Title

Insensitivity to reward shifts in Zebrafish (*Danio rerio*) and implications for assessing affective states.

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Abstract

Theory and empirical findings predict that individuals in a negative affective state are more sensitive to unexpected reward loss and less sensitive to unexpected reward gain compared to individuals in a neutral or positive affective state. We explore the use of sensitivity to reward shifts measured during successive contrast tasks as an indicator of affect in zebrafish (*Danio rerio*). In line with the assumption that exposure to rewarding stimuli induces a relatively positive affective state compared to exposure to stimuli that they do not prefer, we confirmed that zebrafish prefer enriched over barren environments, suggesting that the enriched environment is associated with positive affective states. We trained individuals to swim down a channel for food rewards of differing value and then presented them with unexpected increases or decreases in reward value. Contrary to our hypothesis, individuals conditioned to a high-value reward continued swimming at the same speed when reward value was downshifted, thus showing no successive negative contrast effect and appearing insensitive to reward loss. Individuals whose rewards were upshifted gradually increased their speed, but did not display successive positive contrast effects typical of sensitivity to reward gains. In both cases, housing type did not result in differences in swim time. One potential explanation is that goal-directed control of behaviour is necessary for an animal to show a successive contrast response to unexpected reward gain or loss, and the behaviour of zebrafish in this task was under habitual control, perhaps due to over-training. If so, refinements to task design and training procedures will allow further progress with this assay.

43 Keywords: cognitive bias, reward sensitivity, successive negative contrast, animal
44 affect, fish, environmental enrichment

Introduction

Affective states are increasingly being recognised as a fundamental determinant of an animal's welfare (Dawkins 1990; Mendl et al. 2009), but are difficult to study objectively. A recent innovation in the study of animal affect is to use changes in cognitive function, for example affect-induced 'cognitive biases', as proxy measures of affective states. It is assumed that such states are instantiated in neural activity even if we cannot be sure that they are accompanied by conscious emotional feelings (Anderson and Adolphs 2014; LeDoux 2017; Mendl and Paul 2016). Affect-induced differences in the way individuals make decisions about the valence of an ambiguous stimulus, commonly known as judgment bias, is the most common type of cognitive bias studied in animals (Harding et al. 2004).

A related cognitive measure that has received less attention to date is how animals respond to changes in anticipated reward; their sensitivity to reward shifts. This can be measured in paradigms designed to test for successive negative or positive contrast effects. For example, during operant conditioning studies, individuals who have learnt to make a particular action to obtain a large magnitude reward but then unexpectedly receive smaller rewards, temporarily perform the learnt action more slowly (or otherwise with less efficiency) compared to individuals that have learnt to perform the same action but for the low magnitude reward from the outset (e.g. Capaldi and Lynch 1967; Crespi 1942; Ehrenfreund 1971; Gonzalez et al. 1962).

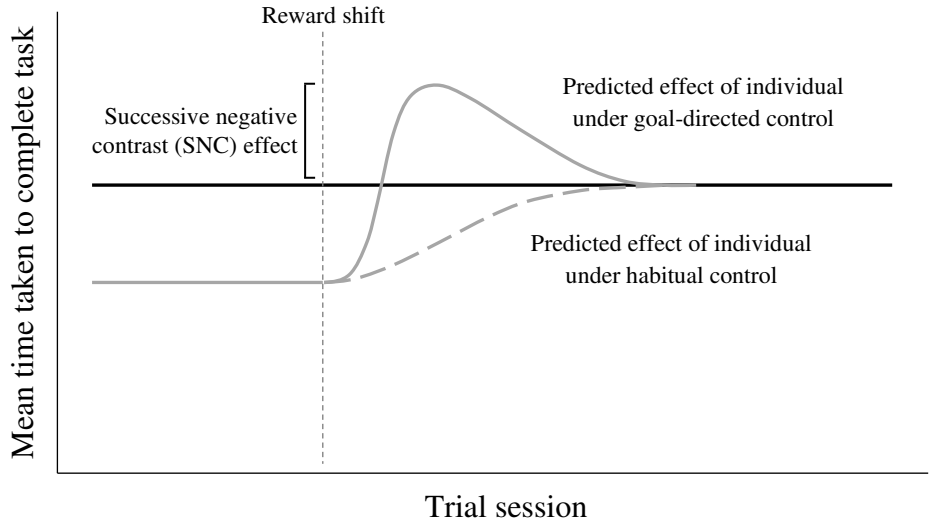
This effect has been termed the frustration, depression or successive negative contrast (SNC) effect (Flaherty 1996). The opposite effect, termed the elation effect or successive positive contrast (SPC) effect, has also been demonstrated (e.g. Benefield et al. 1974; Mellgren 1972; Shanab et al. 1969), although somewhat less reliably.

Individuals appear to vary in their sensitivity to reward shifts, and one factor that may affect this sensitivity is the individual's affective state. For instance, in humans we know that heightened sensitivity to reward loss is associated with anxiety-related disorders such as obsessive-compulsive disorder (Gehring et al. 2000; Hajcak et al. 2004), depression (Beck 1967; Wenzlaff and Grozier 1988), and reduced responsiveness to rewarding stimuli (Clark and Watson 1991; Fowles 1994; Leppänen 2006; Naranjo et al. 2001). Further, anxiolytics (antianxiety agents) have been shown to reduce sensitivity to reward loss (Flaherty et al. 1998; Morales et al. 1992). Therefore, there is potential for sensitivity to reward shifts to be a useful indicator of affective state if SNC and SPC effects can also be elicited in other animal species.

However, SNC or SPC effects are not inevitable responses to unexpected shifts in reward. Amsel (1992) suggested that animals whose behaviour in a SNC task is controlled by anticipation of the outcome of an action – action-outcome learning or goal-directed control (Dickinson and Balleine 1994; Dolan and Dayan 2013) – may experience an affective, frustration-like, response to an unexpected decrease in reward resulting in a SNC effect. However, if an animal's behaviour is under habitual control (for example, due to over-training) resulting in stimulus-response learning with no explicit representation of the outcome, then frustration-like responses to an unexpectedly poor outcome and associated SNC effects are unlikely.

Fig. 1 illustrates these differences in predictions. The key point for our purposes is that an SNC effect needs to be evident in the species of interest before we can go on to consider whether affective states influence the magnitude of this effect and hence generate individual differences in sensitivity to reward shifts that can be used as proxy measures of these states.

(a)


Reward shift
Successive negative contrast (SNC) effect
Predicted effect of individual under goal-directed control
Predicted effect of individual under habitual control
Mean time taken to complete task
Trial session

(b)

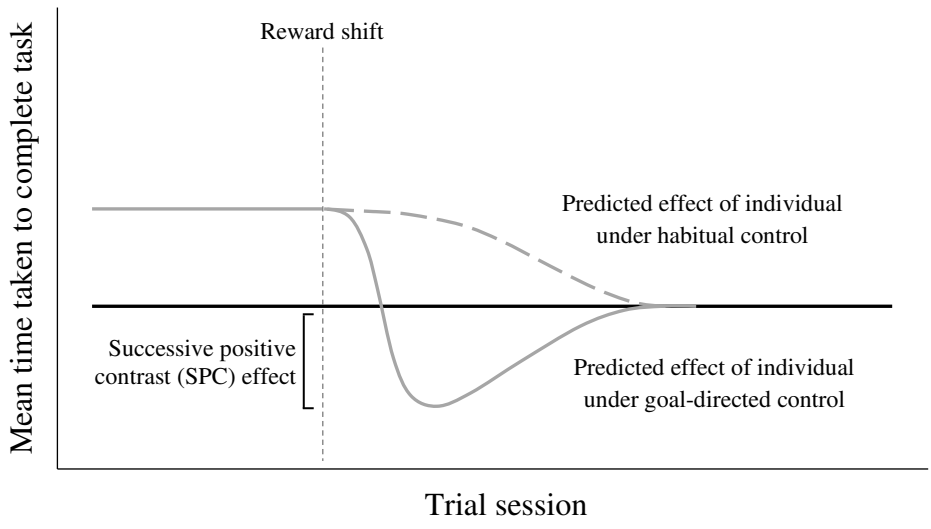

Reward shift
Predicted effect of individual under habitual control
Successive positive contrast (SPC) effect
Predicted effect of individual under goal-directed control
Mean time taken to complete task
Trial session

