Chapter 5

Conditioning an avoidance response in groups of rummy-nose tetra (*Hemigrammus rhodostomus*)

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Abstract

We develop an experimental method to induce controlled perturbations in a group of fish and investigate the propagation of information. We use the paradigm of the shuttle box to condition aversive escape reactions in groups of rummy-nose tetra (*Hemigrammus rhodostomus*) in response to a green light. We show that aversive conditioning can (i) be used in this species, (ii) trigger collective escape reactions and (iii) be transferred from the training set-up to a new setting. We characterise and quantify the aversive conditioning and discuss long-term habituation and forgetting. We discuss these preliminary results in the context of propagation of information in reaction to external stimuli. Our findings suggest that the proportion of conditioned individuals in a group is critical to trigger collective escape reactions in response to external stimuli. Our conditioning experiments open promising possibilities for investigating the collective responses and propagation of
information within groups of fish in response to perturbations mimicking sudden changes in the environment, such as predator attack.

**Contribution of authors**

V.L., C.K.H. and G.T. conceived and designed the study; P.A. and S.F. developed the set-ups. V.L. performed experiments; V.L. analysed data; V.L., C.K.H. and G.T. wrote the paper.

**5.1 Introduction**

Rummy-nose tetra (*Hemigrammus rhodostomus*) is a species of fish that swims in schools (i.e. the collective motion is highly coordinated) with high levels of polarisation (see previous chapter). Fish schools are of particular interest to investigate collective behaviour of animal societies in various ecological contexts, such as, for instance, under predator threat. When a school reacts collectively to an external perturbation, individuals react either directly to the perturbation itself or to startle response of its neighbours (Domenici and Batty, 1994). Therefore, the information responded to differs among group members. Conditioning experiments are of interest in the field of collective behaviour to investigate such phenomena experimentally. This can be done by manipulating the behaviour of a few individuals only by conditioning them to startle in reaction to a stimulus (Pillot et al., 2010, 2011; Miller et al., 2013; Toulet et al., 2015). Here, we use aversive conditioning. This implies that an initially neutral stimulus becomes aversive after repeated pairing with an unconditioned aversive stimulus. For animals in motion, the elicited (trained) escape response is of interest to discover how information propagates in fish schools when a single or a few individuals spot a predator in their neighbourhood. Unfortunately, all previous experiments of aversive conditioning conducted in fish were done with species that do not form fish schools (i.e. groups where individuals are highly coordinated and aligned) (e.g. in zebrafish (*Danio rerio*) (Agetsuma et al., 2012) or goldfish (*Carassius auratus*) (Portavella et al., 2004)). In this paper, we developed experiments with *H. rhodostomus* to condition its escape response in order to investigate the propagation of information within a group of fish in a controlled way. We use aversive conditioning (electric shocks associated with changes in lighting) similar to the classical behavioural paradigm of the shuttle box, already tested in other fish species (Horner et al., 1961; Woodard and Bitterman, 1973; Piront and Schmidt, 1988; Portavella et al., 2004; Pradel et al., 1999; Xu
et al., 2007; Agetsuma et al., 2012). The aversive conditioning involves training fish to escape when a green light is turned on. We subsequently investigate how the proportion of conditioned fish in a group affects its propensity to perform a collective escape. We show in *H. rhodostomus* (i) that individuals learn to escape in response to a green light after aversive conditioning, (ii) that the conditioned escape can be transferred to a new experimental setting and (iii) that a minimum proportion of conditioned individuals in the group is needed to propagate the learnt behaviour.

### 5.2 Material and methods

#### 5.2.1 Animals

A group of 70 rummy-nose tetras (*Hemigrammus rhodostomus*) were purchased from Amazonie Labège (http://www.amazonie.com) in Toulouse, France. The rummy-nose tetra is a tropical freshwater species. Fish were kept in 150 L aquariums on a 12:12 hour, dark:light photoperiod, at 27.5°C (±0.8°C) and were fed *ad libitum* with fish flakes.

#### 5.2.2 Experiment 1: avoidance conditioning in a shuttle box

**Subjects**

Similar experiments (see following sections) with zebrafish have shown that groups of 5 fish learn faster than single individuals or groups of two (Gleason et al., 1977). For this reason and to avoid inactivity of solitary fish, we conducted the conditioning of 6 fish at the same time. 6 fish – thereafter *conditioned fish* – were randomly sampled from the breeding tank in July 2016 and kept in a different breeding tank, under the same conditions as the other fish. All other individuals are labelled as *naïve fish*.

