acta ethologica (2018) 21:169–173
https://doi.org/10.1007/s10211-018-0297-4

ORIGINAL PAPER

Water jet: a simple method for classical conditioning in fish

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Received: 6 June 2018 / Revised: 31 July 2018 / Accepted: 1 August 2018 / Published online: 6 August 2018

Abstract
Classical conditioning in animals is a learning procedure involving a biologically relevant stimulus paired with a previously neutral stimulus. In fish, light and sound are frequently used as previously neutral stimuli for conditioning tests. However, in laboratory experiments with replicates, such stimuli may influence the responses of fish in nearby aquariums. Herein, we developed a simple applicable methodology for classical conditioning in fish that prevents this type of influence. We isolated fish in individual aquariums and introduced a water jet that caused localized water movement, followed by the introduction of a food pellet. These procedures were repeated for each fish for 20 days. After 14 days, all fish were conditioned. Moreover, in subsequent probe trials (memory retention tests) conducted within 32 days after conditioning procedures, fish responded accordingly. These findings corroborate the applicability and usefulness of the method tested herein especially under lab conditions. Therefore, we suggest that a simple water jet is a useful and reliable tool for fish conditioning in future studies.

Keywords Fish learning · Water jet · Classical conditioning · Memory

Introduction

Conditioning is a learning process that includes behavioral changes based on the effects of the paired "stimulus-response" on the central nervous system of animals (Lieberman 2000). Classical conditioning, described in 1927 by Ivan Pavlov, occurs in situations when a certain behavior, naturally expressed in the presence of a biologically relevant stimulus (e.g., salivation in response to savory food), is also expressed in the presence of a previously neutral stimulus after a specific training (e.g., salivation when a specific sound is presented). The basis for this learning process is implicit (subconscious) memory (Kandel et al. 2014), which is a type of long-term memory that remains strongly dependent on the original conditions under which the learning happened (Bailey et al. 1996; Kandel et al. 2014).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10211-018-0297-4) contains supplementary material, which is available to authorized users.

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Classical conditioning experiments are often used to study animal cognition, especially in mammals (Steinmetz et al. 1986, 1989; Kim et al. 1995; Freeman and Rabinak 2004; Boele et al. 2010; Yang et al. 2015). In fish, classical conditioning has been known for decades. Studies have shown that, like mammals, fish are able not only to associate stimuli (Moreira and Volpato 2004; Nilsson et al. 2007; Bratland et al. 2010) but also express memory responses (Yue et al. 2008; Doyle et al. 2017), which is fundamental for any learning process. This fact makes sense since, in most aquatic environments, the complexity of surroundings is associated with the need to locate shelters, feeding areas, predators, etc., thus exemplifying the adaptive value of learning ability in these environments. In this scenario, the occurrence of classical conditioning was recently demonstrated in several studies of fish species, such as zebrafish (*Danio rerio*) (Manabe et al. 2013; Doyle et al. 2017), St. Peter's fish (*Sarotherodon galilgeus*) (Zion et al. 2011a, b), Atlantic salmon (*Salmo salar*) (Bratland et al. 2010), and Dourada (*Sparus aurata*) (Folkedal et al. 2018).

In these studies, the conditioned stimulus is usually some sort of light and/or sound, which is tested in groups of fish aiming to facilitate the management of these animals (Moreira and Volpato 2004; Bratland et al. 2010; Zion and Barki 2012; Folkedal et al. 2018). However, when performing experiments to evaluate individual responses (e.g., understanding learning mechanisms), the effectiveness of this conditioning method may be compromised when fish located in nearby aquariums are tested. This is even more relevant when considering that laboratory experiments may involve animals kept in aquariums or small and/or closed environments (e.g., Yue et al. 2004 and Braubach et al. 2009). In these situations, visual stimuli such as light or acoustic stimuli can be easily noticed by the fish in the aquarium next to the one being conditioned, thus compromising the conditioned response of fish.

