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From fright to anticipation: using aversive light stimuli to investigate reward conditioning in large groups of Atlantic salmon (*Salmo salar*)

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Abstract In this study, we demonstrate how an event that is initially frightening to Atlantic salmon is turned to a positive stimulus through habituation and associative learning. The study was carried out in four commercial sized tanks (5 m^3) with near industry densities (>550 fish, 16 kg m^{-3}), using a delay conditioning procedure with an aversive flashing light as the conditioned stimulus and food reward as the unconditioned stimulus. By using video image analysis of the distribution of the fish in the tanks, the changes in behaviour from trial to trial could be documented in great detail. The current study documents the change in behaviour across the individual conditioning trials, clearly showing the step-by-step nature of the transition. The salmon needed more than 26 trials to become fully habituated to the flashing light but showed clear anticipatory behaviour already after about 19 trials. This demonstrates that the learning process is a combination of habituation and associative learning.

Keywords Farmed Atlantic salmon · Habituation · Associative learning · Conditioning · Stress · Video image analysis

Introduction

The response an animal gives to a stimulus is influenced by both its previous experience with the stimulus and also the associations the animal has made between different stimuli

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and aversive or rewarding events. Farmed fish must adapt to being confined in tanks or cages, and compared to wild fish, they have limited possibilities to flee when frightened. Sudden disturbances from activities on the farm may, therefore, be stressful and lead to impaired appetite and growth and reduced welfare (Ellis 2002; Fernö et al. 2006). Thus, from a management perspective, it is of interest to search for methods to reduce the level of stress released by events that are experienced as frightening. Ideally, such events should be turned to positive experiences. Reward conditioning, i.e. pairing an event with a food reward, has been suggested as a method to reduce the negative sides of sudden stimuli in the farm environment (Fernö et al. 2006).

Few studies have investigated reward conditioning in Atlantic salmon (*Salmo salar*), particularly within an aquaculture context. Tlusty et al. (2008) found that Atlantic salmon can be readily conditioned to acoustic signals that simultaneously sound as food becomes available, and Thomassen and Fjæra (1991) and Lines and Frost (1997) conditioned Atlantic salmon to a light signal announcing food. These studies and observations are, however, based on small groups of fish, and the behaviour of the fish is only briefly described. Important questions about the learning process, like documentation of the changes in habituation behaviour and learning rates, are mostly left unanswered.

The aims of the current study were to investigate whether Atlantic salmon post smolts can learn to associate an initially frightening event (strong light flashes) with a food reward, to determine what behavioural changes develop during the learning process and to find out how the response to the light flashes changes throughout the trials. In order to directly relate these observations to the salmon farming industry, we used commercial sized tanks with relative high densities of fish to mimic a typical farm setting (Fitzgerald et al. 2002).

Materials and methods

The study was carried out at the Institute of Marine Research, Matre Research Station, in July 2006. Atlantic salmon post smolts of the Aquagen strain reared in outdoors tanks and seawater transferred in mid-April 2006 were distributed among four circular indoor tanks (Ø = 3 m, water depth = 75 cm, 5.3 m^3,) and acclimated for 31 days before the start of the study. A representative sample of the fish was weighed and measured at transfer ($n = 303$, fork length 23.8 ± 2.6 (mean ± SD) cm, mass 155.5 ± 49.6 (mean ± SD) g). Approximately 550 fish (83 kg, 15.5 kg m^{-3}) were kept in each tank at the start of the experiment.

The tanks were supplied with seawater (8.3°C, 34.3 PSU) at a constant flow rate of 147 l min^{-1} in order to keep the oxygen saturation above 75% at the outlet. Fluorescent lights (36 W) were positioned above the centre of each tank and were on 24 h a day, while all other light sources were covered. A 24-h light regime was chosen to avoid stressing the fish by turning the lights on and off outside the conditioning trials (Mork and Gulbrandsen 1994). The tanks were divided into a light zone (500 lx at tank centre, 160 lx furthest from light), where food was delivered and a shaded zone ($\leq$50 lx) using a black plastic curtain on one side of the lights (Fig. 1). The purpose of the shaded zone was to give the fish a place to hide when frightened. Food (4-mm dry pellets: 7105 CPK100 ss40, BioMar AS, Norway) was distributed in seven even-sized meals every hour from 8 to 14 o'clock each day (Arvotec feeding units: Arvo-Tec T drum 2000, www.arvotec.fi). The food entrance point into the water was marked with a small black strip of tape at the surface of the water (Fig. 1). Black tape (5 cm wide) also marked three vertical stripes on the tank wall placed 30 cm apart, the first line 30 cm downstream from the food entrance point (Fig. 1). These three stripes indicated the main feeding area of the fish. Video film of this area was

