

Effects of Punishing Target Response During Extinction on  
Resurgence and Renewal in Zebrafish (*Danio rerio*)

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**Highlights**

- Relapse was assessed following punishment versus extinction using zebrafish
- Extinction plus shock punishment decreased responding faster than extinction alone
- Punishment decreased resurgence relative to resurgence in the absence of punishment
- Punishment increased renewal but response rates were not controlled between assessments

**Abstract**

We examined the effects of the presence and absence of punishment on the resurgence and renewal of extinguished operant behavior with zebrafish. With resurgence, food deliveries reinforced target responding in Phase 1 was exposed to shock punishment plus extinction (PUN+EXT) versus extinction alone (EXT) while introducing alternative reinforcement in Phase 2. All contingencies were discontinued in Phase 3. With renewal, target reinforcement during Phase 1 occurred in Context A and then during Phase 2 was exposed to either PUN+EXT or EXT in Context B. All contingencies were discontinued in Context A during Phase 3. During Phase 2 for both resurgence and renewal, decreases in target responding were more rapid with PUN+EXT than EXT. During testing in Phase 3, resurgence was less following PUN+EXT than EXT. In contrast, renewal was greater following PUN+EXT than EXT but differences in response rates at the end of Phase 2 complicated the interpretation. We discuss these differences between resurgence and renewal as due to differences in learning about alternative reinforcement (resurgence) versus more general contextual changes (renewal).

*Keywords:* relapse, resurgence, renewal, punishment, zebrafish

## 1. Introduction

Zebrafish (*Danio rerio*) are an animal model widely used in biomedical research in genetics, neuroscience, pharmacology, and toxicology (Bilotta et al., 2004; Carvan et al., 2004; Eddins et al., 2009; Levin et al., 2003; Reimers et al., 2004; Stewart et al., 2014). In genetics, their features include a fully sequenced genome, high fecundity, transparent embryo/larva, rapid development, and low maintenance cost. As such, zebrafish are suitable for identifying genotype-phenotype relations and examining genetic manipulations in biological processes (e.g., Darland & Dowling, 2001; Kalueff, & Cachat, 2011; Muto et al., 2005). There is, for example, a mutant type of zebrafish whose body remains transparent throughout the life (Antinucci & Hindges, 2016). Zebrafish are a vertebrate animal so that their nervous system is more structurally similar to humans than those of invertebrates traditionally used in genetics (e.g., fruit flies, *Drosophila*; cf. Brembs, 2011; Brembs & Plendl, 2008).

Zebrafish have also been established as an animal model in behavioral science. Zebrafish learn during both preparations of classical conditioning (Blank et al., 2009; Darland & Dowling, 2001; Braubach et al., 2009; de Castro, 2009; Hall & Suboski, 1995a, 1995b; Jesusathan, 2012; Morin et al., 2013; Okamoto et al., 2012; Pradel et al., 2000; Suboski et al., 1990; Xu et al., 2007) and operant conditioning. In the latter, zebrafish show an increase in responding with positive (Manabe et al., 2013a, 2013b) and negative reinforcement (Li, 2013), a decrease in responding with extinction (Kuroda & Mizutani, 2018) and punishment (Kuroda et al., 2019), and discrimination of spatial locations, colors, and shapes (Arthur & Levin, 2001; Colwill et al., 2005; Eddins et al., 2009; Risner et al., 2006).

Understanding relapse of operant behavior could benefit from using zebrafish as an animal model due to the relevance of the species to examining both behavioral and biological factors. Relapse has been modeled under laboratory conditions as the recurrence of a previously reinforced and then extinguished response following some change in the environment. There are several different models of relapse depending on how the environmental change is arranged (Wathen & Podlesnik, 2018).

Resurgence and renewal have been of particular interest clinically due to relevance to understanding relapse of drug use (Bouton, 2019; Cook et al., 2020) and severe problem behavior in individuals diagnosed with developmental disabilities (Briggs et al., 2018; Muething et al., in press).

With resurgence, a target response is reinforced in Phase 1, followed by extinction while introducing a reinforcement contingency for an alternative response in Phase 2. In Phase 3, target responding recurs despite the ongoing extinction contingency upon worsening the consequence of the alternative response. Worsening of the alternative can occur by introducing extinction (e.g., Epstein, 1983), punishing alternative responses (Fontes et al., 2018), or reducing the rate (e.g., Sweeney & Shahan, 2013; Schepers & Bouton, 2015) or magnitude (e.g., Craig et al., 2017; Oliver et al., 2018) of alternative reinforcement.

With renewal, a target response is reinforced under Context A in Phase 1, followed by extinction under Context B in Phase 2. In Phase 3, a return to Context A (ABA renewal) or transitioning to a novel Context C (ABC renewal) results in a recurrence of the target response despite extinction remaining in effect (e.g., Bouton et al., 2011). In general, relapse is a common behavioral phenomenon observed across a wide variety of species including rats (e.g., Podlesnik et al., 2006), pigeons (e.g., Epstein, 1983), monkeys (Mulick et al., 1976), and humans (Kuroda et al., 2016). It also occurs in zebrafish as well (Kuroda et al., 2017a, 2017b).

Reducing the likelihood and severity of relapse is a focus of much preclinical research examining the models of relapse (e.g., Nieto et al., 2017; Shvarts et al., 2020; Wathen & Podlesnik, 2018). Following reinforcement of a target response in Phase 1, punishment of target responding in Phase 2 has received attention as a potential approach for reducing relapse. With resurgence, Nall and Shahan (in press) and Nall et al. (2019) arranged delivery of shock punishment while continuing to reinforce a target response in Phase 2, instead of arranging extinction of target responding. These authors arranged shock delivery as an analog of aversive experiences among those with chronic

substance-use disorders. An alternative response was simultaneously reinforced in that phase. Resurgence still occurred when all the three contingencies were removed in Phase 3. A different approach was examined in Phase 2 by Kestner et al. (2015), who arranged either punishment plus extinction or extinction alone for the target response, in addition to alternative reinforcement. The target response decreased more rapidly with punishment than extinction alone and resurgence was reduced upon removing all contingencies in Phase 3.