In this context, finding other conditioning stimuli that are easily applicable and do not interfere with nearby aquariums is fundamental for experiments with fish conducted in more restricted conditions, such as in laboratories. Considering that the localized movement of the water is a simple applicable stimulus that should not interfere with other aquariums, regardless their size and closeness, herein we evaluated whether such a stimulus can be classically conditioned in the fish Nile tilapia (*Oreochromis niloticus*). We also evaluated whether this response is consistently maintained over time in the animal's memory. We selected Nile tilapia as our study model given that this species is typically social and territorial (Fernandes and Volpato 1993), including complex interactions among individuals and high capability for learning and memory in social and environmental contexts (Ebbesson and Braithwaite 2012; Warburton 2003).

Material and Methods

Animals and maintenance conditions

We used naïve Nile tilapia (*Oreochromis niloticus*), 7.16 ± 0.49 cm (mean ± SD) in length, from a hatchery. The fish were juvenile and could not be sexed. These fish were acclimatized for 30 consecutive days in 500-L tanks (6 fish/L; T°C, 28° ± 1°C; photoperiod, 12L:12D) before the experimental procedures. Tanks were supplied with continuous aeration, biofilters, and PVC pipes that functioned as animal shelters and were siphoned daily. Once a day under these conditions, fish were fed commercial food for tropical fish (40% crude protein; Agromix). The water quality was maintained with pH ~ 6.5 and nitrite and ammonia levels below 0.05 mg/L and 0.5 mg/L, respectively.

Experimental design

We applied a brief water jet to cause localized water movement. Water jet was followed by a food pellet inserted in the same place to condition fish ($n = 10$) to the localized water movement (see video 1 in Supplementary Material). This procedure was repeated four times a day (first conditioning of the day was at a random time) for 20 consecutive days. Thus, the localized water movement caused by the jet application was used as the conditioned stimulus whereas the food pellet was the unconditioned stimulus. In each conditioning test, we recorded the latency response of the animals to the water movement. After this whole conditioning period (20 days), probe trials were performed to test memory retention.

Specific procedures

Ten fish were randomly transferred from tanks to experimental aquariums (20 L; 40 × 20 × 25 cm), which were visually and physically isolated. Such aquariums were equipped with continuous aeration caused by an air pump. Styrofoam plates were used for visual isolation, and aquariums were placed at a distance of at least 5 cm from each other. Such aquariums were siphoned daily, and the water quality was maintained under similar conditions as in the maintenance environment. Experimental aquariums remained on a shelf partially covered with black tarpaulin to prevent animals from perceiving movements of researchers. There were separate holes in the tarpaulin at the height of each aquarium, where individual hoses (diameter of 8 mm) were introduced. Such hoses were not related to the aeration system. The position of the hoses over the aquarium surface was randomized among fish to prevent lateralization influences (previous individual trends on a particular side, as already detected in fish; see Dadda and Bisazza 2016).

In the conditioning tests performed each day (2.5 h interval between daily tests, with the first test of the day randomly starting between 0800 am and 1200 pm), a water jet of 3 ml was manually injected via syringe, gently, into the hoses over the respective aquariums to move the water only in the specific place where the hose originated (see video 1 in Supplementary Material).The jet was always of a same duration (~ 1 s) and of a same intensity. The separation of experimental aquariums by a distance of at least 5 cm prevented possible interference from the localized subtle water vibration from one aquarium to the others. Then, 10 s after water injection, a food pellet was introduced by the same hose. This delay period (10 s) was applied to ensure that fish would be conditioned to the water movement caused by the jet and not by the fall of the pellet. Moreover, we used food pellets as reinforcement to condition fish because although classical conditioning was demonstrated in fish with other reinforcement types (Losey and Margules 1974), food is considered one resource that animals are primarily motivated to reach (Matthews and Ladewig 1994; Galhardo et al. 2011; Houpt 2012) and most frequently used for conditioning fish in other studies (Tlusty et al. 2008; Lindell et al. 2012; Doyle et al. 2017). All the tests were filmed for 5 min and, from these films, we recorded the latency response of each fish to water movement of the jet and the subsequent intake of the food pellet. Fish were considered conditioned when they immediately responded to the water movement on all tests of a specific conditioning day and maintained this response until the last conditioning test day. Fish were not fed between tests.

