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# Providing a food reward reduces inhibitory avoidance learning in zebrafish

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ABSTRACT

As shown in male rats, prior history of subjects changes behavioural and stress-responses to challenges: a two-week history of exposure to rewards at fixed intervals led to slightly, but consistently, lower physiological stress-responses and anxiety-like behaviour. Here, we tested whether similar effects are present in zebrafish (*Danio rerio*). After two weeks of providing *Artemia* (brine shrimp; *Artemia salina)* as food reward or flake food (Tetramin) as control at fixed intervals, zebrafish were exposed to a fear-avoidance learning task using an inhibitory avoidance protocol. Half the number of fish received a 3 V shock on day 1 and were tested and sacrificed on day 2; the other half received a second 3 V shock on day 2 and were tested and sacrificed on day 3. The latter was done to assess whether effects are robust, as effects in rats have been shown to be modest. Zebrafish that were given *Artemia* showed less inhibitory avoidance after one shock, but not after two shocks, than zebrafish that were given flake-food. Reduced avoidance behaviour was associated with lower telencepahalic gene expression levels of *cannabinoid receptor 1* (*cnr1*) and higher gene expression levels of *corticotropin releasing factor* (*crf*). These results suggest that providing rewards at fixed intervals alters fear avoidance behaviour, albeit modestly, in zebrafish. We discuss the data in the context of similar underlying brain structures in mammals and fish.


## 1. Introduction

Recent studies have shown that exposure to rewards for several weeks reduces anxiety- and stress-related physiology and behaviour [e.g. piglets (Dudink et al., 2006) and rats (Ulrich-Lai et al., 2007, 2010)]. Key to these experiments is that access to the rewarding stimulus could be anticipated by the individuals as the rewards were either explicitly announced (piglets) or were given at the same time of the day (rats; conform food anticipatory activity (Liu et al., 2012); see Spruijt et al., 2001 for discussion). In more detail, male rats with a 2-week history of receiving a sucrose solution or access to a sexually receptive female rat as rewards at a fixed time of the day had a slightly, but long-lasting and consistent, lower stress-response and reduced anxiety-like behaviour when tested around the time of scheduled access to the reward (Ulrich-Lai et al., 2007, 2010). Studies have shown that food anticipatory activity exists in fish (Lague and Reebs, 2000a,b) and we hypothesise that such may interfere with behavioural responses to challenges.

Ulrich-Lai et al. (2010) showed that the rewarding effects were dependent on activity in the basolateral amygdala (BLA). In teleost fish, the medial zone of the dorsal pallium (Dm) in the telencephalon corresponds functionally to the mammalian (basolateral) amygdala (Mueller et al., 2011; Mueller, 2012). The Dm is involved anxiety and fear-related avoidance learning (Broglio et al., 2005) as well as in reward-like behaviour (von Trotha and Vernier Bally-Cuif, 2014). Hence also in fish activation of the reward system may interfere with stress-, anxiety- and/or fear-related tasks.

We have recently studied the effects of both stressful and enriched conditions on inhibitory avoidance learning and the expression of telencephalic stress-, anxiety- and fear-related genes herein in zebrafish (*Danio rerio*; Manuel et al., 2014a, 2015). We therefore investigated whether providing a reward could affect inhibitory avoidance learning, a simple learning task in which zebrafish learn to avoid an initially preferred dark compartment to avoid receiving a mild electric shock (Blank et al., 2009) and which is dependent on the Dm (Broglio et al., 2005).

Informal observations showed that zebrafish prefer *Artemia* (brine shrimp; *Artemia salina*) to standard flake food. In addition, many studies have used *Artemia* as food reward in appetitive learning tasks (e.g. Lau et al., 2006; Gerlai, 2011). Thus, on a background of a standard feeding regime with flake food only, one group of zebrafish was given *Artemia* as food reward, once daily at fixed time-points, while a control group received flake food at the same time-point. After two weeks, fish were exposed to the inhibitory

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avoidance paradigm. Based on the mild effects observed in rats (Ulrich-Lai et al., 2010), we assessed inhibitory avoidance learning and gene expression after a single shock (Blank et al., 2009, Manuel et al., 2014b) and two shocks (Manuel et al., 2014a, 2015). A single shock has been shown to be more sensitive to assess subtle effects on individual behaviour than two shocks (Manuel et al., 2014a,b; Manuel, 2015).

## 2. Material and methods

### 2.1. Ethical approval

All experiments were approved by the Animal Ethics Committee of the Radboud University Nijmegen and were conducted in agreement with Dutch law (Wet op de Dierproeven 1996) and European regulations (Directive 86/609/ EEC).

### 2.2. Animals and housing

Twelve-month old zebrafish (mixed sexes) of an in-house bred Tupfel Longfin strain were used. Fish came from a single brood and were split into two equally sized groups (21 fish each). Fish were not fed *Artemia* (*A. salina*) to increase the value of the (new) food reward (*Artemia*) during the experiment.

### 2.3. Experimental groups

Four weeks before the start of the experiment, fish were housed in groups of three or four in 2 L tanks, which were placed in the same room and linked to a single biological filter (300 L). Fish were fed standard flake food (TetraMin, Melle, Germany) at 9:00 h and 15:00 h. After the acclimation period, for a period of two weeks (conform Ulrich-Lai et al., 2010), controls received a pinch of flake food (TetraMin) at 11:00 h, while the experimental group received five drops of *Artemia*.