Fig. 1 Predicted effects of a (a) reward downshift, and (b) reward upshift on time taken to complete a reward-acquisition task. In (a), the group designated by the grey line is routinely rewarded with a higher-value reward, and takes on average a shorter time to complete the task compared to the group designated by the black line, which is routinely rewarded with a lower-value reward. When a reward downshift from the higher-value reward to the lower-value reward (represented by the vertical dashed line) occurs for the grey group, the effects of the downshift on the mean time taken to complete the task are illustrated. For individuals whose behaviour is under goal-directed control, an unexpected decrease in outcome has the potential to induce a frustration-like state resulting in an increase in the mean time taken, past that of individuals conditioned to the lower-value reward from the outset, before eventually reaching equilibrium (solid grey line). The difference in mean time taken is designated the SNC or depression effect. No such effect is predicted for individuals whose behaviour is under stimulus-response habitual control (dashed grey line). In (b), the opposite scenario (a reward upshift) is illustrated

Successive negative contrast has been investigated in a number of species. Of these, studies on mammals such as rats, *Rattus norvegicus* (e.g. Crespi 1942), mice, *Mus musculus* (e.g. Mustaca et al. 2000), opossums, *Lutreolina crassicaudata* and *Didelphis albiventris* (e.g. Papini et al. 1988), domestic dogs, *Canis familiaris* (Bentosela et al. 2009), human babies (e.g. Kobre and Lipsitt 1972), and European starlings, *Sturnus vulgaris* (Flaherty 1996; Freidin et al. 2009) have demonstrated SNC effects. Other non-mammalian vertebrates such as pigeons, *Columbia livia* (e.g. Papini 1997), toads, *Bufo arenarum* (e.g. Muzio et al. 1992; Papini et al. 1995), turtles, *Geoclemys reevesii* (Papini and Ishida 1994) and goldfish, *Carassius auratus* (e.g. Couvillon and Bitterman 1985; Lowes and Bitterman 1967) exhibited a downshift in performance when rewards were reduced but did not perform below the level of controls and hence no SNC effect was observed.

In fish, the majority of early studies were conducted on the goldfish, and all have failed to demonstrate a SNC effect (Couvillon and Bitterman 1985; Gonzalez et al. 1974; Gonzalez et al. 1972; Lowes and Bitterman 1967; Mackintosh 1971), but all studies were conducted on one species, the goldfish, *Carassius auratus*. The authors of these studies also noted that much of the application of frustration theory to non-mammalian vertebrates was poorly understood. Further, in mammals, initial experimental protocols on SPC or elation effects also found it difficult to demonstrate the effect, and it was later found that a more reliable SPC effect could be demonstrated if a number of modifications to the protocol were made, including increasing the difficulty of the task (Mellgren 1971), shifting the reward before the individuals were performing at their physiological limit (Mellgren 1972), or performing a downshift in reward before a subsequent upshift (Maxwell et al. 1976). Thus it seems possible that the scope and execution of previous research on fish could be further refined. Given that millions of fish are held in captivity for research purposes (Reed and Jennings 2010), there is an enormous potential benefit to welfare in developing protocols to understand how husbandry practices influence affect in fish, thus this deserves further attention.

The most commonly used fish species in research is the zebrafish, *Danio rerio*. Zebrafish are used in studies ranging from developmental biology (e.g. Creaser 1934; Grunwald and Eisen 2002) and genetics (e.g. Amsterdam and Hopkins 2006; Kimmel 1989) to drug research (e.g. Berghmans et al. 2005; Rubenstein 2003; Rubenstein 2006). Behavioural studies using zebrafish are less common, possibly because knowledge of the natural biology of the species is far from complete (Spence et al. 2008). Indeed, several authors have identified the development of standardised

145 protocols for husbandry and welfare as one of the key research priorities for zebrafish
146 research (Graham et al. 2018; Spence et al. 2008).

147 Here, we investigated sensitivity to reward shifts in zebrafish. In order to elicit
148 differences in affective state, we used the presence or absence of environmental
149 enrichment. Environmental enrichment has been successfully used to generate
150 differences in affective state in a similar study of sensitivity to reward shifts in rats
151 (Burman et al. 2008), and it is also known to be an important consideration for
152 zebrafish when choosing their habitat (Kistler et al. 2011). Following the assumption
153 that a preferred stimulus is likely to induce a positive affective state (Rolls 2005;
154 Rolls 2006), we investigated whether zebrafish preferred enriched over barren
155 conditions, as reported in previous studies (Kistler et al. 2011), with the intention of
156 then using the preferred condition to induce a relatively positive affective state.
157 Zebrafish were trained to swim down a channel to obtain either high or lower value
158 food rewards, and then reward values were unexpectedly switched, and the effect of
159 this switch on the time taken to complete the action was recorded. We tested the
160 hypothesis that the preferred housing condition induces relatively positive affect and,
161 consequently, minimises sensitivity to reward downshift (SNC) and enhances
162 sensitivity to reward upshift (SPC).

Materials and Methods

This research was approved by the Faculty of Science Animal Ethics Committee, The University of Melbourne (AEC Project #1212695.3).

Animals, housing and husbandry

We used 68 naïve, wild type Tübingen (TU) strain zebrafish obtained from the Australian Regenerative Medicine Institute (ARMI) Monash University, Melbourne, Australia. Ten fish were used in experiment 1 – these had participated in previous behavioural experiments but were naïve to this experiment. The remaining 58 fish were naïve to experimental testing. Twenty-eight fish were used in experiment 2, and 30 fish were used in experiment 3. Only male fish were used as the experiments required an extended period of time in individual housing and lack of access to males can cause female fish to become egg bound (Spence et al. 2008). Fish were obtained at six months of age and experiments were conducted over the next three months.

On arrival, fish were acclimatised to their new environment for seven days in a communal glass tank (25L, 480mm length by 250mm width by 240mm height). Each individual was then transferred into its own tank compartment (henceforth referred to as its home tank), constructed by dividing a 25L tank in half using a plastic mesh partition. Each individual was therefore afforded vision of and limited interaction with at least one other individual through the plastic mesh. Every two home tanks (*i.e.* one 25L tank) contained a biological sponge filter with integrated air bubbler, a water heater, and two sections of PVC pipe to serve as a hide (one in each home tank). This set up was designated the 'barren' housing condition for home tanks. Half of these tanks were supplemented with an additional three PVC pipes and five plastic plants,

similar to the enriched chamber of the habitat preference testing tank (**Fig. 2**b, described below). Tanks with this set up were designated as the structurally 'enriched' housing condition. All tanks were maintained at 26-28°C on a 14:10 day/night light cycle, using deionised water supplemented with 0.625 gm/L of water conditioning salts (Aquasonic Tropical water conditioner) to raise General Hardness to 75-150 ppm and adjusted to a pH of 7-8. Fish were fed two types of food with different nutritional values: spirulina-enhanced brine shrimp (Hikari Bio-Pure Spirulina Brine Shrimp, the higher-value food reward, henceforth referred to as shrimp) and generic flake food (Nutrafin Max Tropical Fish Flakes, the lower value food reward, henceforth referred to as flake), typically once or twice daily, six to seven days a week. The values of these rewards were validated in a previous food preference test as part of our larger study. In this test using the same strain of zebrafish, 28 fish were exposed to two counterbalanced pipettes, one filled with shrimp and one filled with flake, and the number of taps on each pipette as well as the time spent around each pipette analysed. Our preference trials showed a strong preference for shrimp, based on mean number of taps on the pipette ($F_{(1, 27)} = 124.96$, $P < 0.001$) and mean time spent around the pipette ($F_{(1, 27)} = 169.36$, $P < 0.001$), when given a choice between shrimp and flake (manuscript in preparation).

