Environmental diversity constrains learning in *Drosophila melanogaster*

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Abstract

1. Much is known about how enriched environmental diversity affects ability to learn across the months and years that are the developmental periods of large animals.

2. Less is known about how diversity impacts learning across the minutes and hours during which sensory environments of small foraging animals such as insects may vary dramatically.

3. We show that *D. melanogaster* exposed to a diversity of odour-taste associations over a few minutes subsequently learn standard associative learning tasks poorly.

4. This effect is robust to variation in odours used in all parts of experiments.

5. Findings may impact on at least three major research areas in ecology: the relationship between biodiversity and ecosystem functioning, the evolution of floral constancy in pollinators, and the pest-protective effects of mixed species crops.

Introduction

Enriched environmental diversity applied over periods of months or years generally improves the ability of mammals to learn (Gardner *et al.*, 1975; De Jong *et al.*, 2000) but less is known about how experiences over seconds or minutes (relevant to many small foraging animals, for example) affect learning. Most research on how short term exposure to environmental diversity impacts learning is embodied in human cognitive load theory (Sweller, 1988) where learning is assumed to be inefficient when too many 'elements' must be held in working memory, but it is not known if this theory applies generally to other groups such as invertebrates. Only two studies on invertebrates have considered this issue. The first (Johnson *et al.*, 1994) showed that foragers of the ant species *Messor pergandei* and *Pogonomyrmex rugosus* take longer to recognise a novel seed when seed diversity is high. The second (Chittka *et al.*, 1999) explores the possibility that flower constancy in bees may be caused by the inability to retrieve the multiple memories formed in a complex environment. So while our study is

relatively novel in insect science, the relationship between short term learning and environmental diversity is well studied in psychology and there is a great deal of interest more generally in how animals cope with the cognitive demands of a complex natural environment. Most notably the Neural Limitations Hypothesis (Bernays, 2001) states that insects struggle to cope with the attentional demands of a complex resource environment and evolve resource specialisation in response. This hypothesis is well supported (Janz & Nylin, 1997; Bernays, 1998; Egan & Funk, 2006; Tosh *et al.*, 2009) and has been influential in the development of the experiments described in this paper where we consider the relationship between environmental diversity and learning rather than that between environmental diversity and attentional processes.

We show here that *D. melanogaster* exposed to a high diversity of odour-taste associations over a few minutes, subsequently learn standard associative learning tasks over a further few minutes very poorly; those exposed to low diversity learn well. We suggest that the time scale of a few minutes per 'resource' (odour-sugar association) used here is relevant to the natural ecology of *D. melanogaster*. Few relevant studies of the temporal dynamics of *D. melanogaster* foraging behaviour have been carried out in its natural environment, or with a range of suitable and unsuitable resources. Laboratory studies (Hoffmann, 1988; Stamps *et al.*, 2005; Reaume & Sokolowski, 2006) indicate that this organism is relatively immobile, spending several hours on a resource before moving on. On the other hand Tortorici & Bell (1988) demonstrated that when introduced into an approximately 7-cm grid of 25 sugar droplets, *D. melanogaster* sampled a median of about three droplets with a range of approximately 0-20 droplets, in no more than 10 minutes. As these authors point out, the nature of *D. melanogaster* foraging behaviour is likely to depend on its physiological condition. Regardless of the relevance of this study to the natural foraging behaviour of *Drosophila*, the classic *Drosophila* olfactory conditioning protocol we use here is attractive as a model system that can be applied to other insects such as pollinators that certainly do sample multiple resources rapidly. The olfactory conditioning protocol used here is reliable and well used so results can be closely integrated with the vast existing literature on *Drosophila* olfactory learning and memory.

This study is relatively novel so most of our discussion is concerned with elaborating the research areas upon which we think our research results will impact. These include: the relationship between biodiversity and ecosystem functioning (Naeem *et al.*, 1994; Schulze & Mooney, 2012), the evolution of flower constancy in pollinators (Chittka & Raine, 2006), and the reduced pest attack commonly observed on mixed species plant crops (Finch & Collier, 2000). Application of this study to the first and third of these areas also assumes that the timescale we have chosen is appropriate for non-pollinating phytophagous insects. Information on the precise temporal dynamics of host visitation in non-pollinating phytophagous insects is surprisingly scarce, and will vary with species, but stereotypical search behaviour following positive stimuli forms, and extinguishes, in many insects on a cycle of less than 10 minutes, suggesting that the time scale we have chosen is broadly appropriate (Hassell & Southwood, 1978). We note here that we do not definitively establish the mechanism underlying the environmental diversity - learning relationship demonstrated, but cover likely possibilities in the discussion.

