Odour intensity learning in fruit flies

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Animals’ behaviour towards odours depends on both odour quality and odour intensity. While neuronal coding of odour quality is fairly well studied, how odour intensity is treated by olfactory systems is less clear. Here we study odour intensity processing at the behavioural level, using the fruit fly Drosophila melanogaster. We trained flies by pairing a MEDIUM intensity of an odour with electric shock, and then, at a following test phase, measured flies’ conditioned avoidance of either this previously trained MEDIUM intensity or a LOWer or a HIGHer intensity. With respect to 3-octanol, n-amylacetate and 4-methylcyclohexanol, we found that conditioned avoidance is strongest when training and test intensities match, speaking for intensity-specific memories. With respect to a fourth odour, benzaldehyde, on the other hand, we found no such intensity specificity. These results form the basis for further studies of odour intensity processing at the behavioural, neuronal and molecular level.

**Keywords**: fruit fly; olfaction; odour intensity; associative learning; recognition; benzaldehyde

1. INTRODUCTION

Animals use odours for finding food and for detecting predators, competitors or social interaction partners. For all these functions, they potentially rely on both odour quality and odour intensity: while the quality of an odour can, for example, signal edibility, its intensity can be used to track down the food. Although neuronal coding of odour quality is fairly well studied in various species (e.g. reviewed by Buck 1996; Galizia & Menzel 2000; Laberge & Hara 2001; Laurent et al. 2001; Korsching 2002; Mainen 2006; Johnson & Leon 2007; Gerber et al. 2009), how odour intensity is treated by olfactory systems is less clear. This needs to be resolved to fully appreciate the richness of olfactory behaviour and to reach an understanding of olfaction detailed enough to permit, for instance, the implementation into a biologically inspired artificial chemosensor.

We therefore study odour intensity processing, using the fruit fly Drosophila melanogaster. Like other insects, the fruit fly is a favourable system for such research as its olfactory system shares its basic molecular and cellular architecture with vertebrates, but comprises far fewer cells (reviewed by Hildebrand & Shepherd 1997; Strausfeld & Hildebrand 1999; Davis 2004; Ache & Young 2005; Bargmann 2006). The fruit fly olfactory system is particularly well studied at the molecular and cellular level, and recently also at the physiological level (reviewed by Vosshall 2000; Hallem et al. 2006; Dahanukar et al. 2005; Benton 2006; Fiala 2007; Vosshall & Stocker 2007; Gerber et al. 2009) and is particularly accessible to transgenic intervention (Brand & Perrimon 1993; Phelps & Brand 1998; Duffy 2002). Also, fruit flies have a fairly rich repertoire of olfactory behavior: for example, having experienced an odour together with electric shock, they later strongly avoid this odour (Tully & Quinn 1985). Other, non-trained odours only partially elicit such conditioned avoidance (T. Niewalda 2009, Universität Würzburg, personal communication). Because such generalization is typically only partial, flies must to some extent recognize odour quality. That is, it matters to the flies whether the odour at test has the same quality as the trained one. Here we ask whether odour intensity is also recognized: do the flies care whether the odour at test has the same intensity as during training?

In brief, we trained flies with pairings of electric shock with an odour (figure 1) at a particular MEDIUM intensity. After training, we tested conditioned avoidance in different groups of flies that were offered either the previously trained MEDIUM or a LOWer or a HIGHer intensity. If the flies include intensity information in their memory trace (i.e. ‘a MEDIUM intensity of this odour predicts shock’), they will show strongest conditioned avoidance when the previously trained MEDIUM intensity is indeed presented at test (figure 2a). Alternatively, if the memory trace does not contain any intensity information (i.e. ‘this odour predicts shock’), the flies will show stronger conditioned avoidance when more of the previously trained odour is presented; that is, the HIGHest intensity will induce the strongest conditioned avoidance at test (figure 2b). Clearly, this type of experiment specifically probes whether the flies ‘spontaneously’ integrate intensity information into their memory trace, without explicitly being cued to do so. Using such rationale, we probed for intensity learning with respect to the four odours that have been regularly used in neurogenetic analyses of Drosophila olfactory learning.
2. MATERIAL AND METHODS

(a) Flies

*Drosophila melanogaster* of the Canton-Special wild-type strain were maintained as mass culture at 25°C and 60 to 70 per cent relative humidity, under a 14:10 h light: dark cycle. On the day prior to the experiments, 1- to 4-day-old flies were collected in fresh food vials and kept overnight at 18°C and 60 to 70 per cent relative humidity.