Experiment 1 – Habitat preference trials

This trial aimed to determine whether zebrafish show a distinct preference for more structured (enriched) environments over less structured (more barren) environments. Ten zebrafish naïve to this experiment were used. Fish were tested individually in one of two 25L, 480mm length by 250mm width by 240mm height testing tanks. The testing tanks were partially separated into three chambers: one structurally complex section with plastic plants and PVC pipe sections (designated the enriched chamber),

one empty section nearly identical to the barren home tanks apart from the functional tank furniture described above (designated the barren chamber), and a small middle chamber containing a heater and water filter where feeding took place once daily. Small gaps (20mm diameter) allowed travel between chambers (Fig. 2).

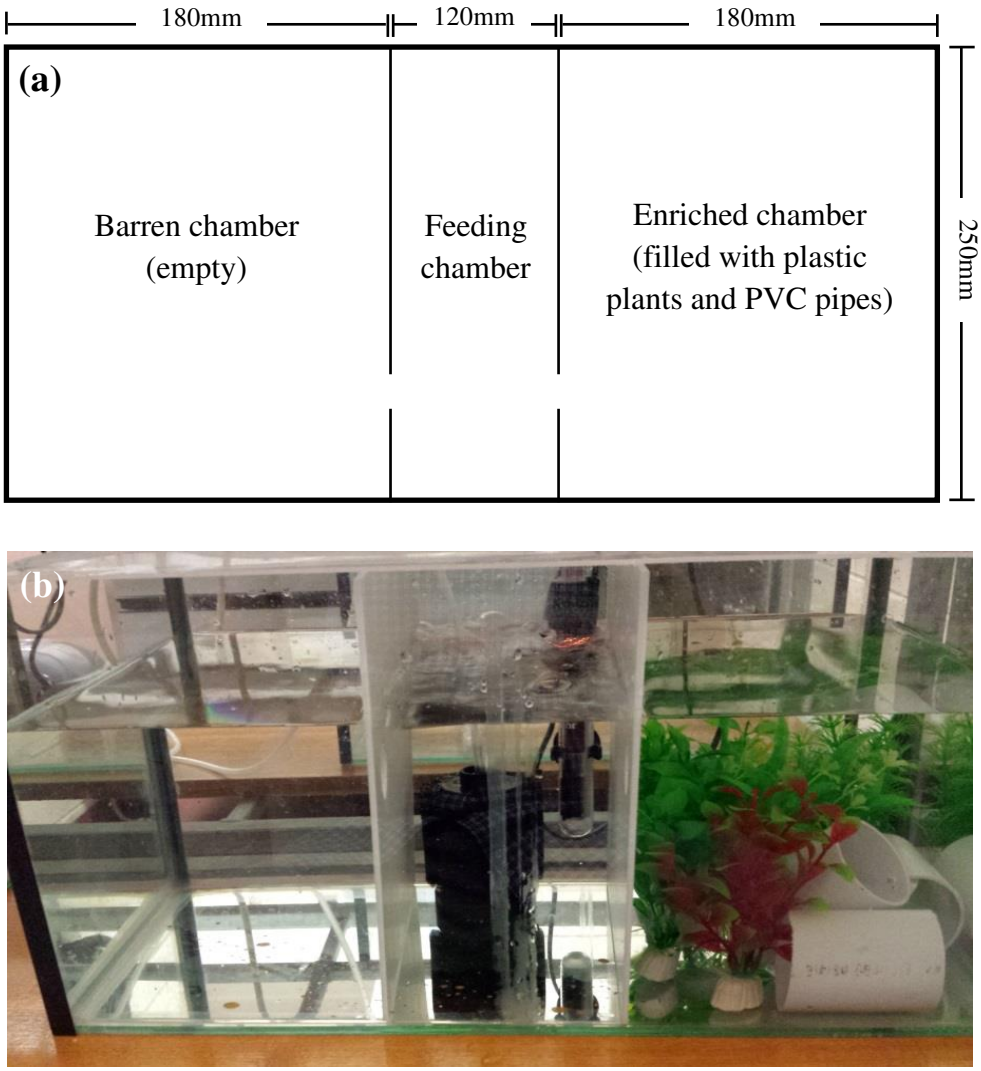


Fig. 2 (a) Diagram of top view, and (b) Photo of front view of the habitat preference trial testing tank. The testing tank was partially separated into three sections: one structurally complex section with plastic plants and PVC pipe sections (enriched), one empty section (barren), and a small middle chamber containing a heater and water filter where feeding took place once daily. Small gaps (20 mm) allowed travel between sections

Fish were acclimatised to their barren home tanks for a minimum of two weeks prior to habitat preference testing. During testing, fish were placed individually into a testing tank for three consecutive days – the first two days to allow fish to habituate to the tank set up before the 24-hour trial on the last day. While fish were in the testing tank, all chambers were accessible and the normal daily feed was delivered only in the middle chamber to eliminate possible bias towards the barren or enriched chambers due to the feeding regime. After the two-day habituation period, a 24-hour video recording was taken from the front of the tank (as seen in **Fig. 2**b) using an infrared surveillance camera system (Techview QV-3048 4 channel DVR kit, 0.25" CMOS colour cameras). Fish were fed in the central chamber at least half an hour prior to the start of this recording period, and no further food was given until after the completion of the recording period. The chamber the fish was located in was recorded every 15-minutes by reviewing the videos.

Preparation of fish for the sensitivity to reward shift experiments

Two groups of 28 fish were used in two separate experiments. In both experiments, fish were habituated to their home tanks, which were either enriched or barren (as described in Animals, housing and husbandry), for at least two weeks prior to the start of the experiment. During this time, fish were fed a mixture of shrimp and flake using multi-pipettes (Eppendorf Multipette 4780, 10µL dose). After this habituation period, fish were pseudo-randomly assigned to either higher-value (shrimp) or lower-value (flake) rewards. Therefore, each experiment had four groups of 7 fish using a 2 x 2 factorial design based on housing environment and reward value:

1. Enriched/shrimp;
2. Enriched/flake;

3. Barren/shrimp;
4. Barren/flake.

After the initial habituation period, fish were fed only their designated food reward at all times until the conclusion of the experiments.

In order to habituate fish to the experimental tank, they were individually transferred to the experimental tank using a plastic container (120mm diameter by 90mm depth), for five minutes once a day for three consecutive days. Fish readily swam into the container whenever it was introduced and displayed no signs of distress (apart from one individual, subsequently removed from the experiment, see Results), thus we believe it was unlikely that this procedure greatly influenced the fish's affective state.

The experimental tank was made up of a one metre long, 150mm diameter PVC pipe cut in half lengthways to form a half-cylindrical channel. Both ends of the PVC pipe were sealed with additional acrylic boards and the resultant channel filled with water.

Fish were allowed to swim freely and were fed their designated food reward while habituating in the experimental tank. Feeding helped fish to habituate to the tank more quickly (e.g. Bilotta et al. 2005; Galhardo et al. 2011) and was presumed to reduce the likelihood of any stress occurring due to the experimental tank itself, or the act of transferring individuals for the actual experiment, which might confound the data.