### 2.4. Inhibitory avoidance protocol

Inhibitory avoidance learning and tissue collections were done as previously described (Gorissen et al., 2015; Manuel et al., 2014a,b, 2015). In short, on day 1 all fish were individually exposed to the inhibitory avoidance paradigm ($n = 21$ for both flake and *Artemia*) around the same time they would normally receive the additional feed (11.00 h). Fish received a shock upon entering the dark area and none were sacrificed. On day 2, fish were reintroduced into the experimental tank and their latencies to enter the dark area were recorded. Fish in the one-shock condition (about half the number of fish in each treatment group ($n = 9$ for flake; $n = 10$ for *Artemia*)) did not receive a shock when entering the dark area; fish ($n = 19$) were sacrificed for gene expression analysis (Manuel et al., 2014a). Fish in the two-shock condition (the other half in each treatment group ($n = 12$ flake; $n = 11$ *Artemia*)) received a second shock however (regardless of their avoidance behaviour (Manuel et al., 2014b). On day 3, these latter fish ($n = 12$ flake; $n = 11$ *Artemia*) were reintroduced in the experimental tank and their latencies to enter the dark area were recorded. None of these fish received a shock when they entered the dark area, but were sacrificed for gene expression analysis. On day 2 and 3 fish were sacrificed two hours after testing (Manuel et al., 2014a,b). The telencephalon was dissected from the brain, stored and processed for qPCR analysis as described previously (Gorissen et al., 2015; Manuel et al., 2014a,b, 2015) to assess the expression levels of stress, anxiety and fear-related genes (see Table 1 for genes).

### 2.5. Statistical analysis

Statistical analyses were performed using IBM SPSS 21 for Mac (IBM, Armonk, USA). Latency times (measured by stopwatch) were analysed by non-parametric tests as a cut-off point of 180 s was used (Gorissen et al., 2015; Manuel et al., 2014a,b, 2015). As we did not tag individual fish, and hence could not follow the behaviour of individual fish over days, data between days were treated as independent groups (Gorissen et al., 2015; Manuel et al., 2014a,b, 2015). Differences within a feeding regime over days were tested with the Kruskal–Wallis test ($H$-values), while the Jonkheere–Terpstra test ($J$-values) was used to test for significant trends across days. Effect sizes were calculated and are indicated as '$r$-values' in case of the Kruskal–Wallis test and Jonckheere–Terpstra test. Mann–Whitney $U$-tests were used as post-hoc tests. Differences in telencephalic gene-expression levels after one and two shocks were analysed separately using Student's $t$-test or Mann–Whitney $U$-test, depending on whether data were normally distributed or not. Effect sizes were calculated and are indicated as '$r$-values'. Means (or medians in case of non-parametric tests) were considered statistically different when $P < 0.05$.

## 3. Results

### 3.1. Inhibitory avoidance learning

For flake-fed fish the latencies on day 2 ($p < 0.001$) and day 3 ($p < 0.0001$) were significantly higher than on day 1, while no significant difference was present between the latencies on day 2 and day 3 ($H(2) = 12.129$; $J = 650.50$; $Z = 4.305$; $P < 0.0001$; $r = 0.59$; Fig. 1). For *Artemia*-fed fish the latency on day 3 was significantly higher than the latencies on day 1 ($p < 0.001$) and day 2 ($p < 0.01$), while no significant difference was present between the latencies on day 1 and day 2 ($H(2) = 12.217$; $J = 644.00$; $Z = 3.194$; $P = 0.001$;

**Table 1**
Telencephalic gene expression analysis from flake-fed and *Artemia*-fed zebrafish following a single or two shocks. Values are listed as means (±1 s.d.) of the relative and normalised expression (conform Vandesompele et al., 2002) to *elongation factor 1α* and *ribosomal protein L13*) for each group. Groups showing significant differences ($P \leq 0.05$) are shown in, bold non-significant differences are listed in grey. Statistical analysis was performed between flake and Artemia groups after one or two shocks; no comparison was made between one and two shocks within the flake or *Artemia* group. $n = 10$–11 for one shock and $n = 11$–12 for two shocks.

| Name (abbr.) | 1 Shock | | 2 Shock | |
|---|---|---|---|---|
| | *Flake* | *Artemia* | *Flake* | *Artemia* |
| *Brain derived neurotrophic factor (bdnf)* | 0.76 (0.17) | 0.68 (0.12) | 0.77 (0.14) | 0.82 (0.17) |
| *Cocaine and amphetamine regulated transcript 4 (cart4)* | 0.55 (0.27) | 0.58 (0.15) | 0.71 (0.31) | 0.53 (0.16) |
| *Serotonin receptor 1ab (htr1ab)* | 0.87 (0.22) | 0.57 (0.11) | 0.63 (0.19) | 0.77 (0.20) |
| *Cannabinoid receptor 1 (cnr1)* | 0.87 (0.22) | 0.57 (0.11) | 0.63 (0.19) | 0.77 (0.20) |
| *Neorogenic differentiation (neurod)* | 1.00 (0.13) | 1.04 (0.11) | 1.00 (0.24) | 1.00 (0.17) |
| *Corticotropin releasing factor (crf)* | 0.81 (0.15) | 1.05 (0.23) | 0.96 (0.27) | 1.02 (0.28) |
| *Corticotropin releasing factor-binding protein (crf-bp)* | 0.68 (0.27) | 0.89 (0.19) | 0.78 (0.30) | 0.62 (0.18) |
| *Glucocorticoid receptor-α (gr-α)* | 1.21 (0.36) | 1.25 (0.28) | 1.11 (0.29) | 1.19 (0.24) |
| *Glucocorticoid receptor-β (gr-β)* | 0.79 (0.20) | 0.59 (0.28) | 0.75 (0.28) | 0.71 (0.18) |
| *Mineralocorticoid receptor (mr)* | 1.01 (0.20) | 1.11 (0.29) | 0.80 (0.22) | 0.96 (0.18) |