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Behavioral studies of stimulus learning in zebrafish larvae

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ABSTRACT

Within a week of fertilization, a zebrafish larva has developed a robust behavioral repertoire that includes the ability to learn about noncontingent stimuli. I begin this paper with a brief review of the t1-t2 framework in which groups receive different experiences at the first time point (t1) followed by a common assessment at the second time point (t2) and the strengths of this framework for studying stimulus learning. I then describe assays that have been implemented within the t1-t2 framework to demonstrate stimulus learning in the developing zebrafish. I discuss how these assays have been used to address three fundamental questions about stimulus learning: What are the conditions for stimulus learning? What is the content of stimulus learning? How is stimulus learning reflected in behavior? For each of these three questions, I also identify those issues regarding stimulus learning in the developing zebrafish that warrant further analysis at the behavioral level.

1. Introduction

Learning that occurs during exposure to a stimulus is not only important for subsequent recognition of that stimulus but is also relevant to learning about predictive relations between stimuli in Pavlovian conditioning and among stimuli, responses and outcomes in instrumental conditioning. The repeated presentation of a noncontingent stimulus is generally regarded as the simplest procedure for studying learning and is a ubiquitous feature of many tasks used to examine a broad range of phenomena including habituation, sensitization, imprinting, the mere exposure effect, perceptual learning, latent inhibition, expectancy violation, priming and recognition memory. Deficits on some of these stimulus repetition tasks have been reported in humans with diseased (Braff et al., 1992; Geyer et al., 1990), damaged (Cowell et al., 2006) and aging brains (Duff et al., 2011; Viggiano et al., 2008). Studies of stimulus learning in animals like *C. elegans*, *Drosophila* and the zebrafish (*Danio rerio*) are invaluable not only for revealing key fundamental psychological principles that govern the acquisition, representation and use of information but also because of their potential to help identify the molecular and genetic mechanisms underlying stimulus learning deficits and to aid in the development of therapeutic interventions.

The primary goals of this paper are to (re-)acquaint the reader with the t1-t2 framework in which groups receive different experiences at the first time point (t1) followed by a common assessment at the second time point (t2) and the strengths of this framework for studying stimulus learning, to review some examples of its application to stimulus learning in the developing zebrafish, and to highlight the need for additional studies to address three fundamental questions about stimulus learning: What are the conditions for stimulus learning? What is the content of stimulus learning? How is stimulus learning reflected in behavior? Comprehensive answers to these questions at the behavioral level are central to developing a theoretical account of stimulus learning which may in turn help guide the search for the underlying mechanisms and the interpretation and amelioration of task-related deficiencies in stimulus learning.

2. The t1-t2 framework

A necessity that is all too often overlooked in the design of experiments to study learning is to separate the exposure phase (t1) during which learning may occur from a subsequent test phase (t2) in which that learning is assessed. Failure to observe this basic requirement can lead to a variety of unwarranted assumptions and interpretative challenges. For example, studies that compare acquisition curves across groups trained under different conditions obviously confound these two phases. This can lead to erroneous conclusions about the source of observed differences in the acquisition trajectories (see Davis, 1970; Davis and Wagner, 1968; Rescorla and Holland, 1976; Rescorla, 1988).

The t1-t2 framework formulated by Rescorla and Holland (1976) guards against this confound between learning and performance factors by stipulating that only the conditions in the exposure phase (t1) should differ across subjects; the conditions in the test phase (t2) should be the same for all subjects. Under this framework, the critical observation for inferring learning is differential behavior in a common test at t2 as a result of the differential experiences at t1. Table 1 illustrates the basic

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Table 1
Between-subject design for demonstrating stimulus learning. Group E receives presentations of a stimulus (S1) at t1 but Group C does not (—). Both groups are then tested with S1 at t2. Learning is indicated if there is a difference in their behavior towards S1 in the test. Note that the t1-t2 framework does not dictate any specific type of assessment at t2, only that it be identical for all subjects (Rescorla and Holland, 1976). Note also that this between subject design reflects only the most basic control condition which may not be optimal for every S1 experience.