Experiment 2 – Sensitivity to reward loss

After fish were habituated to the experimental tank over three days, trials commenced the next day. Fish were individually transferred to the holding area at one end of the experimental tank using the plastic container. A small removable plastic barrier separated the fish from the rest of the channel. Each trial started when the barrier was

lifted and the trial ended when the individual crossed the finishing line on the other end of the channel (**Fig. 3**). The duration of each trial is henceforth referred to as the swim time. Trial times were determined by a stationary observer using a stopwatch. A multi-pipette tip (the same one used to feed fish during the habituation period) was also attached behind the finishing line as a motivator for fish in learning the task. Fish were rewarded with their respective reward (one brine shrimp or a small piece of flake food (approximately 10mg)) typically within half a second of crossing the finishing line (**Fig. 3**).

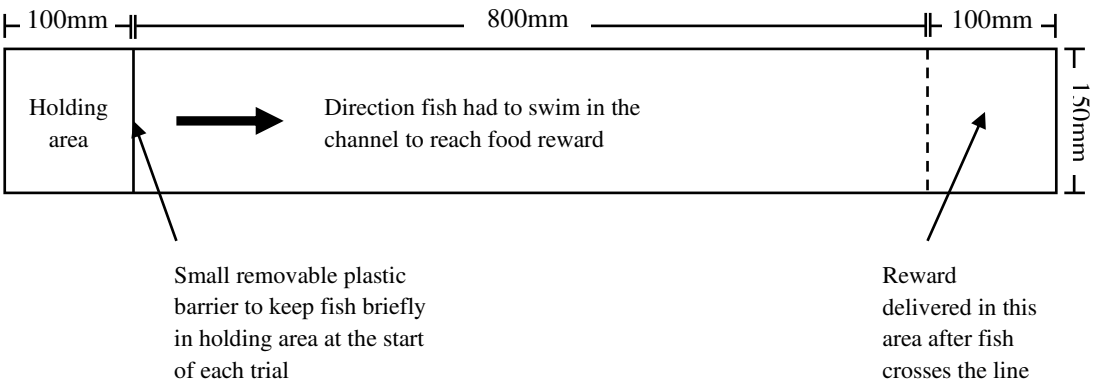


Fig. 3 Diagram of sensitivity to reward shift experimental tank (top view). Fish were kept in the holding area, separated from the rest of the channel by a removable plastic divider. At the start of each trial, the divider was lifted and the time taken for the fish to swim to the finishing line at the other end of the channel was recorded. Fish were rewarded with their respective food reward upon crossing the finishing line

Six trials per day were conducted for each individual. This number was chosen, on the basis of fish responses in previous experiments, to obtain enough trials but without posing unnecessary risk of satiation to food rewards. However, once fish were

returned to their home tanks, additional food was provided up to the usual daily amount. Trials were conducted for 11 days or until a statistically significant difference (via general linear mixed model, see Statistical analysis section below) in the times taken to swim the length of the channel between the shrimp and flake groups was observed over 4 consecutive days, whichever was shorter. A cut-off was designated for the pre-reward shift trial period to minimise the risk of over-training, and because we expected a difference in swim times to manifest readily within a few days.

Once either of these criteria was met, all of the fish originally trained on shrimp (i.e., enriched/shrimp and barren/shrimp groups) had their food rewards downshifted from shrimp to flake. Enriched/flake and barren/flake groups acted as control groups and continued to be rewarded with flake. Daily trials continued for the next six days to determine whether this downshift in reward produced any differences in the time taken to swim the length of the channel to the feeding area.

Experiment 3 – Sensitivity to reward gain

This experiment was conducted on a different group of naïve individuals. The experimental procedure was identical to that of experiment 2, except in this case fish originally trained on flake (i.e., enriched/flake and barren/flake groups) were upshifted to the shrimp reward during the reward shift phase. Enriched/shrimp and barren/shrimp groups now acted as control groups and continued to be rewarded with shrimp.

In addition, after a further 13 days of testing, all rewards for fish during trials ceased, and trials continued for another 13 days to determine how quickly behavioural extinction would occur once the task was no longer rewarded. Behaviours under habitual control are generally more resistant to behavioural extinction than goal-

directed behaviours (Bitterman 1969; Gonzalez et al. 1967), so this experiment would provide useful information on the underlying control of the conditioned behaviour.

Statistical analysis

All data were statistically analysed using IBM SPSS v23. We assessed the normality and homogeneity of data graphically (Zuur et al. 2010). All data fulfilled the assumptions of normality and homogeneity of variance.

Habitat preference trials were analysed using a paired *t*-test with individuals as replicates to compare the proportions of instances where fish were recorded in the barren versus enriched chambers of their tank during day, night, and combined periods. A linear mixed model (LMM) was also used to compare proportions of recordings within barren and enriched chambers across the day and night periods, with time period (day or night), habitat type (enriched or barren) and their interaction as fixed effects, and individuals as a random effect. Instances where fish were recorded in the middle chamber were excluded from all analyses.

For sensitivity to reward shift trials, prior to any shifts in reward, mean swim times were analysed each day using a LMM with habitat type (enriched or barren), original reward (shrimp or flake) and their interaction as fixed effects, and individuals nested within habitat type by original reward as a random effect, to determine if there was a significant difference in mean swim times between individuals trained on shrimp and flake respectively. Swim times for each trial day were aggregated for each individual, and mean swim times for each reward shift phase, including the day before each shift (*i.e.* experiment 1 – days 6-12; experiment 2 – days 11-24 and 24-37, see Results), were analysed using a LMM, with habitat type (enriched or barren), original reward (shrimp or flake), trial day and their interactions as fixed effects, and individuals

336 nested within habitat type by original reward as a random effect, to determine
337 sensitivity to reward shift.

Results

Experiment 1 – Habitat preference trials

During each 24-hour habitat preference trial, each fish's position was observed 96 times. Of these, 56 observations were during the day (14-hours) and 40 observations were at night (10-hours). **Fig. 4** presents the proportions of observations in each chamber for each individual.