(b) Odours

As odours, we used 3-octanol (OCT), *n*-amylacetate (AM), 4-methylcyclohexanol (MCH) and benzaldehyde (BA; all from Fluka, Steinheim, Germany, except AM, which is from Merck, Darmstadt, Germany; CAS 589-98-0, 628-63-7, 589-91-3, 100-52-7). Odours were diluted in solvent paraffin oil (Merck; CAS 8012-95-1); the ‘relative concentration’ of an odour is operationally defined as dilution relative to pure odour throughout this paper. Differences in such relative concentration did lead to behaviourally relevant differences in intensity for all odours used (figure 3). Throughout, we applied 350 µl of either the odour solution at the specified relative concentration or the solvent paraffin oil in 1-cm-deep Teflon containers of 15 mm diameter.

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Figure 1. The learning paradigm. One group of flies was trained such that the odour was paired with the shock, and the solvent paraffin oil was presented alone (top); for a second group, we used a reciprocal training regimen (bottom). Each group was then tested for choice between the odour and solvent in a T-maze. For each group, an odour preference (PREF) was calculated based on the distribution of the flies. The difference between the PREF values of the reciprocally trained groups then gives the learning index. Negative learning indices demonstrate conditioned avoidance of the odour. Note that for half of the cases the sequence of events was as depicted (Odour–Shock / Solvent or Solvent–Shock / Odour), whereas for the other half of the cases (not drawn here) the sequence was reversed (i.e. Solvent / Odour–Shock or Odour / Solvent–Shock).

Figure 2. Experimental strategy to probe for intensity learning. We trained flies using a designated MEDIUM intensity of an odour. In a subsequent test, different groups of flies were then offered either the trained MEDIUM intensity or a LOWer or a HIGHer intensity. Two possible experimental outcomes are sketched. (a) Intensity learning: if the flies learn that specifically the MEDIUM intensity of the odour predicts shock, they will show strongest conditioned avoidance when offered the same MEDIUM intensity at test (dashed grey line). (b) No intensity learning: if the flies do not include the intensity parameter in their memory trace, stronger conditioned avoidance will be found when the intensity at test is HIGHer.
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**c) Learning paradigm**

Experiments were performed at approximately 25 °C and 70 to 80 per cent relative humidity, under the light from a 50 W light bulb placed approximately 50 cm above the experimental setup (Schwaerzel et al. 2003; Yarali et al. 2008). Flies were trained and tested in groups of approximately 100. Training started by loading the flies into the set-up (figure 1; 0:00 min). The odour was presented from 1:00 min on for 1 min; the electric shock followed at 1:15 min as 12 pulses of 100 V; each pulse was 1.2 s long and was followed by the next pulse with an onset-to-onset interval of 5 s. The solvent was then presented from 3:00 min on, also for 1 min. In half of the cases, contrary to the above, the training started with the no-shock treatment. Once this training was completed (9:00 min), flies were transferred to a T-maze, where they could choose between the odour and the solvent arm. After an additional 2 min, the arms of the maze were closed and flies on each side were counted. A preference index (PREF) was calculated as

\[
PREF = \frac{\#_{\text{Odour}} - \#_{\text{Solvent}}}{\#_{\text{Total}}} \times 100. \tag{2.1}
\]

In this equation, \( \# \) indicates the number of flies in the respective maze arm. For every group trained as above (i.e., Odour−Shock / Solvent [or Solvent / Odour−Shock]), another group was trained reciprocally (Solvent−Shock / Odour [or Odour / Solvent−Shock]; figure 1; in the figure as well as below, the subgroups that received the respective other sequence of training events are omitted for clarity). As these kinds of group received identical treatment except for the contingency between odour and shock during training, any difference between their olfactory preferences must reflect associative learning. This difference is quantified as a learning index

\[
PREF_{\text{Odour−Shock}} / PRE_{\text{Solvent−Shock}} / Odour = \frac{PREF_{\text{Odour−Shock}}}{PREF_{\text{Solvent−Shock}} / Odour}. \tag{2.2}
\]

In this equation, the subscripts of PREF represent the respective training regimen. Learning indices thus range from −100 to 100, positive values indicating conditioned approach and negative values conditioned avoidance.