In contrast to resurgence, punishment of target responding in Context B during Phase 2 does not appear to reduce renewal following a context change. Similar to the resurgence procedures in Nall and Shahan (in press) and Nall et al. (2019), Bouton and Schepers (2015) arranged punishment for a target response while maintaining food reinforcement in Context B. Both ABA and ABC renewal occurred following Context B with punishment. ABA renewal following punishment has been replicated with drug reinforcers, including alcohol (Marchant & Kaganovsky, 2015; Marchant et al., 2016) and cocaine (Pelloux et al., 2018). It remains unclear, however, how the magnitude of renewal effects differs between punishment plus extinction and extinction alone in Context B.

All of the previous studies examining effects of punishment on relapse used rats as experimental subjects, thereby limiting the generality of the experimental findings. Zebrafish have high potential for integrative research between biomedical and behavioral studies. Thus, the goal of the present study was to examine resurgence and renewal depending on whether punishment plus extinction or extinction alone reduces responding in zebrafish. Wild-type zebrafish served as experimental subjects because we demonstrated their operant behavior is susceptible to relapse (Kuroda et al., 2017a, 2017b) and to decreases in responding with punishment (Kuroda et al., 2019). With resurgence, we systematically replicated Kestner et al.'s (2015) methods with zebrafish and extended these procedures to examine ABA renewal.

## **2. General Method**

## 2.1. Subject

A total of 20, 8-9 month old, experimentally naïve wild-type zebrafish (National Bioresource Project of Japan, Riken Center for Brain Science) served as subjects. Each fish was housed individually in an aquarium (transparent plastic container; 13.0-cm wide by 16.8-cm long by 11.3-cm high) connected to a pump and tank where water was filtered, aerated, and maintained at 28 degrees Celsius with a heater. Water was kept at a pH level of 7.5 and half of the water in the tank was replaced weekly. The home aquariums were set next to each other so that the fish were visible to each other except during sessions, as described below. When a fish produced fewer than 30 reinforcers (decapsulated brine shrimp eggs) in a session, postsession feedings of supplemental food (Kyorin, Hikari Labo 270) occurred 30 min after sessions. The aquarium room maintained a 14hr:10hr light-dark cycle (lights on at 6:00 a.m.).

## 2.2. Apparatus

A fully automated device described elsewhere (Manabe et al., 2013a) was used in each experiment for measuring zebrafish behavior and for delivering reinforcers. The device, which consisted of sensors and a feeder fixed on a Plexiglas plate, was placed in the home aquarium of each fish during sessions. Each sensor consisted of a 2-mm diameter polymeroptical fiber (Mitsubishi Rayon Co., LTD, Esca CK80), a fiber sensor (Keyence FU-23X), an amplifier (Keyence, FS-V21X), and a tri-color LED (Oput-Supply OSTA5131A-C). The tip of the sensor was set 5 mm below the water surface and 5 mm away from the front wall. The tip could be illuminated red, green, or blue. A response was registered when the fish approached within 5 mm below the sensor tip. The next response was not registered until the fish left the 5-mm area in order to prevent repeatedly recording responses while remaining in the area. The number of sensors varied across experiments.

The feeder consisted of a servo motor (Futaba S3005), a brass tube with the inner diameter of 600  $\mu\text{m}$ , a 400- $\mu\text{m}$ -diameter piano wire inserted in the brass tube, a vibrator, and a white LED (i.e., the

feeder light). During food presentations, the feeder light turned on immediately upon a response, the servo motor pulled the piano wire, the vibrator operated, decapsulated brine shrimp eggs were loaded into the brass tube, and the servo motor pushed the eggs out of the tube and into the water – this series of events occurred in approximately 0.5 s. The feeder light then flashed 5 times at a 0.2-s interval. Thereafter, all the devices remained inoperative until 5 s had elapsed since the onset of reinforcement; thus, food-presentation time was 5 s. The location of the feeder on the Plexiglas plate varied across experiments.

A shock-delivery system described elsewhere (Kuroda et al, 2019) consisted of an alternating-current (AC) transformer, an RC switch with a MOSFET (Pololu), a solid-state relay (Panasonic AQZ 202), a potentiometer (i.e., adjustable resistor; 5-kiloohm max), a fuse, and two 8 cm by 5 cm aluminum panels. The transformer reduced the voltage from 100 to 6.3 V AC (60 Hz), which served as the base voltage in a circuit for the shocker throughout the experiment. The amperage was set at 2.7 mA throughout the experiment, which was slightly above the threshold (2.6 mA) for zebrafish as assessed in a pilot study and below those *not* producing decreases in unconditioned or conditioned activity (i.e., 5 mA; Kenney et al., 2017). During shock presentations, the RC switch was turned on, which in turn activated the solid-state relay. The activated relay completed the circuit, generating shock under water through the two aluminum-panel electrodes set on the side walls of the aquarium, right next to the sensors described above. The relay was deactivated 1 s later. The activation/deactivation of the solid-state relay was silent, thereby producing no other exteroceptive stimuli. Thereafter, all the devices remained inoperative for 1 s; thus, shock-presentation time was 2 s.

A partition covering each side of the aquarium minimized visual distraction during sessions. The color of the partition varied across experiments. A Lenovo® laptop computer with Visual Basic™ 2015 controlled the experiment and recorded data through an I/O board (Pololu, Mini Maestro servo controller).