	Input (t1)	Assessment (t2)
Group E	S1	S1
Group C	—	S2

between-subject design for studying learning about a noncontingent stimulus proposed by Rescorla and Holland (1976); an appropriate control for determining learning about a stimulus is to compare behavior to that stimulus in a common test at t2 between animals that were exposed to that stimulus at t1 (Group E) and those that were not (Group C). A significant difference in their behavior at t2 would provide evidence that Group E learned about the stimulus at t1. Rescorla and Holland (1976) characterized this exposure operation as providing two pieces of information about the stimulus: that it exists and that it has certain properties.

A second notable benefit of adopting the t1-t2 framework is that it avoids a problem inherent in the more common approach towards stimulus learning; that of reliance on a change in behavior between the initial and terminal presentations of a stimulus to infer that learning has occurred. This concern is readily apparent when using animals undergoing rapid development because maturational changes per se may affect stimulus processing or response production. However, behavior may also change due to acute motivational fluctuations that are unrelated to experience with the stimulus. For example, in a larval zebrafish experiment, there might be gradual shifts in ambient conditions such as water temperature or quality, or effects of prolonged isolation from conspecifics, of an increasing level of food deprivation (hunger) or of circadian activity levels. Thus, the observation of a decrease in the response to a repeatedly presented stimulus per se is totally inadequate to support the widespread assertion it all too often encourages that within-session learning has occurred. There are also more general reasons to avoid measuring learning as a change in behavior over time. First, it perpetuates the faulty assertion that there is no learning in the absence of a change in behavior (Leaton and Tighe, 1976; Rescorla, 1988; Soderstrom and Bjork, 2015). Second, it encourages the misguided belief that the amount of learning is directly proportional to the magnitude of the response decrement observed at t1 (Davis, 1970; Davis and Wagner, 1968; Rescorla, 1988; Rescorla and Holland, 1976).

3. The larval zebrafish

Much has already been written about the merits of the zebrafish for research in developmental neurobiology, toxicology, pharmacology and genetics (Ali et al., 2011; Bailey et al., 2013; Fontana et al., 2018; Gerlai, 2014; He et al., 2014; Richendrfer et al., 2014; Shams et al., 2018; Stewart et al., 2014; Patton and Zon, 2001). Highlights typically noted are its small size, hardiness in the laboratory, daily availability of large numbers of externally fertilized eggs, the optical transparency and rapid development of the embryo, its fully sequenced genome, amenability to genetic manipulation and the availability of numerous transgenic and knockout lines. Zebrafish development is extremely rapid by any vertebrate standard. In the first 24 h post-fertilization (hpf), the embryo, visible through its protective chorion, has developed eyes, a beating heart, and tail movements. Within 5 days of fertilization, the early larval zebrafish has an inflated swim bladder and well-developed visual and motor systems that enable it to hunt and capture live prey, to forage and to avoid potential predators such as adult zebrafish.

Researchers have already taken advantage of the behavioral repertoire of the early larval zebrafish to study sensory and motor development. This is particularly evident in the assessment of visual ability and functional recovery from visual impairments for which there are now several robust behavioral assays. The optokinetic reflex (OKR), a stereotypical eye movement, can be reliably elicited and measured at 4 dpf by rotating a drum of alternating black and white stripes around the immobilized larva (Brockerhoff et al., 1995; Easter and Nicola, 1996). At 4 and 5 dpf, unrestrained larvae will orient clockwise during the presentation of a clockwise rotating colored cross and counterclockwise when the direction of the rotating cross is reversed (Thorn et al., 2017). At 6 and 7 dpf, the optomotor response (OMR) can be accurately measured by presenting a moving line grating to free-swimming larvae. Larvae will swim in the same direction as the grating (Neuhauss et al., 1999; Orger et al., 2000; Orger and Baier, 2005).