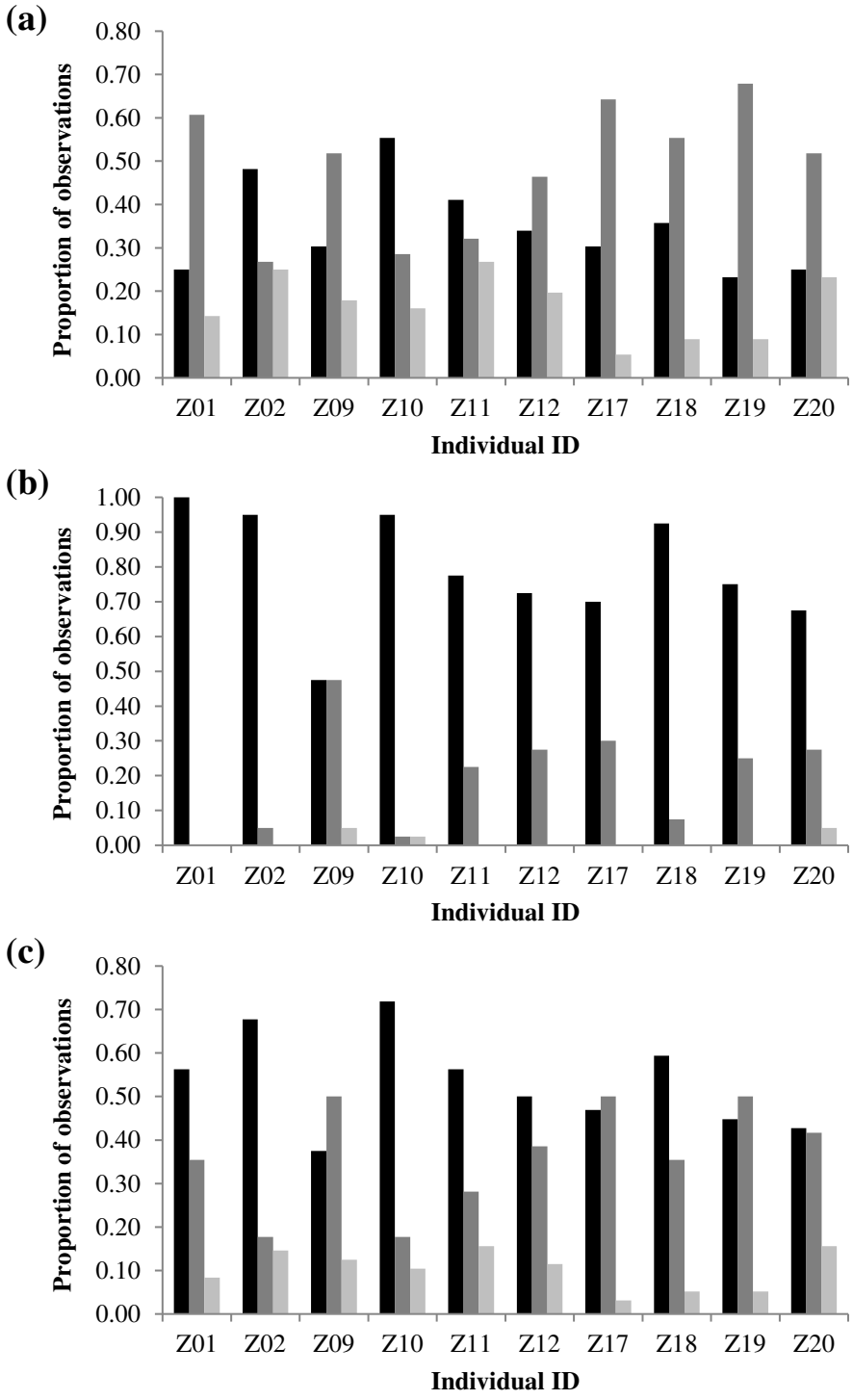
There was a significant preference for the enriched chamber over the barren chamber, both when the combined day and night observations were used (mean ± SE for the enriched chamber = 0.53 ± 0.04 versus 0.10 ± 0.01 for the barren chamber, $t_9 = 11.73$, $P < 0.001$), and when only day (mean ± SE for the enriched chamber = 0.35 ± 0.03 versus 0.17 ± 0.02 for the barren chamber, $t_9 = 5.47$, $P < 0.001$) or night (mean ± SE for the enriched chamber = 0.79 ± 0.05 versus 0.01 ± 0.01 for the barren chamber, $t_9 = 14.17$, $P < 0.001$) observations were used. In addition, there was a stronger preference for the enriched chamber at night compared to the day ($F_{(1, 27)} = 95.86$, $P < 0.001$, also see **Fig. 4**).

Experiment 2 – Sensitivity to reward loss

This experiment lasted 16 days. Within 3 days of the start of training, individuals trained on shrimp had significantly faster mean swim times than individuals trained on flake ($F_{(1, 24)} = 6.49$, $P = 0.018$), though mean swim times did decrease for both groups from an initial mean of 14 seconds as individuals learned the task. This effect persisted for the next 3 days (day 4: $F_{(1, 24)} = 7.93$, $P = 0.010$; day 5: $F_{(1, 24)} = 7.58$, $P = 0.011$; day 6: $F_{(1, 24)} = 8.98$, $P = 0.006$). There was no effect of habitat type (enriched

or barren) or interaction effect between food reward and habitat type on mean swim times during this initial training.

On day 7 onwards, groups trained on shrimp were unexpectedly rewarded with flake instead (*i.e.* a reward loss). Analysis of the reward shift phase, including the day before the reward downshift (*i.e.* days 6-12) demonstrated that individuals originally trained on shrimp continued to have significantly faster mean swim times than individuals trained on flake, despite the fact that both groups were receiving identical flake rewards during this period ($F_{(1, 24)}$ = 6.67, P = 0.016). Mean swim times also decreased significantly during this period ($F_{(6, 144)}$ = 4.29, P = 0.001). The difference in mean swim times between fish originally trained on shrimp and fish trained on flake persisted during an additional set of trials conducted 4 days after day 12 (day 16: $F_{(1, 24)}$ = 7.85, P = 0.010, **Fig. 5**). The habitat type did not have an effect on mean swim times ($F_{(1, 24)}$ = 0.00, P = 0.997). All other interaction effects were not significant.



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375 **Fig. 4** Proportion of observations in each of the three chambers (■ Black: enriched; ■ dark grey:
376 middle; and ■ light grey: barren) for each individual during the (a) day period, (b) night period, and (c)
377 combined day and night periods of the habitat preference trial

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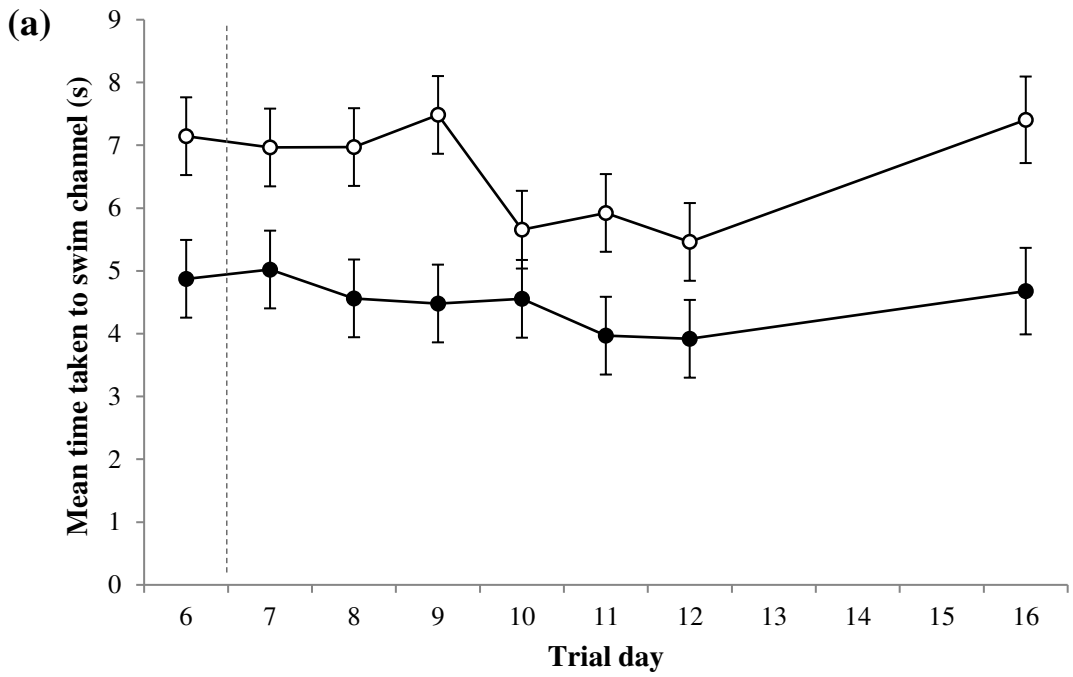
(a)
Mean time taken to swim channel (s)
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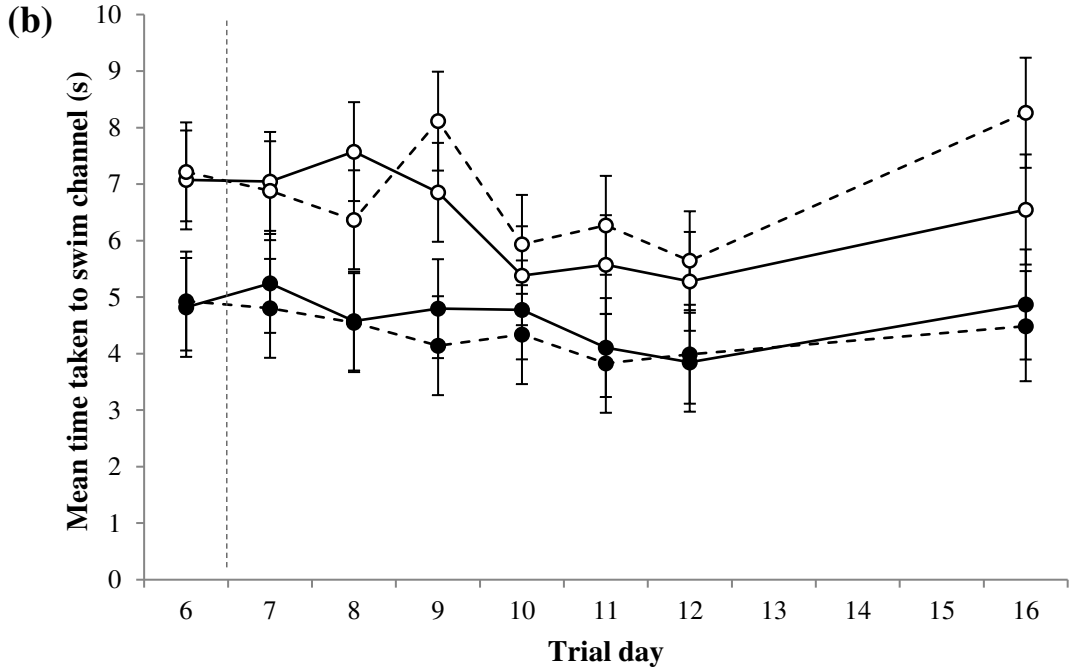
Trial day


