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1 **Title**

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

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20 **Abstract**

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

42

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

45 **Introduction**

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

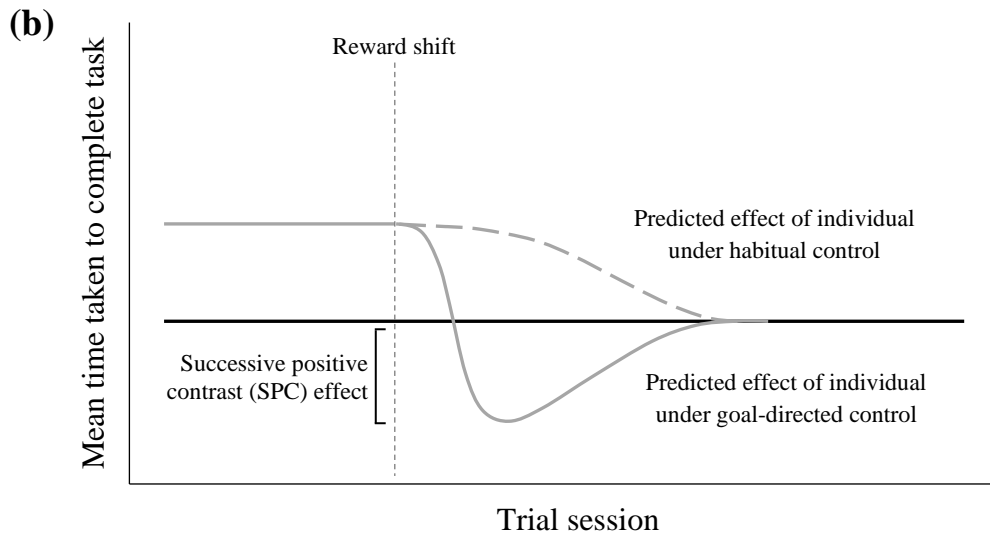
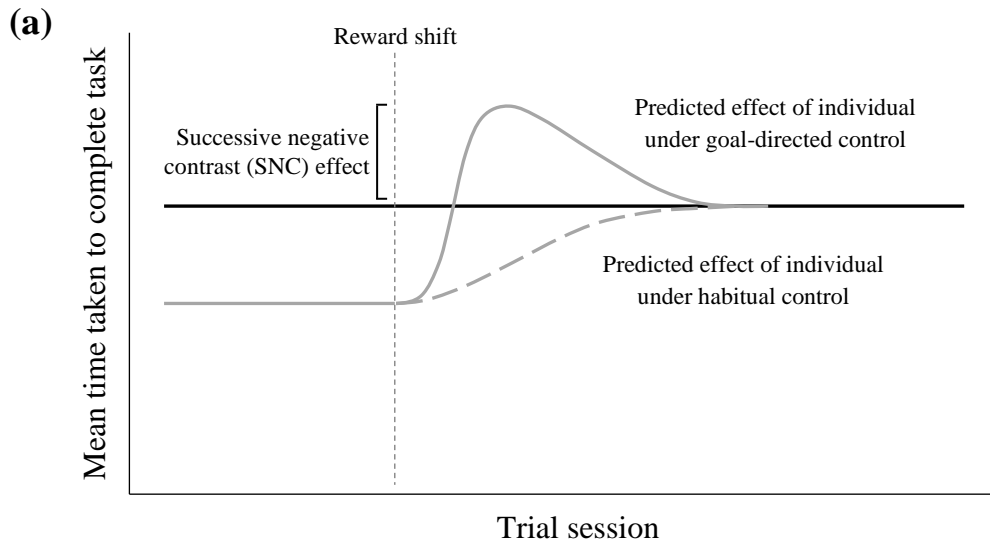
56 A related cognitive measure that has received less attention to date is how animals
57 respond to changes in anticipated reward; their sensitivity to reward shifts. This can
58 be measured in paradigms designed to test for successive negative or positive contrast
59 effects. For example, during operant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

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

69 Individuals appear to vary in their sensitivity to reward shifts, and one factor that may
70 affect this sensitivity is the individual's affective state. For instance, in humans we
71 know that heightened sensitivity to reward loss is associated with anxiety-related
72 disorders such as obsessive-compulsive disorder (Gehring et al. 2000; Hajcak et al.
73 2004), depression (Beck 1967; Wenzlaff and Grozier 1988), and reduced
74 responsiveness to rewarding stimuli (Clark and Watson 1991; Fowles 1994; Leppänen
75 2006; Naranjo et al. 2001). Further, anxiolytics (antianxiety agents) have been shown
76 to reduce sensitivity to reward loss (Flaherty et al. 1998; Morales et al. 1992).
77 Therefore, there is potential for sensitivity to reward shifts to be a useful indicator of
78 affective state if SNC and SPC effects can also be elicited in other animal species.