Here we use the appetitive olfactory conditioning protocol of Krashes & Waddell (2010) with one addition to investigate the importance of environmental diversity on the ability of *D. melanogaster* to learn olfactory-gustatory associations. Thus instead of simply exposing flies to CS+ and CS- (conditioned odours with and without sugar reward) as is customary, we firstly expose the flies to four additional olfactory-gustatory parings. In half of our experiments we vary these pairings within the few minutes prior to undertaking the standard learning assay. In the other half, this prior experience is invariant across the same period. We thus test the impact of environmental variation on the ability of flies subsequently to learn a standard olfactory conditioning task.

Methods

Flies used were the Dahomey wild-type (see Reuter *et al.*, 2008)). Prior to the present study flies had been maintained for four years in CRTs laboratory in a cage population of 1000-2000 individuals fed liberally on the Jazz Mix medium (Fisher Scientific, AS-153) at 25ºC and a 12h light / 12h dark photoperiod.

A high-diversity experience prior to the standard learning task was simulated by exposing flies to four different odours within a single trial (4 x 2-min periods), with two of these associated with an unconditioned sugar stimulus (+) and the other two associated with the absence of such a stimulus (-) (Figures 1& 2). In the low-diversity treatment, prior to the standard learning, flies were exposed four times to the same odour-taste association. The particular odour-taste association used was changed between trials (replicates) such that across trials, flies were exposed to all the odour-taste associations experience by flies in the high-diversity trials (Figures 1 & 2). We also considered whether variation in the odours used in different parts of the study significantly impacted the main experimental effect demonstrated (Figure 1). We ran standardised learning tasks without pre-treatment to determine the effect of pre-treatment *per se* on the ability to learn the standardised task. These data were not included in the factorial statistical analysis described below because they render that analysis non-factorial; however, means and 95% confidence intervals were created for this treatment and included in Figure 3 to allow visual comparison with other treatments.

To understand this experimental design better, we ask the reader to consider the biological analogy of the experimental design. Consider a *D. melanogaster* fly foraging on a number of different fruit species, perhaps lying discarded in the back room of a grocers shop or a delivery area of an outdoor market (alternatively readers can consider a pollinating insect flitting between flowers of different plant species or a herbivorous insect sampling different plants in a meadow in its search for something to eat or lay its eggs on). The odours we present to flies are analogous to the smell of the fruit, and the sugar/plain papers we present concurrently with the odour are analogous to the taste of the fruit. Sugar paper + odour represent a 'host' fruit that is suitable for the fly, and plain paper + odour represent a non-host that is unstimulating to the fly. We envisage the fly foraging on four different fruit species for several minutes and then moving to a different area of the room where two completely new fruit species lie discarded, one of which is a host and one of which is a non-host. The fly then forages on these fruits for a few minutes, learning their odours so that in the future it may return more efficiently to the host fruit and avoid the non-host. The 'pre-treatment' phase of our experiment is analogous to the flies foraging on the four fruit species, and the 'standard-task' phase of

our experiments are analogous to the fly subsequently foraging on the two fruits. The scenario where flies forage initially on four fruits we refer to as the HD (high-diversity) treatment. We compare the ability of these flies to learn the odours of the two fruits in the standard-task phase with flies that have initially foraged only on single fruit species, the LD - low diversity - treatment. Ultimately we are interested in whether this initial foraging on a variety of fruits constrains the subsequent ability of the fly to learn the odours of the two fruits. Lastly, we change all the identities of the four and two fruits on which the fly forages to determine if the precise identity of fruit species used in different phases of foraging impacts the ability of the fly to learn the standard task.

