

# Larval zebrafish display dynamic learning of aversive stimuli in a constant visual surrounding

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Balancing exploration and anti-predation are fundamental to the fitness and survival of all animal species from early life stages. How these basic survival instincts drive learning remains poorly understood. Here, using a light/dark preference paradigm with well-controlled luminance history and constant visual surrounding in larval zebrafish, we analyzed intra- and intertrial dynamics for two behavioral components, dark avoidance and center avoidance. We uncover that larval zebrafish display short-term learning of dark avoidance with initial sensitization followed by habituation; they also exhibit long-term learning that is sensitive to trial interval length. We further show that such stereotyped learning patterns is stimulus-specific, as they are not observed for center avoidance. Finally, we demonstrate at individual levels that long-term learning is under homeostatic control. Together, our work has established a novel paradigm to understand learning, uncovered sequential sensitization and habituation, and demonstrated stimulus specificity, individuality, as well as dynamicity in learning.

[Supplemental material is available for this article.]

Learning while being exposed to a stimulus (i.e., nonassociative learning) is of great importance in that it triggers intrinsic constructs for subsequent recognition of that stimulus and provides a foundation for associative learning (e.g., learning about relations between stimuli in Pavlovian conditioning and stimuli responses-outcomes in instrumental conditioning). Nonassociative learning precedes associative learning in the evolutionary sequence and involves a broad range of behavioral phenomena including habituation, sensitization, perceptual learning, priming, and recognition memory (Pereira and van der Kooy 2013; Ioannou and Anastassiou-Hadjicharalambous 2018).

As the simplest learning form, habituation is defined as the progressively reduced ability of a stimulus to elicit a behavioral response over time (Glanzman 2009; Rankin et al. 2009; Thompson 2009). Such a response reduction is distinguished from sensory adaptation and motor fatigue and is often considered adaptive in that it helps animals to filter out harmless and irrelevant stimuli (Rankin et al. 2009). Since an early study of EEG arousal in cats (Sharpless and Jasper 1956), the habituation phenomenon has been widely documented in invertebrates such as *C. elegans* (Rankin and Broster 1992; Rose and Rankin 2001; Giles and Rankin 2009; Ardiel et al. 2016) and *Aplysia* (Glanzman 2009) as well as in vertebrates such as rodents (Bolivar 2009; Salomons et al. 2010; Arbuckle et al. 2015), zebrafish (Best et al. 2008; Wolman et al. 2011; Roberts et al. 2016; Randlett et al. 2019; Pantoja et al. 2020) and humans (Bornstein et al. 1988; Coppola et al. 2013).

Accompanying habituation is a process termed sensitization, which in contrast enhances responses to a stimulus over time (Kalivas and Stewart 1991; McSweeney and Murphy 2009; Robinson and Becker 1986). This counterpart of habituation may also be adaptive if it helps animals avoid potentially risky or costly situations (Blumstein 2016; King et al. 2007). Like habituation,

sensitization has also been documented in a phylogenetically diverse set of organisms (Cai et al. 2012; Kirshenbaum et al. 2019; Tran and Gerlai 2014; Watkins et al. 2010), suggesting an evolutionarily conserved biological underpinning for both processes. Furthermore, these simple learning forms are observed in various functional outputs of nervous systems ranging from simple reflexes (Blanch et al. 2014; Pantoja et al. 2020; Pinsker et al. 1970; Randlett et al. 2019) to complex cognitive phenotypes (Bolivar 2009; Leussis and Bolivar 2006; Thompson and Spencer 1966) and may represent deeper neurobiological constructs associated with anxiety-memory interplay (Morgan and LeDoux 1995; Ruehle et al. 2012; Sullivan and Gratton 2002). Therefore, understanding basic building blocks of habituation and sensitization is essential to fully understand complex behaviors.

Habituation and sensitization have been reported with short-term (intrasession) and long-term (intersession) mechanisms in a number of systems (Rankin et al. 2009; Thompson 2009). Short-term mechanisms sensitize or habituate a response within a session (Meincke et al. 2004; Leussis and Bolivar 2006; Rahn et al. 2013; Byrne and Hawkins 2015). In contrast, long-term mechanisms retain memory formed in previous session and use it to modify behavioral responses in a subsequent session (Rankin et al. 2009).

Although both short- and long-term learning and memory have been demonstrated in young larval zebrafish (Wolman et al. 2011; O'Neale et al. 2014; Roberts et al. 2016; Randlett et al. 2019), so far, most paradigms use unnatural stimuli and are designed without integrating sensitization and habituation in the same paradigm. The latter limitation is crucial as the influential dual-process theory, proposed by Groves and Thompson (1970),

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suggests that the two learning forms interact to yield final behavioral outcomes and therefore assessment of only one process might be confounded by alteration in the other process (Meinke et al. 2004).