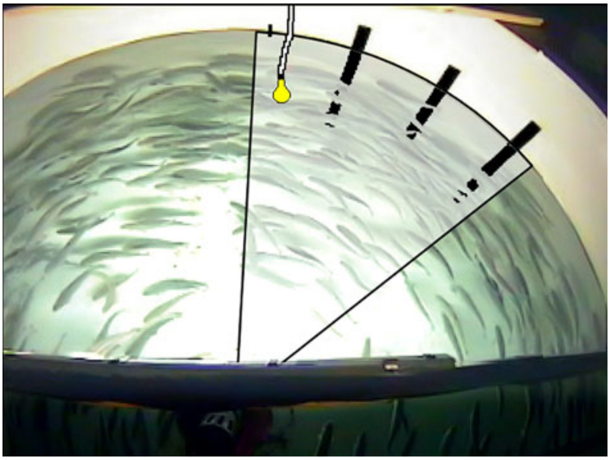

Fig. 1 The four fish tanks were divided into a light half (*upper part of image*) and a shaded half (*lower part of image*) by a *black plastic sheet* hanging on one side of the lights. Feed was delivered into the light half of each tank. The feed entrance point into the water was marked by a small *black stripe* on the wall. Three larger vertical stripes marked the main feeding area as seen from an underwater camera positioned at the centre of the tank. The sector indicates the field of view of the camera. The CS light bulb was positioned directly above the feed entrance point

recorded in each tank during all feeding sessions. The underwater cameras (VN-SVUC-IR, Scan Secure AS, Norway) were positioned at a depth of 20 cm in the centre of the tanks and were controlled via the GeoVision GV-800 Video Capture Card and the GV-800 Multicam Surveillance System (GeoVision Inc., Taipei, Taiwan).

Conditioning procedure

In order to increase feeding motivation, and thus responsiveness to the conditioning procedure, the fish were fed approximately 70% of the amount eaten when given feed in mild excess the final two days of the acclimation period, i.e. 0.7 × (food into tank − food in feed collector at outlet). The experiment lasted for eight days. During this period, all the meals were part of the conditioning procedure. The conditioning procedure used aversive light flashes (1 s on and 2 s off) from a light bulb (21 W) hanging 50 cm above the food entrance point as the conditioned stimulus (CS) and food as the unconditioned stimulus (US, reward). The CS started 30 s before the US and lasted 10 s after the onset of the US (delay conditioning). Each feeding session lasted a total of 300 s. Programmable relays (Ocean Controls, KT-5074APC Printer Port Relay Board Assembled, www.oceancontrols.com.au) activated the CS and the US. Unfortunately, the relays failed to activate (both CS and US) for 8 of the 56 planned conditioning trials, leading to only two trials (and meals) on Day 2, and five trials on Days 3 and 4. Recordings were activated 30 s prior to the CS and stopped 1 min past feeding.

Video image analyses

To register how the fish distributed vertically in the feeding area during the conditioning trials, the film recordings of the feeding area in each tank were analysed as described by Stien et al. (2007). In short; for each video image frame, an automatic image analysis procedure identified those parts of the three vertical black stripes (Fig. 2) that were not

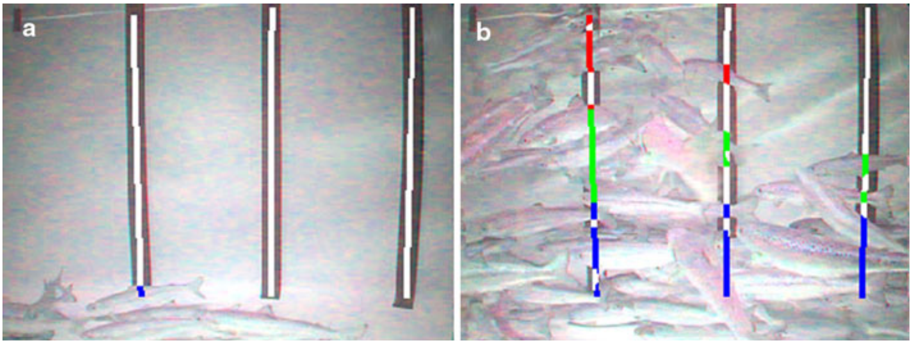

Fig. 2 Field of view of the camera. **a** Nearly, no fish in the feeding area, **b** feeding fish. The *white pixels* are the pixels identified by the video analysis procedure as representing the *black stripes* on the tank wall (predefined by user). The parts of the lines that are covered by fish are divided into upper (*red*), middle (*green*) and lower (*blue*) parts of the lines, thus, providing a measure of relative fish distribution in the upper, middle and lower parts of the feeding area for each video image frame