**d) Statistics**

The data were analysed using non-parametric statistics throughout. For global comparisons between multiple groups, Kruskal–Wallis tests (KW test) were used. For comparing scores between pairs of groups, we used Mann–Whitney U tests (U test). When multiple pairwise comparisons were made, a Bonferroni correction kept the experiment-wide error rate at 5 per cent by dividing the critical \( p \)-value by the number of tests (e.g., \( p < 0.05 / 2 \) if two tests are applied). All statistics were performed on a PC with STATISTICA (Statsoft, Tulsa, OK, USA). We report the statistics in the figure captions.

**e) Experimental design**

For each odour, we performed two experiments. The first experiment characterized the dose dependency of olfactory learning and retrieval with respect to that odour. That is, between groups, we varied the relative concentration of the odour; importantly, for each group, the relative concentration at test equaled the one used for training. Based on this experiment, we designated three relative concentrations of the respective odour as, LOW, MEDIUM or HIGH intensity. For MCH we used a fourth intensity, VERY LOW.

The second experiment then used these intensities to probe for intensity learning. We trained the flies with the designated MEDIUM intensity; then, at test, different groups of flies were offered either the same MEDIUM intensity or a LOTer one or a HIGHER one (or VERY LOW for MCH). If during training the flies learned that specifically the MEDIUM intensity of the odour predicted shock, they would show the strongest conditioned avoidance when that particular intensity was offered at test (as sketched in figure 2a). Contrarily, if the flies did not form any intensity-specific memory for the odour, they would show the strongest conditioned avoidance at test when the intensity was the HIGHEST (figure 2b).

**3. RESULTS**

**a) Intensity learning for 3-octanol, n-amylacetate and 4-methylcyclohexanol**

Learning scores improved with increasing relative concentration of OCT, within the range we looked at, arguing that the different relative concentrations we used support different perceived intensities (figure 3a(i)). We assigned intensities as LOW, MEDIUM and HIGH according to the learning scores they supported (figure 3a(ii)).

We then used these three intensities to test whether flies learn about OCT intensity. We trained the flies with the designated MEDIUM intensity and varied the intensity between groups during the test. When the MEDIUM intensity was used for the test, we found clearly stronger conditioned avoidance compared with either LOW or HIGH intensity (figure 4a). Thus, flies showed the strongest conditioned avoidance to that odour intensity they have been trained with, suggesting that they do indeed include intensity information in their memory.

Two further odours, AM (figures 3b(i,ii) and 4b) and MCH (figures 3c(i,ii) and 4c), yielded the same pattern of results. Thus, we conclude that flies do form intensity-specific memories for AM, MCH and OCT.

**b) No intensity learning for benzaldehyde**

Concerning the fourth odour, BA, we found that learning scores improve with increasing relative concentration within the covered range (figure 3d(i)); assignment of intensities as LOW, MEDIUM and HIGH followed the learning scores they support (figure 3d(ii)).

We then trained flies using the designated MEDIUM intensity of BA and, at test, offered them either this MEDIUM intensity or the LOW or the HIGH intensity. Despite having been trained with MEDIUM, flies avoided the HIGH intensity more strongly at test, whereas conditioned avoidance of LOW did not statistically differ from that of MEDIUM (figure 4d(i)).