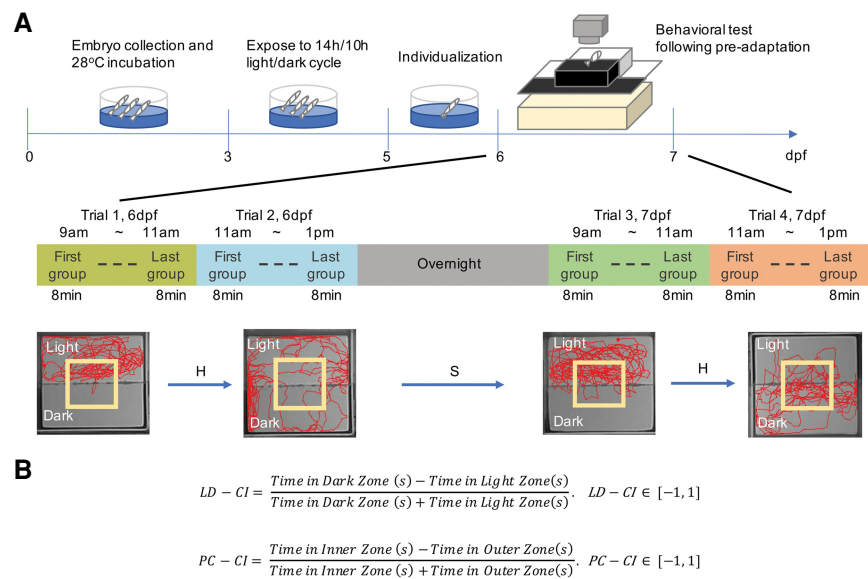
In this study, we examined stimulus learning in a large population of larval zebrafish using a light/dark preference paradigm over four trials across 2 d. Light/dark preference as a motivated behavior is observed across the animal kingdom (Serra et al. 1999; Bourin and Hascoët 2003; Gong et al. 2010; Lau et al. 2011). Larval zebrafish display distinct motor behaviors that are sensitive to the intensity of both preadapted and current photic stimuli (Burgess and Granato 2007; Burgess et al. 2010; Facciol et al. 2019). In our paradigm with well-controlled luminance history and constant visual surrounding, larval zebrafish generally display dark avoidance and center avoidance (also known as thigmotaxis) with heritable individual variability and are considered fear- or anxiety-related (Steenbergen et al. 2011; Schnörr et al. 2012; Bai et al. 2016; Wagle et al. 2017; Dahlén et al. 2019). From an ethological perspective, the extent of avoidance is likely a readout of the circuitry that balances anti-predation (i.e., avoid the dark and the center) and free exploration (i.e., approach the dark and the center). As described below, we have uncovered stimulus-specific temporal dynamicity of learning (both short term and long term), as well as individual differences in learning that are under homeostatic control.

## Results

### The dark avoidance behavior displays intratrial sensitization followed by habituation

We developed a population of 1680 healthy wild-type EK larval zebrafish. Each individual was preadapted to a well-controlled luminance background, then introduced into the center of a half-light and half-dark choice chamber and video recorded in four trials of 8 min each. During each trial, animals were exposed to a constant visual surrounding and had the freedom to navigate the entire arena, thereby mimicking possible encounters in nature. The intertrial time interval was ~2 h between Trials 1–2 and 3–4, whereas the intertrial interval was ~22 h between Trials 2 and 3. Two behavioral responses were analyzed: The dark avoidance behavior was measured by the light/dark choice index (LD-CI) and the center avoidance behavior was quantified by the periphery/center index (PC-CI) (Fig. 1). Previous studies have shown that while individual variability exists, larval zebrafish generally find the dark more aversive than the light environment, and the center more aversive than the periphery of the arena. These preferences likely reflect anti-predatory responses (Treit and Fundytus 1988; Prut and Belzung 2003; Schnörr et al. 2012; Bai et al. 2016; Wagle et al. 2017; Dahlén et al. 2019).

Both types of behavioral responses could potentially be subjected to nonassociative learning, resulting in short- and long-term memories that modify the original responsiveness to aversive stimuli. We first assessed short-term learning by characterizing behav-