After the 20th conditioning test day, we performed probe trials (memory retention tests) on days 2, 4, 8, 16, and 32 after the whole conditioning period to evaluate whether the fish response of association between the water movement and the food pellet was maintained over time. The selected time intervals to evaluate fish's memory of conditioning were based on Doyle et al. (2017). For these probe trials, fish were exposed to the stimulus to which they were conditioned (water movement caused by 3 ml of water jet), but without receiving the associated food reward (a food pellet). Such tests were always conducted only once per day and at random times in each day. This was done to prevent possible influences of circadian components on fish response. These probe trials were also filmed for 5 min and, from these films, we recorded the latency response of the animals after the water movement. Fish were fed between probe trials, but not in the test days.

Data analysis

We used the data on latency response to the water movement during each conditioning tests to construct the learning curve per test day (20 days) and per test (4 tests per day = 80 tests total). We also calculated the percentage of conditioned fish on each conditioning day (considering the 4 tests of a same day). Such results were daily and cumulatively expressed over time. In probe trials, we evaluated whether the response to water movement remained unambiguously immediate throughout the days after the animals were conditioned.

Results

All tested fish were conditioned by the water movement caused by the water jet. The first conditioned fish ($n = 2$; 20%) expressed this response on the seventh test day (Fig. 1). In only 10 test days, more than half of the animals ($n = 6$; 60%) were already conditioned to water movement (Fig. 1). From the 14th test day, all fish were already expressing clearly conditioned responses to water movement (Fig. 1). The learning curve of fish, considering the average data of individuals on each conditioning test or each conditioning day (4 tests per day) is represented in Fig. 2. Moreover, the latency response for each individual fish to water movement over the days of conditioning is shown in Supplementary Material Fig. 1.

Considering the probe trials, fish always responded positively. That is, on each test day (at 2, 4, 8, 16, and 32 days after the whole conditioning period), the response to the water movement caused by water jet was always immediate for all tested individuals.

Discussion

Herein we have demonstrated a new simple methodology to perform classical conditioning in fish, which can be applied without causing any interference from the application of the stimulus in small and nearby aquariums. This methodology involves the localized movement of water as a conditioned stimulus and food as an unconditioned stimulus. Based on our results, in 2 weeks of conditioning tests, all fish learned to associate these stimuli. In addition, all individuals were able to retain and recover associative memory within 32 days after conditioning procedures, thus indicating the retention of the conditioned response in fish's memory. Together, these findings indicate that this new methodology is applicable and useful for the conditioning of Nile tilapia fish under lab conditions.

Classical conditioning is a learning process often employed in studies involving cognitive issues (Moreira et al. 2004; Nilsson et al. 2007; Braubach et al. 2009; Nordgreen et al. 2009; Roy and Bhat 2016; Kenney et al. 2017) or even to facilitate management and/or other animal husbandry interventions (Bratland et al. 2010; Zion and Barki 2012; Folkedal et al. 2018). The methodology used herein, based on water movement as a conditioned stimulus, is simple and inexpensive to apply for classical conditioning of fish. Such methodology requires only readily available materials, and thus could be used in a simple way in experiments involving

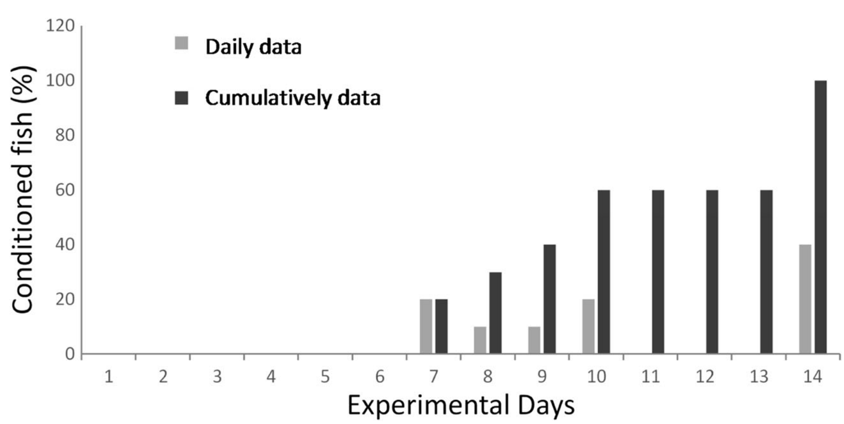

Fig. 1 Percentage of conditioned fish (as defined in the "Material and methods" section) in each test day (daily and cumulatively data). All fish (100%) were already conditioned after day 14