To extend and confirm these results, we trained three further groups with the designated LOW intensity of BA and found the same pattern of results: both MEDIUM and HIGH intensity at test were avoided more strongly than the LOW intensity, which had been presented during training (figure 4d(ii)). Thus, regardless of which BA intensity was present at training, flies avoided HIGHER intensities more strongly at test, directly arguing against an intensity-specific memory trace with respect to BA.
Figure 3. Dose–effect curves for olfactory learning. For the indicated odours, we characterized the dose–effect relationship of olfactory learning. With respect to each odour, we then designated relative concentrations as LOW, MEDIUM or HIGH intensity, supporting different levels of learning scores (for MCH, a VERY LOW intensity was also chosen). In part (i) of (a–d), box plots represent the median as the middle line, 25, 75% and 10, 90% as box boundaries and whiskers, respectively. Statistics refer to KW tests (*p < 0.05). In part (ii) of (a–d), the respective median learning indices are plotted on a truncated y-axis, across a logarithmic scale for the relative odour concentration. Statistics refer to U tests. n.s.: p > 0.05/2, *p < 0.05/2 (Bonferroni correction; see §2). (a(i)) Learning scores improved with increasing relative OCT concentration (H = 23.44, d.f. = 4, p < 0.05, n = 8, 22, 24, 15, 24). (a(ii)) We designated the relative concentration of 0.00001 as LOW, 0.0001 as MEDIUM and 0.001 as HIGH intensity; these supported increasingly better learning scores (LOW versus MEDIUM: U = 101.00, p < 0.05/2). (b(i)) For AM also, learning scores improved with increasing relative odour concentration (H = 19.71, d.f. = 3, p < 0.05, n = 16, each). (b(ii)) We designated the relative concentrations of 0.00001, 0.0001 and 0.01 as LOW, MEDIUM and HIGH intensities, respectively. MEDIUM supported better learning scores than LOW (U = 49.00, p < 0.05/2), whereas a trend for HIGH to support better learning scores than MEDIUM failed to reach significance (U = 83.00, p = 0.09). (c(i)) MCH also supported better learning scores as its relative concentration increased (H = 77.04, d.f. = 4, p < 0.05, n = 16, 39, 40, 32, 32). (c(ii)) We chose the relative concentration of 0.0003 as VERY LOW, 0.001 as MEDIUM and 0.01 as HIGH intensity; additionally, by interpolating, we chose the relative concentration of 0.00054 as LOW. HIGH supported better learning scores than MEDIUM (U = 250.00, p < 0.05/2), which in turn worked better than VERY LOW (U = 188.00, p < 0.05/2). (d(i)) Learning scores also improved with increasing relative concentration of BA (H = 64.22, d.f. = 5, p < 0.05, n = 16, 24, 24, 24, 24, 16). (d(ii)) We assigned the relative concentrations of 0.0001, 0.00054 and 0.001, respectively, as LOW, MEDIUM and HIGH intensities. These supported increasingly better learning scores (LOW versus MEDIUM: U = 132.00, p < 0.05/2; MEDIUM versus HIGH: U = 145.00, p < 0.05/2).
relative odour concentration at test/at training