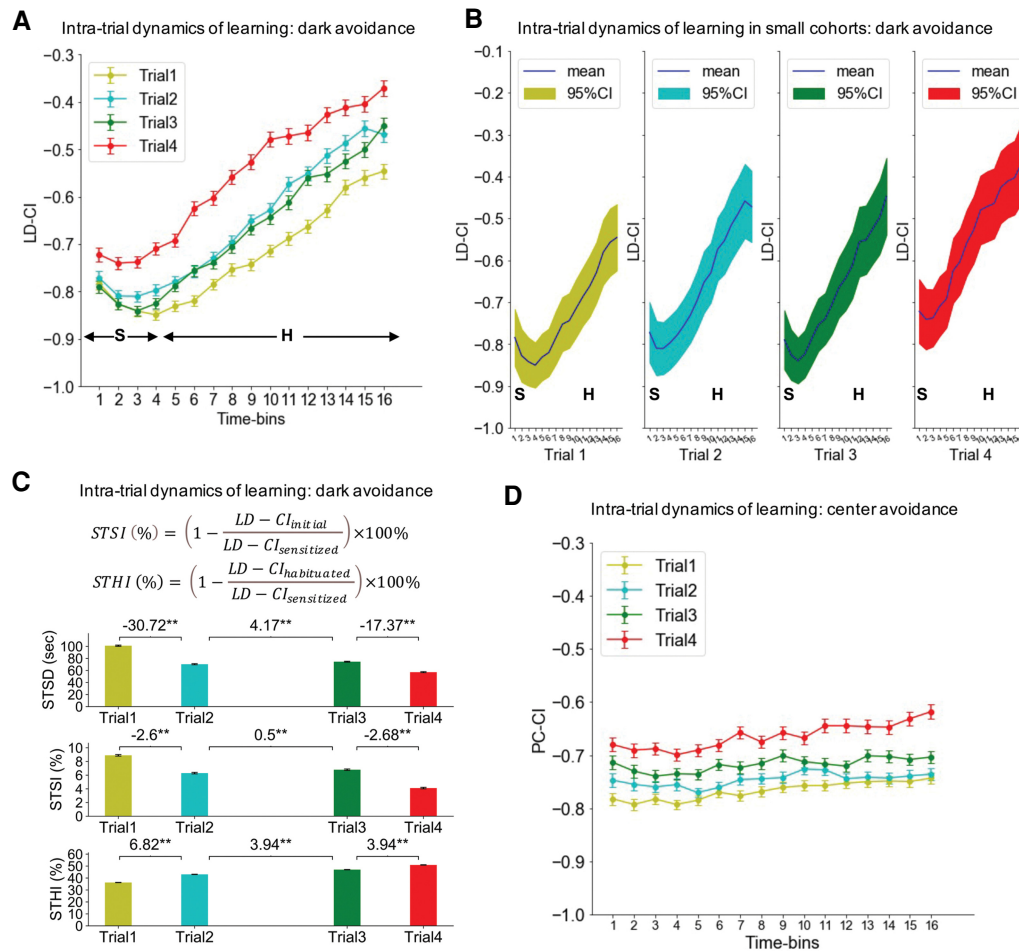


**Figure 1.** Schematic design of the behavioral assay for characterizing stimulus learning in larval zebrafish. (A) Larvae were tested in a chamber (4 cm [L] × 4 cm [W] × 1.5 cm [H]) with a physical light/dark division (top panel) and a virtual center–periphery division (indicated by the 1-cm × 1-cm yellow square border in the bottom panel). Individual behavior was recorded in four 8-min trials scheduled from 6 dpf to 7 dpf. Groups of 16 individuals at a time were recorded for an 8-min trial and up to 192 individuals were tested in a 2-h session. The early trials on each day were performed from 9 a.m. to 11 a.m. followed by the later trials from 11 a.m. to 1 p.m. Experiments were repeated weekly to test a total of 1680 larvae and the movement recordings were subsequently digitized to compute the time spent in the divided zones. (Bottom panel) The example trajectory illustrates an individual’s habituation (H) and sensitization (S) learning depending on the intertrial time interval. (B) Equations for quantifying the light/dark choice index (LD-CI) and the periphery–center choice index (PC-CI).

ioral dynamics within a trial. We profiled each trial with sixteen 30-sec time bins and took the population ( $n=1680$ ) mean of LD-CI and PC-CI for each bin to uncover intratrial dynamics of the two behaviors, respectively. We found a decline of LD-CI in early time bins followed by a progressive increase throughout the rest of time bins (Fig. 2A). The early decline of LD-CI indicated an increase of dark avoidance (i.e., sensitization), whereas the later increase of LD-CI demonstrated a decrease of dark avoidance (i.e., habituation). Furthermore, increased dark avoidance in the sensitization period coincided with an increase of swim velocity that was stabilized during the subsequent habituation period (Supplemental Fig. S1A). Intriguingly, such an increase of swim velocity was also observed in a uniformly illuminated environment (Supplemental Fig. S1B), similar to previously observed transfer inhibition of locomotor activity (Yokogawa et al. 2012). Together, these results demonstrate that larval zebrafish display short-term learning of dark avoidance by an initial sensitization followed by habituation.

To determine whether this phenomenon is observable in smaller cohorts or whether it is only detectable in the large cohort of 1680 individuals, we performed 1000 iterations of random subsampling of 200 individuals and calculated the subpopulation ( $n=200$ ) mean of LD-CI for each 30-sec time bin (see the Materials and Methods). Similar to the entire population, most of the 1000 small cohorts underwent sensitization in early time bins before they transitioned to habituation (Fig. 2B). This analysis reinforces that larval zebrafish learn dark stimulus through an initial short sensitization followed by a subsequent habituation, which can be consistently detected in cohorts as small as 200 individuals.