### 2.3. Procedure

Sessions were conducted during the light period of the room's light-dark cycle, around the same time of the day, seven days a week. Each session lasted for 20 min except during magazine and response training. Session time excluded food- and shock-presentation time. All variable-interval (VI) and variable-time (VT) schedules used in the present experiment consisted of ten intervals derived from Fleshler and Hoffman's (1962) distribution, randomly selected without replacement. Each experiment started with magazine training in which reinforcers (decapsulated brine shrimp eggs) were delivered independently of responding according to a VT 20-s schedule (i.e., the mean of intervals between successive food deliveries was 20 s). Magazine-training sessions ended after 40 food deliveries. The fish completed magazine training when they consumed the reinforcer within 4 s after the food delivery for more than 20 times in a session. Sensors and the shocker were not placed under water during magazine training. Response training followed in which a target response (approach to a sensor) was reinforced with the food delivery, as described below.

### 2.4. Data Analysis

A two-way repeated-measures ANOVA was conducted in each experiment with consecutive sessions ("Session") and the set of conditions with punishment plus extinction versus extinction alone in Phase 2 ("Set") as within-subject independent variables and target responding as a dependent variable. When comparing target and control responding, the Set was replaced by another within-subject independent variable "Target vs. Control." The analysis included data from the last five training (Phase 1) sessions, the first five extinction (Phase 2) sessions, and the five relapse-test (Phase 3) sessions in each experiment. The sphericity assumption was met by setting compound symmetry structure for the covariance matrix when the ANOVA was conducted using the PROC MIXED function on SAS®. The PROC MIXED did not generate effect sizes ( $\eta_p^2$ ) so that their approximated estimates were computed using a method described by Tippey and Longnecker (2016).

In Phase 1, response rate (i.e., responses per min) was the dependent variable included in the ANOVA. In Phase 2, we calculated for each subject the proportion of target response rate in each of the first five sessions relative to the mean of the last five session of Phase 1 and used the proportion as the dependent variable. In Phase 3, we calculated the difference in response rate in each session relative to the last session of Phase 2 separately for target and control responding. We determined the presence of relapse if target responding increased relative to the last session of Phase 2 *and* if the magnitude of increase was greater than that for control responding. Then we examined how relapse changed following Phase 2 with punishment plus extinction or extinction alone, using the difference in target response rate relative to the last session of Phase 2 in the ANOVA.

### **3. Experiment 1**

Experiment 1 was a systematic replication of Kestner et al.'s (2015) study using zebrafish as experimental subjects. We examined whether resurgence would be reduced during testing in Phase 3 following punishment plus extinction for target responding, compared to extinction alone, in Phase 2.

#### 3.1. Method

##### *3.1.1. Subject and apparatus*

Ten zebrafish (four males and six females) served as subjects. The apparatus consisted of three sensors, two feeders, and a shocker. The middle sensor was placed on the midline of the front wall of the aquarium whereas left and right sensors were placed at the left and right corners of the front wall of the aquarium, with each being 5.4 cm away from the midline of the wall. The left and right sensors served as the target and alternative sensors, respectively, for a half of the fish; counterbalanced for the other half. The tips of the target and alternative sensors were illuminated red and green, respectively, for a half of the fish; counterbalanced for the other half. The middle sensor served as the control sensor for all of the fish and was not illuminated throughout the experiment. The feeders were located 2.7 cm to the left and right of the middle sensor. In a test with 100 operations, the left and right feeders

delivered a mean of 19.7 eggs ( $SD = 6.97$ ) and a mean of 22.4 eggs ( $SD = 5.40$ ) per operation, respectively.

### *3.1.2. Procedure*

*3.1.2.1. Training.* Following magazine training, each approach to the target sensor was reinforced with food on a fixed-ratio (FR) 1 schedule. Response-training sessions ended after 40 reinforcer deliveries. When subjects finished a response-training session within 20 min, the reinforcement schedule was switched from an FR 1 to a VI 1 s to a VI 10 s, with the latter being in effect for three 20-min sessions. In VI schedules, responding during intervals had no programmed consequence and the first response after the intervals resulted in food deliveries.

*3.1.2.2. Experimental conditions.* Following the training, a half of the fish was exposed to Phase 1, Phase 2 with extinction alone (EXT), and Phase 3. Subsequently, the fish returned to the training and then proceeded to Phase 1, Phase 2 with punishment plus extinction (PUN+EXT), and Phase 3. The order of experimental conditions was counterbalanced for the other half of the fish.

*3.1.2.2.1. Phase 1.* The target response was maintained in a VI 20-s schedule of reinforcement. Alternative and control responses were recorded but had no programmed consequence. Phase 1 lasted for 10 sessions.

*3.1.2.2.2. Phase 2 with EXT.* The target response was extinguished while the alternative response was reinforced according to a VI 10-s schedule. The control response had no programmed consequence. This phase was in effect for eight sessions.

*3.1.2.2.3. Phase 2 with PUN+EXT.* This phase was similar to Phase 2 with EXT except that a VI 60-s schedule of punishment with electric shock also was in effect for target responding. This phase lasted for eight sessions.

*3.1.2.2.4. Phase 3.* Extinction was in effect for target, alternative, and control responding. This phase lasted for five sessions.

### 3.2. Results and Discussion

Fig. 1 shows responses per min for target, alternative, and control responses each across sessions, separately for the set of Phases 1-3 with PUN+EXT in Phase 2 and the set with EXT. The former and the latter sets will hereafter be referred to as PUN+EXT set and EXT set, respectively. In Phase 1, target response rates were considerably higher than alternative and control response rates in both the PUN+EXT and EXT sets, indicating reinforcement effects on the target response. Control response rates were slightly but consistently higher than alternative response rates in that phase, which likely resulted from the physical proximity of the control (middle) sensor to the target sensor compared with the alternative sensor. Comparing the PUN+EXT and EXT sets, Phase 1 started with similar rates of target responding but the rates differentiated by the end of that phase. Using data from the last five sessions of Phase 1, a two-way repeated-measures ANOVA indicated the effect of Set,  $F(1, 9)=21.75$ ,  $p=.001$ ,  $\eta_p^2=.18$ , but there was no effect of Session,  $F(4, 36)=1.09$ ,  $p=.37$ ,  $\eta_p^2=.04$ , and no interaction effect,  $F(4, 36)=0.66$ ,  $p=.63$ ,  $\eta_p^2=.03$ . Thus, the baseline target response rate was significantly higher in the PUN+EXT set than in the EXT set but was nevertheless greater in only four out of ten fish. Mean obtained reinforcers per min from the last five sessions of Phase 1 were 1.94 ( $SD = 0.39$ ) and 1.81 ( $SD = 0.43$ ) in the PUN+EXT and EXT sets, respectively.