(a) Intensity learning for OCT, AM and MCH but not for BA. Statistics refer to U tests. n.s.: \( p > 0.05/3 \), \( * p < 0.05/3 \), except in (c) and (d(iii)), where n.s.: \( p > 0.05/3 \), \( * p < 0.05/3 \). Other details as in the legend of figure 3. (a) Having been trained with a MEDIUM intensity of OCT, flies showed the strongest conditioned avoidance when offered the same MEDIUM intensity at test (MEDIUM versus LOW: \( U = 107.00, p < 0.05/2 \); MEDIUM versus HIGH: \( U = 72.00, p < 0.05/2 \), \( n = 20, 24, 24 \)). (b) Similarly, after training with a MEDIUM intensity of AM, the same MEDIUM intensity induced stronger conditioned avoidance than a HIGHER intensity (\( U = 96.00, p < 0.05/2 \)). Conditioned avoidance did not statistically differ between LOW and MEDIUM (\( U = 189.00, p = 0.23 \)). The adjacent LOW intensity induced as strong conditioned behaviour as MEDIUM (\( U = 290.00, p = 0.16 \)). Sample sizes are \( n = 24, 31, 24 \). (d(i)) Despite having been trained with a MEDIUM intensity of OCT, flies showed stronger conditioned avoidance to the HIGH intensity at test (\( U = 492.00, p < 0.05/2 \); conditioned avoidance to MEDIUM and LOW did not differ statistically (\( U = 1030.00, p < 0.05/3 \)). The adjacent LOW intensity induced as strong conditioned behaviour as MEDIUM (\( U = 290.00, p = 0.16 \)). Sample sizes are \( n = 24, 34, 24 \). (d(ii)) Similarly, although they were trained with a LOW intensity of BA, flies avoided both the MEDIUM (\( U = 220.00, p < 0.05/2 \)) and the HIGH intensity (\( U = 196.00, p < 0.05/2 \)) more strongly at test. Sample sizes are \( n = 48, 48, 40 \). (d(iii)) The median learning indices from (d(iii)) are plotted on a truncated y-axis against a logarithmic scale for BA intensity at test. Under the conditions of LOW testing intensity, a MEDIUM training intensity supported stronger BA memories than a LOW training intensity (\( U = 364.00, p < 0.05/3 \)). Also, learning scores uncovered by the HIGH testing intensity were lower after training with LOW than after training with MEDIUM (\( U = 177.00, p < 0.05/3 \). (e) Semi-schematic summary of the data in (a–d). With respect to each odour, we express the various relative concentrations used at test as multiplicative of the training concentration (i.e. multiplicative of the relative concentration designated as the MEDIUM intensity). These values are plotted on the x-axis using a logarithmic scale. Then, with respect to each odour, we take the median learning score obtained when MEDIUM training intensity is offered at test and define this as -1 (grey circle; by definition the same for all odours). All other median learning scores are then accordingly normalized and are plotted as ‘normalized learning indices’ on the y-axis. For OCT (purple), AM (blue) and MCH (green), the best learning scores are obtained when the relative odour concentration matches between training and test (dashed grey line), showing intensity learning for these odours. Contrarily, for BA (red), flies show the best learning score when the highest relative concentration is offered at test, arguing against intensity learning of BA.
Interestingly, despite a mismatch between training and testing intensity, when trained with the MEDIUM intensity but tested with LOW, flies had better scores than when trained and tested with LOW. This demonstrates that training with MEDIUM establishes stronger memories for BA than training with LOW does; critically, this difference in memory strength shows under conditions of equal testing intensity (figure 4d(iii)).

Thus, intensity affects both the establishment and the recall of BA memory: for a given testing intensity, scores were higher when the training intensity was higher. And, for a given training intensity, scores were higher for higher testing intensities. Most important, it was irrelevant to the flies whether the intensities at training and test matched. Thus, as far as BA is concerned, flies apparently do not include intensity information in their olfactory memory.

4. DISCUSSION

(a) Exception and rule in Drosophila intensity learning

Do flies spontaneously integrate the information about odour intensity into their memory? Yes and no. With respect to OCT, AM and MCH, flies did show intensity learning, but for BA we found no intensity learning using the present experimental rationale (figure 4e). Using somewhat modified experimental designs, intensity learning for pentyl acetate, 6-methyl-5-hepten-2-one (see fig. 4 in DasGupta & Waddell 2008) and iso-AM (fig. 6a in Masek & Heisenberg 2008) has previously been found. Thus, it seems that BA is the ‘odd one out’ with respect to intensity learning. Indeed, BA has long been suggested to be peculiar: loss of function of the abnormal chemo-sensory jump 6 gene (CG9151) abolishes maxillary palp electrophysiological responses to BA (Ayer & Carlson 1992), but not to four other tested odours. Loss of function of the *pentagon* gene in turn disturbs the flies’ jump response to BA, but not to three other tested odours (Helfand & Carlson 1989). Furthermore, after surgical removal of the antenna and the maxillary palps, flies still avoid BA, while avoidance of two other odours is lost (Keene et al. 2004). In addition, activity of the DPM neuron seems to be required for the formation of BA memory, while such activity is required with respect to two other tested odours only during the consolidation of memory (Keene et al. 2004). Interestingly, in honeybees also intensity learning has been demonstrated for some (1-hexanol and 2-octanone: Wright et al. 2005) but not for other odours (linalool: Pelz et al. 1997; geraniol: Wright et al. 2005). In both flies and bees, the neuronal determinants as well as ecological relevance of these discrepancies between odours remain clouded.

(b) Under which conditions may high–low differential training be suitable to measure intensity learning?