To further characterize the intratrial dynamic learning of dark avoidance, we calculated three parameters for every subsampled



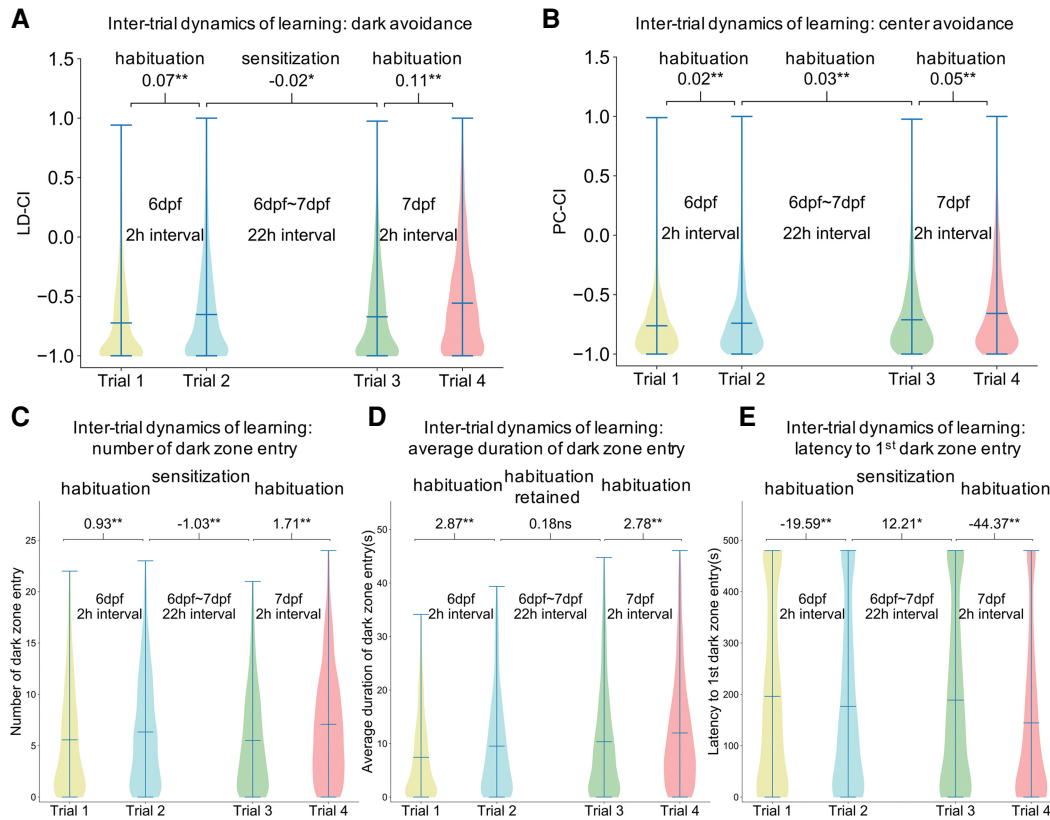
**Figure 2.** Larval zebrafish display dynamic short-term learning of dark avoidance with initial sensitization followed by habituation. (A) Intratrial kinetics of LD-CI. In all four trials, LD-CI was initially decreased during the first several bins followed by a continuous increase over the rest bins, indicating that dark avoidance was first sensitized for a short period before it was habituated. (B) A collection of intratrial kinetics of LD-CI in small cohorts of 200 individuals. A total of 1000 iterations of random subsampling demonstrated the sequential occurrence of sensitization and habituation is a characteristic detectable in small cohorts of 200 individuals. (C) Quantitative characterization of intratrial kinetics. Three parameters were computed (see the Materials and Methods) to quantify intratrial kinetics of LD-CI. Comparisons between trials were performed with vectors of each parameter. Paired two-tail *t*-test: (\*\*\*)  $P < 0.01$ , (\*)  $P < 0.05$ . (D) Intratrial kinetics of PC-CI. Compared to LD-CI, changes of PC-CI was observed with a much lower magnitude, suggesting a minimal effect of habituation on aversive response to the arena center. (H) Habituation, (S) sensitization, (STSD) short-term sensitization duration, (STSI) short-term sensitization index, (STHI) short-term habituation index, (LD-CI) light/dark choice index, (PC-CI) periphery–center choice index. A paired two-tailed *t*-test was used for C. For A and D,  $n = 1680$  individuals. For B and C,  $n = 1000$  iterations. Each iteration sampled 200 individuals.

population: (1) short-term sensitization duration (STSD), (2) short-term sensitization index (STSI), and (3) short-term habituation index (STHI) (Fig. 2C). These analyses uncovered that Trial 1 had the longest STSD (99 sec), which also resulted in the highest STSI (9.7%) and the lowest STHI (36.3%) (Fig. 2C). Across the four trials, cohorts displayed a decrease of both STSD and STSI upon a stimulus reexposure after 2 h (between Trials 1–2 and Trials 3–4), while a retest after an overnight break (between Trials 2 and 3) resulted in an increase of both parameters. In contrast, the STHI showed steady increase across the four trials.

Intriguingly, the dynamic learning of dark avoidance (i.e., sensitization followed by habituation) was not observed with regard to the center avoidance behavior, which underwent a marginal change within the trial, suggesting minimal short-term learning of the periphery–center stimulus (Fig. 2D). Taken together, larval zebrafish display dynamic short-term learning in a stimulus-specific manner: When faced with the light/dark stimulus, larval zebrafish display initial sensitization followed by habituation.