obstructed by fish from the field of view of the camera. This was then compared with the known extent of the stripes, and so the percentage of fish coverage in the feeding area could be calculated. To obtain a measure of vertical distribution in the tanks, the stripes were divided into three equal vertical sections; upper, middle and lower (Fig. 2). The percentage coverage of the three vertical parts of the stripes was used to indicate the relative density of fish in the upper, middle and lower parts of the water column. The frame rate of the videos was eight frames s^{-1}, this means that the image analysis procedure provided estimates of the relative density of fish at the three different heights eight times per s of the recordings, i.e. 3,120 times per recording.

Statistical methods

The resulting data from the experimental setup were analysed statistically with the software package Statistical Analysis System (SAS) for Windows (ver. 8e). First, the percentage coverage values of the stripes were arcsine transformed $\sin^{-1}[(0.01 \times P)^{0.5}]$ to facilitate statistical comparison by means of *t*-test (Crawley 2007). Differences in mean percentage coverage of the stripes between defined periods of the condition procedure (the period before the CS, the 10-s period after the onset of the CS and the 10-s period before the start of the US) were then tested for individual trials or per day using paired *t*-test (proc *t*-test SAS). Due to the high frequency of sampling by the image analysis method, the mean percentage coverages were constructed for each of the four tanks based on a high number of observations (mean of a 10-s period is a mean of 80 observations). For easy visibility in the figures, the curves illustrating the percentage coverage of the stripes were fitted using data from all four tanks using the penalized least square method (proc tpspline, SAS).

Results

First exposure to the CS (trial 1)

The fish reacted to the first exposure of the light flashes (CS) by immediately fleeing from the feeding area (solid black line in Fig. 3), and the fish did not return to the feeding area

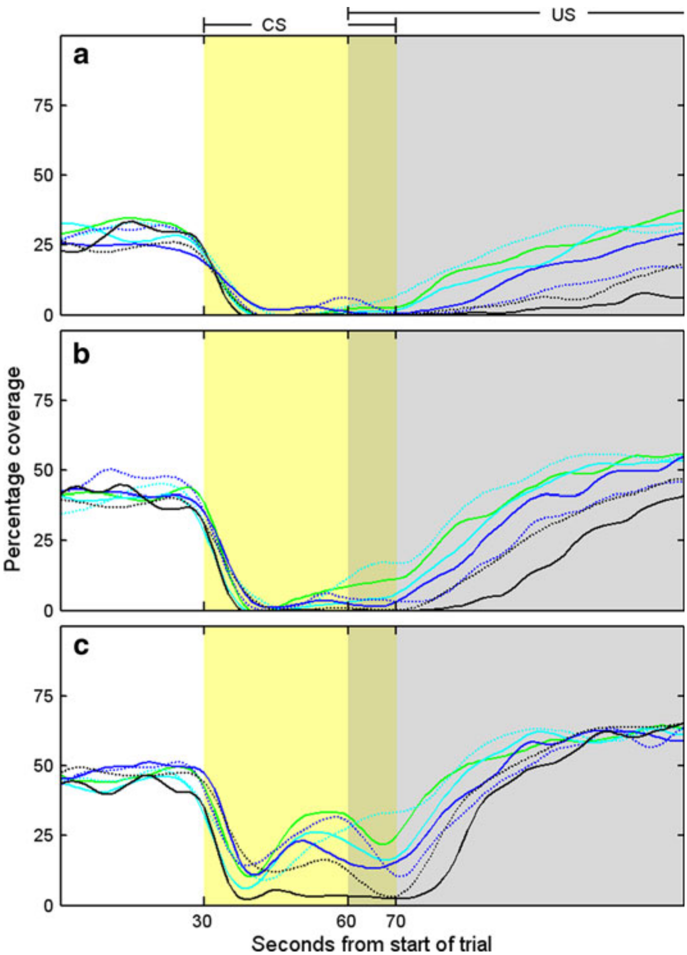

Fig. 3 Trial-to-trial changes in fish distribution over the first day. Percentage coverage of the **a** upper **b** middle and **c** lower parts of the *stripes* on the tank walls for trial 1 (*black solid line*), 2 (*black dotted line*), 3 (*blue solid line*), 4 (*blue dotted line*), 5 (*cyan solid line*), 6 (*cyan dotted line*) and 7 (*green solid line*) on the first day. *Curves* are fitted using data from all four tanks using the penalized least square method (proc tpspline, SAS) for easy visibility. Notice the gradual change from trial to trial