More recently, there has been considerable interest in how the behavior of early larval zebrafish changes with experience and, more specifically, in probing their basic learning abilities. Colwill and Creton (2011) reported that 5 dpf larvae were more exploratory and less thigmotactic when re-exposed to an environment that they had been exposed to at 4 dpf. Experience-related effects have also been noted in prey capture efficiency. Carrillo et al. (2016) found that capture rates of *Paramecium* improved with practice in the dark. They suggested that the young zebrafish larvae had learned to use information about water flow during the first month post-fertilization.

Investigators have also devised methods to study more conventional forms of associative and nonassociative learning in the larval zebrafish. To date, assays for studying associative learning are fairly limited and the results have been mixed. Valente et al. (2012) were unsuccessful in using electric shock to demonstrate classical fear conditioning or operant avoidance conditioning in larvae younger than 3 and 4 weeks, respectively. Others, however, have reported evidence of classical conditioning in 6–8 dpf larvae (Aizenberg and Schuman, 2011; Hinz et al., 2013). Aizenberg and Schuman (2011) used illumination of an LED as a signal for a tactile poke to the side in gel-restrained subjects. Acquisition of a conditioned tail response was observed in the paired group but not in the unpaired and CS alone control groups. More importantly, this conditioning effect was detected 30 min later when all groups were tested with the CS alone. Evidence for nonassociative learning in very young zebrafish larvae has, in contrast, been less elusive and the assays offer greater promise for high throughput (Ahmad et al., 2012; Fero et al., 2011; Roberts et al., 2013).

4. Stimulus learning in the larval zebrafish

All of the studies reviewed in this section used a t1-t2 design to examine learning about a repeatedly presented stimulus in early larval zebrafish. They documented that learning as a difference at t2 in the behavior elicited by the stimulus in the exposed group relative to a control group. The learning resulting from this stimulus exposure operation has traditionally been characterized as nonassociative and a specific product of that learning, the reduced ability of the exposed stimulus to elicit a response, is widely referred to as habituation.

4.1. Demonstrations

Remarkably, only a few studies have adopted the t1-t2 framework to demonstrate stimulus learning during early larval development (O'Neale et al., 2014; Roberts et al., 2011, 2016; Wolman et al., 2011). Roberts et al. (2011) used a between-subject design and implemented the conventional no treatment control condition outlined in Table 1. In one experiment, they exposed 6–8 dpf larvae to an auditory/vibrational stimulus comprised of 10 blocks of 900 stimuli (1-ms long, 109 dB, 200 Hz ramp wave, every 1 s) with a 5 min interblock interval. During subsequent test presentations of the stimulus 15 min later, they observed a reduction in the C-start reflex (a characteristic C-bend escape

response that occurs with a short onset latency of < 12 ms) to that stimulus in the exposed larvae compared to control larvae that were spared the exposure treatment.

In our original demonstration of stimulus learning (Experiment 1 in O'Neale et al., 2014), we exposed 7 dpf larvae individually to one of two visual displays projected on a screen below the larvae. For Group B, a gray disk moved horizontally back and forth (bounced) across one half of the agarose well; for Group S, an identical gray disk was also presented but its position was fixed (stationary) at the midpoint of the pathway traversed by the bouncing disk. After 12 5-min presentations of the visual display alternating with 5 min presentations of the uniform white background, both groups were tested with the bouncing gray disk for 5 min. Images were taken every 6 s and were subsequently processed using an ImageJ macro to extract the x, y coordinates of the larva's centroid (a position corresponding to a point on the head behind the eyes). We calculated the displacement of the larva between pairs of consecutive images. The larva was recorded as still if it moved less than 0.3 mm in the 6-sec interval (a conservative value that represented no visible displacement when the images were inspected by eye). This measure of immobility indexes the freezing component of the startle response elicited by a potentially threatening moving stimulus (see section 4.3.3). During testing, the percentage of still observations was significantly lower in Group B than in Group S during the bouncing disk; there was no such difference between the groups in the immediately preceding 5 min on the white background. In a subsequent experiment, we confirmed this result in 5 dpf and 6 dpf larvae (Experiment 2 in O'Neale et al., 2014).