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

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



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

107

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

120

121 In fish, the majority of early studies were conducted on the goldfish, and all have
122 failed to demonstrate a SNC effect (Couvillon and Bitterman 1985; Gonzalez et al.
123 1974; Gonzalez et al. 1972; Lowes and Bitterman 1967; Mackintosh 1971), but all
124 studies were conducted on one species, the goldfish, *Carassius auratus*. The authors
125 of these studies also noted that much of the application of frustration theory to non-
126 mammalian vertebrates was poorly understood. Further, in mammals, initial
127 experimental protocols on SPC or elation effects also found it difficult to demonstrate
128 the effect, and it was later found that a more reliable SPC effect could be
129 demonstrated if a number of modifications to the protocol were made, including
130 increasing the difficulty of the task (Mellgren 1971), shifting the reward before the
131 individuals were performing at their physiological limit (Mellgren 1972), or
132 performing a downshift in reward before a subsequent upshift (Maxwell et al. 1976).
133 Thus it seems possible that the scope and execution of previous research on fish could
134 be further refined. Given that millions of fish are held in captivity for research
135 purposes (Reed and Jennings 2010), there is an enormous potential benefit to welfare
136 in developing protocols to understand how husbandry practices influence affect in fish,
137 thus this deserves further attention.

138 The most commonly used fish species in research is the zebrafish, *Danio rerio*.
139 Zebrafish are used in studies ranging from developmental biology (e.g. Creaser 1934;
140 Grunwald and Eisen 2002) and genetics (e.g. Amsterdam and Hopkins 2006; Kimmel
141 1989) to drug research (e.g. Berghmans et al. 2005; Rubenstein 2003; Rubenstein
142 2006). Behavioural studies using zebrafish are less common, possibly because
143 knowledge of the natural biology of the species is far from complete (Spence et al.
144 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).

163 **Materials and Methods**

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

166 **Animals, housing and husbandry**

167 We used 68 naïve, wild type Tübingen (TU) strain zebrafish obtained from the
168 Australian Regenerative Medicine Institute (ARMI) Monash University, Melbourne,
169 Australia. Ten fish were used in experiment 1 – these had participated in previous
170 behavioural experiments but were naïve to this experiment. The remaining 58 fish
171 were naïve to experimental testing. Twenty-eight fish were used in experiment 2, and
172 30 fish were used in experiment 3. Only male fish were used as the experiments
173 required an extended period of time in individual housing and lack of access to males
174 can cause female fish to become egg bound (Spence et al. 2008). Fish were obtained
175 at six months of age and experiments were conducted over the next three months.

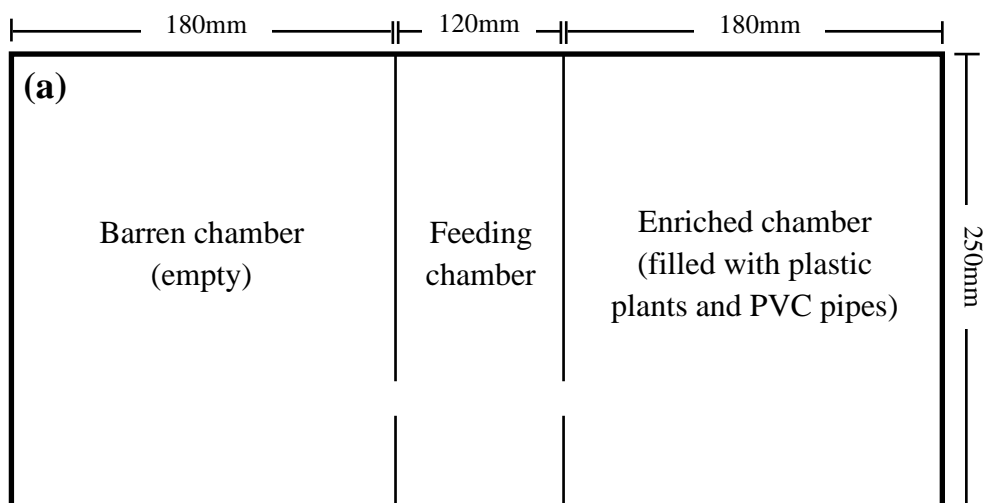
176 On arrival, fish were acclimatised to their new environment for seven days in a
177 communal glass tank (25L, 480mm length by 250mm width by 240mm height). Each
178 individual was then transferred into its own tank compartment (henceforth referred to
179 as its home tank), constructed by dividing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

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

204 **Experiment 1 – Habitat preference trials**

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

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



215

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

221

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

235 **Preparation of fish for the sensitivity to reward shift experiments**

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

- 244 1. Enriched/shrimp;
- 245 2. Enriched/flake;

246 3. Barren/shrimp;

247 4. Barren/flake.