until several seconds after the light flashes had ceased, even though feeding had started (Fig. 3). As the fish returned, they first moved into the lower part of the tanks, then to the middle, but only very slowly to the upper part (Fig. 3).

Return to the feeding area

By the second trial, some fish were already returning to the feeding area in the lower part of the water column during the CS (black dotted line in Fig. 3c), and throughout the first day (trials 1–7), the fish coverage during the CS, the overlap CS/US and the US increased slowly. In the middle part of the water column, a small increase in coverage during the overlapping CS/US could be observed from trial to trial, as well as during the US (Fig. 3b). Almost no

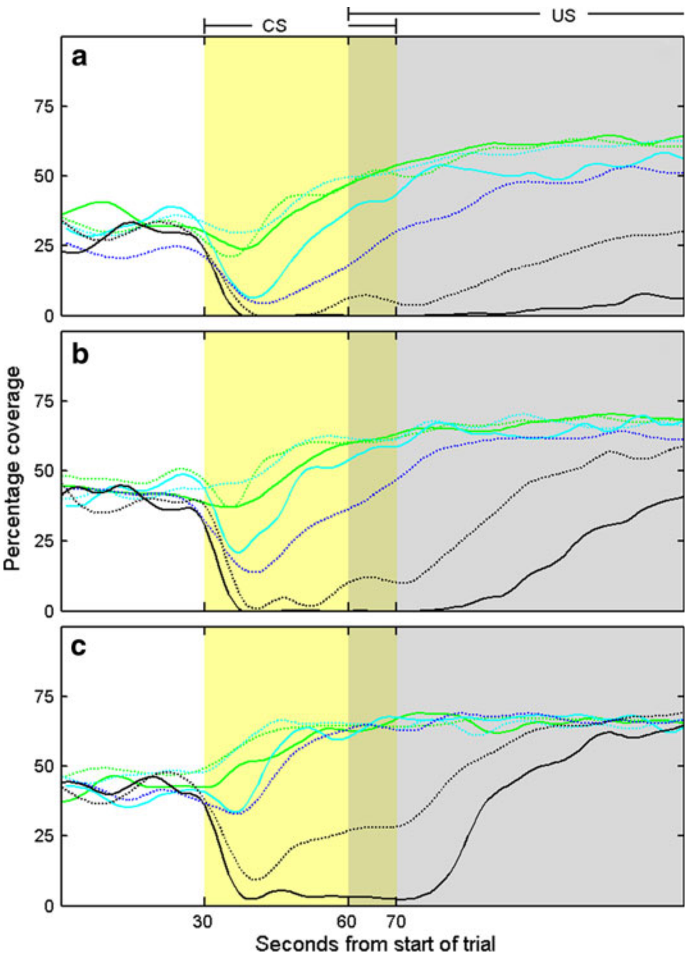

Fig. 4 Day-to-day changes in fish distribution over the whole experiment: Percentage coverage of the **a** upper, **b** middle and **c** lower parts of the *stripes* on the tank walls for the first trial on day 1 (*black solid line*), day 2 (*black dotted line*), day 3 (*blue solid line*), day 4 (*blue dotted line*), day 5 (*cyan solid line*), day 6 (*cyan dotted line*), day 7 (*green solid line*) and day 8 (*green dotted line*). *Curves* are fitted based on the data from all four tanks using the penalized least square method (proc tpspline, SAS) for easy visibility. Notice the gradual change from day to day

fish were found in the upper part of the water column during the CS in any trials on day 1 (Fig. 3a). There was, however, a marked increase during the US throughout the first day (Fig. 3a). The coverage of the upper, middle and lower parts of the stripes reached their maximum, faster and faster as the trials progressed (Fig. 4). Except for the first trial on day 1, the fish coverage reached or surpassed the pre-CS coverage during the US, and from day 4 onwards, the coverage also surpassed the pre-CS level before the onset of the US (Fig. 5).