**Conditioning apparatus**

Fish were trained using a shuttle box modified from Horner et al. (1961). The shuttle box (46×23×21 cm) is made of white plastic (polyvinyl chloride, PVC) (Figure 5.1A). The two compartments (20×15×20 cm) have 84 green light-emitting diodes (LEDs) set on 4 rows of 7 LEDs for each panel (Figures 5.1B-C). The LEDs of each compartment can be turned on independently from those of the other compartment. Compartments are separated by a trapezoidal hurdle 7.5 cm high. Two metallic plates conduct electricity in each compartment. Compartments are filled with 4.5 cm of white gravels and white ceramic balls to enrich the environment of fish, in line with
policies for animal welfare. The water comprised 50% of water purified by reverse osmosis and 50% of water treated by activated carbon, heated at $27^\circ\text{C}$. Its level is set at 3 cm above the hurdle (i.e. 10.5 cm in each compartment). Training is monitored by a webcam (Logitech QuickCam Pro 9000). Light and electric stimuli are both delivered manually.

**Conditioning procedure**

Before and after the training session, the group of 6 conditioned fish is let in the set-up without any stimulus for respectively 15 min (habituation) and 5 min. Water is changed after each experiment.

**Acquisition**

Training sessions were performed on a regular basis (77 sessions in total) from July 2016 to July 2017 with the same 6 fish $^1$. Each training session consisted of 20 trials (intertrial time of 2 min). In each trial, at least one of the 6 fish had to cross the hurdle to avoid mild electric shocks (7 V, 2.7 mA, measured on the electrodes) (unconditioned stimulus, US) administered via

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$^1$One of the 6 fish died, 10 months after the beginning of the experiment. The death did not occur during an experiment and no wounds were noticed. Experiments were thus performed with the other 5 fish thereafter.
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electrodes 3 s\(^2\) after onset of a light signal (green light, conditioned stimulus, CS). The light signal was always turned on in that compartment where the majority of the individuals were located. A trial is labelled as a Success if at least one fish crossed the hurdle before the onset of electric shocks (i.e. within 3 s after onset of the CS) and Failed in all other cases. If there is no fish crossing the hurdle within 15 s after the onset of electric shocks, all stimuli (CS + US) are turned off and the trial is labelled as Failed. The proportion of trials labelled as Success over the 20 trials will be referred to as the proportion of escapes.

10 training sessions without US have been conducted on groups of 6 naïve fish randomly sampled from the breeding tank to measure the proportion of escapes due to the spontaneous exploration of the apparatus (Control).

Our dataset consists in the output (Failed or Success) of 200 trials for naïve fish and 1540 trials for conditioned fish.

**Ethical use of animals**

To minimise the stress induced by the experiments with aversive conditioning, we carefully followed the Three Rs principle (Replacement, Reduction and Refinement) (Russel and Burch, 1959). The Three Rs are a guiding principle for ethical use of animal in testing, that recommends to Replace experiments that kill or harm animals with alternative techniques, to Reduce the number of animals used, and to Refine experiments to reduce suffering. We use aversive conditioning to Replace empirical experiments that would involve predators hunting their preys. Regarding Reduction, only 6 individuals have been used during the whole conditioning, conducted over almost a year. As for Refinement, the power of the electric shocks has been set to the minimum power (7 V, 2.7 mA, measured on the electrodes) triggering a reaction in fish visible by eye. Individuals were carefully observed every day for wounds or abnormal behaviour. Visible injuries are endpoints, i.e. an animal with wounds would be removed from the experiment. Experiments have been conducted in a different room than that of the breeding tanks. Thus, experiments were not visible by fish in the breeding tank, in agreement with policies for animal welfare.

All experiments comply with the European legislation for animal welfare. Our experiments have been approved by the Ethics Committee for Animal Experimentation of the Toulouse Research Federation in Biology (CEEA N°1).