### The dark avoidance behavior displays long-term habituation or sensitization in an intertrial interval-dependent manner

Long-term habituation, which lasts from hours to days, was measured by intertrial comparisons of behavioral indices. While intertrial dynamics could be glimpsed from data presented in Figure 2, A and C, we further calculated average choice indices for each of the four trials and compared them between consecutive trials. As shown in Figure 3A, larvae showed less dark avoidance in a second trial (Trial 2, LD-CI =  $-0.65 \pm 0.009$ ) conducted 2 h after the first trial (Trial 1, LD-CI =  $-0.72 \pm 0.008$ ), suggesting a 2-h memory-mediated habituation. A similar habituation phenomenon was also observed when comparing Trial 4 with Trial 3 at 7 dpf (Trial 3, LD-CI =  $-0.67 \pm 0.008$ ; Trial 4, LD-CI =  $-0.56 \pm 0.009$ ). However, when comparing the two trials with an overnight interval (i.e., Trial 3 vs. Trial 2), we uncovered a slight but significant increase of dark avoidance, suggesting a 22-h memory-mediated sensitization. These results indicate that the habituation memory



**Figure 3.** Long-term stimulus learning exhibit different effects depending on intertrial interval. (A) Population mean of LD-CI significantly increased over two successive trials performed with a 2-h interval, indicative of habituation. Trial 2 versus Trial 1, mean LD-CI diff. = 0.07, (\*\*) $P < 0.01$ ; Trial 4 versus Trial 3, mean LD-CI diff. = 0.11, (\*\*) $P < 0.01$ . A decrease of mean LD-CI was detected across the two trials performed with an overnight interval, indicative of sensitization. Trial 3 versus Trial 2, mean LD-CI diff. =  $-0.02$ , (\*) $P < 0.05$ . (B) Population mean of PC-CI were observed with slight yet still distinguishable increments for the two 2-h intervals (Trial 2 vs. Trial 1, mean PC-CI diff. = 0.02, (\*\*) $P < 0.01$ ; Trial 4 vs. Trial 3, mean PC-CI diff. = 0.05, (\*\*) $P < 0.01$ ) as well as the overnight interval, indicative of habituation (Trial 3 vs. Trial 2, mean PC-CI diff. = 0.03, (\*\*) $P < 0.01$ ). (C–E) Two components positively correlated with LD-CI, the number of dark zone entry (C) and average duration of dark entry (D), were increased significantly over the 2-h intervals. Trial 2 versus Trial 1, mean diff. of number of dark zone entry = 0.93, (\*\*) $P < 0.01$ , mean diff. of average duration of dark zone entry = 2.87, (\*\*) $P < 0.01$ ; Trial 4 versus Trial 3, mean diff. of number of dark zone entry = 1.71, (\*\*) $P < 0.01$ , mean diff. of average duration of dark zone entry = 2.78, (\*\*) $P < 0.01$ . Like the LD-CI, the population also demonstrated a detectable overnight decrease in its number of dark zone entry (Trial 3 vs. Trial 2, mean diff. =  $-1.03$ , (\*\*) $P < 0.01$ ) while the average duration of dark entry was not detected with a change of significance (Trial 3 vs. Trial 2, mean diff. = 0.18, [ns] $P > 0.05$ ). (E) As a negatively correlated component, latency to the first dark zone entry was significantly decreased over the 2-h intervals and increased over the 22-h interval. Trial 2 versus Trial 1, mean diff. =  $-19.59$ , (\*\*) $P < 0.01$ ; Trial 4 versus Trial 3, mean diff. =  $-44.37$ , (\*\*) $P < 0.01$ ; Trial 3 versus Trial 2, mean diff. = 12.21, (\*) $P < 0.05$ . A paired two-tailed *t*-test is used for all panels with  $n = 1680$  individuals.

was disrupted or obscured by the sensitization memory during the overnight time interval. Intriguingly, such a time-dependent learning outcome closely mirrored the observed dynamicity in STSD and STSI (see Fig. 2C), suggesting that the long-term effects of stimulus learning appear to abide by short-term learning performance.

In contrast to dark avoidance, long-term habituation of center avoidance steadily progressed across all four trials (Fig. 3B), with a magnitude that is, however, smaller than that of dark avoidance. Given the intriguing patterns of habituation and sensitization of dark avoidance behavior, in subsequent sections, we will focus on this behavior; wherever applicable, we will also analyze center avoidance as a comparison.

To verify whether the observed behavioral differences between trials are truly due to learning rather than a simple difference in test timing, we compared the first groups and last groups of Trial 1 that were tested  $\sim 2$  h apart (Fig. 1A). No significant differences in their choice indices were detected (Supplemental Fig. S2), indicating that the observed behavioral differences between trials are a result of previous experience.

### Two subcomponents of dark avoidance undergo long-term habituation or sensitization in an intertrial interval-dependent manner

The complex trait of dark avoidance could be dissected into several subcomponents. Accordingly, the dynamics of dark avoidance across the four trials could be explained by plasticity in one single subcomponent or in multiple subcomponents. To further investigate the learning rules of dark avoidance, we quantified intertrial changes of three subcomponents: (1) the number of dark zone entry, (2) average duration of dark zone entry, and (3) the latency to the first dark zone entry. Pairwise correlation analysis (Pearson's *r*) revealed a significant correlation among all components, albeit the correlation between "the number of dark zone entry" and "average duration of dark zone entry" was weak (Supplemental Fig. S3).

