunlap and Stephens 2014). The evolution of specialized and prepared learning will depend on how the regulatory pathways are interconnected and on how strong selection acts on uncoupling correlated responses (Beldade et al. 2002; Allen et al. 2008), which has not been studied within a cognitive-behavioral context. Our findings provide new avenues for such further experimental efforts on the evolution of cognition.

## Conclusion

Lines of parasitoid wasps selected for improved ability to learn color-reward associations consistently demonstrated better associative learning than control lines, independent of the learning context. The enhanced associative learning ability also extended to the males of the selected lines even though the learning task applied in the selection regime was female-specific. These changes in associative learning ability occurred in the absence of relative volumetric changes in brain neuropils between selected and control lines. In conclusion, selection on associative visual learning ability can result in a change in overall associative learning ability that is independent of the sensory modality of the stimuli in the learning task used during selection.

## AUTHOR CONTRIBUTIONS

Methodology, M.L., C.L.L., and K.M.H.; Analysis, M.L. and J.E.; Writing—original draft, M.L. and J.E., Writing—review and editing, M.L., K.M.H., C.L.L., H.M.S., and J.E.; Supervision, J.E. and H.M.S.

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## DATA ARCHIVING

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