The classic conditioning protocol used in a modified form here (Krashes & Waddell, 2010) is inspired by Tully & Quinn (1985), with sugar reward replacing electric shock as the unconditioned stimulus. In the Tully & Quinn (1985) protocol:

"100 flies were placed in a tube whose internal surface was comprised of an electrifiable copper grid. The flies were subsequently exposed to odor A [the conditioned stimulus, CS] for one minute in the presence of 12 pulses of electric shock (CS+) followed by a 1-min exposure to odor B in the absence of electric shock (CS -). Here, the odors were pulled into the tube by vacuum such that all flies were exposed to both the odorant and shock. After training, the flies were tested in a T-maze apparatus where they were required to choose between two arms containing either odor A or odor B. A performance index was calculated by determining the fraction of flies avoiding the CS+ minus the fraction that avoided the CS-." (McGuire *et al.*, 2005) (square brackets added by us).

This protocol has been at the heart of most of the vast body of work investigating the neural and molecular mechanisms underlying learning and memory in *Drosophila* up to the present day (Keene & Waddell, 2007; Masse *et al.*, 2009). However, some authors have pointed out that this protocol is not particularly ecologically realistic, in particular the electric shock (Krashes & Waddell, 2008), and have replaced the unconditioned stimulus with sugar (Krashes & Waddell, 2010), thus assaying the ability of flies to form olfactory-gustatory associations, which is ecologically relevant to *Drosophila* and many other insects.

We used the olfactory appetitive conditioning protocol of Krashes and Waddell (2010) (see also Huetteroth *et al.*, 2015; Owald *et al.*, 2015), modified (see below and SI) to include four odour-taste presentation chambers, used prior to presenting a standardised learning task. After acclimating approximately 100 flies to the learning apparatus in a 'stimulus free' chamber for 2 mins, they were exposed to another chamber with odour-infused air and lined with dry, sugar-saturated paper (+ve) or plain paper (-ve) for another two mins. The flies were moved to another three such chambers, each for two mins, before undertaking a standard learning task where one odour, not yet experienced, was paired with +ve stimulus for two mins and another (also not yet experienced) was paired with –ve stimulus for two mins. Finally flies were moved to a choice chamber where the odours presented in the standard learning task were blown into the chamber from opposite directions and the flies allowed to choose an odour. This experiment was repeated, reversing the odour-paper associations during the standardised test, and learning-score indices calculated as standard (see SI, and Krashes & Waddell, 2010). A learning-score index of 1 indicates perfect learning, while an index of 0 implies no learning. The procedures described were repeated 8 times per treatment (n = 8).

All odours and their abbreviations are explained in Figure 1. The two sets of two odours used for the two standard learning tests, 4M-3O and EA-IA, can be learned by *D. melanogaster* using appetitive olfactory conditioning (Schwaerzel *et al.*, 2003; Krashes & Waddell, 2010). The two sets of four odours used for learning pre-treatment are predominantly components of fruit odour and show behavioural, electroantennal, or olfactory receptor neuron activity in *D. melanogaster* (de Bruyne *et al.*, 2001; Zhu & Park, 2003; Hallem *et al.*, 2004).

We analysed data using a general linear model (GLM) including all main effects and all interactions, using the learning-index score as the dependent variable. Our three fixed-factor main effects were: learning task (type 1 or type 2, differentiated on the basis of odours used), prior treatment diversity (low or high diversity) and prior treatment type (type 1 or type 2, differentiated on the basis of odours used)(Figure 1). The assumptions of this statistical technique were analysed by visual inspection of normal probability plots, a plot of residuals vs fitted values, and a plot of residual vs observation order. Untransformed data appeared largely to meet the assumptions of the GLM, but

common transformations were undertaken to determine if these could improve fit. None of these improved the fit, and generally substantially worsened it, so we used the untransformed data. The raw data and residual plots (from untransformed and transformed dependent variable) are provided in the Supplementary Information.

Results

Learning of the standard task was undertaken more efficiently when the diversity of treatments experienced prior to the standard learning task was low. This effect was highly significant ($F_{(1,56)}$ = 13.4, P = 0.0006), and did not vary with the particular odours used for pre-treatment ($F_{(1,56)}$ = 2.05, P = 0.16), nor with the particular odours used for the standard learning task ($F_{(1,56)}$ = 0.072, P = 0.79) (Figure 3). Thus the effect of diversity appear robust to variation in the particular odour components of the experimental system.