(b)
Mean time taken to swim channel (s)
0
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Trial day

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Fig. 5 (a) Mean time taken for zebrafish to swim the channel on each trial day for shrimp and flake reward groups in experiment 2 (post-reward downshift phase), and (b) the same data, but with food reward groups further separated into barren and enriched habitat groups. In both figures, the vertical dashed line between trial days 6 and 7 marks the day that shrimp reward groups had rewards downshifted from shrimp to flake, ● black markers represent pre-shift shrimp reward groups, ○ white markers represent pre-shift flake reward groups, and error bars denote standard error. In (b), dashed lines represent barren habitat groups, while solid lines represent enriched habitat groups

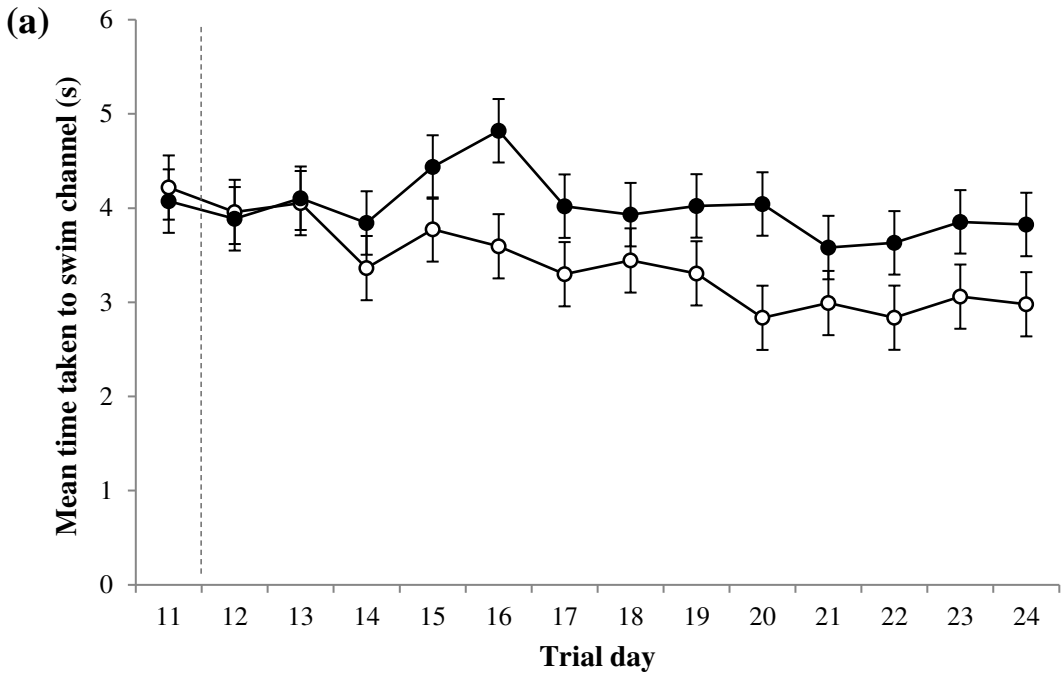
Experiment 3 – Sensitivity to reward gain

This experiment was conducted over 37 trial days. At the start of the experiment, one individual failed to habituate to the experimental tank, displaying erratic behaviour and symptoms of distress. This fish was replaced after trial day 2. In addition, two individuals died during the experimental period due to accidents, one on day 4 and one on day 13. The individual on day 4 was replaced, but the individual that died on day 13, which belonged to the shrimp/enriched treatment group, was not replaced because the experiment had already progressed substantially. Therefore, the shrimp/enriched treatment group had a sample size of 6 compared to 7 in the other treatment groups from day 13 onwards.

Although the pre-reward shift phase of this experiment was conducted identically to that of experiment 2, individuals trained on shrimp in this experiment did not have significantly faster mean swim times than individuals trained on flake after 11 days of conditioning (day 11: $F_{(1, 24)} = 0.12$, P = 0.74). There were also no interaction effects or effect of habitat type during this period. We proceeded with the reward upshift on day 12, where all individuals trained on flake were unexpectedly rewarded with shrimp (*i.e.* a reward gain). Analysis of the first reward shift phase, including the day

before the reward upshift (*i.e.* days 11-24) demonstrated that mean swim times decreased significantly during this period ($F_{(13,\ 300.72)}$ = 4.15, P < 0.001). Groups where the reward was upshifted from flake to shrimp appeared to decrease in mean swim times compared to groups trained on shrimp from the outset (Fig. 6), but this difference was not significant (trial day × food reward: $F_{(13,\ 300.72)}$ = 1.51, P = 0.111). The habitat type did not have an effect on mean swim times ($F_{(1,\ 24.57)}$ = 0.04, P = 0.839). All other main and interaction effects were not significant.

From day 25 onwards, all food rewards during the experiment were ceased to investigate the rate of behavioural extinction. Analysis of the second reward shift phase, including the day before the reward downshift (*i.e.* days 24-37) demonstrated that mean swim times increased significantly during this period ($F_{(13,\ 289.31)}$ = 15.65, P < 0.001, **Fig. 7**). The effect of initial food reward was not significant ($F_{(1,\ 24.36)}$ = 0.13), and all other main and interaction effects were not significant.