4.2. But is it learning?

As discussed in section 2, the t1-t2 design controls for the contribution of maturational and various unrelated motivational factors to a difference between exposed and control subjects during testing at t2. However, two other non-learning explanations, sensory adaptation and effector (motor) fatigue, have traditionally been considered for a decrement in responding to a repeatedly presented stimulus. Using a t1-t2 design per se does not rule out these two explanations for a difference in behavior at t2 between the exposed and control subjects. The typical procedure for separating these non-learning accounts from a learning explanation for a response decrement is to examine the effect of a novel stimulus presented shortly before t2. If responding to the exposed stimulus recovers at t2, it would appear that the subject is not only able to detect that stimulus but that there is no impediment preventing a response to it. An alternative strategy to dishabituation is to demonstrate the persistence of the response decrement over a time period long enough to allow for the dissipation of any sensory adaptation or motor fatigue effects. Using both of these strategies, Roberts et al. (2016) have demonstrated habituation of the C-start reflex to an auditory/vibrational stimulus detected 18 h after exposure and the dishabituation of that response by the application of a novel stimulus, lightly touching the larva with a broom bristle, 15 min before testing.

There are several reasons why explanations in terms of sensory adaptation or motor fatigue seem somewhat implausible for the results of our stimulus learning studies. First, in our assay, repeated exposure to the bouncing disk resulted in more activity rather than less during testing at t2 compared to the control condition suggesting that motor fatigue is not a factor. Second, our methodology discouraged sensory adaptation effects. For example, the stimulus was not repetitively applied to the same sensory receptors and we alternated stimulus presentations with 5 min periods of no visual stimulus. Third, we have more direct evidence against a sensory adaptation account from a related study using an identical exposure phase except that the bouncing disk was red. We switched from gray to red to optimize image analysis by splitting color channels to remove the red disk (see Pelkowski et al., 2011). We found that 7 dpf larvae were located significantly more often at the edge of the well during presentations of the bouncing disk than during its absence suggesting that their ability to detect the movement of the bouncing disk was not muted by sensory adaptation (Lovato et al., 2016). Lastly, using our assay with a red bouncing disk, we have obtained evidence of stimulus learning in 7 dpf larvae when the test was administered one hour after exposure to the stimulus. The test data from this unpublished experiment are shown in Fig. 1.

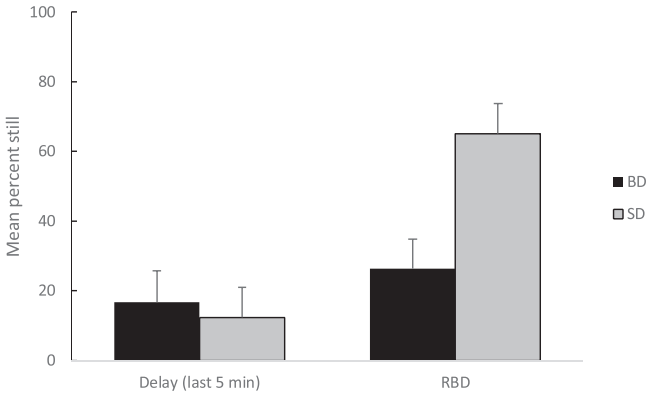

Fig. 1. Experiment 1: Stimulus learning with a 1 h t1-t2 interval. Mean (± SEM) percent still during testing (t2) with a red bouncing disk. Subjects were 7 dpf zebrafish larvae randomly assigned to one of two groups. During exposure training (t1), Group BD (n = 24) received 8 5-min presentations of a red bouncing disk alternating with 5 min presentations of a white background; Group SD (n = 24) received 8 5-min presentations of a red stationary disk alternating with 5-min presentations of a white background. A one hour delay separated the exposure phase from the test phase. Both groups were exposed to a white background during the delay and to a 5-min presentation of a red bouncing disk during the test. See Supplementary Materials for additional details regarding methods. Mean percent still and SEMs are plotted separately for Groups BD and SD during the last 5 min of the 1 h delay (left bars) and during the 5 min test with the red bouncing disk (RBD, right bars). There was no significant difference in mean percent still between the two groups in the last 5 min of the delay, $F(1,46) = 1$, $p > .10$. However, mean percent still was significantly lower in Group BD relative to Group SD during the test, $F(1,46) = 13.1$, $p < .001$, indicating that stimulus learning persisted for at least one hour.

