

Short report

Rapid, accurate time estimation in zebrafish (*Danio rerio*)D.T. Cerutti^a, J. Jozefowicz^{b,c,*}, J.E.R. Staddon^{d,e}^a California State University – East Bay, United States^b Université Lille Nord de France, France^c Universidade do Minho, Portugal^d Duke University, United States^e The University of York, United States

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ABSTRACT

Zebrafish were tested in an appetitive Pavlovian delayed conditioning task. After an intertrial interval of $k \cdot T$ s ($k = 11.25$; $T = 8, 16$ or 32 s), a small, translucent vertical pole was illuminated (CS) for T s. Food was presented at $T/2$ s. Pole-biting response latencies from CS onset were a linear function of the food delay $T/2$, with slope approximating unity (*proportional timing*), and standard deviation proportional to latency (*scalar timing*). Response latencies tracked changes in food delays even when they changed every other day. These findings are significant because the zebrafish genome has recently been sequenced, opening the door to studies in the genetics of interval timing.

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1. Introduction

The zebrafish, a small (3–5 cm) cyprinid, is becoming the primary model of vertebrate ontogeny. Its development is rapid, its larval structures are transparent to view and its genome has been sequenced. Many genes regulating organ development and cellular functions have been identified, including those involved in the expression of sensory-motor processes (Bang et al., 2002; Driever et al., 1994; Fadool et al., 1997; Granato et al., 1996; Guo, 2001; Linney et al., 1999; Mullins et al., 1994). Recently, attention has been directed to developing behavioral screens to study the genetic and developmental bases of learning and memory in these animals (i.e. Arthur and Levin, 2001; Bilotta et al., 2005; Eddins et al., 2009; Gómez-Laplaza and Gerlai, 2010; Sisson and Gerlai, 2010; Pather and Gerlai, 2009; Valente et al., 2012; Williams and Messer, 1998; Williams et al., 2002).

Interval timing, the ability to adjust behavior to temporal stimuli on the order of seconds to minutes, plays a key role in reinforcement learning. The ability is widespread among species including birds, rodents, and fish (Bateson, 2003; Drew et al., 2005; Lejeune and Wearden, 1991; Richelle and Lejeune, 1980). It is increasingly well understood in mammals and birds (Staddon and Cerutti, 2004). It can be studied in two ways, operant or classical (Pavlovian)

conditioning (Zielinski, 1966; Vogel et al., 2003; Staddon and Cerutti, 2004). Most work has been done using operant techniques. For example, an animal can be rewarded with food for the first response after T s, a fixed-interval (FI) T schedule. In this case, many mammal and bird species soon learn to wait before making the first response in each interval for a fixed fraction of the interval (*proportional timing*). Moreover, the standard deviation of waiting times is typically a constant fraction of their mean (*scalar timing*) (Gibbon, 1977; Schneider, 1969; Zeiler and Powell, 1994). Timing behavior is adaptive because it limits the target response to times when it is likely to be most effective.

Existing studies of fish suggest that their interval timing is not as good as in mammals and birds (Higa and Simm, 2004; Talton et al., 1999). But a recent study with goldfish (Drew et al., 2005), using classical rather than operant conditioning and an aversive stimulus (electric shock) rather than food, has shown that in this simpler situation, averaged data from groups of fish show adequate interval timing. Similar data for zebrafish have begun to emerge: Sumpre et al. (2008) have reported tail flips and neuronal activity in the tectum adjusted to the temporal interval between successive presentation of a light flash in larval zebrafish as young as 4 days old. Lee et al. (2010) observed that, in a Pavlovian conditioning task in which a 5-s visual stimulus was paired with a shock, activity peaked in the last second before shock delivery. No study has systematically investigated timing abilities in zebrafish nor do we know whether timing in zebrafish is scalar or not.