It should be noted that in our LD treatments, half of the replicates have had no sugar reward prior to undertaking the standard learning task. All HD flies, on the other hand, receive sugar exposure. Assuming that odour learning occurs more effectively when paired with a reward than when paired with a neutral stimulus, it is possible that those LD replicates that have received sugar exposure are similarly constrained in their learning behaviour to HD flies, and the higher overall ability of LD flies to learn is caused simply by those replicates that have had no sugar exposure and so have had no opportunity to learn an odour. To investigate this further, we separated these components of the LD treatment (Figure 4), and found no evidence that the +ve and –ve constituent replicates of the LD treatments contribute differently to the overall mean and CIs. The most parsimonious interpretation is that short-term temporal diversity of experience determines the subsequent ability to learn associatively.

Discussion

Before discussing the general implications of our study it is worth discussing the limitations of our experiments. We have only demonstrated the effect in one species and in a quite abstract form. The work should be repeated with additional species and under more natural conditions. Learning scores

are also a little low (Krashes & Waddell, 2008) although still significantly positive in the absence of prior treatment (the 95% CIs do not overlap zero). The study informs on effects of diversity at a very fine temporal and spatial scale, and it would be useful to know how provision of an enriched sensory environment throughout larval and/or adult development affects efficiency of learning in adult insects. Finally, we have not definitively established the class of phenomenon to which the principal effect is attributable, but we strongly suspect it is some sort of diversity-related effect on proactive interference (Reaume *et al.*, 2011). The work by Reaume *et al.*(2011) is one of the most detailed studies of proactive interference in *D. melanogaster* to date. They demonstrated that proactive interference occured when an olfactory learning task A+B- (where A and B are different learnable odours) is preceded by the reciprocal association, B+A-, but that this interference faded with time. When an A+B- was preceded by a C+D- association (i.e. completely different odours used), no proactive interference occurred. This is interesting as the latter experiment is analogous to our study, with the main exception being that we assayed four odour-unconditioned-stimulus associations prior to the standard learning test. As the authors of this previous study used an aversive, mechanical shock unconditioned stimulus, this suggests that the impact of environmental diversity on learning in *D. melanogaster* may vary with the nature of the unconditioned stimulus. Lastly, it would be informative to know whether environmental diversity principally impacts memory formation or retrieval during or after the standard learning task.

As learning in insects impacts fitness through increased resource-use efficiency (Dukas & Bernays, 2000; Egas & Sabelis, 2001), the effect we have shown here, if general, could lead to decreased resource-use efficiency with environmental diversity, and so increased resource productivity i.e. a positive biodiversity-ecosystem functioning relationship (Reiss *et al.*, 2009). Alternatively, some insects, such as pollinators, enhance resource productivity. Decreased behavioural efficiency of pollinators with increased plant diversity could, therefore, potentially decrease accumulation of plant biomass if plants go unfertilised (Worm & Duffy, 2003). Additionally, while diversity over the short term constrains learning in *Drosophila*, over the longer term it could improve learning as it does in mammals (Gardner *et al.*, 1975; De Jong *et al.*, 2000). In mammals,

environmental enrichment leads to anatomical and electrophysiological changes in the hippocampus, which is responsible for memory formation (van Praag *et al.*, 2000). It is conceivable that long term exposure to environmental diversity could induce analogous changes to brain structures such as the mushroom body that are responsible for learning and memory in insects (Dukas, 2008). Considerably more work under more natural conditions will be required to establish if and how the effect demonstrated here influences biodiversity-ecosystem function relationships.

Other authors have suggested that memory retrieval in pollinators might be impeded by a diverse resource environment, so driving the evolution of flower constancy (Chittka *et al.*, 1999). The present article indicates that resource diversity affects the formation, as well as, the retrieval of memories, which could provide an extremely potent driver of the evolution of flower constancy in pollinators. We see no reason why such mechanisms could not be responsible for driving the wider phenomenon of specialised niche width in insects (see also Bernays, 2001).