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

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

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

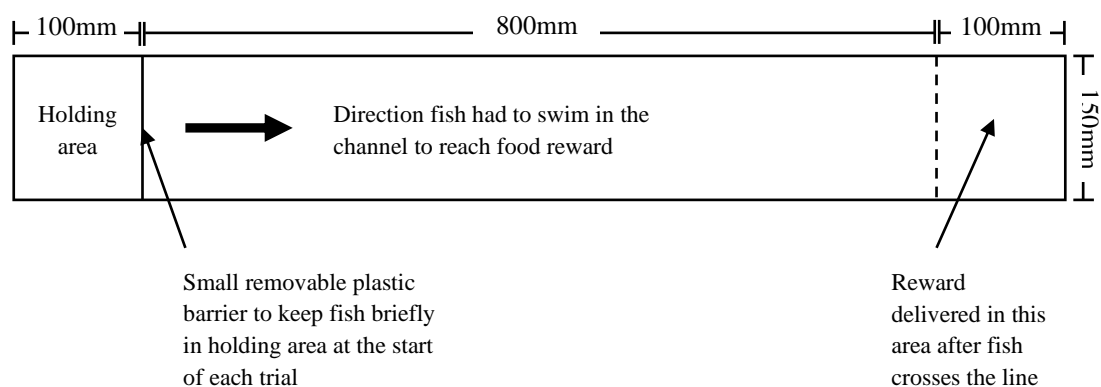
259 Fish were allowed to swim freely and were fed their designated food reward while
260 habituating in the experimental tank. Feeding helped fish to habituate to the tank more
261 quickly (e.g. Bilotta et al. 2005; Galhardo et al. 2011) and was presumed to reduce the
262 likelihood of any stress occurring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

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

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

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

277



278

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

284

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

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

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

301 **Experiment 3 – Sensitivity to reward gain**

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

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

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

314 **Statistical analysis**

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

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

326 For sensitivity to reward shift trials, prior to any shifts in reward, mean swim times
327 were analysed each day using a LMM with habitat type (enriched or barren), original
328 reward (shrimp or flake) and their interaction as fixed effects, and individuals nested
329 within habitat type by original reward as a random effect, to determine if there was a
330 significant difference in mean swim times between individuals trained on shrimp and
331 flake respectively. Swim times for each trial day were aggregated for each individual,
332 and mean swim times for each reward shift phase, including the day before each shift
333 (*i.e.* experiment 1 – days 6-12; experiment 2 – days 11-24 and 24-37, see Results),
334 were analysed using a LMM, with habitat type (enriched or barren), original reward
335 (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.