The present study looked at interval timing in individuals as well as small groups of zebrafish over a range of interval values in an

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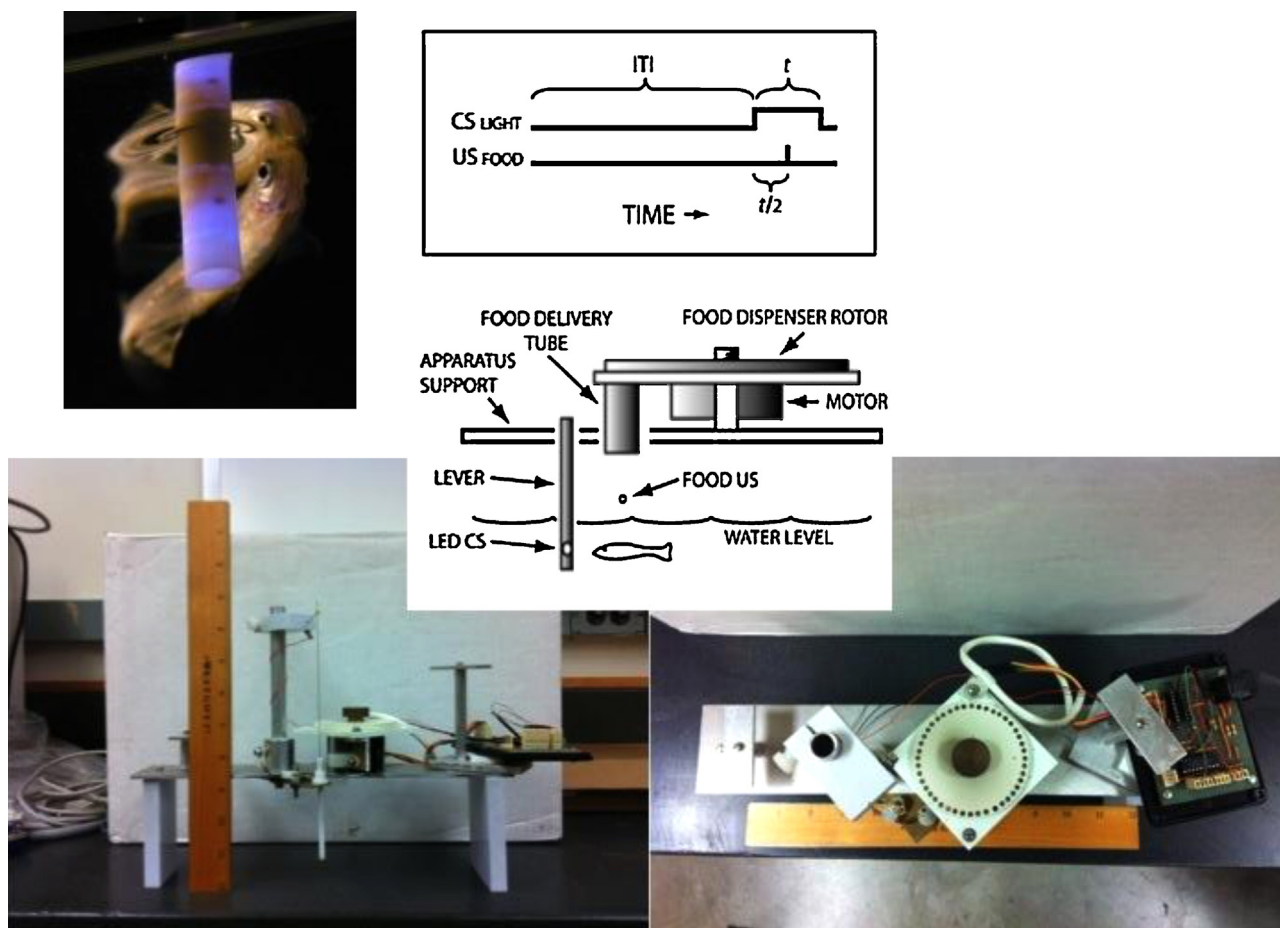


Fig. 1. Procedure and apparatus employed in the Pavlovian delay-conditioning task.

appetitive, classical conditioning situation. We arranged repeated pairings of a localized light (a *conditioned stimulus*: CS) with food (an *unconditioned stimulus*: US), in a paradigm known as delay conditioning or *autoshaping* (Brown and Jenkins, 1968). After a few CS–US pairings, pigeons, rats and many other organisms begin to direct behavior that normally occurs in the presence of the US (e.g., food) to the CS. In pigeons, for example, presenting a brief light followed by food produces classically conditioned pecking (*conditioned response*: CR) to light (Gamzu and Williams, 1973; Williams and Williams, 1969). Autoshaping is useful for studying classical conditioning in a small organism like a zebrafish because the CR is a large easily detected skeletal response toward the CS. In our study, we recorded bites that the fish directed to a subminiature lamp illuminated just before food delivery.