In Phase 2, target response rates decreased, alternative response rates increased, and control response rates remained near-zero in both PUN+EXT and EXT sets (see Fig. 1). To assess whether PUN+EXT decreased target responding more effectively than EXT, Fig. 2 shows proportions of target response rate in each Phase-2 session relative to the mean of the last five sessions of Phase 1. Using data from the first five sessions in Phase 2, the ANOVA indicated that the effects of Set,  $F(1, 9)=8.69$ ,  $p=.016$ ,  $\eta_p^2=.08$ , and Session,  $F(4, 36)=16.48$ ,  $p<.0001$ ,  $\eta_p^2=.41$ , but no interaction effect,  $F(4, 36)=0.60$ ,  $p=.66$ ,  $\eta_p^2=.02$ . Thus, extinction led to decreases in target responding across sessions in Phase 2 and punishment further decreased the responding. Mean obtained alternative reinforcers per

min averaged over all Phase-2 sessions were 1.56 ( $SD = 1.26$ ) and 1.78 ( $SD = 0.96$ ) in the PUN+EXT and EXT sets, respectively. In the PUN+EXT set, obtained punishers per min averaged over all Phase-2 sessions was 0.12 ( $SD = 0.17$ ).

Fig. 3 shows the difference in response rate in Phase 3 relative to the last session of Phase 2 separately for target and control responding. In both PUN+EXT and EXT sets, target responding in Phase-3 sessions increased relative to the last Phase-2 session and the magnitude of the increase was greater than that of control responding, suggesting resurgence. An ANOVA for the PUN+EXT set indicated the effect of Target vs. Control,  $F(1, 9)=13.02$ ,  $p=.006$ ,  $\eta_p^2=.12$ , but no effect of Session,  $F(4, 36)=0.95$ ,  $p=.444$ ,  $\eta_p^2=.04$ , and no interaction effect,  $F(4, 36)=0.25$ ,  $p=.909$ ,  $\eta_p^2=.01$ . Likewise, an ANOVA for the EXT set indicated the effect of Target vs. Control,  $F(1, 9)=17.08$ ,  $p=.003$ ,  $\eta_p^2=.15$ , but no effect of Session,  $F(4, 36)=0.89$ ,  $p=.479$ ,  $\eta_p^2=.04$ , and no interaction effect,  $F(4, 36)=0.68$ ,  $p=.611$ ,  $\eta_p^2=.03$ .

When comparing target responding between the PUN+EXT and EXT sets, an ANOVA indicated the effect of Set,  $F(1, 9)=5.53$ ,  $p=.043$ ,  $\eta_p^2=.05$ , but no effect of Session,  $F(4, 36)=1.42$ ,  $p=.25$ ,  $\eta_p^2=.06$ , and no interaction effect,  $F(4, 36)=0.26$ ,  $p=.90$ ,  $\eta_p^2=.01$ . Thus, punishment of target responding in Phase 2 decreased the magnitude of resurgence. No correlation was found between mean target response rates averaged over the last five sessions of Phase 1 (calculated for each fish in each set of conditions) and those rates averaged over the five Phase-3 sessions,  $r(18)=0.06$ ,  $p=.80$  (Pearson's correlation).

Target responding more rapidly decreased with PUN+EXT than EXT in Phase 2. Subsequently, resurgence was reduced following punishment in Phase 2. Therefore, the present experiment replicated Kestner et al.'s (2015) findings using zebrafish as experimental subjects instead of rats. Other than the difference in species, the present experiment differed from Kestner et al. by using a within-subjects design while they used a between-subjects design. Despite different experimental designs and species,

the same experimental findings strongly support the generality of these findings. It also adds to the validity of using zebrafish as an animal model for studying resurgence in general (Kuroda et al., 2017a, 2017b) including examinations of biological factors (e.g., genes).

Instead of punishment, zebrafish associating shocks with contextual features (e.g., Kenney et al., 2017) might account for the rapid decrease in target responding in Phase 2 and reduced resurgence in Phase 3 through conditioned suppression. Merely presenting shock could decrease operant responding independently from the operant contingency (e.g., Hake & Azrin, 1965). These effects unlikely are due to conditioned suppression because we previously showed with zebrafish that electric shocks identically presented were specific to responses producing shock and did not affect concurrently available but unpunished responses (Kuroda et al., 2019). Thus, decreased target responding in Phases 2 and 3 likely reflect effects of the punishment contingency rather than conditioned suppression.

One of the limitations of the present experiment was that target response rates in Phase 1 differed between the PUN+EXT and EXT sets. Although this was something difficult to control *a priori*, it may have influenced the results (see General Discussion). Another limitation was that our procedure did not eliminate a potential effect of order of exposure by the PUN+EXT and EXT sets despite counterbalancing. Such an order effect could be minimized, for example, by using a multiple schedule having components with and without a punishment contingency in Phase 2. We did not use multiple schedule because an unpublished study indicated that it takes a fairly large number of sessions (i.e., more than 30 sessions) to establish stimulus control in zebrafish under similar conditions. Thus, the development of procedures for establishing stimulus control quickly and reliably in this species could offer a superior approach to examine the influence of punishment on relapse.