Our analysis uncovered that while 2-h habituation was observable for all three subcomponents, only "the number of dark zone entry" and "latency to the first dark zone entry" showed a significant 22-h sensitization (Fig. 3C–E). These results suggest that all three subcomponents develop habituation with a possibly



overlapping mechanism. Larval zebrafish can retain 22-h habituation memory (in the case of “average duration of dark zone entry”) but can also replace it with sensitization (in the case of the other two subcomponents), which might be adaptive if it helps the animals avoid potentially risky or costly situations.

### Stimulus learning occurs with individual variability

While stimulus learning displayed stereotypical patterns as we have shown at the population and subpopulation levels, we also observed a considerable spread in distribution across individuals. To further explore the underlying principles, we developed a long-term learning index (LTLI) that quantifies behavioral changes at individual levels between two successive trials (Fig. 4A; Eq. 2, below). A value of LTLI greater than 1 indicates habituation while less than 1 implies sensitization of that behavior. Individuals with the minimum LD-CI value of  $-1$  in either or both trials cannot be properly characterized with the LTLI equation and were therefore excluded for plotting the distribution curve (see below). Nevertheless, they were still classifiable as habituated or sensitized individuals depending on their behavioral differences between successive trials. Of note, individuals with the minimum LD-CI value of  $-1$  in successive trials were separately categorized as nonlearners.

Although the population mean indicates a pervasive long-term habituation or sensitization depending on trial interval lengths (Fig. 3), the spread distribution curve of LTLI uncovered individuality in stimulus learning (Fig. 4B–D). The LTLI distribution of dark avoidance deviates from a gaussian distribution by displaying a higher frequency with values close to both extremes (i.e., “shoulders” observed in the blue graphs in Fig. 4B–D). This represented the individuals that displayed a LD-CI close to the lower limit ( $-1$ ) in one of the two consecutive trials (indicating an immediate withdrawal from the dark zone after entry).

In the trials separated by a 2-h interval, we found  $>56\%$  of the population underwent habituation learning at both 6 dpf and 7 dpf compared with a  $<40\%$  observed with sensitization learning (Fig. 4E,G). In contrast to 2-h intervals, reexposure after an overnight interval resulted in a reversed scenario where more individuals showed sensitization (49.76%) as opposed to habituation (46.73%) (Fig. 4F). A small portion (3.15%–4.46%) of nonlearners were always detected throughout all trials.

Unlike the deviated learning distribution of dark avoidance, learning of center avoidance showed a near normal distribution throughout all four trials with a slight bias toward the habituation side (orange graphs in Fig. 4B–D). Percent of individuals that displayed habituation or sensitization learning remained steady across the trials (Fig. 4H–J). In addition,  $<1\%$  of the population were detected as nonlearners. These observed differences further support the notion that learning of the two avoidance behaviors are governed by different principles.

### Individual learning is under a homeostatic control

Next, we wondered how an individual’s learning performance varies across trials. In other words, will a habituated individual defined in Trials 1–2 continue the same type of learning in subsequent trials (Trials 2–3 and Trials 3–4)? To address this question, we constructed a transition diagram that illustrates an individual’s probability of displaying a certain learning type following its previous learning type. Two transitions of learning performance can be defined in our four-trial paradigm: First transition compares learning that occurred during Trials 1–2 with Trials 2–3, and second transition compares learning that occurred during Trials 2–3 with Trials 3–4 (Fig. 4K). For the first transition, a majority of previously habituated individuals (65.3%) were transitioned into a sensitization learning (Fig. 4L). Likewise, a majority of previously sensitized individuals (61.2%) were transitioned to habituation learning

behavior. A similar trend was observed for the second transition: A preceding habituation drove a preference for subsequent sensitization (53.9%), and a preceding sensitization was followed by preferential habituation (70.3%). In addition, nonlearners were transitioned into habituation learners in the following trial with a probability (67.8%–69.3%) about twice as high as those remaining as a nonlearner (30.7%–32.2%).

The fact that learning behavior tends to switch to the opposite type as opposed to maintaining the same type was also observed with respect to the center avoidance behavior (Fig. 4M). Together, such an alternation between different learning patterns suggests an underlying homeostatic process that keeps the extent of avoidance behavior within a steady range for optimal adaptation to environmental changes.

While a majority of individuals displayed homeostatically balanced learning patterns, it was worth noting that a small percentage of individuals did not. For instance, across the four trials, an estimated  $\sim 3\%$  individuals ( $39.46\% \times 33.3\% \times 25.6\%$ ) continued to become sensitized toward the dark stimulus, whereas  $\sim 9\%$  individuals ( $56.07\% \times 34.7\% \times 46.1\%$ ) continued to become habituated toward the dark stimulus. Thus, these rarer individuals likely represent opposite sides of the spectrum underlying individual learning patterns.