The primary focus of this study was the timing of CRs relative to CS onset. Our experiment studied delay intervals of 4, 8, and 16 s. The purpose of the experiment was to explore whether the zebrafish would show proportional and scalar timing under steady-state conditions (phase 1), to see how quickly they learned, and to examine their memory dynamics under rapidly changing conditions (phase 2).

2. Materials and method

2.1. Subjects

Subjects were 10 adult zebrafish (*Danio rerio*), Z1–Z10, purchased from a local tropical fish supplier. They ranged in size from 20 to 25 mm at the start of the study. Fish were kept individually

in glass 10-gallon fish tanks, in which they lived and were tested. They were fed freeze-dried bloodworms (insect larvae) in daily experimental sessions. An automatic lighting system provided illumination on a 14:10 h on-off cycle.

2.2. Apparatus

The general testing procedure and apparatus are shown in Fig. 1. Illumination of a submerged miniature blue light (3-mm bright blue LED) was used as the CS while presentation of a 1 mm section of a bloodworm (freeze-dried insect larva) was used as the US. The lamp was embedded at the tip of a white translucent pole submerged 10 mm below the surface of the water; food was dropped about 10 mm from the pole. A computer arranged for timing of stimulus events, operated the food dispenser, and recorded pole displacements. A response was recorded automatically by the computer whenever the pole was moved by a fish.

2.3. Procedure

2.3.1. Phase 1

Fish Z1 to Z8 served as subjects in Phase 1 of the study. Sessions consisted of CS–US cycles as shown at the top of Fig. 1. There were three conditions corresponding to a CS–US delay between CS onset and the delivery of the US of 4, 8 or 16 s. The CS duration was always twice the CS–US delay (8 s in the 4-s CS–US delay condition, 16-s in the 8-s CS–US delay condition, and 32-s in the 16-s CS–US delay condition). The ratio of the inter-trial interval (ITI) duration to the CS duration was held at 11.25 for all conditions, so as to

Table 1
Order of the conditions for the various fish in Phase 1.

Fish	First condition	Second condition
Z1–Z2	8-s CS–US delay	16-s CS–US delay
Z2–Z4	8-s CS–US delay	4-s CS–US delay
Z5–Z6	16-s CS–US delay	8-s CS–US delay
Z7–Z8	4-s CS–US delay	8-s CS–US delay

avoid changes in the strength of conditioning that occur when this ratio varies (Gibbon and Balsam, 1981). This phenomenon has been observed in all the species generally studied in the learning laboratory, that is to say rats, pigeons and humans. It is usually regarded as a fundamental principle of associative learning and hence, we should expect it to apply to zebrafish too, even though this has never been tested. Hence, the ITI was 90 s in the 4-s CS–US delay condition, 180 s in the 8-s CS–US delay condition, and 360 s in the 16-s CS–US delay condition. In all conditions, test trials in which the US was omitted were presented randomly throughout, occurring once every six trials on average.

The experimental design included within- and between-subject features. Each fish was trained in two conditions. Table 1 shows the order of conditions for each fish. For the first condition, each fish was trained in daily sessions consisting of 20 CS–US cycles until the first CR was recorded (a pole displacement while the CS was presented), and trained another 30 20-trial sessions thereafter. They

were then moved to the second condition, in which they received 30 daily 20-trial sessions.

2.3.2. Phase 2

Fish Z6 and Z7 from Phase 1 and two naïve fish (Z9, Z10) served as subjects. Training for the naïve fish was arranged as in Phase 1 using the 8-s CS–US delay condition only (with an ITI of 180 s, a CS of 16 s, and a US delay of 8 s). They began experimental conditions five days after they showed the first CR (displacement of the pole) to the blue light. The experiment proper arranged two-day exposures to each CS–US delay conditions in the order: 8 s, 16 s, 8 s, and 4 s; twice repeated (a total of 16 days of testing). ITI and CS durations for each US delay changed proportionately as in Phase 1. Each session was composed of 20 CS–US trials.