Habituation to light signals

As described earlier, the initial reaction of the fish to the CS was to move down towards the bottom of the tank and away from the flashing light in the feeding area (fright reaction).

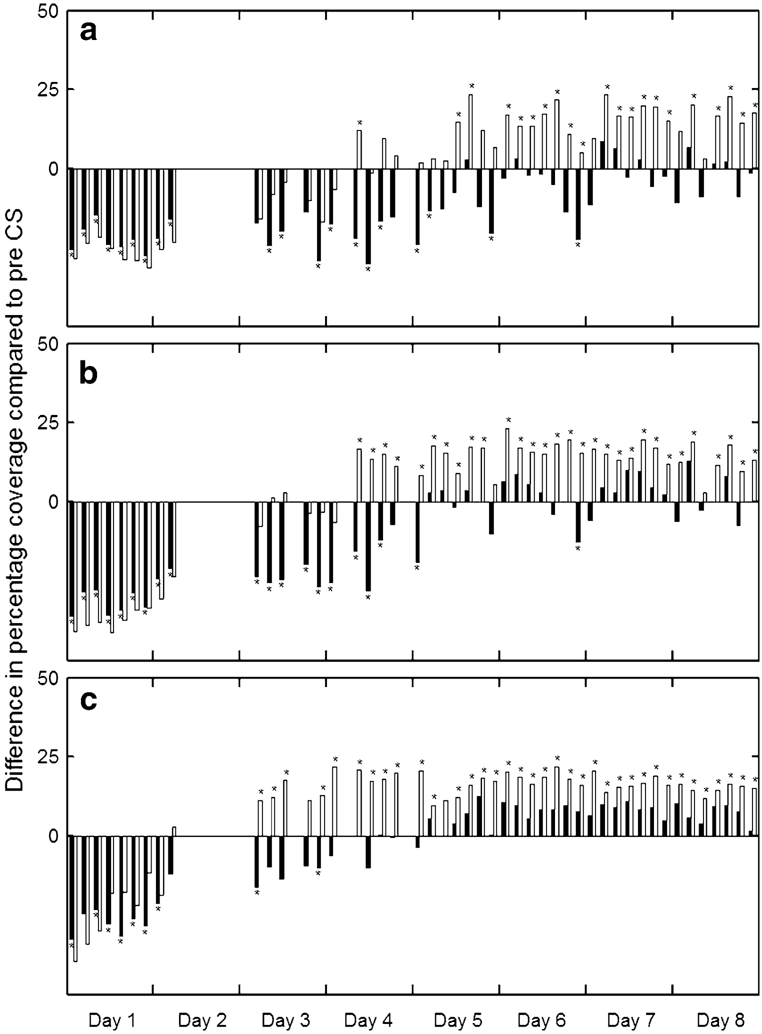

Fig. 5 Fright reaction (*black bars*) measured as the mean difference between the percentage coverage of the stripes in the period before the CS (*light flashes*) and the percentage coverage of the stripes in the 10-s period immediately after the onset of the CS for the upper (**a**), middle (**b**) and lower (**c**) parts of the tanks. Anticipation (*white bars*) measured as the mean difference between the percentage coverage of the stripes before CS and the percentage coverage of the stripes in the 10-s period before the start of the US (feeding). This figure shows the results from all the trials in the study. Significant fright ($P < 0.05$) is indicated by '*' on the *black bars*, while significant anticipation is indicated by '*' on the white bars (paired *t*-test, proc *t*-test, SAS). Loss of fright is habituation, while increased anticipation means that the fish are gathering in the feeding area before feed arrives, i.e. associative learning. Notice the gradual loss of fright and the gradual gain of anticipatory behaviour

This effect is clearly demonstrated in the distinct significant difference between the percentage coverage of the lower, middle and upper parts of the stripes during the first 10 s of the CS compared to the respective percentage coverage pre-CS (Fig. 5a–c; Table 1). This difference first disappears for the lower part of the stripes (day 4), then from the middle part (day 5) and last for the upper part (day 6–7, >26 trials) (Fig. 5a–c; Table 1).