338 **Results**

339 **Experiment 1 – Habitat preference trials**

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

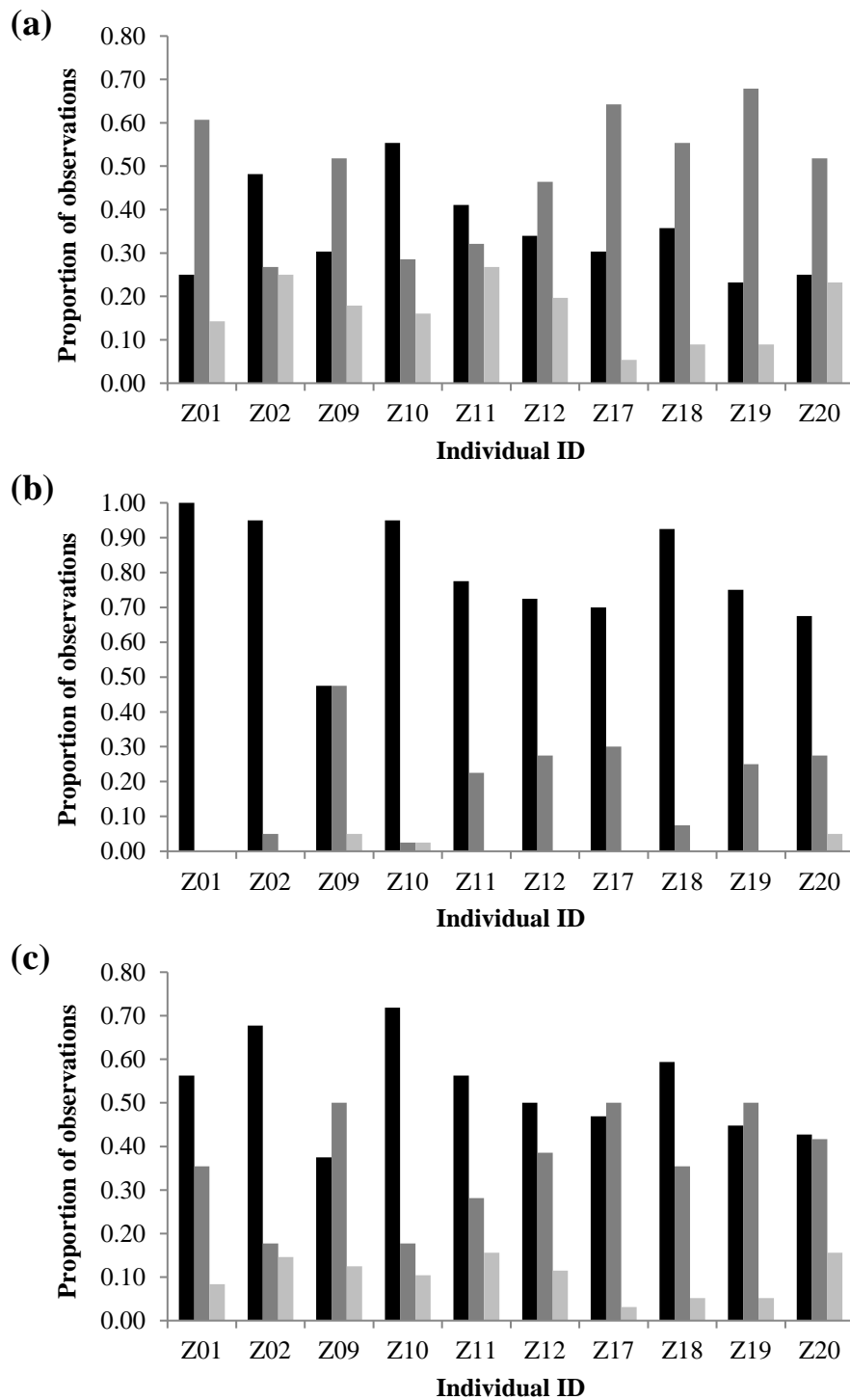
344 There was a significant preference for the enriched chamber over the barren chamber,
345 both when the combined day and night observations were used (mean \pm SE for the
346 enriched chamber = 0.53 ± 0.04 versus 0.10 ± 0.01 for the barren chamber, $t_9 = 11.73$,
347 $P < 0.001$), and when only day (mean \pm SE for the enriched chamber = 0.35 ± 0.03
348 versus 0.17 ± 0.02 for the barren chamber, $t_9 = 5.47$, $P < 0.001$) or night (mean \pm SE
349 for the enriched chamber = 0.79 ± 0.05 versus 0.01 ± 0.01 for the barren chamber, $t_9 =$
350 14.17 , $P < 0.001$) observations were used. In addition, there was a stronger preference
351 for the enriched chamber at night compared to the day ($F_{(1, 27)} = 95.86$, $P < 0.001$, also
352 see **Fig. 4**).

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

354 This experiment lasted 16 days. Within 3 days of the start of training, individuals
355 trained on shrimp had significantly faster mean swim times than individuals trained
356 on flake ($F_{(1, 24)} = 6.49$, $P = 0.018$), though mean swim times did decrease for both
357 groups from an initial mean of 14 seconds as individuals learned the task. This effect
358 persisted for the next 3 days (day 4: $F_{(1, 24)} = 7.93$, $P = 0.010$; day 5: $F_{(1, 24)} = 7.58$, $P =$
359 0.011 ; day 6: $F_{(1, 24)} = 8.98$, $P = 0.006$). There was no effect of habitat type (enriched