(a)
Mean time taken to swim channel (s)
0
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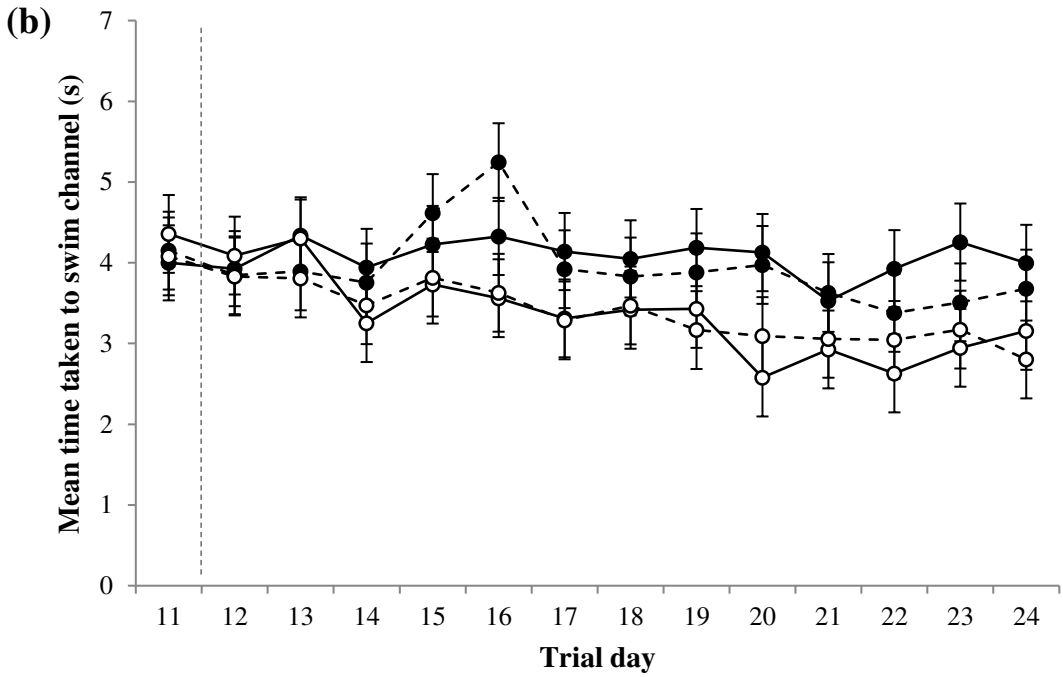
Trial day


(b)
Mean time taken to swim channel (s)
0
1
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Trial day

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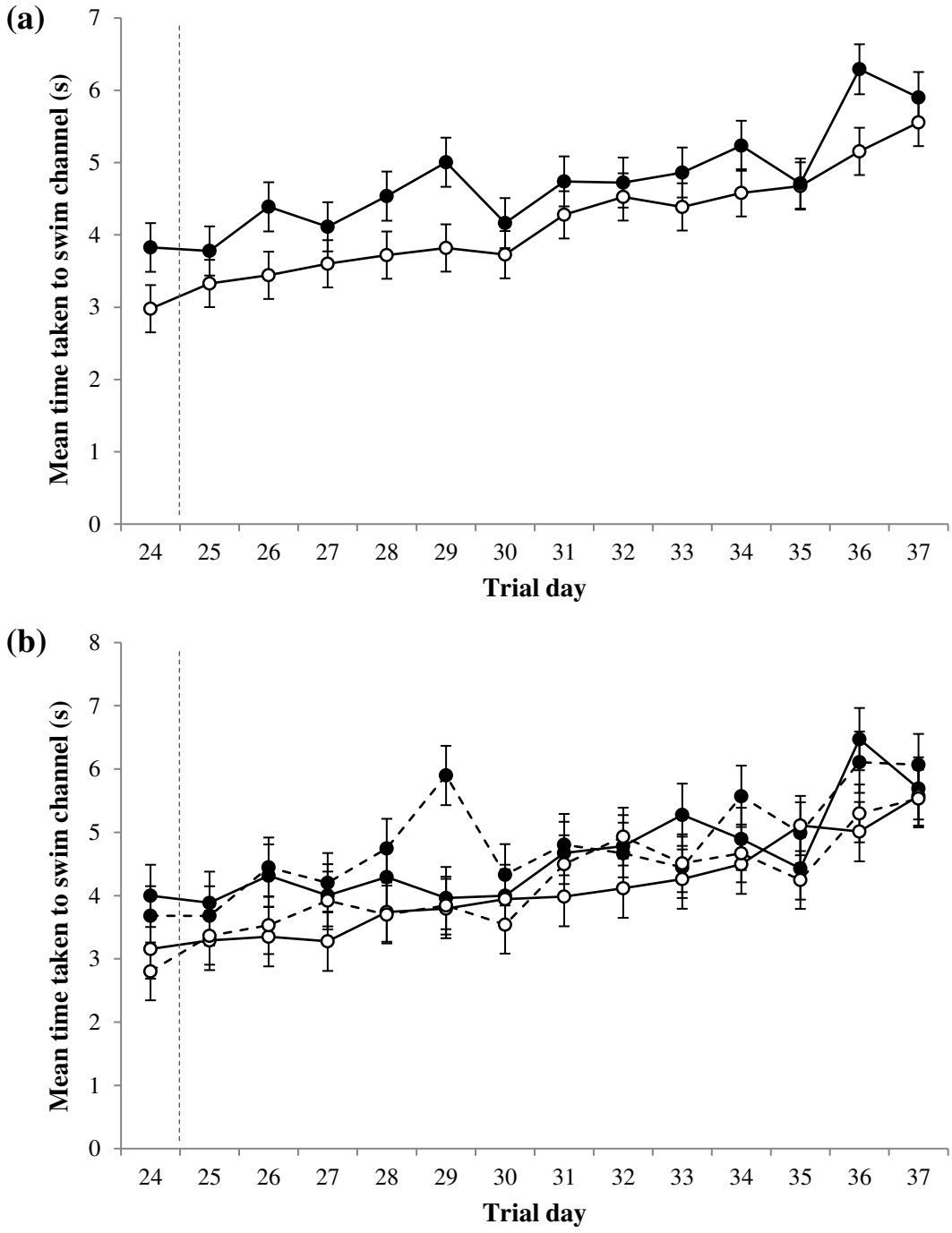
Fig. 6 (a) Mean time taken for zebrafish to swim the channel on each trial day for shrimp and flake reward groups in experiment 3 (post-reward upshift phase), and (b) the same data, but with food reward groups further separated into barren and enriched habitat groups. In both figures, the vertical dashed line between trial days 11 and 12 marks the day that flake reward groups had rewards upshifted from flake to shrimp, ● black markers represent pre-shift shrimp reward groups, ○ white markers represent pre-shift flake reward groups, and error bars denote standard error. In (b), dashed lines represent barren habitat groups, while solid lines represent enriched habitat groups



427 **Fig. 7** (a) Mean time taken for zebrafish to swim the channel on each trial day for shrimp and flake
428 reward groups in experiment 3 (behavioural extinction phase), and (b) the same data, but with food
429 reward groups further separated into barren and enriched habitat groups. In both figures, the vertical
430 dashed line between trial days 24 and 25 marks the day that all rewards during trials were ceased, ● black markers represent pre-shift shrimp reward groups, ○ white markers represent pre-shift flake
431 reward groups, and error bars denote standard error. In (b), dashed lines represent barren habitat groups,
432 while solid lines represent enriched habitat groups
433

434

Discussion

Our aim was to investigate whether assumed positive or negative affective states in zebrafish generated increased sensitivity to reward (food) gain or loss respectively using a successive contrast paradigm. We followed the assumption that exposure to rewarding stimuli, those that animals choose to access, would induce a relatively positive affective state compared to exposure to stimuli that they do not prefer (Rolls 2005). After having established that zebrafish showed a clear preference for an enriched over a barren environment, we did not find that zebrafish in barren environments showed a stronger response to reward loss and a weaker one to reward gain as predicted. One reason for this may have been that zebrafish behaviour in the successive contrast task was under habitual control, thus minimising the likelihood of any influence of affective state.

Experiment 1 – Habitat preference trials

The habitat preference trials showed that when given a choice, zebrafish had a clear preference for enriched (structured) environments over barren (empty) environments. This preference was also more pronounced at night. This provides important empirical evidence for environmental preferences in zebrafish as few studies have been conducted previously (e.g. Hamilton and Dill 2002; Kistler et al. 2011); much of the non-empirical information available originates from the non-scientific aquarist 'grey' literature, and is generally derived from either anecdotal observations or assumed based on knowledge of the natural habitat of similar fish species.