\(^2\)The CS-US interval had first been set to 5 s and then to 3 s after the 7 first experiments, to elicit faster and stronger avoidance reactions.
Table 5.1: Experimental conditions tested in Experiment 2 with the new environment. \(1vs4\) and \(5vs0\) refer to the number of conditioned vs naïve fish.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Replicates</th>
<th>Trials per replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>(1vs4)</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>(5vs0)</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

5.2.3 Experiment 2: test in a new environment

Experimental set-up

The square experimental tank (120×120 cm) was made of glass and was set on top of a box to reduce vibrations. The room was heated at the same temperature as the water in the tank to avoid gradients of temperature. The tank was surrounded by four opaque white curtains and illuminated homogeneously by four LED light panels. Inside the experimental tank, a ring-shaped corridor was filled with 7 cm of water of controlled quality (50% of water purified by reverse osmosis and 50% of water treated by activated carbon) heated at 28.8\(^\circ\)C (±0.7\(^\circ\)C). The corridor was 10 cm wide with a circular outer wall of radius 35 cm. The shape of the circular inner wall was conic and its radius at the bottom was 25 cm. The conic shape was chosen to avoid the occlusion on videos of fish swimming too close to the inner wall. The outer wall has 2 rows of the same LEDs as used in the shuttle box, equally spaced by 1 cm. Only 35 cm of the entire diameter (thus 70 LEDs) can be turned on (CS).

Experimental procedure for testing

These experimental tests were conducted after the conditioning experiments presented in the previous section. Conditioned fish were trained for 10 trials of the conditioning experiment a few hours before an experiment was done in the new set-up. Per day, fish participated only in a single experiment. Experiments concerned groups of 5 fish and were done in the ring-shaped tank. Trajectories were recorded by a high-speed camera (R&D Vision) recording from above the set-up at 45 Hz or 180 Hz, both in high resolution (2000×2048 pixels). Groups swim spontaneously for 20 min, with trajectories recorded at 45 Hz, until the CS is turned on for the first time. At the onset of the CS, the camera automatically switches to record fish positions at 180 Hz. The CS is turned on for 3 s and trajectories are recorded subsequently for 30 s, still at 180 Hz. Several trials are performed
on each group of fish, with an intertrial duration of 1 min. After the first trial, fish swim spontaneously for 1 min, recorded at 45 Hz before the CS. Three conditions are tested, the Control condition, with 5 naïve fish, a condition with 4 naïve fish and 1 conditioned (1vs4) and a condition with 5 conditioned fish (0vs5). Number of replicates and trials are reported in Table 5.1.

For each trial, we measure the number of fish that turn at least once in the direction opposite to the CS in the first 3 s following the onset of the CS (i.e while the light is on) and during the 3 s seconds after (i.e. when the light turns off). These two quantities estimate the strength of the collective reaction: if all individuals react fast and strongly to the CS, we expect them to turn while the light is on. We also measure whether all individuals are swimming in the direction opposite to the CS 6 s after its onset or not. If yes, we count one collective escape for the respective trial. This quantity is used to disentangle non-coordinated behaviours (e.g. where all individuals make U-turns several times and eventually the group does not swim away from the CS) to collective escape behaviours (i.e all group members make one clear U-turn and the group swims away from the CS). In the condition with one conditioned fish and 4 naïve ones (1vs4), we monitor if the conditioned fish turned first. The conditioned fish was recognised by eye by the operator. No tagging was used, because of the difficulty to tag small fish without altering their swimming behaviour.

We removed trials where not all fish were aligned in the same direction just before the CS is turned on (4 trials out of 55 in the condition Control, 8 trials out of 45 in the condition 1vs4 and 0 out of 20 in the condition 5vs0).
5.3 Results

5.3.1 Experiment 1

We use logistic regressions (with R (R Core Team, 2016)) to investigate the effect of the conditioning experiments on the escape response of fish. We model the probability $p$ that the binary result $r$ of each trial (Failed ($r = 0$) or Success ($r = 1$)) is 1 as a function of the index of the trial $i$, the number of days $d$ since the beginning of the conditioning for the considered group and of $c$ that indicates whether the group is a control ($c = 0$) or a conditioned one ($c = 1$). We start by evaluating the full model:

$$\text{logit}(p) = \beta_0 + \beta_1 i + \beta_2 d + \beta_3 c + \beta_4 id + \beta_5 ic + \beta_6 cd,$$  \hspace{1cm} (5.1)
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Figure 5.3: Results of the conditioning experiments (page 132). A). Proportion of escapes in control and conditioned conditions. Open circles and dots show the proportion of escapes of each experiment. The colour of the dots stand for the time since the beginning of the experiments. B). Time series of the proportion of escapes in 20 trials. An empty diamond stands for a change of protocol, from a duration of 5 s of CS to 3 s of CS before onset of electric shocks (to trigger stronger aversive responses). The cross indicates the death of one of the 6 individuals. Subsequent training sessions were conducted with the remaining 5 individuals. Colour of the dots are the same as in (A). The first point of the time series is averaged over the first conditioning replicate and all control replicates. Red line and grey shade represent the model (Eq. 5.2) predictions and its 95% confidence interval. C). Performance of the group averaged over all training sessions as a function of the trial. Red line and grey shade represent the model (Eq. 5.2) predictions and its 95% confidence interval. D). Proportion of escapes against time since last training session, with exponential fit. Points stand for the proportion of escapes averaged over all experiments with the same amount of days since the previous conditioning experiments and vertical bars stand for the standard error. There is no bar when there is only one data point.