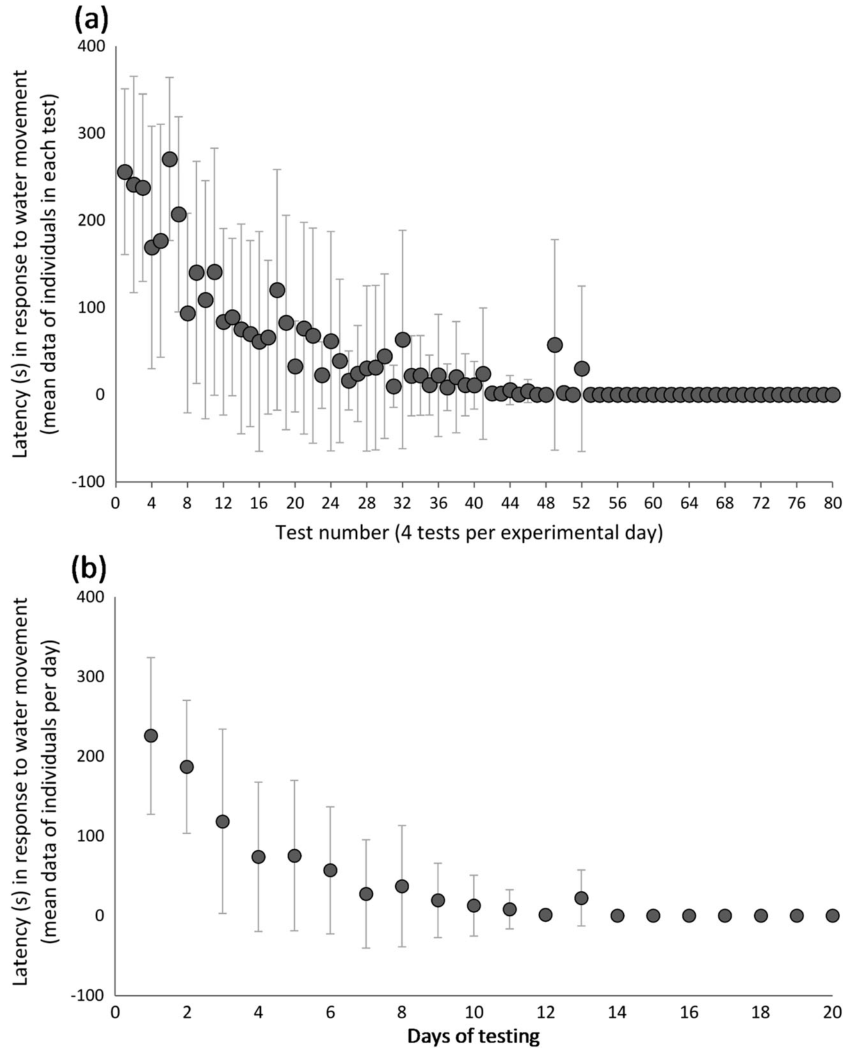

Fig. 2 Learning curves for fish response (latency) to water movement, the conditioned stimulus, after the application of the water jet. **a** Data considering all conditioning tests performed over 20 consecutive days (mean ± SD). **b** Data considering conditioning daily averages (4 tests per day; mean ± SD) of latency response over the 20 consecutive days

cognitive issues or to facilitate management of these animals. The demonstration of the applicability of this new methodology is based on the facts that all fish were conditioned in two weeks of experimentation (see Figs. 1 and 2) and responded accordingly in all probe trials.