3. Results and discussion

3.1. Phase 1

All fish rapidly acquired the conditioned pole-biting response to the blue light, irrespective of the initial training condition. The development of the light CR followed the pattern described by Pavlov, with short-latency CRs early in training, followed by the lengthening of CR latency. Stable test-trial performances were characterized by one or a few pole responses at the time that food was scheduled for delivery. However, visual observation of the fish

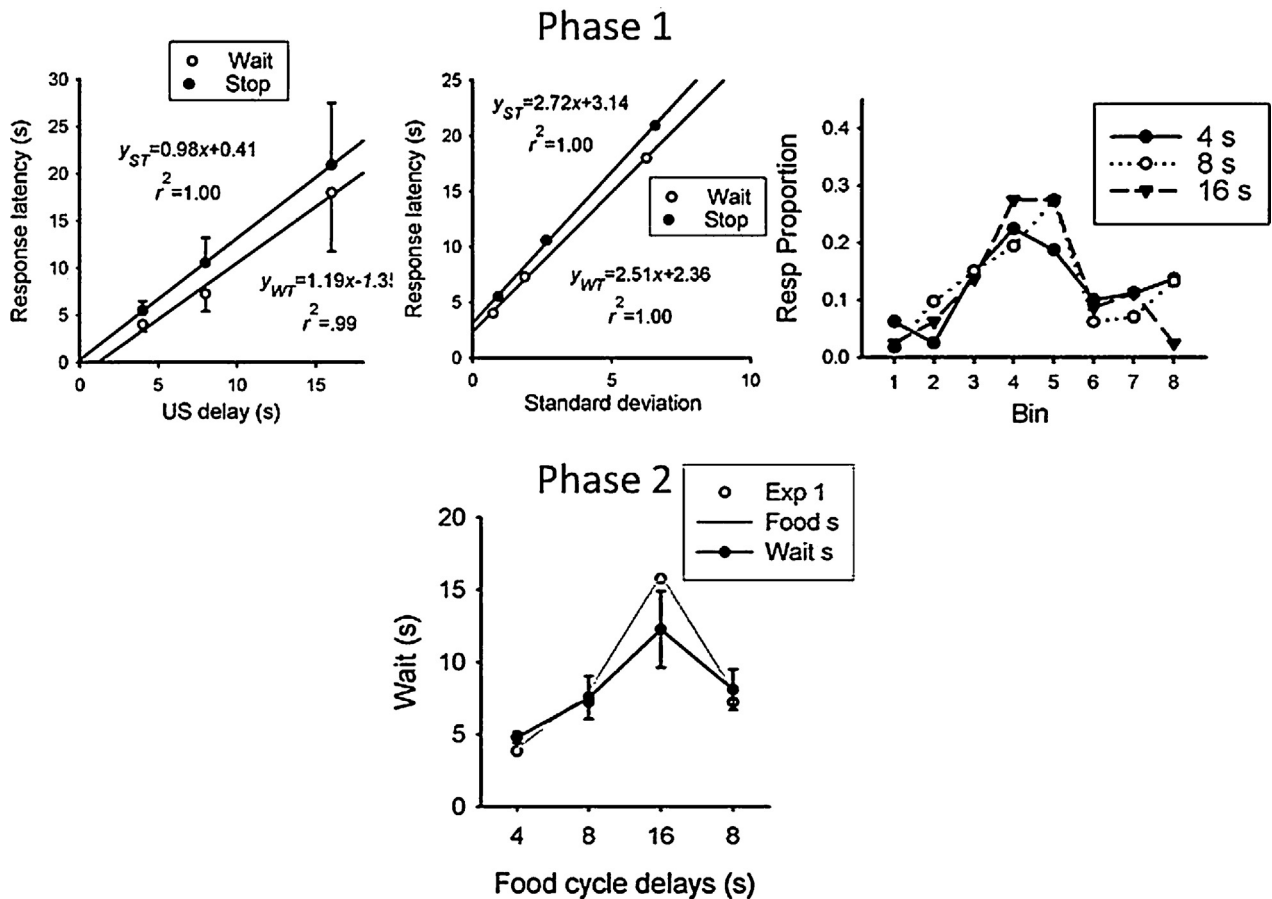


Fig. 2. Top panels: averaged results of steady-state procedures in Phase 1. The Rightmost plot shows mean wait (WT—unfilled points) and stop (ST—filled points) plotted against US delay. Error bars are standard deviations. The center plot shows wait times plotted against the standard deviation of wait. Lines, equations, and r^2 values in upper and middle plots are for best fitting linear functions. The leftmost plot shows normalized proportions of total responses in the three delay conditions (bins are 1/8 of CS duration). For all plots, data are from test trials only; each point is the average of the performance of each fish over the last ten sessions in a condition. Lower panel: averaged results of dynamic manipulations in Phase 2. Unfilled data points show average wait data from Phase 1; filled data points show average wait times for each two-day exposure to a US delay in Phase 2. The gray line plots the actually scheduled US delay. Phase 2 data are from test trials only, averaged over the two-session exposure to the condition; error bars are standard deviations.

indicated that CS illumination was always accompanied by rapid pole approach and vigorous swimming close to the pole.

The zebrafish CRs to the illuminated pole showed *proportional timing* of both start (first response) and stop (last response) times. These data are shown in the upper left panel of Fig. 2. Data are from test trials only. Each point is the average of the performance of each fish over the last ten sessions in a condition. Best-fitting linear functions show slopes close to unity and near zero intercepts. The start function is displaced only slightly below the stop function, reflecting low response rates and a “bout-like” pattern of responding. Hysteresis effects were negligible: performance at a given delay value was more or less independent of the preceding delay value, whether smaller or larger. There were negligible between-subject differences: all the fish showed behavior consistent with the group averages in Fig. 2.