Table 5.2: Exponential of the estimated logistic regression coefficients (odd ratios and confidence intervals) of the model shown in Equation 5.2. We also report the $p$-values of the Wald statistic that tests for each $\beta_j$ the null hypothesis $\beta_j = 0$ (no significant effect of the $j^{th}$ variable). Hierarchical stepwise likelihood ratio tests give the same significances. For $\beta_1$ to $\beta_3$, values greater than 1 indicate positive correlation while values less than 1 indicate negative correlation between $p$ and the respective explanatory variable.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>2.5%</th>
<th>97.5%</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\exp(\hat{\beta}_0)$</td>
<td>0.073</td>
<td>0.044</td>
<td>0.116</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$\exp(\hat{\beta}_1)$</td>
<td>1.062</td>
<td>1.040</td>
<td>1.084</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$\exp(\hat{\beta}_2)$</td>
<td>12.281</td>
<td>7.918</td>
<td>19.802</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$\exp(\hat{\beta}_3)$</td>
<td>0.999</td>
<td>0.9998</td>
<td>0.9999</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
where \( \beta_j, j \in [0, 6] \) are the regression coefficients. We search the model that minimises the Akaike information criterion (AIC) from the full model (AIC\textsubscript{full} = 2231). The AIC addressed the trade-off between the goodness of fit of the model and the number \( j - 1 \) of explanatory variables. We find that

\[
\logit(p) = \beta_0 + \beta_1 i + \beta_2 c + \beta_3 id,
\]

(hereafter called the model) minimises the AIC (AIC\textsubscript{mod} = 2228). We report the exponential of the estimated regression coefficients (odd ratios) in Table 5.2. This model predicts correctly the output of 63% of all trials and all coefficients \( \beta_j, j \in [0, 3] \) are significantly different from 0 (Table 5.2).

Our results show that \textit{H. rhodostomus} can learn the task of the shuttle box, in a social context. The proportion of escape responses (avoidance of the CS) is statistically larger in the conditioned group (0.57 ± 0.02, mean±standard error) than in the control groups (0.125 ± 0.03) (Figure 5.3A and \( \exp(\hat{\beta}_3) = 12.281 \), Table 5.2). Control groups reach a non-null proportion of escapes because they randomly swim in the shuttle box and may cross the barrier during the CS, either by accident or because fish are afraid of the CS. We qualitatively assess an effect of the CS on the behaviour of the naïve fish which is different from the behaviour of conditioned fish. In general, groups of naïve fish become excited during CS but without any avoidance behaviour – they are even sometimes attracted towards the LEDs that emit the light. The time series of the proportion of successful responses of the conditioned group shows that there is a decrease in reaction to the stimulus (Figure 5.3B with the model fit and \( \exp(\hat{\beta}_3) = 0.999 \)). The learning of the conditioning occurs within a month (purple dots) and the best performances are achieved until November (blue dots). From January to July (green and yellow dots), despite 27 conditioning experiments, it was not possible to reach the performances obtained in the other months. It seems that fish experience long-term habituation, even though our protocol cannot properly test it (i.e. we should test the specificity of habituation to the stimulus to exclude a fatigue effect). Although fish react less to the CS in the last sections of the time series, the achieved performance is, on average, still higher than the controls. Within a training session, fish perform significantly better after several trials, typically 3 (Figure 5.3C and \( \exp(\hat{\beta}_1) = 1.062 \)). This effect decreases with the number of days since the beginning of the experiments, possibly because of the long-term habituation previously discussed (interaction term \( \beta_3 id \) of the model, \( \exp(\hat{\beta}_3) = 0.999 \)).

In the models presented in Equations 5.1 and 5.2, we did not use the
Table 5.3: $P$-values of the likelihood ratio tests performed to assess the significance of the effects of the condition and of the trial to predict the occurrence of collective escapes from binomial generalised linear mixed effect models.