The practice of planting different species or varieties of plant together, a common practice in small-scale and subsistence farming, can provide protection from insect pests (Letourneau *et al.*, 2011). A commonly cited mechanism posits that insect pests may simply land on anything that is green, and in a diverse background many will land on non-hosts. This will cause them to take off again without receiving positive stimulation, and in time this can lead to reduced plant infestation (Finch & Collier, 2000). Using similar arguments to those made above for biodiversity-ecosystem functioning relationships, we suggest that insect learning may contribute to this phenomenon, with reduced learning ability in diverse backgrounds limiting the ability of insects to locate and utilise hosts efficiently.

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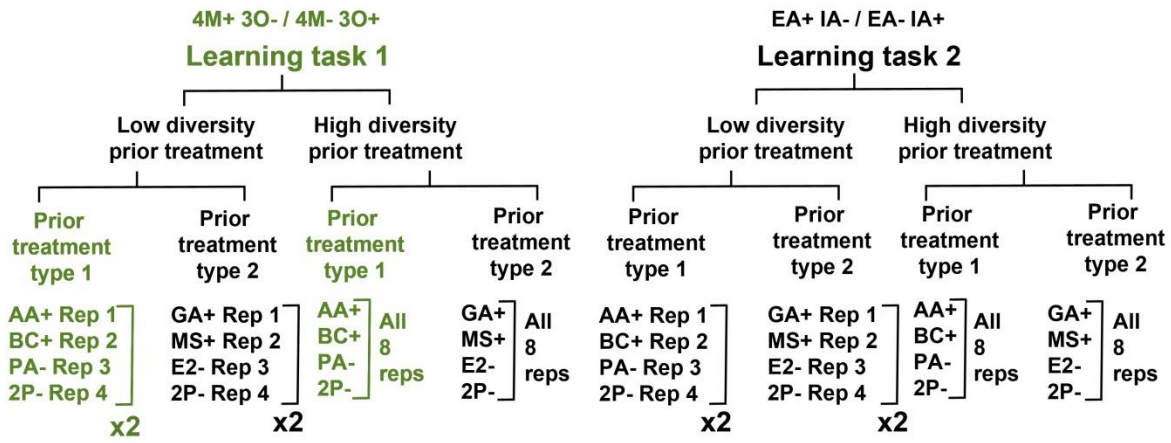
Figure 1. Factorial structure of the main part of the experiment. Symbols and abbreviations: +, the unconditioned stimulus (sugar on filter paper); -, absence of the unconditioned stimulus (filter paper without sugar); 4M, odour 4-Methylcyclohexanol; 3O, odour 3-Octanol; EA, odour Ethyl Acetate; IA, odour Isoamyl Acetate; AA, odour Amyl Acetate; BC, odour *B* –Caryophyllene; PA, odour Phenethyl Acetate; 2P, odour 2-Phenylethanol; GA, odour Geranyl Acetate; MS, odour Methyl Salicylate; E2, odour Ethyl 2 Methylbutyrate; 2P, odour 2-Pentyl butyrate. Experimental procedures involved in the low vs high diversity prior treatment comparison shown in green are shown in Figure 2.

Figure 2. Details of the experimental procedures involved in the comparison highlighted in green in Figure 1. All other low prior experiential diversity vs high prior experiential diversity comparisons are the same but use different odours. Approximately 100 flies are used in each replicate. AA, odour Amyl Acetate; BC, odour *B* –Caryophyllene; PA, odour Phenethyl Acetate; 2P, odour 2-Phenylethanol; 4M, odour 4-Methylcyclohexanol; 3O, odour 3-Octanol.

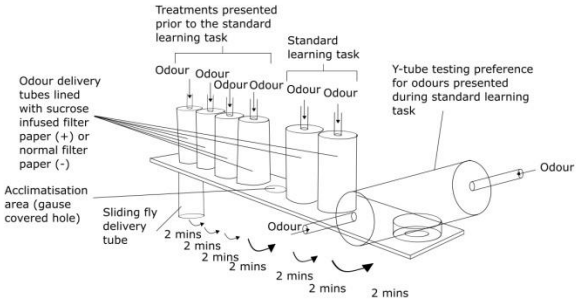
Figure 3. Learning index scores of the flies subject to the various treatments outlined in Figure 1. Relevant terms from the GLM analysis are shown. 'No prior treatment' is not included in the GLM. The treatments highlighted in green are those highlighted in green in Figure 1 and those shown in Figure 2.