4.3. The three Cs: conditions, content and conduct

The accumulating evidence is strong that early larval zebrafish are capable of learning about a noncontingent stimulus. However, from a behavioral perspective, comparatively little is known about the details of this learning process in the larval zebrafish. In this section, I discuss how the three fundamental questions that Rescorla (1980) posed about learning in general can be applied to stimulus learning in the developing zebrafish and I identify those areas that need to be investigated further.

4.3.1. What are the conditions for stimulus learning?

An examination of the determinants of stimulus learning has generally asked two questions about acquisition: What is the effect of the number of stimulus presentations and what is the effect of the rate of stimulus presentations? The consensus to emerge is that more stimulus exposures and longer interstimulus intervals (ISIs) at t1 produce stronger learning effects at t2 (Rescorla and Holland, 1976). Data from the larval zebrafish are consistent with these outcomes (O'Neale et al., 2014; Roberts et al., 2011, 2016; Wolman et al., 2011). For example, O'Neale et al. (2014) varied the number of 5-min presentations of the bouncing disk or the stationary control disk across different groups of 7 dpf larvae. They recorded significantly less freezing during testing with the bouncing disk in the larvae exposed to 4 or 8 5-min presentations of the bouncing disk relative to their respective control groups but no significant difference after 1 5-min presentation. Wolman et al. (2011)

manipulated the ISI. They exposed 6 dpf larvae in groups of 20 to a 2 -h habituation procedure consisting of a series of 480 1-s dark flashes with an ISI of 15 s. For one group (spaced), the series was split into 4 blocks of 120 flashes punctuated by 10 min intervals; for the other group (massed), the stimuli were presented in a single block. All larvae were then tested with a series of 10 1-s dark flashes with an ISI of 60 s. Larvae that had received a spaced pattern of stimulation showed a longer-lasting response decrement (dark flash failed to elicit an O-bend response) than those that had received a massed pattern of stimulation. Additional studies of the effect of the ISI in zebrafish that are modeled more closely on the design of the rodent studies by Davis (1970) and related work examining intertrial interval effects in associative learning (e.g., Lattal, 1999) are necessary to establish the generality of the finding that stimulus learning is superior with spaced presentations.

Two questions have generally been posed about the conditions leading to the removal of stimulus learning: What is the effect of extending the t1-t2 interval and what is the effect of an intervening stimulus between t1 and t2? Both spontaneous recovery of habituated responses and their dishabituation by a novel stimulus have been reported in studies using larval zebrafish. These studies did not rely on the traditional definition of spontaneous recovery as a change in behavior between the end of stimulus exposure (t1) and the beginning of the test (t2), an approach that has been justly criticized by Rescorla (2004) but instead made comparisons across groups at t2. It should be noted, however, that the full complement of improved designs recommended by Rescorla (2004), which necessitate adjustments in when training or testing is carried out, may not have been used. Roberts et al. (2011) observed spontaneous recovery of the habituated response in 6–8 dpf zebrafish larvae with t1-t2 intervals ranging from minutes to hours, depending on the pattern and duration of the stimulus exposure period. Wolman et al. (2011) also found spontaneous recovery of the short-latency C-start reflex in 5 dpf larvae with a 3 min t1-t2 interval. Moreover, they obtained dishabituation of this response using a tactile stimulus (head touch) although a dark flash was ineffective. As noted in Section 4.2, dishabituation of the C-start reflex was obtained by Roberts et al. (2016) using a tactile stimulus in 6–8 dpf larvae. Best et al. (2008) reported that a 2 s light pulse dishabituated the startle response to an auditory stimulus in 6–9 dpf larvae.