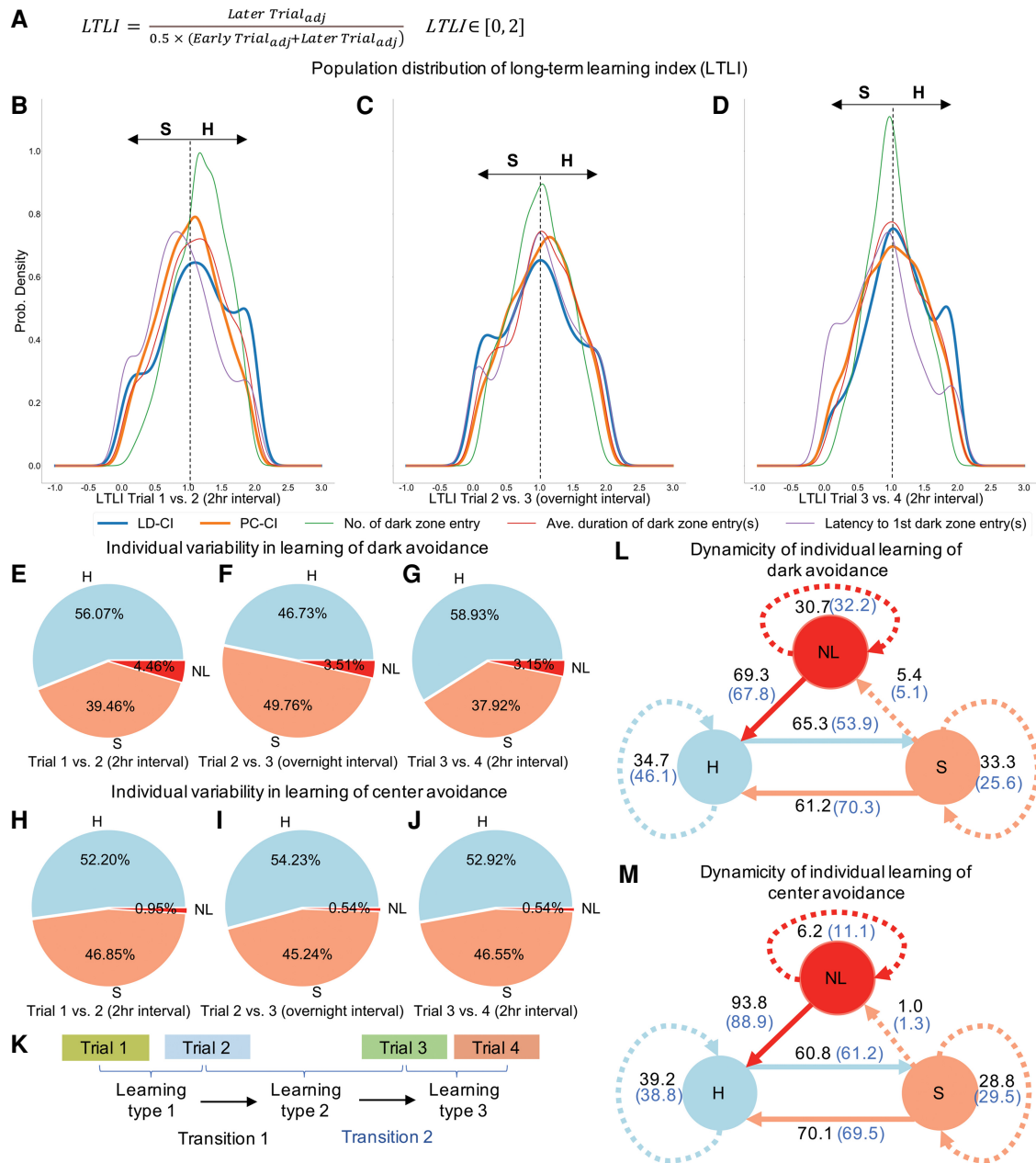
### Long-term learning is more apparent with increased sample size

In an effort to assess the importance of sample size for identifying long-term learning patterns, we examined the power for detecting intertrial differences in dark avoidance behavior (LD-CI) in subsamples of the full data set (Fig. 5). For detecting long-term habituation (i.e., LD-CI changes between Trials 1–2 and Trials 3–4), the power increased exponentially from 0.53 with a sample size of 100 to the maximum of 1.0 with a sample size of 500. As for the overnight sensitization, the power increases in a linear manner from 0.22 with 100 larvae to 0.56 with 1600 larvae. Our results suggest a necessity of using a sufficient sample size in order to detect long-term learning patterns, as a larger sample not only minimizes the standard error but also more closely approximate the entire population (Everitt and Skrondal 2002).

### Discussion

Larval zebrafish have demonstrated learning and memory capabilities in both nonassociative (Best et al. 2008; Wolman et al. 2011; Roberts et al. 2013; O’Neale et al. 2014; López-Schier 2019; Randlett et al. 2019) and associative (Lee et al. 2010; Valente et al. 2012; Hinz et al. 2013; ) settings. In this study, using a large population of larval zebrafish, we have uncovered dynamic stimulus learning in two avoidance behaviors. The distinct patterns of changes in dark avoidance and center avoidance indicate that learning is stimulus-specific. This observation also excludes other forces such as sensory adaptation or motor fatigue in driving behavioral changes, which should otherwise generalize across stimuli (Rankin et al. 2009; Randlett et al. 2019). The notion that learning drives avoidance behavioral changes is further supported by the observation that the locomotor activity (e.g., swim velocity) does not display similar patterns of changes during habituation (Supplemental Fig. S1).