The demonstration of this new methodology for classical conditioning is even more relevant considering the problems involved with traditional stimuli that are frequently used to condition fish. Light or sound is frequently used as conditioned stimuli for fish. Despite this, such stimuli can be difficult to isolate, thus favoring interference in the experimental procedures for lab experiments involving individualized fish in small and nearby aquariums. For example, Yue et al. (2004) used light as a conditioned stimulus in experiments on rainbow trout (*Oncorhynchus mykiss*), and had to transfer fish between aquariums during the tests to isolate the stimulus. This fact, besides representing a source of stress for fish (Wendelaar-Bonga 1997), allowed testing only six fish per day. Nilsson et al. (2007) conditioned Atlantic codfish (*Gadus morhua*) with a light stimulus, which hampered the isolation during the experiment of these animals, who were then removed from tanks and anesthetized to apply an individual marking. Furthermore, sound frequencies that easily propagate in the water and are also frequently used to condition fish in large environments (see review Zion and Barki 2012) may bias the conditioning response of fish when in smaller environments. Given that localized water movement is easily isolated from other aquariums, the conditioning methodology proposed herein prevents the problems described above, and can serve as a useful tool for fish conditioning.

When animals learn something new, one method of evaluating the learning consolidation is by assessing the memory of individuals. Memory was reported in fish (Yue et al. 2004; Nilsson et al. 2007; Doyle et al. 2017) and has been demonstrated in relation to different stimuli and contexts. Herein, we demonstrated that Nile tilapia remember localized water movement as a conditioned stimulus and respond accordingly for up to 32 days. This fact demonstrates the applicability of the methodology employed. Thus, we conclude that the simple movement of water caused by a small jet is an applicable and useful methodology for conditioning fish, which is especially advantageous when conditioning is performed in more restricted environments as labs, when possible interferences caused by such other stimuli as light or sound can hamper the performance of experiments.

Financial support This project was funded by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) through a scholarship for the first author.

Compliance with ethical standards

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

Ethical approval All procedures performed in this research were in accordance with the Ethical Principles for Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on the Use of Animals (CEUA) at the Biosciences Institute of UNESP, Botucatu campus (SP-Brazil) (Protocol # 699-CEUA).

References

Bailey C, Bartsch D, Kandel E (1996) Toward a molecular definition of long-term memory storage. Proc Natl Acad Sci U S A 93(24): 13445–13452

Boele H, Koekkoek S, De Zeeuw C (2010) Cerebellar and extracerebellar involvement in mouse eyeblink conditioning: the ACDC model. Front Cell Neurosci 3:1–13

Bratland S, Stien L, Braithwaite V, Juell J, Folkedal O, Nilsson J, Oppedal F, Fosseidengen J, Kristiansen T (2010) From fright to anticipation: using aversive light stimuli to investigate reward conditioning in large groups of Atlantic salmon (*Salmo salar*). Aquac Int 18(6): 991–1001

Braubach O, Wood H, Gadbois S, Fine A, Croll R (2009) Olfactory conditioning in the zebrafish (*Danio rerio*). Behav Brain Res 198(1):190–198

Dadda M, Bisazza A (2016) Early visual experience influences behavioral lateralization in the guppy. Anim Cogn 19:949–958

Doyle J, Merovitch N, Wyeth R, Stoyek M, Schmidt M, Wilfart F, Fine A, Croll R (2017) A simple automated system for appetitive conditioning of zebrafish in their home tanks. Behav Brain Res 317:444–452

Ebbesson L, Braithwaite V (2012) Environmental effects on fish neural plasticity and cognition. J Fish Biol 81(7):2151–2174

Fernandes M, Volpato G (1993) Heterogeneous growth in Nile tilápia: social stress and carbohydrate metabolism. Physiol Behav 54:319–323

Folkedal O, Fernö A, Nederlof M, Fosseidengen J, Cerqueira M, Olsen R, Nilsson J (2018) Habituation and conditioning in gilthead sea bream (*Sparus aurata*): effects of aversive stimuli, reward and social hierarchies. Aquac Res 49(1):335–340

Freeman J, Rabinak C (2004) Eyeblink conditioning in rats using pontine stimulation as a conditioned stimulus. Integr Physiol Behav Sci 39(3):180–191

Galhardo L, Almeida O, Oliveira R (2011) Measuring motivation in a cichlid fish: an adaptation of the push-door paradigm. Appl Anim Behav Sci 130:60–70