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

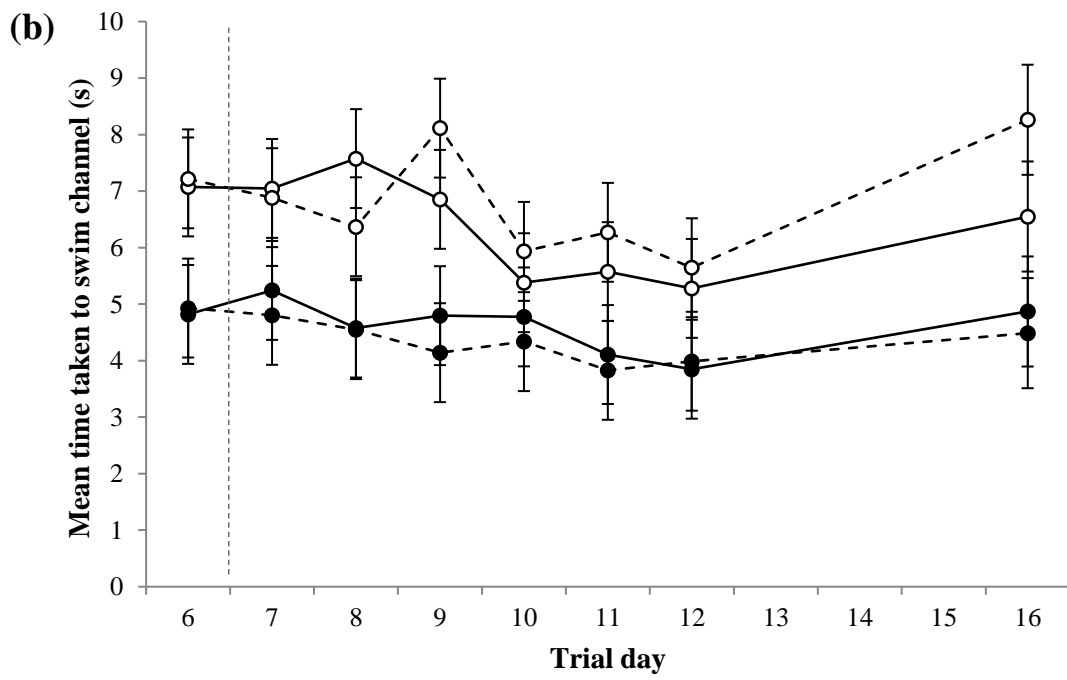
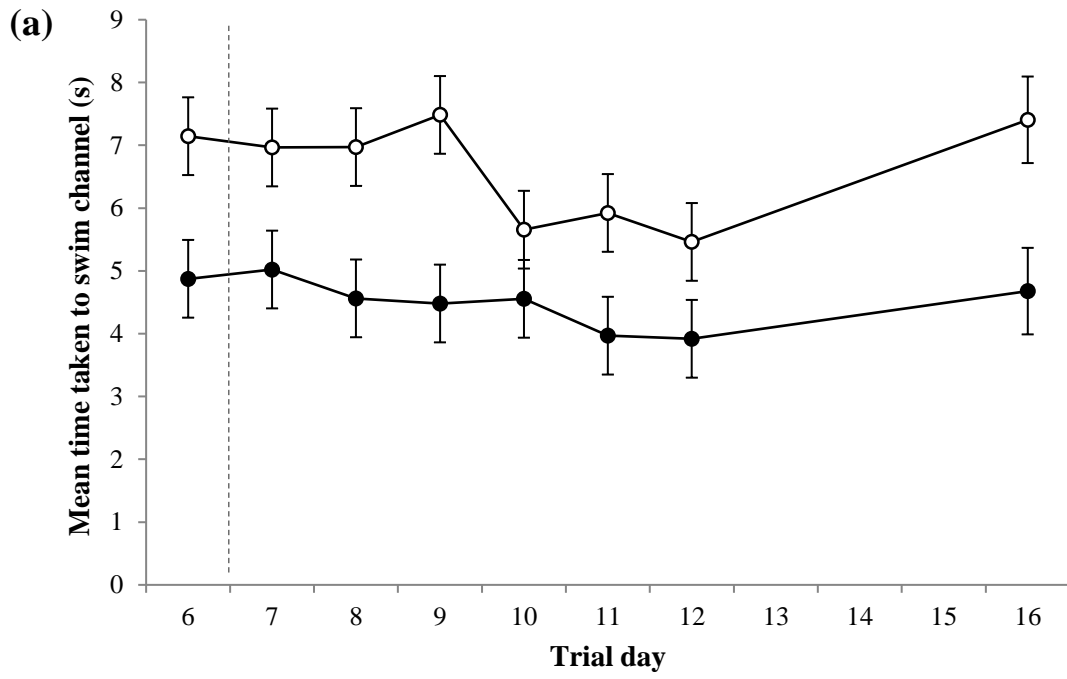
362 On day 7 onwards, groups trained on shrimp were unexpectedly rewarded with flake
363 instead (*i.e.* a reward loss). Analysis of the reward shift phase, including the day
364 before the reward downshift (*i.e.* days 6-12) demonstrated that individuals originally
365 trained on shrimp continued to have significantly faster mean swim times than
366 individuals trained on flake, despite the fact that both groups were receiving identical
367 flake rewards during this period ($F_{(1, 24)} = 6.67$, $P = 0.016$). Mean swim times also
368 decreased significantly during this period ($F_{(6, 144)} = 4.29$, $P = 0.001$). The difference
369 in mean swim times between fish originally trained on shrimp and fish trained on
370 flake persisted during an additional set of trials conducted 4 days after day 12 (day 16:
371 $F_{(1, 24)} = 7.85$, $P = 0.010$, **Fig. 5**). The habitat type did not have an effect on mean
372 swim times ($F_{(1, 24)} = 0.00$, $P = 0.997$). All other interaction effects were not
373 significant.