#### **4. Experiment 2**

Experiment 2 was similar to Experiment 1 except that we assessed the effects of punishing target responding on ABA renewal, instead of resurgence.

## 4.1. Method

### 4.1.1. *Subject and apparatus*

Ten zebrafish (four males and six females), different from those used in Experiment 1, served as subjects. The apparatus consisted of two sensors, a single feeder, and a shocker. The sensors were placed at the left and right corners of the front wall of the aquarium, with each being 5.4 cm away from the midline of the wall. The left and right sensors served as the target and control sensors, respectively, for a half of the fish; counterbalanced for the other half. The tips of the target and control sensors were illuminated red and green, respectively, for a half of the fish; counterbalanced for the other half. The feeders were located on the midline of the front wall. In a test with 100 operations, the feeder delivered a mean of 21.91 eggs ( $SD = 5.39$ ) per operation.

### 4.1.2. *Procedure*

Following the trainings described in Experiment 1, a half of the fish were exposed to Phase 1, Phase 2 with extinction alone (EXT), and Phase 3. Then the fish returned to the training and then proceeded to Phase 1, Phase 2 with punishment plus extinction (PUN+EXT), and Phase 3. The order of the experimental conditions was counterbalanced for the other half of the fish. The control response was recorded but had no programmed consequence throughout the experiment.

*4.1.2.1. Phase 1.* The target response was maintained in a VI 20-s schedule of reinforcement. The color of partition covering each side of the aquarium wall was white for all the fish, serving as Context A. This phase lasted for 10 sessions.

*4.1.2.2. Phase 2 with EXT.* Extinction was in effect for target responding. The partition color was black and, in addition, a green silicon sheet was placed underneath the aquarium for all the fish, constituting Context B. This phase was in effect for five sessions.

4.1.2.3. *Phase 2 with PUN+EXT.* This phase also occurred in Context B and was similar to Phase 2 with EXT. The only difference was that a VI 60-s schedule of punishment with electric shock also was in effect for target responding. This phase lasted for five sessions.

4.1.2.4. *Phase 3.* Extinction was in effect for target responding in Context A. This phase lasted for five sessions.

Note that we did not counterbalance the stimuli constituting Contexts A and B across subjects. We used a white partition covering each side of the aquarium as Context A and a black partition with a green silicon sheet underneath the aquarium as Context B for all the fish in the present experiment. This was because, in our previous study of renewal with zebrafish (Kuroda et al., 2017b), that configuration led to greater renewal effects than the reversed configuration in our previous study of renewal with zebrafish. It was preferred to maximize the magnitude of renewal when the direction of change in the magnitude as a result of punishment was unknown prior to the experiment.

## 4.2. Results and Discussion

Fig. 4 shows responses per min for target and control responding across sessions, separately for the PUN+EXT and EXT sets. In Phase 1, target response rates were considerably higher than control response rates, indicating reinforcement effects on the target response. An ANOVA using data from the last five sessions of Phase 1 indicated the effect of Set,  $F(1, 9)=13.67$ ,  $p=.005$ ,  $\eta_p^2=.12$ , but no effect of Session,  $F(4, 36)=0.67$ ,  $p=.61$ ,  $\eta_p^2=.03$ , and no interaction effect,  $F(4, 36)=0.57$ ,  $p=.69$ ,  $\eta_p^2=.02$ . Thus, baseline target responding was higher in the PUN+EXT than EXT set but was nevertheless greater in only four out of ten fish. Mean obtained reinforcers per min from the last five sessions of Phase 1 were 1.86 ( $SD = 0.22$ ) and 1.74 ( $SD = 0.24$ ) in the PUN+EXT and EXT sets, respectively.

Fig. 5 shows proportions of target response rate in each Phase-2 session relative to the mean of the last five sessions of Phase 1. The proportions consistently were lower in the PUN+EXT than EXT set. An ANOVA indicated the effects of Set,  $F(1, 9)=55.77$ ,  $p<.0001$ ,  $\eta_p^2=.36$ , and Session,  $F(4,$



36)=5.21,  $p=.002$ ,  $\eta_p^2=.18$ , but no interaction effect,  $F(4, 36)=1.49$ ,  $p=.22$ ,  $\eta_p^2=.06$ . Thus, extinction decreased target responding across sessions in Phase 2 and punishment further decreased it.

Fig. 6 shows the difference in response rate in Phase 3 relative to the last session of Phase 2, separately for target and control responding. In both PUN+EXT and EXT sets, target responding increased relative to the last Phase-2 session and the magnitude of the increase generally was greater than that for control responding, suggesting ABA renewal. An ANOVA for the PUN+EXT set indicated the effect of Target vs. Control,  $F(1, 9)=28.23$ ,  $p=.001$ ,  $\eta_p^2=.22$ , but no effect of Session,  $F(4, 36)=2.08$ ,  $p=.103$ ,  $\eta_p^2=.08$ , and no interaction effect,  $F(4, 36)=1.08$ ,  $p=.379$ ,  $\eta_p^2=.04$ . An ANOVA for the EXT set indicated the effect of Target vs. Control,  $F(1, 9)=11.14$ ,  $p=.009$ ,  $\eta_p^2=.10$ , the effect of Session,  $F(4, 36)=3.27$ ,  $p=.022$ ,  $\eta_p^2=.12$ , and an interaction effect,  $F(4, 36)=3.57$ ,  $p=.015$ ,  $\eta_p^2=.13$ .