Figure 4. Low diversity treatments shown in Figure 3 are replotted here next to their constituent replicates, half of which are sugar exposed (+) and half of which are not exposed to sugar (-). As discussed in the main text, a substantial deviation in + and – within each LD treatment could indicate that the main findings are driven by differential sugar exposure rather that temporal diversity of experience prior to the standard learning task. We find no evidence for substantial deviation between + and – indicating that differential sugar exposure is unlikely to be a cause of results.

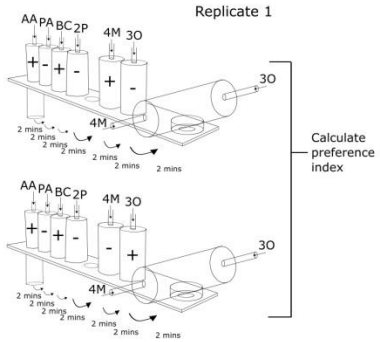
Figure 1


4M+ 3O- / 4M- 3O+
Learning task 1
Low diversity prior treatment
High diversity prior treatment
Prior treatment type 1
Prior treatment type 2
Prior treatment type 1
Prior treatment type 2
AA+ Rep 1
BC+ Rep 2
PA- Rep 3
2P- Rep 4
x2
GA+ Rep 1
MS+ Rep 2
E2- Rep 3
2P- Rep 4
x2
AA+
BC+
PA-
2P-
All 8 reps
GA+
MS+
E2-
2P-
All 8 reps
EA+ IA- / EA- IA+
Learning task 2
Low diversity prior treatment
High diversity prior treatment
Prior treatment type 1
Prior treatment type 2
Prior treatment type 1
Prior treatment type 2
AA+ Rep 1
BC+ Rep 2
PA- Rep 3
2P- Rep 4
x2
GA+ Rep 1
MS+ Rep 2
E2- Rep 3
2P- Rep 4
x2
AA+
BC+
PA-
2P-
All 8 reps
GA+
MS+
E2-
2P-
All 8 reps

Outline of the apparatus

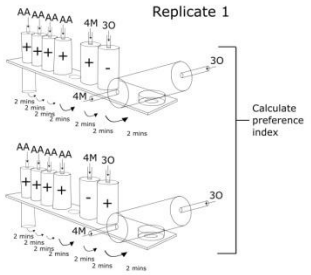


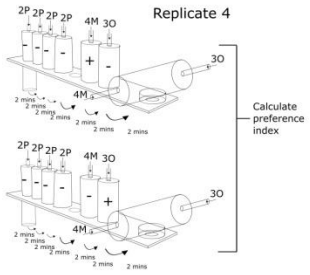
A high diversity of experience prior to the standardised learning task

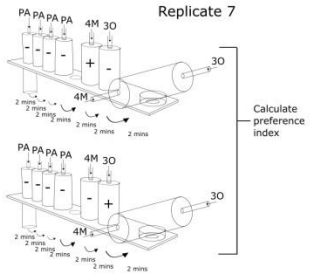


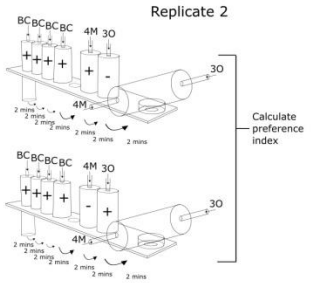
Repeat a further 7 times, randomising order/position of treatments within the 3 phases (prior experience, standard learning task, preference trial) of each trial.

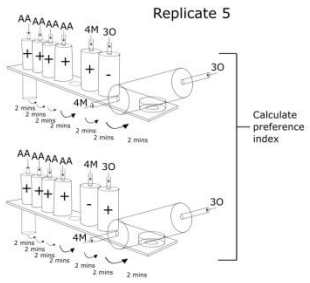
A low diversity of experience prior to the standardised learning task