374

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

378



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

387

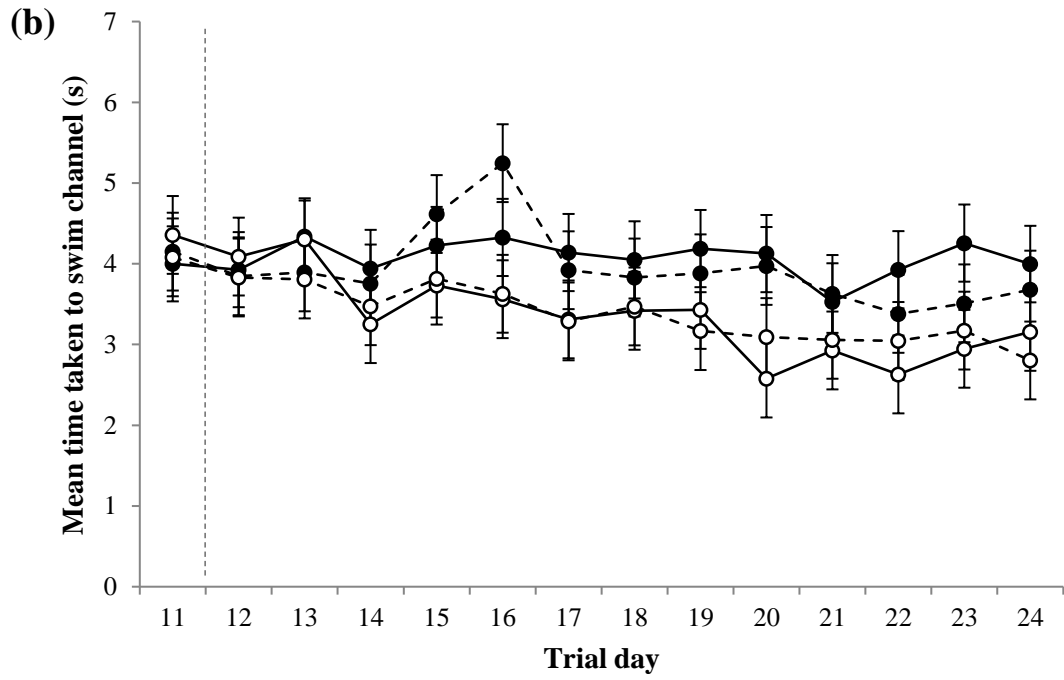
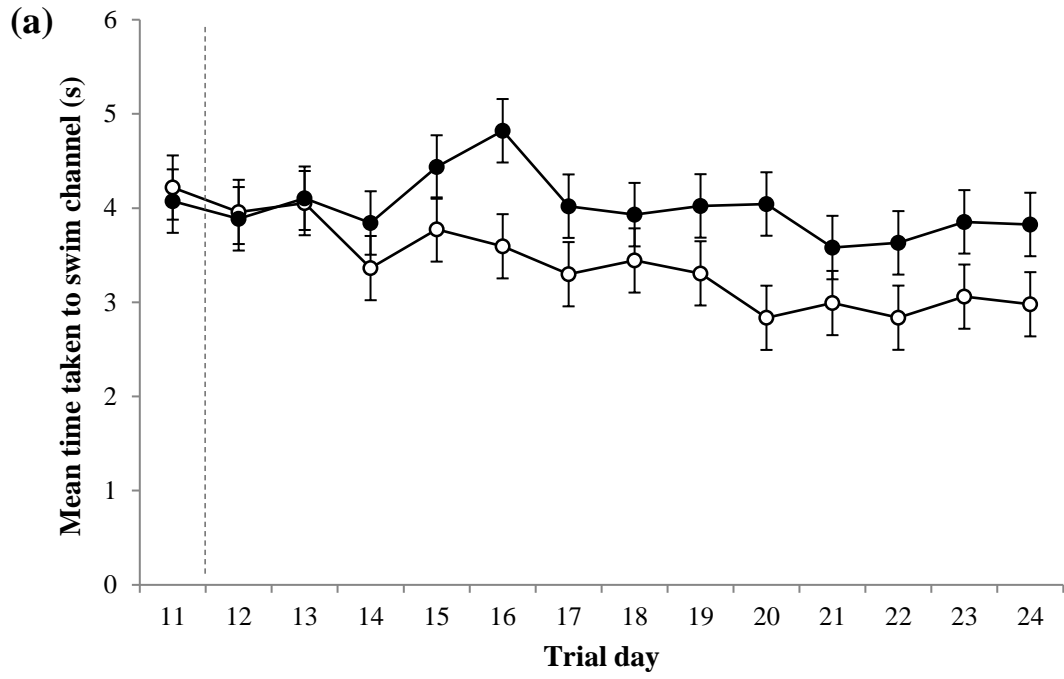
388 **Experiment 3 – Sensitivity to reward gain**