When comparing target responding between the PUN+EXT and EXT sets, an ANOVA indicated the effects of Set,  $F(1, 9)=7.69$ ,  $p=.022$ ,  $\eta_p^2=.07$ , and Session,  $F(4, 36)=5.52$ ,  $p=.001$ ,  $\eta_p^2=.19$ , but no interaction effect,  $F(4, 36)=1.10$ ,  $p=.37$ ,  $\eta_p^2=.04$ . Thus, renewal increased following PUN+EXT, relative to EXT, in Phase 2. Nonetheless, the results should be interpreted carefully given the difference in target response rate in the last session of Phase 2 between the PUN+PUN and EXT sets as discussed below (see Fig. 4). No correlation was found between mean target response rates in the last five sessions of Phase 1 (calculated for each fish in each set of conditions) and those rates in the five Phase-3 sessions,  $r(18)=-0.16$ ,  $p=.50$  (Pearson's correlation).

The present findings are the second example demonstrating operant renewal in zebrafish (see Kuroda et al., 2017b) and the first to demonstrate renewal with zebrafish following punishment contingencies. Several researchers observed ABA renewal following punishment in Context B in Phase 2 with rats (Bouton & Schepers, 2015; Marchant & Kaganovsky, 2015; Marchant et al., 2016; Pelloux et al., 2018). These studies compared a group of rats receiving punishment to a control group continuing to receive reinforcement in which presentations of punisher were yoked to the experimental

group, independently of responding. In contrast, the present experiment compared PUN+EXT to EXT, producing decreases in responding in both conditions. Therefore, the present findings expand both the species and the conditions in which operant renewal occurs and demonstrate that renewal effects following punishment are robust.

As with Experiment 1, however, it is important to address conditioned suppression as a potential explanation of the punishment effects in Context B and renewal upon returning to Context A in the present experiment. Specifically, shock presentations might have become associated with the contextual features comprising Context B to suppress responding through conditioned responses evoked by the context absent when returning to Context A (e.g., Bouton & Swartzentruber, 1986). There are a couple lines of argument against the present findings resulting from conditioned suppression. First, the shock value arranged in the present study (i.e., 2.7 mA) was less than those in other studies with zebrafish failing to produce conditioned suppression (i.e., 5 mA; Kenney et al., 2017) or suppressing other concurrently available responses (Kuroda et al., 2019). Second, other research shows little evidence of conditioned suppression through simple contextual fear conditioning. Bouton and Schepers (2015) arranged two different responses to be reinforced during Phase 1, such as lever pressing in Context A intermixed with sessions of chain pulling in Context B. In Phase 2, responses were switched and punished in the opposite context (Context A: chain; Context B: lever). During testing, either Context A or Context B were presented and response rates were consistently greater in the contexts arranged in Phase 1 during reinforcement only. Thus, the contexts did not generally suppress all operant behavior but appeared to result from context mediating the effects of the specific punishment contingencies. Nevertheless, including controls to assess conditioned suppression as a potential explanation would strengthen the interpretations of the present findings being due to the specific punishment contingency.

A limitation of the present experiment was the difference in target response rate in the last session of Phase 2 between the PUN+EXT and EXT sets. Following a common definition of relapse (e.g., Shvarts et al., 2020), we defined renewal as an increase in target responding in Phase 3 relative to the last session of Phase 2. This definition led us to conclude that punishment of target responding in Phase 2 increases renewal effects. Nonetheless, Fig. 4 shows that target response rates in Phase 3 overlap between the PUN+EXT and EXT sets. In this case, the experimental results can be reinterpreted as that punishment more rapidly decreased target responding in Phase 2 but target response rates did not differ in Phase 3. This raises a concern over the common definition of relapse, especially, when assessing the effect of an experimental manipulation on the magnitude of relapse as in the present study. It remains unclear how relapse data should be analyzed in such a case when there are differences in target response rate at the end of Phase 2 (Bouton & Trask, 2016; Craig & Shahan, 2016). In general, conclusions about differences in relapse are least ambiguous when continuing Phase 2 until target response rates are undifferentiated between experimental manipulations.

## **5. General Discussion**

The present experiments examined resurgence and renewal in zebrafish following reductions in responding with PUN+EXT versus EXT. Responding decreased more rapidly with the presentation of electric shock than in its absence in both resurgence and renewal preparations, providing additional evidence of punishment effects with electric shock on zebrafish operant behavior (Kuroda et al., 2019). Subsequently, resurgence occurred after the removal of alternative reinforcement (Experiment 1) and renewal after a return to the training context (Experiment 2), replicating the results of our previous studies (Kuroda et al., 2017a, 2017b). Between the two present experiments, magnitudes of relapse differed between resurgence and renewal tests. For resurgence, the magnitude was smaller following PUN+EXT relative to EXT, similar to Kestner et al. (2015) with rats. In contrast, the magnitude of renewal appeared greater following PUN+EXT than EXT. The latter was the first to suggest an

increase in renewal as a result of punishment relative to extinction in any species. Nevertheless, systematic replications in which target response rates are undifferentiated between the two sets of conditions are required for firmer conclusions.

The direction of change differed between resurgence and renewal, perhaps, because punishment led to different learning processes in those preparations. With resurgence, subjects learn in Phase 2 that target responding leads to punishment while alternative responding leads to reinforcement. The discontinuation of alternative reinforcement in Phase 3 does not provide a salient signal that the punishment contingency is also removed. Thus, the response-suppression effect of punishment likely continues until subjects contact the absence of the contingency between target responding and the punisher. This interpretation is supported by the more gradual increase in target responding in Phase 3 following the removal of punishment compared with the typical test of resurgence in the absence of punishment in Phase 2. With renewal, in contrast, punishment is associated only with Context B and never with Context A. Thus, returning to Context A in Phase 3 directly signals both the absence of a punishment contingency and the presence of a reinforcement contingency for target responding. A somewhat different perspective is that the punishment contingency contributed to enhancing the difference of Context B from Context A relative to extinction alone. Greater differences between Context B and the testing context (A or C) produce greater renewal during testing (e.g., Todd et al., 2012).