The present experimental design allows probing whether flies integrate information about odour intensity into their olfactory memory, without being explicitly cued to do so. Two previous studies (Xia & Tully 2007; Masek & Heisenberg 2008; see also Borst 1981 with respect to sugar reward learning) probed for intensity learning using an experimental design intended to force the flies to use intensity information: the authors trained one group of flies such that a low intensity of the odour was paired with shock, whereas a high intensity was presented alone (low–Shock / high); another group of flies was trained as high–Shock / low. Both groups were then tested for their preference between the low and the high intensity. The authors concluded that intensity learning took place based on the difference between the preferences of these two groups: namely, the flies trained with high–Shock / low avoided the high intensity more than the low–Shock / high-trained flies. Such interpretation may, but need not, be misleading. Take the case of BA: using the same testing intensity, a relatively higher intensity at training later allows stronger conditioned avoidance than a relatively lower intensity at training (figure 4d(iii)); comparing between the red curve and the pink curve); in other words, the higher training intensity establishes a stronger memory trace. Regarding a differential training design, both reciprocal groups may then avoid the high intensity proportional to the strength of the aversive memory they had formed for the odour during training. Such memory would be stronger when the high intensity is paired with shock than when the low intensity is paired with shock. Thus, the group trained as high–Shock / low would avoid the high intensity more strongly than the group trained as low–Shock / high, although intensity-specific memory may not have been formed. Thus, potentially, such strategy is not actually measuring intensity learning—unless it is shown within the respective experimental series that the chosen odour intensities are learned equally well.

It is worth noting that, after differential training, intensity learning may alternatively be probed for by separately looking at the high versus low preference scores of each reciprocal group (e.g. fig. 5c in Masek & Heisenberg 2008). That is, one can test whether flies that have been trained as low–Shock / high, when given the choice between high and low intensities, avoid the low intensity more strongly in comparison with a baseline situation. But what would be the most reasonable measure of baseline? Should one use the distribution of naive, untreated flies when determining ‘baseline’ preferences and that using the preference of naive, untreated flies may be misleading.

(c) Mechanism of intensity learning in Drosophila

Regarding a possible site of an odour intensity memory trace, local interneurons (Ng et al. 2002; Sachse & Galizia 2003; Wilson & Laurent 2005; Shang et al. 2007) or multi-glomerular projection neurons (Lai et al. 2008) appear likely candidates, as they seem to monitor the total antennal lobe activity level. As a short-term memory trace for odour quality is arguably localized to
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the mushroom bodies (reviewed by Gerber et al. 2004), according to this scenario memory traces for quality and intensity are stored in separate neurons. Alternatively, both kinds of memory trace may be entangled within the mushroom bodies (with the above-mentioned caveats in mind, see Xia & Tully 2007). Typically, only a small fraction of mushroom body Kenyon cells is activated by a given odour (Wang et al. 2004; Turner et al. 2008; but see Voeller 2009); different intensities of an odor might induce sufficiently non-overlapping activity patterns to support intensity-specific memory traces (fig. 3 in Wang et al. 2004; but see Voeller 2009).

It is also tempting to speculate about distinct intracellular mechanisms of quality and intensity learning. In typical differential conditioning experiments, two odours are used without adjusting their intensities for equal learnability; thus, learning scores probably reflect both quality and intensity learning. In such experiments, even null mutations of various ‘learning genes’ typically cause only partial deficits (e.g. Godenschwege et al. 2004; Michaels et al. 2005 with respect to the synapsin gene), suggesting that either quality or intensity learning might be differentially impaired (with the above-mentioned caveats in mind, see Xia & Tully 2007; Masek & Heisenberg 2008). Indeed, Masek & Heisenberg (2008) found, using high-low differential training, that the rut2080 mutant is unimpaired in intensity learning while showing the previously reported defect in quality learning; however, given the recent observation of Pan et al. (2009) that in the rut2080 mutants approximately 30 per cent of rut-mRNA remains, the suggestion that intensity learning is independent of rut function is called into question.

To conclude, neither the neuronal nor the molecular mechanisms of intensity coding and intensity learning are yet understood. As an initial step towards filling this gap, we here analyse intensity learning at the behavioural level, using a simple experimental design, which as far as we see is clear of confounds. We find that flies form intensity learning; the neurobiological and ecological implications of this finding are discussed below.

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Brand, A. H. & Perrimon, N. 1993 Targeted gene expression using a simple experimental design, which as far as we see is clear of confounds. We find that flies form intensity-learning; the neurobiological and ecological implications of this finding are discussed below.