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

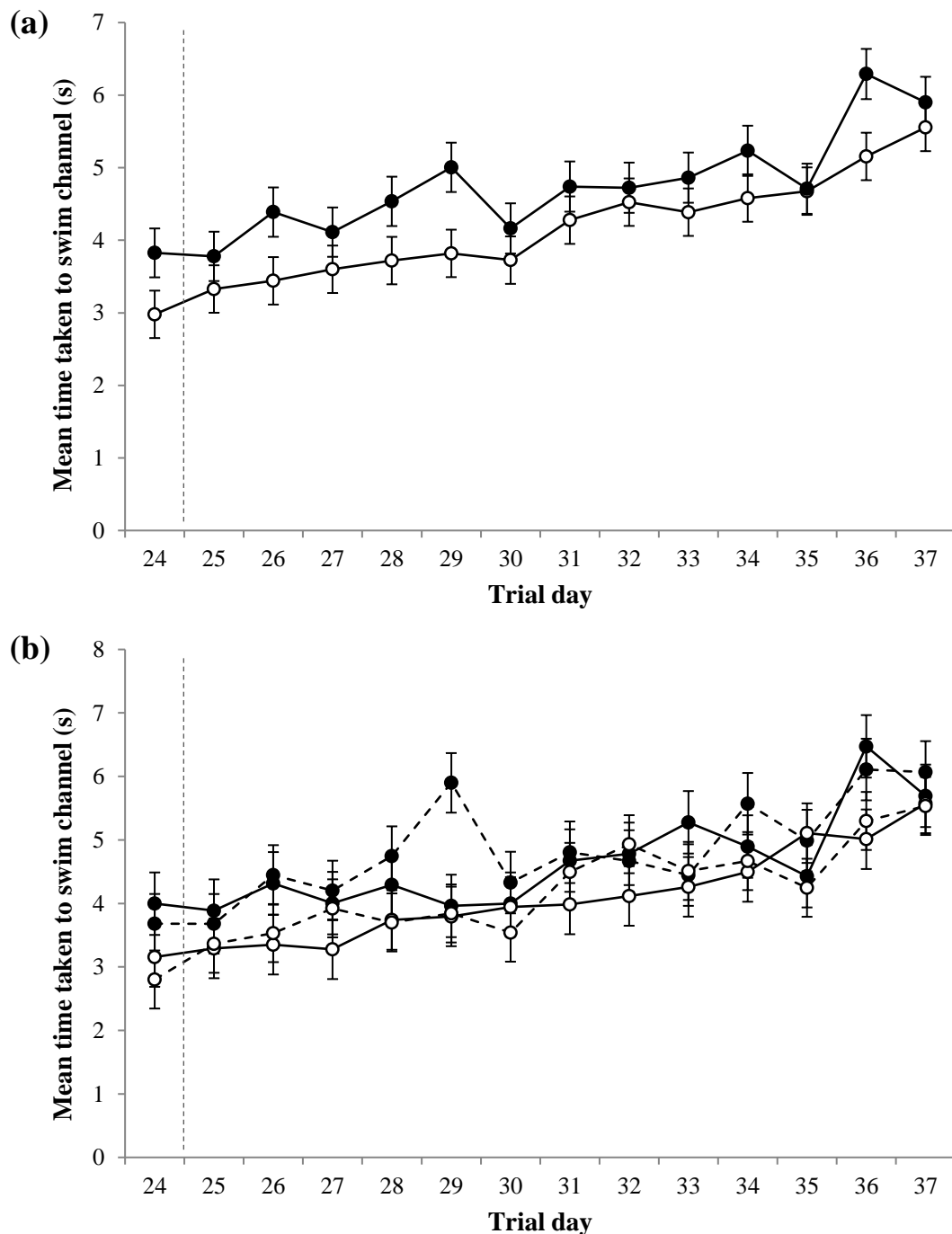
398 Although the pre-reward shift phase of this experiment was conducted identically to
399 that of experiment 2, individuals trained on shrimp in this experiment did not have
400 significantly faster mean swim times than individuals trained on flake after 11 days of
401 conditioning (day 11: $F_{(1,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