Table 1 Habituation measured as the mean difference between the percentage coverage of the stripes in the 10-s period before the CS (light flashes) and the percentage coverage of the stripes in the 10-s period after the onset of the CS

	Day	Habituation			Associative learning		
		Mean difference	Arcsine transformation	$P < t$	Mean difference	Arcsine transformation	$P > t$
Upper part of tanks	1	−22.3	−0.353	<0.001	−26.8	−0.506	1.000
	2	−18.5	−0.274	<0.001	−24.3	−0.429	1.000
	3	−20.9	−0.320	<0.001	−11.2	−0.147	1.000
	4	−20.3	−0.250	<0.001	3.5	−0.001	0.502
	5	−12.5	−0.138	<0.001	9.1	0.084	0.002
	6	−6.6	−0.082	0.012	13.9	0.141	<0.001
	7	0.3	−0.015	0.239	17.4	0.168	<0.001
	8	−2.8	−0.045	0.057	11.2	0.150	<0.001
Middle part of tanks	1	−31.8	−0.414	<0.001	−37.7	−0.546	1.000
	2	−22.4	−0.280	<0.001	−26.5	−0.382	1.000
	3	−23.9	−0.288	<0.001	−2.0	−0.022	0.866
	4	−17.7	−0.195	<0.001	10.0	0.088	0.002
	5	−2.9	−0.046	0.030	16.7	0.126	<0.001
	6	0.8	0.005	0.590	17.6	0.174	<0.001
	7	4.4	0.036	0.993	15.2	0.149	<0.001
	8	0.7	0.002	0.529	12.4	0.127	<0.001
Lower part of tanks	1	−27.5	−0.317	<0.001	−24.5	−0.286	1.000
	2	−26.8	−0.202	0.002	−6.3	−0.114	0.897
	3	−16.5	−0.130	<0.001	13.0	0.129	<0.001
	4	−3.3	−0.051	0.035	19.6	0.195	<0.001
	5	3.7	0.015	0.806	15.0	0.155	<0.001
	6	8.4	0.081	1.000	18.5	0.184	<0.001
	7	8.4	0.075	1.000	16.7	0.170	<0.001
	8	6.9	0.058	1.000	15.0	0.149	<0.001

Associative learning measured here as the mean difference between the percentage coverage of the stripes before the CS and the percentage coverage of the stripes in the 10-s period before the start of the US (feeding). The mean differences following arcsine transformation to facilitate *t*-test are also shown. As long as the habituation comparison was significantly negative (paired *t*-test, proc *t*-test, SAS), the fish fled at start of CS. When the associative learning comparison becomes significantly positive, there was an increase in the number of fish in the feeding area before the US compared to before the CS. The comparison is done for the upper, middle and lower parts of the tanks

Conditioning

From day 4 (>19 trials), the fish coverage of the stripes in the 10-s period before the start of the US increased to at least pre-CS level for all parts of the stripes (Fig. 5a–c; Table 1). The response was initially greatest in the lower part of the water column (Fig. 5c; Table 1), but from day 5, the increase in stripe coverage was significant in all three parts of the water column, showing that the fish gathered in the feeding area even before the start of the US. Notice also that there was a period where the fish both fled from and were attracted to the CS (day 4–6, Fig. 5; Table 1).

Discussion

Were the light flashes initially aversive?

The relatively strong light flashes (21 W) used in the current experiment caused a strong fleeing response on first exposure. Mork and Gulbrandsen (1994) have previously observed that turning lights on or off leads to increased activity and causes salmon to move downward, and Mueller et al. (2001) observed the strong avoidance responses to intense strobe lights in Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) fry.

In the present study, the fish reacted to the light flashes (CS) by immediately fleeing from the feeding area, and the return to the feeding area was initially slow even during feeding, demonstrating that the sudden light flashes were aversive for the naive fish. This point is strengthened by the fact that feeding motivation was relatively high due to restricted feeding which would normally make the fish more willing to take risks to obtain food (Milinski 1993).

Habituation to light signals

From day 6 (>26 trials), the salmon no longer exhibited any signs of fleeing at the start of the light flashes, and from this point, they appear to have been fully habituated to the signal. Interestingly, in the study by Tlusty et al. (2008), salmon stopped showing aversive responses more quickly. The aversion to the acoustic signal was lost in as few as 6–9 trials (2–3 days), suggesting that salmon find the light signal used in the present experiment more stressful/frightening than the acoustic signal (250 Hz tone at 134 dB) used in the study by Tlusty et al. (2008). Fast changes in light level are probably more correlated with predator attacks than sounds, and therefore inherently trigger stronger flight responses. However, owing to different life stages, group sizes and strains of salmon in these two experiments, the results cannot be directly compared.