Hamilton and Dill (2002) found that zebrafish preferred to forage under overhead cover, but the presence of vegetation did not affect foraging behaviour. Kistler *et al.*

458 (2011), on the other hand, found a strong preference for structured environments,
459 which was similar to the results of our study. Our habitat preference test also adopted
460 some design features from Kistler *et al.* (2011), with a couple of improvements.
461 Firstly, fish in our habitat preference test were assessed individually, as opposed to in
462 groups of 6-9 as used by Kistler *et al.* (2011). Zebrafish are a schooling species (Kerr
463 1963; Spence et al. 2008), thus fish are likely to exhibit more pronounced habitat
464 preferences when they are not part of the safety of a group. Our design also
465 eliminated the potential biases in space use exerted by dominant individuals within a
466 group (e.g. Larson et al. 2006). Secondly, in our study, fish observations were
467 recorded at regular intervals throughout the full 24-hour period, in contrast to Kistler
468 *et al.* (2011) where in total only 16 observations were made over four days, all during
469 daylight hours. Our design allowed us to identify a stronger preference for enriched
470 habitats at night, which has not been reported previously. This is consistent with both
471 the biology of wild zebrafish, which are primarily active diurnally (Baganz et al.
472 2005; Plaut 2000) and thus are likely to experience a higher risk of predation while
473 inactive at night, as well as their natural habitat (Engeszer et al. 2007; McClure et al.
474 2006; Spence et al. 2008). Following the assumption that preferred/worked for stimuli
475 induce positive affective states, our study therefore provides supportive evidence that
476 enriched (structured) environments are likely to improve the welfare of captive
477 zebrafish.

478 **Experiment 2 – Sensitivity to reward loss**

479 In experiment 2, all individuals trained on shrimp were unexpectedly rewarded with
480 flake instead from day 7 onwards. We predicted that if zebrafish behaviour was under
481 goal-directed control involving anticipation of outcomes, the reward shift would be
482 perceived as an unexpected loss, and could cause mean swim times to increase

483 beyond that of individuals trained on flake from the outset of the experiment, i.e. a
484 depression or SNC effect. If so, we also hypothesised that individuals in the barren
485 treatment group, which were presumed to be in a putatively negative affective state
486 relative to individuals in the enriched treatment group, would be more sensitive to the
487 reward loss, resulting in higher mean swim times than the enriched treatment group.

488 However, no SNC effect was observed. The downshift of reward did not appear to
489 affect the mean swim times of shrimp-to-flake groups; individuals behaved as if
490 shrimp was still the food reward, and had significantly faster mean swim times
491 compared to individuals trained on flake from the outset throughout the remainder of
492 the experiment, including after a 4-day break. One possible explanation is that
493 behaviour at the point of reward downshift was under habitual control and hence fish
494 behaved in a stimulus-response fashion with no expectations of the outcomes of their
495 actions and thus did not perceive a reward loss. This might occur due to over-training
496 (Thorndike 1911) and, with time, the value of actions would be expected to slowly
497 update because the reward associated with them has changed, leading to similar
498 behaviour in both treatment groups (Dolan and Dayan 2013). The apparent lack of
499 this effect after 10 days is somewhat surprising, suggesting that the response had
500 become quite routinised. The sample size of our experiment was similar to both
501 Burman et al. (2008)'s reward shift study on rats (which detected significant effects of
502 similar experimental treatments) and previous reward shift studies on goldfish (e.g.
503 Couvillon and Bitterman 1985).

504 Our findings corroborate those of previous studies on goldfish. For example, Lowes
505 and Bitterman (1967) found that goldfish shifted from a large reward (40 worms) to a
506 small reward (4 worms) continued to perform identically to fish conditioned to the
507 large reward from the outset, while fish shifted from a small reward to a large reward

gradually increased in performance to match the level of fish conditioned to the large reward from the outset, without a SPC effect. This latter result supports the notion that the behaviour is under habitual control. Other studies on goldfish have shown that individual performance decreased gradually over a varying number of trial sessions to that of individuals conditioned to the small reward from the outset, without a SNC effect (Couvillon and Bitterman 1985; Gonzalez et al. 1962; Mackintosh 1971). Therefore, our study provides additional empirical evidence that responses to reward shifts in fish studied in these paradigms may be under habitual control.

The habitual nature of the behaviour exhibited here does not provide a useful indicator of affective state. This also explains why habitat type did not have any impact on mean swim times during either of the experiments, despite there being a clear preference for enriched habitats during the habitat preference trials. If we assume that control of behaviour shifts from goal-directed to habitual with increasing repetition of a task, there is scope for further refinement of SNC training protocols, in particular by shortening the pre-shift training period in an attempt to maintain goal-directed control and hence the potential for perceived reward loss and SNC.

Experiment 3 – Sensitivity to reward gain

In experiment 3, because shrimp was considered a higher-value reward, we predicted that this would generate the opposite effect of experiment 1; that is, mean swim times of flake-to-shrimp groups would decrease, with the enriched treatment group decreasing to a larger extent compared to the barren treatment group as the enriched treatment group was presumed to be more sensitive to reward gain.

Surprisingly, in contrast to our findings for experiment 1, individuals trained on shrimp in experiment 3 did not perform significantly faster than individuals trained on

flake during the pre-reward upshift phase, despite experimental conditions being identical in both experiments. We can think of no systematic explanation for this difference. However, after the reward upshift, mean swim times of flake-to-shrimp groups did appear to decrease (although this decrease was not statistically significant), highlighting the fact that the mean swim times during the pre-reward shift phase was not due to a physiological limitation in swim speed. This mean swim time was also maintained throughout the remainder of the experimental phase as compared to the temporary shift predicted. This result again provides more support for a habitual account as opposed to one assuming goal-directed control accompanied by a temporary SPC-like affective response to perceived reward gain. Although faster mean swim times could have been indicative of a SPC effect, it was difficult to conclude this given that there were no differences in swim speed prior to the reward shift. Further, the maintenance of this faster mean swim time was uncharacteristic of a SPC effect, which is often short-lived (Flaherty 1996).

When all rewards were discontinued in experiment 3, individuals continued to perform the trained task of swimming from one end of the channel to the other end consistently throughout the extinction trial period of 13 days. At the end of this trial period, individuals were still swimming, on average, quicker than average times on day 4 of the trial, even though none of the previous 78 trials were rewarded. This behavioural extinction period was much longer than what would be expected of goal-directed behaviours, at least in goldfish (e.g. Gonzalez et al. 1967), and provides further evidence that the fish were acting habitually during the trials, and in a highly routinized way. Previous comparative studies on goldfish and mice have also demonstrated that habitual behaviours are more resistant to behavioural extinction compared to goal-directed behaviours (Bitterman 1969; Gonzalez et al. 1967).

Conclusions

The fact that provision or denial of preferred enrichment, assumed to induce relatively positive and negative affective states respectively, did not influence performance during the sensitivity to reward shift experiments was likely to be because zebrafish behaviour in this experiment was driven largely by habit rather than expectation. Our research adds to accumulating evidence that fish do not generally respond to shifts in rewards via the same mechanisms as mammals, and therefore responses to shifts in rewards are unlikely to be a reliable measure of affective state in fish. However, 'over-training' during the experiment could have favoured habitual control of behaviour, and therefore subsequent research should consider minimising the amount of training done before reward shifts occur, or systematically investigating the effects of different training durations on contrast effects. Additionally, obstacles may be introduced within the channel to increase the mean swim time and resolution when detecting treatment effects. It should also be emphasised that this does not imply the lack of existence of affective states in fish; rather, it highlights our inability to probe affective states via this particular experimental protocol.

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Compliance with ethical standards

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The authors declare that they have no conflict of interest.

This article does not contain any studies with human participants performed by any of the authors.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (The University of Melbourne, AEC Project 1212695.3).

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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