Several candidate mechanisms offer viable explanations for these dishabituation effects. One popular account is that an effective dishabituator activates the state system which sensitizes the larva potentiating its response to any stimulus. To support this possibility, studies would need to include control groups that received no stimulus exposure at t1 but were tested at t2 with or without the dishabituator presented before t2. A difference between these two controls would confirm that the dishabituator was in fact a sensitizing stimulus. It would be of interest to examine if novel stimuli lacking the propensity to activate the state system can nevertheless induce recovery of the habituated response (Wagner, 1976; Whitlow, 1975). Such a finding would be consistent with Wagner's (1976, 1981) memory model of habituation which asserts that a dishabituator functions by displacing the representation of the repeatedly exposed stimulus from a limited capacity short-term memory. It is also possible that an effective dishabituator leaves aftereffects that alter perception of the exposed stimulus so that it is unrecognizable or that alter the context in which the exposed stimulus has been presented. In all of these cases, presentation of the exposed stimulus at t2 following a dishabituator would be expected to elicit a response.

4.3.2. What is learned about the stimulus?

Learning about a stimulus has usually been conceptualized as the construction of an internal representation which incorporates information about the specific sensory properties of the stimulus (see Delamater, 2012). The most straightforward prediction derived from this perspective regarding noncontingent stimuli is that the learning detected at t2 should depend upon the degree of similarity between the stimulus tested at t2 and the stimulus presented at t1; the more similar the stimuli are, the greater the learning detected. However, it should also be noted that there is an interesting exception to this simple rule. In the rat acoustic startle procedure, Davis and Wagner (1968) found that habituation at t2 to a weak stimulus (108 dB) was actually greater if the intensity of the stimulus presented at t1 was 120 dB rather than 108 dB. To our knowledge, there has been no demonstration of stimulus specificity of habituation and no analysis of the effects of stimulus intensity on habituation in the larval zebrafish. This is an area that warrants further investigation.

We have recently begun to explore the potential for stimulus specificity using our visual learning assay. Zebrafish are tetrachromatic with normal color and ultraviolet vision. By 5 dpf, they have a cone-dominated retina with circuits for tetrachromatic color vision that survey the horizon and lower visual field (Zimmermann et al., 2018) and a well-developed visual system although acuity continues to improve even after 7 dpf (Gestri et al., 2012; Chhetri et al., 2014). Innate color preferences have been described in 5 dpf zebrafish larvae with blue preferred to red (Park et al., 2016). To find out if zebrafish larvae might learn about the color of the bouncing disk in our assay, we exposed different groups of 7 dpf larvae to one of four conditions at t1: a red bouncing disk (Group 1), a blue bouncing disk (Group 2), a red stationary disk (Group 3), and a blue stationary disk (Group 4). All larvae received 12 5-min presentations of the visual stimulus alternating with 5 min periods of the uniform white background alone. They were then presented with the red bouncing disk at t2. The design is shown in Table 2.

If hue is encoded in the stimulus representation, mean percent still should be greater during testing with the red bouncing disk in the group exposed to a blue bouncing disk at t1 (Group 2) than in the group exposed to the red bouncing disk at t1 (Group 1). The data from the test are shown in Fig. 2. There was no significant difference during testing between these two groups ($F < 1$). There was also no significant difference during testing between the two groups exposed to the red (Group 3) or blue (Group 4) stationary disks ($F < 1$). A separate analysis confirmed that stimulus learning had nevertheless occurred; the mean percent still was significantly lower in the larvae exposed to a bouncing disk at t1 (Groups 1 and 2 combined) relative to the larvae exposed to a stationary disk at t1 (Groups 3 and 4 combined), $F(1, 46) = 8.9$, $p < .005$. Comparable analyses of mean percent still during the 5 min period on the white background immediately preceding testing revealed no statistically significant differences. There was no significant difference in mean percent still between the two groups exposed to the red (10.6 ± 5.4) or blue (13.5 ± 6.9) stationary disks ($F < 1$) or between the two groups exposed to the red (20.5 ± 10.6) or blue (4.9 ± 2.4) bouncing disks, $F(1,22) = 2.1$, $p > .10$, or between the larvae exposed to the bouncing disk (Groups 1 and 2 combined) or the stationary disk (Groups 3 and 4 combined), $F(1,46) = 1.2$, $p > .10$.