Conditioning

In the current study, the fish became positively conditioned to the CS after about 19 trials. In the study by Thomassen and Fjæra (1991), salmon were positively conditioned to a blinking light after 72–144 trials, and in the study by Lines and Frost (1997), the majority of the fish moved towards the light after as few as 10 trials. The conditioning procedure in the study by Thomassen and Fjæra (1991) is in some respects similar to the one reported here, but they used a shorter interstimulus interval (CS started only 9 s before US vs. 30 s before in this study), a shorter interval between trials (72 vs. 2–7 trials per day) and the light bulb was switched on when it was not blinking. Unlike the current study, the focus of Thomassen and Fjæra (1991) was to determine whether light signalling before feeding could influence the growth of the fish, and thus, their study gave only a very brief account of fish behaviour.

Lines and Frost (1997) used CS bulbs of much lower intensity (0.4 vs. 21 W) and a shorter interstimulus interval (light started flashing 5 s before feed delivery and lasted throughout the entire feeding period). These differences, possibly along with the much smaller groups sizes (8 vs. >550 in the present study), probably underlie the more rapid conditioning seen in their experiment (ten trials). Similarly, in the study by Tlusty et al.

(2008), the tone signal and the feeding started at the same time, and the tone signal continued throughout the entire feeding period. There are also potential differences between the background of the fish and the biological conditions used in the different experiments.

Concurrent fright and anticipation

During days 4–6, the fish still fled at the start of the CS (light flashes) but soon returned and gathered in the feeding area even before the start of US (feeding). This demonstrates the duality of the learning mechanisms into habituation and conditioning. Habituation can be said to be non-associative learning (the fish become accustomed to the light flashes), while conditioning is associative learning (the fish learn an association between the light flashes and feed). Habituation is viewed as an automatic process in the brain decreasing the strength of a reflex upon multiple exposures to a stimulus (Lieberman 2000). Associative learning may involve two different learning systems (Lieberman 2000). In the first (stimulus substitution), the animals behave as if a CS paired with food actually is the food (Hearst and Jenkins 1974). In the other (anticipation), the animals behave as if a paired CS is a signal of forthcoming food (Boakes 1977). In the first case, the CS elicits responses automatically, and the animal should approach the CS in the same way it approaches the US (here food), while in the second case, anticipations and cognition guide the responses (Lieberman 2000). That the fish first fled and then returned to position themselves in a fixed position down current from the feed entrance point suggests that they were waiting for food to arrive, i.e. anticipating forthcoming food. Active anticipation has also been suggested in other aquaculture species such as cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) (Nilsson 2008; Nilsson et al. 2008). Further studies where the CS is positioned spatially separated from the US may resolve which type of associative learning is occurring.

Relevance to industry

Most of the initially frightening stimuli and handling events occurring in fish farms are not really dangerous to the farmed fish, but the fish are not aware of this and react instinctively with acute stress responses (Fernö et al. 2006). There should, therefore, be clear positive effects of reward conditioning fish within a farming context: For example, Schreck et al. (1995) showed that conditioning fish can reduce transportation stress in juvenile Chinook salmon. In fish farms, many husbandry procedures like the removal of dead fish using dip nets, people passing the tanks and other activities making aversive sounds or sudden light changes, which lead to acute stress and probably in many cases, reduced feed intake and growth. If we, for example, train the fish to associate human activity near the tanks with food rewards, the acute stress response should be replaced by anticipatory behaviour. This is typically observed in farms where the fish are hand fed or fed with feeding canons from boats. A Pavlovian conditioning procedure can relatively easily be automatically generated using movement sensors or microphones that detect the human activity and send signals to a feeding computer which then gives out a small meal to all tanks. Activation of the animals' reward systems in the brain will also (by definition) be positive for the fish wellbeing and welfare (van den Bos et al. 2002). Conditioning may also function as a method for assessing motivational state and welfare (Spruijt et al. 2001). This is currently being investigated in ongoing studies at our laboratory.

Conclusions

It is clear from the present study that large groups of Atlantic salmon post smolts are able to habituate to an initially frightening event and form a positive association between the event and the availability of food. Rewarding stressful events can, thus, be used in order to reduce fish stress caused by disturbances and activities at a fish farm. The study also demonstrates the step-by-step nature of the learning process, and that the process is a combination of habituation and associative learning.

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