<table>
<thead>
<tr>
<th></th>
<th>Only Condition</th>
<th>Only Trial</th>
<th>Null Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Model</td>
<td>0.4</td>
<td>0.03*</td>
<td>0.03*</td>
</tr>
<tr>
<td>Only Condition</td>
<td>0.02*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only Trial</td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
</tbody>
</table>

number of days since the previous experiment as an explanatory variable, because the value for the control experiments and the first experiment of the conditioning experiments would be arbitrary (i.e. infinity, encoded for instance as 1000 days). Such an arbitrary value would have influenced the significance of the statistical tests performed. We find that there is an effect of the number of days since the previous experiment on the performance over the 20 trials of a focal experiment (Figure 5.3D). Namely, we find a decay of the proportion of escapes that can be modelled as

$$p = a \exp(bt),$$  \hspace{1cm} (5.3)

where $a$ is the degree of learning, $-b$ is the inverse of the rate of forgetting (in days) and $t$ the time since the previous conditioning experiments (in days). We find $a = 0.64$ and $b = -0.009$. The value for $a$ corresponds to the performance of the group at the beginning of our protocol (blue dots in Figure 5.3B). It is worth to note that we do not find any significant correlation between the number of days since the beginning of the conditioning $d$ and the number of days since the previous conditioning $t$ ($p$-value = 0.25), excluding a confounding effect of $d$ and $t$ on the proportion of escapes $p$. In other words, it seems that our results indicate both habituation and forgetting.

### 5.3.2 Experiment 2

Figure 5.4 shows a collective escape away from the CS performed by the group of conditioned fish: before the onset of the CS, the group is polarised (Figure 5.4A); at the onset of the CS, one individual reacts instantaneously, performing a U-turn (Figure 5.4B) which propagates to the other group members (Figure 5.4C) and the group swims in the opposite direction in less than 2 s after the light is turned on (Figure 5.4D-F).

We used R (R Core Team, 2016) and the packages \texttt{lme4} (Bates et al.,
A $t = -1 \text{s}$

B $t = 0 \text{s}$

C $t = 1 \text{s}$

D $t = 2 \text{s}$

E $t = 3 \text{s}$

F $t = 4 \text{s}$
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Figure 5.4: Snapshots from the escape behaviour of a group with 5 conditioned fish (page 136). Snapshots are taken every second from 1 second before the onset of the CS to 4 seconds after. The white arrow on (B) shows the fish that responds first to the CS.

We used R packages glmer (Bates et al., 2015) and lsmeans (Lenth, 2016) to perform a binomial generalised linear mixed effects analysis of the occurrence of collective escapes in trials as a function of the condition of the replicate (control, 1 conditioned versus 4 naïve (1vs4) or 0vs5) and the index of the trial (1 to 10). Namely, we test (i) an effect of the condition, to investigate whether the proportion of conditioned individuals in the group may change the propensity to initiate a collective escape and (ii) an effect of habituation or fatigue to the CS. As random effect, we use intercept for the experiment as well as by-experiment random slopes to account for the non-independence of the responses within a condition, since there are several trials per tested group of fish. P-values were obtained by likelihood ratio tests of the full model with the fixed effects against models with only one of each of the fixed effect. We also perform the likelihood ratio tests of the models with only one of each of the fixed effects against the null model with intercept and random effect only.

Both tests show a significant effect of the condition (control, 1vs4 or 0vs5) on the occurrence of collective escapes and no significant effect of the trial (Table 5.3). We find that the CS is not neutral on the behaviour of the naïve groups. naïve fish perform collective escapes in 16% of all trials (Figure 5.5A). In 71% of all trials there are at least 3 fish that make individual U-turns (Figure 5.5B). However, when all group members are conditioned, groups perform collective escapes in 75% of all trials (Figure 5.5A). Here, the response is in general collective and fast: in 80% of all trials all group members have turned within the three seconds after the CS is turned on (Figure 5.5C). In short, although groups of naïve fish react to the CS, with fluctuations in the heading of individuals they react more slowly and not collectively, in contrast to the groups composed only of conditioned fish.