This pattern of results is inconclusive about whether or not the larvae learned about the color of the moving disks. It is possible, for instance, that color was encoded as part of the stimulus representation but that learning generalized from the blue disk to the red disk based on

Table 2
Basic design for demonstrating specificity of stimulus learning. Groups 1 and 2 received presentations of a bouncing disk (B) at t1 while Groups 3 and 4 received presentations of a stationary disk (S). For Groups 1 and 3 the disk was red (R) and for Groups 2 and 4 the disk was blue (B). All four groups were then tested with a red bouncing disk (RB) at t2.

	Input (t1)	Assessment (t2)
Group 1	RB	RB
Group 2	BB	RB
Group 3	RS	RB
Group 4	BS	RB

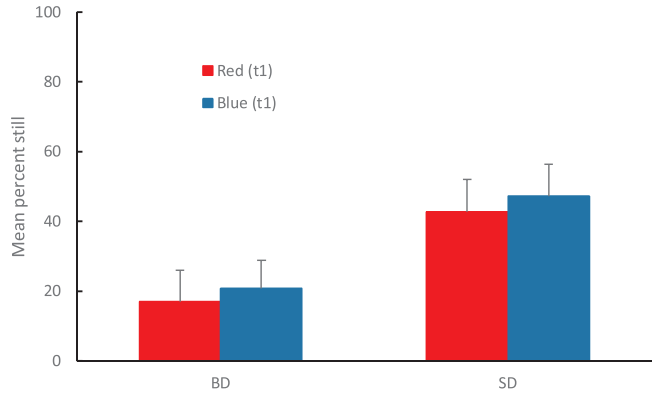

Fig. 2. Experiment 2: Specificity of stimulus learning. Mean (± SEM) percent still (percent displacements of larval centroids between two consecutive images smaller than 0.3 mm) during testing (t2) with a red bouncing disk. The 7 dpf larvae had been exposed in training (t1) to a bouncing disk (BD, left bars) that was either red (Group 1, n = 12) or blue (Group 2, n = 12) or to a stationary disk (SD, right bars) that was either red (Group 3, n = 12) or blue (Group 4, n = 12).

their shared or common attributes such as the speed, size, shape and trajectory of the bouncing disks. If these shared features were more salient than hue, the magnitude of generalization would be profound. Alternatively, the assessment may have been insensitive to detecting a difference between the groups exposed to the red and blue bouncing disks. This issue of stimulus specificity should be explored further by manipulating the value of these shared features between the exposure stimulus and the test stimulus, by increasing the t1-t2 interval to increase assay sensitivity, or by using another index such as new learning about the exposed stimulus (see 4.3.3).

Finally, there have been a number of reports that stimulus learning may be context specific (e.g., Evans and Hammond, 1983; Hall and Channell, 1985). Studies typically demonstrate this point by showing that when testing is conducted in a novel context, responding to the previously exposed (habituated) stimulus recovers. One explanation of these effects asserts that an association is learned between the context and the noncontingent stimulus (Wagner, 1976, 1981); another account, based on configural theory (e.g., Pearce, 1987, 1994; Rescorla, 1972) proposes that contextual features are integrated with the stimulus features to form a configural representation. Both accounts predict recovery of responding if the previously exposed stimulus is tested in a different context. They differ, however, in their predictions for the effects of context extinction, an approach that is immune to the complications of generalization decrement, competing responses and sensitization associated with the context change approach (Honey et al., 1992). The associative account, but not the configural account, predicts that repeated exposure to the context in the absence of the stimulus will also result in recovery of the response to the habituated stimulus (Wagner, 1976). To date, there has been no evaluation of the role of context in stimulus learning in the larval zebrafish.