Future studies might further isolate processes involved in relapse with punishment contingencies by assessing combinations of resurgence and renewal procedures. Resurgence and renewal procedures differ in terms of the presence/absence of alternative reinforcement and changes in contextual stimuli. Some researchers have combined resurgence and renewal procedures to assess individual contributions of these factors to overall relapse (e.g., Kincaid et al., 2015; King & Hayes, 2016; Podlesnik & Kelley, 2014; Podlesnik et al., 2019; Sweeney & Shahan, 2015; Trask & Bouton,

2016). These studies generally indicate that resurgence is greater in the presence than absence of changes in contextual stimuli. This approach might also be useful for examining how punishment interacts with contexts in determining the magnitude of relapse. Does arranging extinction, alternative reinforcement, and punishment with versus without contextual changes in Phase 2 influence the size and pattern of relapse in Phase 3? Specifically, target responding is reinforced under Context A in Phase 1; PUN+EXT is in effect for target responding while introducing alternative reinforcement either under Context A or B in Phase 2; finally, all contingencies are removed in Context A in Phase 3. If resurgence is greater with the change in contextual stimuli than without the change, then that would indicate that the change in contextual stimuli, rather than the presence/absence of alternative reinforcement, is responsible for the difference in the direction of change in resurgence and renewal following punishment.

The present study used wild-type zebrafish as experimental subjects but it certainly is possible to examine the present procedures with genetically modified zebrafish fish to assess biological factors in relapse (e.g., Antinucci & Hindges, 2016). Previous studies with rodents identified neurobiological factors influencing relapse. For example, Pelloux et al. (2018) reported that inactivation of basolateral amygdala with GABA receptor agonists, muscimol and baclofen, increased ABA renewal of cocaine self-administration in rats following Context B with punishment and reinforcement but decreased the renewal following Context B with extinction alone (see Marchant et al., 2019 for a review). These findings indicate that the same pharmacological intervention can affect renewal differently depending on how responding is reduced in Context B. Punishment involves multiple regions in the brain including forebrain circuits and midbrain dopamine circuits (Jean-Richard-Dit-Bressel et al., 2018). Using a calcium-imaging technique for monitoring the whole brain of zebrafish at single-cell resolutions (Li, 2013), for example, zebrafish with transparent body (Antinucci & Hindges, 2016) could allow for the direct observation of neurobiological mechanisms governing punishment, drug

effects, and contextual changes. The use of zebrafish as an animal model in relapse research opens the door to the examination of how environmental factors interact with biological systems in determining relapse.

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**Figure Captions**

**Fig. 1.** Responses per min across all sessions in the experimental conditions in Experiment 1. Target, alternative, and control response rates are shown separately for the set of Phases 1-3 with punishment and extinction for target responding in Phase 2 (“PUN+EXT”) and for the set with extinction alone (“EXT”). These rates are indicated with different symbols and different types of lines. The error bar represents SEM.

**Fig. 2.** Proportions of target response rates in Phase 2 relative to the mean of the last five sessions of Phase 1 in Experiment 1. The filled and unfilled circles represent target responding in the set of Phases 1-3 with punishment and extinction for target responding in Phase 2 (“PUN+EXT”) and for the set with extinction alone (“EXT”), respectively. The horizontal dashed line is for a reference, which is, 1.0.

**Fig. 3.** Differences in response rate between the last Phase-2 session and the five Phase-3 sessions in Experiment 1. Target and control response rates are shown separately for the set of Phases 1-3 with punishment and extinction for target responding in Phase 2 (“PUN+EXT”) and for the set with extinction alone (“EXT”). These rates are indicated with different symbols and different types of lines. The error bar represents SEM.

**Fig. 4.** Responses per min across all sessions in the experimental conditions in Experiment 2. Target and control response rates are shown separately for the set of Phases 1-3 with punishment and extinction for target responding in Phase 2 (“PUN+EXT”) and for the set with extinction alone (“EXT”). These rates are indicated with different symbols and different types of lines. The error bar represents SEM.

**Fig. 5.** Proportions of target response rates in Phase 2 relative to the mean of the last five sessions of Phase 1 in Experiment 2. The filled and unfilled circles represent target responding in the set of Phases 1-3 with punishment and extinction for target responding in Phase 2 (“PUN+EXT”) and for the set with extinction alone (“EXT”), respectively. The horizontal dashed line is for a reference, which is, 1.0.

**Fig. 6.** Differences in response rate between the last Phase-2 session and the five Phase-3 sessions in Experiment 2. Target and control response rates are shown separately for the set of Phases 1-3 with punishment and extinction for target responding in Phase 2 (“PUN+EXT”) and for the set with extinction alone (“EXT”). These rates are indicated with different symbols and different types of lines. The error bar represents SEM.