405 before the reward upshift (*i.e.* days 11-24) demonstrated that mean swim times
406 decreased significantly during this period ($F_{(13, 300.72)} = 4.15$, $P < 0.001$). Groups
407 where the reward was upshifted from flake to shrimp appeared to decrease in mean
408 swim times compared to groups trained on shrimp from the outset (Fig. 6), but this
409 difference was not significant (trial day \times food reward: $F_{(13, 300.72)} = 1.51$, $P = 0.111$).
410 The habitat type did not have an effect on mean swim times ($F_{(1, 24.57)} = 0.04$, $P =$
411 0.839). All other main and interaction effects were not significant.

412 From day 25 onwards, all food rewards during the experiment were ceased to
413 investigate the rate of behavioural extinction. Analysis of the second reward shift
414 phase, including the day before the reward downshift (*i.e.* days 24-37) demonstrated
415 that mean swim times increased significantly during this period ($F_{(13, 289.31)} = 15.65$, P
416 < 0.001 , **Fig. 7**). The effect of initial food reward was not significant ($F_{(1, 24.36)} = 0.13$),
417 and all other main and interaction effects were not significant.



419 **Fig. 6** (a) Mean time taken for zebrafish to swim the channel on each trial day for shrimp and flake
 420 reward groups in experiment 3 (post-reward upshift phase), and (b) the same data, but with food reward
 421 groups further separated into barren and enriched habitat groups. In both figures, the vertical dashed
 422 line between trial days 11 and 12 marks the day that flake reward groups had rewards upshifted from
 423 flake to shrimp, ● black markers represent pre-shift shrimp reward groups, ○ white markers represent
 424 pre-shift flake reward groups, and error bars denote standard error. In (b), dashed lines represent barren
 425 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, ●
431 black markers represent pre-shift shrimp reward groups, ○ white markers represent pre-shift flake
432 reward groups, and error bars denote standard error. In (b), dashed lines represent barren habitat groups,
433 while solid lines represent enriched habitat groups

434

435 **Discussion**

436 Our aim was to investigate whether assumed positive or negative affective states in
437 zebrafish generated increased sensitivity to reward (food) gain or loss respectively
438 using a successive contrast paradigm. We followed the assumption that exposure to
439 rewarding stimuli, those that animals choose to access, would induce a relatively
440 positive affective state compared to exposure to stimuli that they do not prefer (Rolls
441 2005). After having established that zebrafish showed a clear preference for an
442 enriched over a barren environment, we did not find that zebrafish in barren
443 environments showed a stronger response to reward loss and a weaker one to reward
444 gain as predicted. One reason for this may have been that zebrafish behaviour in the
445 successive contrast task was under habitual control, thus minimising the likelihood of
446 any influence of affective state.

447 **Experiment 1 – Habitat preference trials**

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

456 Hamilton and Dill (2002) found that zebrafish preferred to forage under overhead
457 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

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

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

524 **Experiment 3 – Sensitivity to reward gain**

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

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

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

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

557 **Conclusions**

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

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577 **Compliance with ethical standards**

578 This research was funded by the Margaret Catto Award and K. Handasyde.

579 The authors declare that they have no conflict of interest.

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

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

586

587 **Data availability statement**

588 The datasets generated during and/or analysed during the current study are available

589 from the corresponding author on reasonable request.

590

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