4.3.3. How is stimulus learning reflected in conduct?

This section addresses two questions about the performance aspects of stimulus learning: What determines the topography of the response elicited by a noncontingent stimulus and how can stimulus learning be measured behaviorally? The simple answer to the first question is that the stimulus elicits an innate or genetically hard-wired response whose topography is a product of natural selection. For example, the orienting response or what is it reflex is elicited by relatively novel and innocuous stimuli and is thought to aid in the processing of that stimulus (Pavlov, 1927). Startle responses are elicited by unexpected and more noxious stimuli; the function of these defensive reflexes is to aid in predator escape and avoidance of potential injury or harm (Eaton, 1984). With respect to the second question, several techniques may be used to index stimulus learning. The most common measure is to quantify a change in the response elicitation power of the stimulus. Alternatively, a change in the ability of the stimulus to function as an effective signal or outcome can be assessed. To date, only the first approach has been used to measure stimulus learning in larval zebrafish.

In the t1-t2 studies described in this paper, three different responses were measured in larval zebrafish. Glanzman and his colleagues (Roberts et al., 2011, 2016) recorded the classic C-start reflex, a characteristic escape response of teleost fish. This response depends on stimulation of the Mauthner neuron and is triggered within 12 msec of the application of an auditory/vibrational stimulus. The O-bend response measured by Wolman et al. (2011) is a unique response to a decrease in illumination and is thought to enable the larva to maintain orientation towards light which is related to food availability (Burgess and Granato, 2007). The precise temporal coupling between these two responses and the delivery of their respective stimulus events makes them highly amenable to analysis of the cellular and molecular mechanisms that support alterations in their elicitability as a result of stimulus exposure and learning.

In a previous study, we examined the swimming patterns of 7 dpf larvae exposed to a red bouncing ball (Pelkowski et al., 2011). During a 30 s period analyzed at 5 frames per second, we observed larvae turn rapidly or slowly away from the bouncing ball, swim erratically until reaching the edge of the well, or lie still in the upper or lower halves of the well. In our studies of stimulus learning, which use a 6-s time point sampling procedure, percent observations lying still has provided a robust index of stimulus learning across development (5 dpf to 7 dpf) and various acquisition parameters in our studies (O'Neale et al., 2014). Given the likelihood that this response measure reflects freezing, an adaptive behavioral state to avoid detection by a potential threat, it is of some interest to examine the relation of this behavior to thigmotaxis or edge hugging which is also considered to reflect a state of anxiety or fear (Champagne et al., 2010; Maximino et al., 2010; Miller et al., 2010; Pellow et al., 1985; Richendrfer et al., 2012). We have obtained mixed results using thigmotaxis to measure stimulus learning. O'Neale et al. (2014) reported that thigmotaxis scores did not reveal evidence of stimulus learning using a gray disk and a wide range of parameters. Larvae were just as likely to be observed on the edge of the well during testing regardless of their exposure conditions (moving or stationary disk). However, in the two new experiments reported here, we did find evidence of stimulus learning using thigmotaxis scores (see Supplementary Materials for details). The source of this variability in the sensitivity of thigmotactic behavior for detecting learning effects remains to be determined.

5. Conclusion

In this paper, I have laid out an argument for adoption of a t1-t2 framework in studies of learning about noncontingent stimulus presentations. I described several protocols using this framework to study stimulus learning in the larval zebrafish and reviewed what we know and do not yet know about the conditions, content and expression of that learning. Profiling the behavioral characteristics of this learning process is an important component of leveraging other attributes of the zebrafish for dissecting the genetic and cellular mechanisms of information acquisition, retention and use. To this end, it will also be important to evaluate the accumulated behavioral data within a theoretical context. Although there are many models of stimulus learning (see Peeke and Herz, 1973a, 1973b; Rescorla and Holland, 1976; Tighe and Leaton, 1976), the dual process model (Groves and Thompson, 1970) and the dual memory model (Wagner, 1976, 1981) have tended to dominate accounts of changes in the response elicitation power of a noncontingent stimulus. It would be judicious not only to consider their relative merits in both behavioral and neurobiological analyses of stimulus learning in the developing zebrafish but to be mindful of other theoretical approaches and the substantial literature they have inspired.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.beproc.2019.04.005.

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