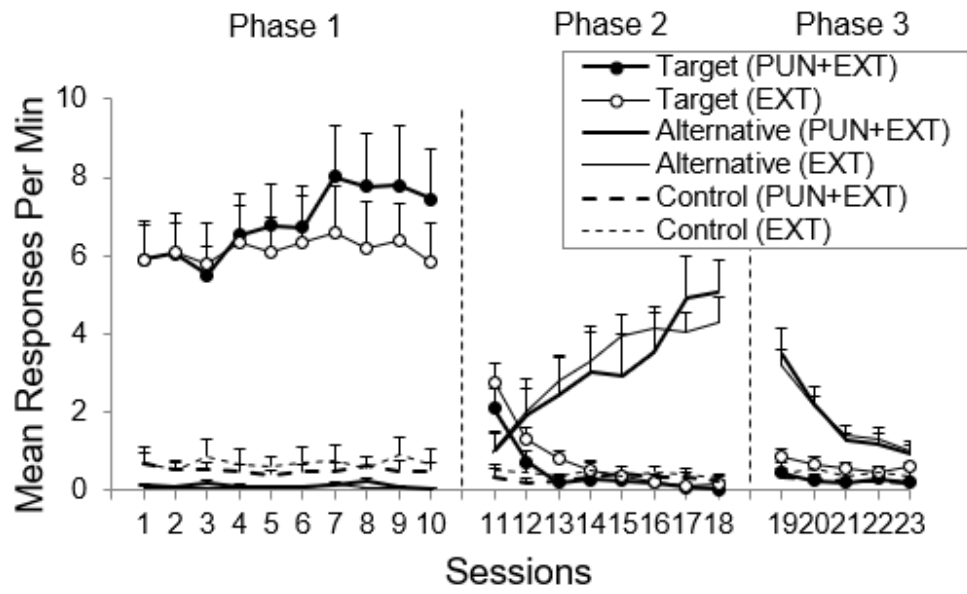


Fig. 1

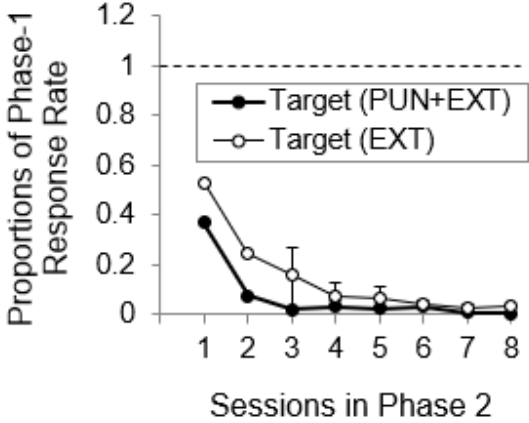


Fig. 2

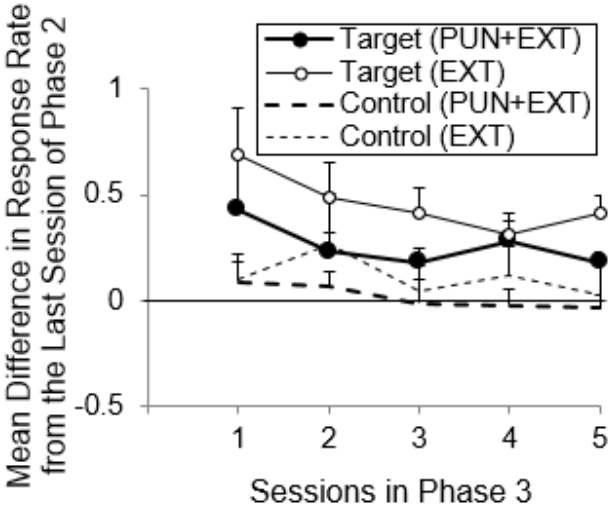


Fig. 3

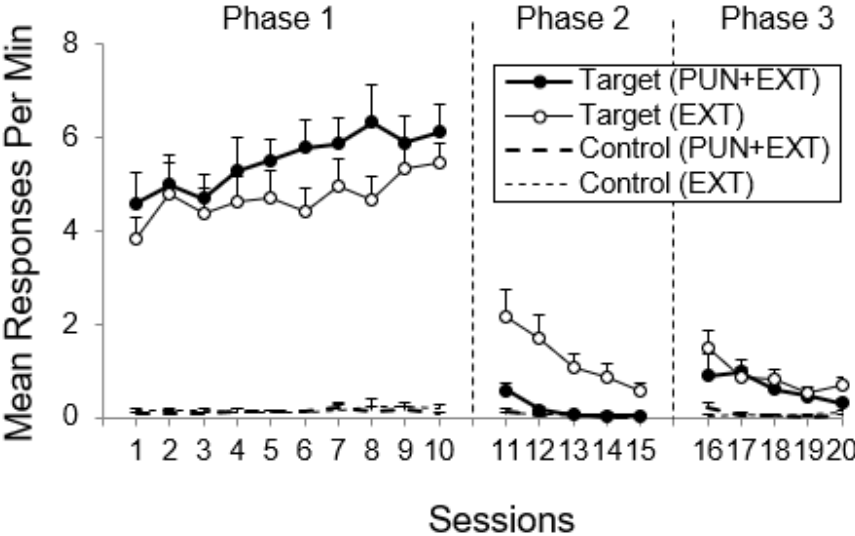


Fig. 4

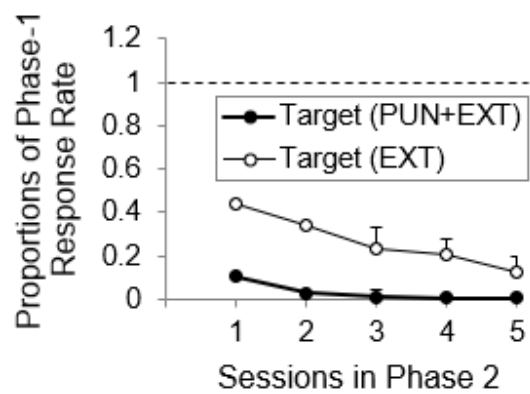


Fig. 5

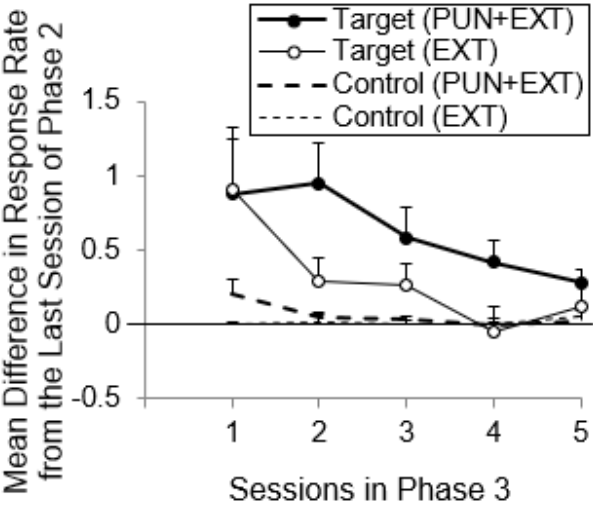


Fig. 6