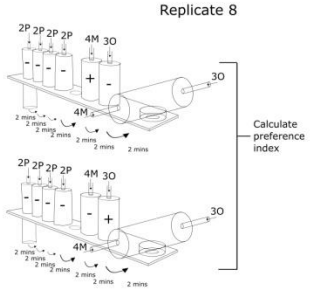


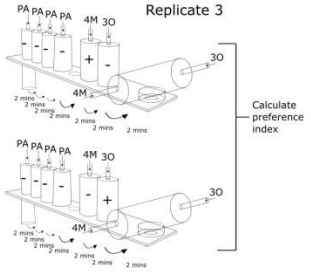


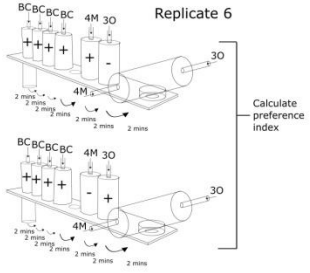








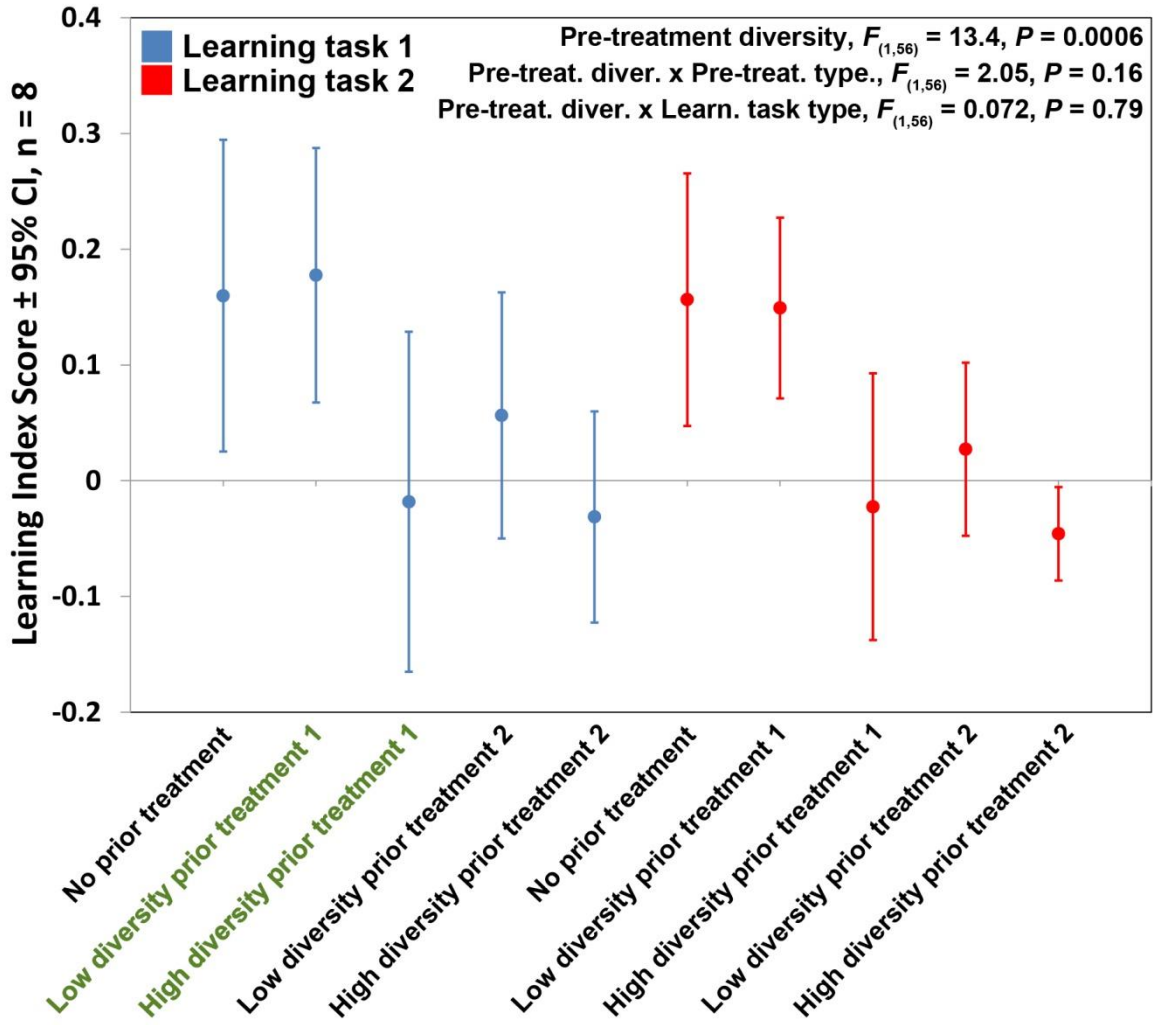




Order/position of treatments is randomised within the 3 phases (prior experience, standard learning task, preference trial) of each trial.