The occurrence of collective escapes when only one fish is conditioned (condition 1vs4) does not significantly differ from the occurrence of collective escapes in pure groups of naïve fish (pairwise Tukey method, p-value = 0.3). However, fish in this condition tend to react more and faster than naïve fish (Figures 5.5B and C). We find that the conditioned fish turns in 78% of all trials, turns first in 43% of all trials (always during the CS) and initiates 54% of the collective escapes performed in the 1vs4 condition.
Figure 5.5: The conditioning in the new set-up. A). The average proportion of collective escapes as a function of the number of conditioned fish in the group. We report results averaged over replicates (dots) and conditions (empty circles) as well as the model predictions. The model used for reference is the binomial generalised linear mixed model with only the condition as fixed effect. Noise is added to the x-coordinates of the points, to improve visualisation. B). Bar chart of the number of individual escapes per trial for each condition. C). Bar chart of the number of individual escapes per trial that occur during the CS.
5.4 Discussion

Our experiments are, to our knowledge, the first conditioning experiments ever tried with *H. rhodostomus*. We show that it is possible to train *H. rhodostomus* to escape using an active aversive paradigm. We find that this conditioned behaviour can be successively used in a new environment. The possibility to condition *H. rhodostomus* opens opportunities to investigate collective behaviour with obligate schoolers (in contrast to experiments with zebrafish or goldfish).

The duration of the CS before the onset of electric shocks (3 s) is very short compared to previous studies (7.5 s (Agetsuma et al., 2012), 10 s (Horner et al., 1961; Woodard and Bitterman, 1973; Portavella et al., 2004), 12 s (Pradel et al., 1999; Xu et al., 2007), or 20 s (Piront and Schmidt, 1988, although with a different protocol)). This has to be taken into account to assess the conditioning success of *H. rhodostomus* (which was on average close to 63% during the first half of the year) compared to the other studies, achieving learning criterion of 70% (Portavella et al., 2004) or 80% (Pradel et al., 1999; Piront and Schmidt, 1988). This choice was made to elicit stronger (i.e. fast) aversive reactions in response to the CS.

It seems that our training leads to habituation because the performance of the conditioned group decreased after the first 48 experiments conducted over a period of 4 months. Even if our protocol was not designed to test habituation, our results suggest that this decrease in the performances of the conditioned groups was not the consequence of a lapse in fish memory: fish kept on performing fast aversive responses, although less often during the later conditioning experiments.

To explain the decay of the conditioned escapes as a function of the time since the previous training session, we make the assumption that the proportion of escapes during a replicate $p$ can be modelled as $p = a \exp(bt)$, with $a$ the degree of learning, $-b$ the inverse of the rate of forgetting (in days) and $t$ the time since the previous conditioning experiments. Such a curve is similar to curves referred to as *forgetting curves*, after the seminal work of Herman Ebbinghaus in experimental psychology (Ebbinghaus, 1913, Chapter VII). Interestingly, we find that the exponential fit in our experiments is close to the fits proposed for forgetting curves in humans, modelled as combinations of exponential functions or power laws (Wickelgren, 1974; Rubin et al., 1999; Murre and Dros, 2015). Although our results concern a group of fish in contrast to an individual, it is likely that the collective performance is correlated with the level of learning of individuals. Future research with an experimental protocol dedicated to investigate the forgetting of conditioned behaviour could focus on the dependence of $b$ to
the number of times the group has experienced the conditioning $m$, in order to account for an overlearning effect (we predict that $b$ decreases with $m$) as well as the dependence of $a$ to $m$, to account for long-term habituation (we also predict that $a$ decreases with $m$, in agreement with the present findings). Such research may be of interest to understand the macroscopic scale of memory in *H. rhodostomus* and its underlying mechanisms.

As for the set of experiments that concerns the new environment, a ring-shaped tank and the propagation of the induced perturbation, we found that the occurrence of fast, collective escape behaviours increases when the proportion of conditioned fish in the group increases. Our results might suggest that there is a critical proportion of trained fish required to elicit a collective escape but this has to be confirmed. Future work will concern the development of a tracking software that would take the change of luminosity into account to keep track of fish positions during the emission of the light, which is badly done by current tools. Given the time resolution of our videos (180 fps after the onset of the CS), data with the positions of fish will help to compare qualitatively and quantitatively the collective behaviours and the propagation of information between the different conditions tested in our experiments. The spatio-temporal propagation of information after the CS will be analysed, using the domino-like propagation of information that occurs during spontaneous collective U-turns as benchmark (see previous chapter). Further experiments with more conditions (i.e. with all possible ratios of conditioned versus naïve fish in a given group size) will help to elucidate the question of the critical proportion.

Our conclusions remain uncertain because of the small number of conditioned fish in our experiments. We made the choice to train only a few fish for ethical reasons, given that it was the first time conditioning experiments were tried with this species. The results of our experiments being promising, we emphasise that our results need to be reproduced experimentally with a greater number of conditioned groups (e.g. 10 groups of 5 conditioned fish).