The *Weber-law* (scalar) property of timing, standard deviation in wait and stop measures proportional to to-be-timed interval, is shown in the upper middle panel of Fig. 2. The functions relating wait and standard deviation are linear, demonstrating scalar timing. Indeed, all properties of the wait-time distributions scaled with the to-be-timed interval (time-scale invariance), as shown by the superimposition of the three distributions in the upper right panel of Fig. 2. Taken together, these findings show exceedingly accurate interval timing in zebrafish, not inferior in its precision to data from rats, pigeons, and humans.

3.2. Phase 2

Wait data from the two-day US-delay manipulation are presented in the lower panel of Fig. 2 as filled data points (average wait data from Phase 1 are shown for comparison as unfilled data points). In the 4-s and 8-s CS-US delay conditions, the wait time after only two days of exposure to the temporal contingency was identical to the one observed in Phase 1, where the animals had been trained to asymptote. In the 16-s CS-US delay condition, wait time undershoots both the programmed delay (gray line) and the steady-state data from Phase 1 (unfilled point). Hence, even though the animals adapted very quickly to the new temporal contingency, the adaptation was not yet at the asymptotic level, contrary to what was observed in the 4-s and 8-s CS-US delay conditions. This indicates that the zebrafish were not merely responding to the last CS-US delay but were integrating information over several trials. As in Phase 1, scalar timing is seen in proportionally larger standard deviation with larger US delays. The rapid adjustments in timing seen in this experiment are comparable to findings with goldfish employing a shock US (Drew et al., 2005), and consistent with other species responding for food reinforcement (Ludvig and Staddon, 2004; Staddon and Higa, 1999; Staddon et al., 2002; Wynne and Staddon, 1988, 1992).

4. General discussion

These experiments show precise proportional and scalar timing in the zebrafish (Phase 1) and an integrative memory process for temporal information very similar to that widely demonstrated for mammals and birds (Phase 2). The fact that this tiny fish shows behavior often thought to be unique to “higher” animals is truly remarkable. The utility of the zebrafish as a general genomic model for vertebrate development should make this newfound ability of great interest to students of brain function in vertebrates.

Acknowledgements

Dan Cerutti conducted this work. He designed all the apparatus used to test the zebrafish in this study. It bears witness to his

ingenuity as an experimenter and genius tinkerer. Dan died unexpectedly of a heart attack in January 2011. This paper is dedicated to his memory. Thanks to Marvin Lamb for sending us the pictures of the testing apparatus.

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