Figure 2

405 Figure 3



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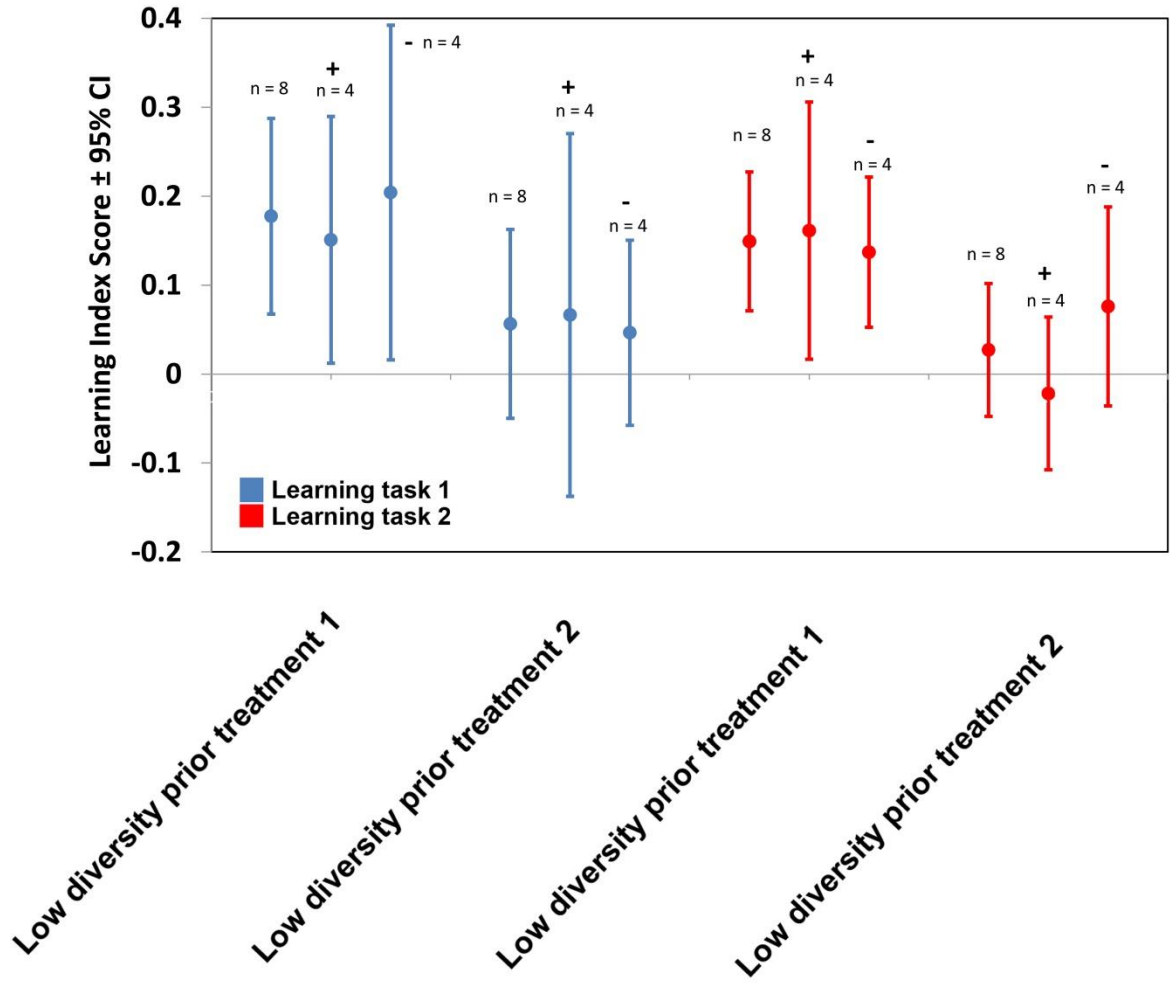
414 Figure 4



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