Houpt K (2012) Motivation for cribbing by horses. Anim Welf 21:1–7

Kandel E, Schwartz J, Jessel T, Siegelbaum S, Hudspeth A (2014) Princípios de neurociências, 5th edn. McGraw-Hill-Education, Porto Alegre, pp 1256–1273

Kenney J, Scott I, Josselyn S, Frankland P (2017) Contextual fear conditioning in zebrafish. Learn Mem 24(10):516–523

Kim J, Clark R, Thompson R (1995) Hippocampectomy impairs the memory of recently, but not remotely, acquired trace eyeblink conditioned responses. Behav Neurosci 109(2):195–203

Lieberman D (2000) Learning: behavior and cognition. Wadsworth, Belmont

Lindell S, Miner S, Goudey C, Kite-Powell H, Page S (2012) Acoustic conditioning and ranching of black sea bass Centropristis striata in Massachusetts USA. Bull Fish Res Age 35:103–110

Losey G, Margules L (1974) Cleaning symbiosis provides a positive reinforcer for fish. Science 184(4133):179–180

Manabe K, Dooling R, Takaku S (2013) Differential reinforcement of an approach response in zebrafish (*Danio rerio*). Behav Process 98: 106–111

Matthews L, Ladewig J (1994) Environmental requirements of pigs measured by behavioral demand-functions. Anim Behav 47:713–719

Moreira P, Volpato G (2004) Conditioning of stress in Nile tilapia. J Fish Biol 64(4):961–969

Nilsson J, Kristiansen T, Fosseidengen J, Fernö A, Van den Bos R (2007) Learning in cod (*Gadus morhua*): long trace interval retention. Anim Cogn 11(2):215–222

Nordgreen J, Janczak A, Hovland A, Ranheim B, Horsberg T (2009) Trace classical conditioning in rainbow trout (*Oncorhynchus mykiss*): what do they learn? Anim Cogn 13(2):303–309

Roy T, Bhat A (2016) Learning and memory in juvenile zebrafish: what makes the difference - population or rearing environment? Ethology 122(4):308–318

Steinmetz J, Rosen D, Chapman P, Lavond D, Thompson R (1986) Classical conditioning of the rabbit eyelid response with a mossy-fiber stimulation CS: I. Pontine nuclei and middle cerebellar peduncle stimulation. Behav Neurosci 100(6):878–887

Steinmetz J, Lavond D, Thompson R (1989) Classical conditioning in rabbits using pontine nucleus stimulation as a conditioned stimulus and inferior olive stimulation as an unconditioned stimulus. Synapse 3(3):225–233

Tlusty M, Andrew J, Baldwin K, Bradley T (2008) Acoustic conditioning for recall/recapture of escaped Atlantic salmon and rainbow trout. Aquaculture 274(1):57–64

Warburton K (2003) Learning of foraging skills by fish. Fish Fish 4(3): 203–215

Wendelaar-Bonga S (1997) The stress response in fish. Physiol Rev 77(3):591–625

Yang Y, Lei C, Feng H, Sui J (2015) The neural circuitry and molecular mechanisms underlying delay and trace eyeblink conditioning in mice. Behav Brain Res 278:307–314

Yue S, Moccia R, Duncan I (2004) Investigating fear in domestic rainbow trout, *Oncorhynchus mykiss*, using an avoidance learning task. Appl Anim Behav Sci 87(3–4):343–354

Yue S, Duncan I, Moccia R (2008) Investigating fear in rainbow trout (*oncorhynchus mykiss*) using the conditioned-suppression paradigm. J Appl Anim Welf Sci 11(1):14–27

Zion B, Barki A (2012) Ranching fish using acoustic conditioning: has it reached a dead end? Aquaculture 344-349:3–11

Zion B, Barki A, Grinshpon J, Rosenfeld L, Karplus I (2011a) An automatic fishing machine based on acoustic conditioning. Aquac Eng 45(2):87–91

Zion B, Barki A, Grinshpon J, Rosenfeld L, Karplus I (2011b) Retention of acoustic conditioning in St Peter's fish *Sarotherodon galilaeus*. J Fish Biol 78(3):838–847