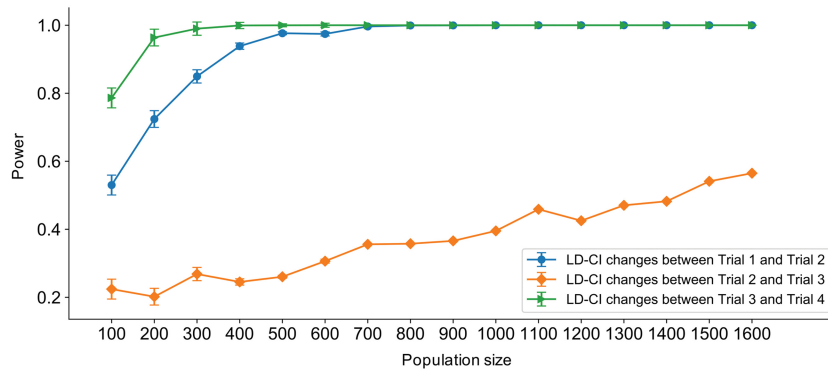
We have shown that short-term learning of dark avoidance starts with sensitization followed by habituation. While such a sequential occurrence of sensitization and habituation has been previously reported in rats (Payne and Anderson 1967; Szabo and Kolta 1967; Davis 1972; Geyer and Braff 1987) and in humans (Geyer and Braff 1987; Ornitz and Guthrie 1989), to our knowledge, this is the first demonstration of such a dynamic learning



**Figure 4.** Long-term stimulus learning occurs with individual variability and under homeostatic control. (A–D) Long-term learning index (LTLI) computed at the individual level (A) unraveled learning performance with individuality in LD-CI (blue), PC-CI (orange), number of dark zone entry (green), average duration of dark entry (red), and latency to the first dark zone entry (purple) (B–D). (E–G) Based on the LTLI of LD-CI, the entire population were divided into three groups corresponding to three distinct learning types of dark avoidance: habituation (“H,” blue), sensitization (“S,” orange), and no learning (“NL,” red). (E) With the first learning process at 6 dpf, more individuals display a habituation learning (56.07%) than sensitization learning (39.46%). (G) A similar but more biased situation was observed in the third learning process at 7 dpf. (F) In contrast, the second learning process was detected with a reversed scenario where learning was biased toward sensitization (49.76%) as opposed to habituation (46.73%). In each of the three learning processes, ~3%–4% of the population were detected as nonlearners. (H–J) Using the same criteria, the population were divided into three groups that differentially learned center avoidance. Unlike the learning of dark avoidance, the portions of the sensitized and habituated individuals remained steady with the non-learning behavior detected in <1% of the population. (K–M) Three learning types were assigned to an individual based on its differences of LD-CI in two successive trials (K). A transition diagram was constructed for each avoidance behavior to illustrate individual learning dynamicity. Each of the three possible preceding learning types are represented by a circle filled with the color corresponding to the pie chart. Conditioned on a certain preceding learning type, a solid arrow is used to indicate the major transition while other minor transitions were symbolized by dashed arrows. Transition probabilities were designated with each arrow for the first (black) and second (blue) transition. Throughout all four trials, a preceding learning type is more likely to be transitioned to the opposite learning type than to be maintained as the same. This tendency applies for learning of both dark (L) and center (M) avoidance.

pattern in larval zebrafish. In many cases, sensitization takes place during the first few presentations of a stimulus (McSweeney and Murphy 2009). Our observation that sensitization precedes habit-

uation in all four trials concurs well with this notion. The initial predominance of sensitization over habituation is thought to represent the influence of a novel aversive stimulus on the central



**Figure 5.** Power of detecting intertrial behavioral differences improves with increased population size. Power for detecting the statistical significance of LD-CI changes over the trials was analyzed with population size ranging from 100 to 1600. Changes between Trial 1 and Trial 2 at 6 dpf (blue) and between Trial 3 and Trial 4 at 7 dpf (green) became exponentially detectable with the increment of the population size and plateaued after the size reached 500. For the detection of overnight sensitization between Trial 2 and Trial 3 (orange), the power was linearly improved to <60% as the population size increased to the maximum.

nervous system (Groves and Thompson 1970; Ornitz and Guthrie 1989) and is relevant to certain human disorders such as schizophrenia (Meincke et al. 2004). While the velocity increase that coincided with the initial sensitization of dark avoidance (Supplemental Fig. S1A) is indicative of increased excitability of motor neurons often driven by sensitization (Thompson and Spencer 1966), the similar movement pattern was also observed in larvae transferred to a uniformly illuminated arena (Supplemental Fig. S1B), suggesting novelty and handling in addition to the dark visual stimulus drive the initial sensitization of dark avoidance. Indeed, similar motor activity changes attributed to transfer inhibition have been previously reported (Yokogawa et al. 2012).

Through iterative population subsampling, we have further shown that sensitization followed by habituation within a trial is detectable in a smaller cohort of 200 individuals (Fig. 2B). More importantly, the iterations enable us to quantify intratrial learning performances with multiple indices. Remarkably, the duration of sensitization in each trial (STSD) mirrors the patterns of long-term habituation: A shortened STSD in the subsequent trial predicts intertrial habituation, whereas a lengthened STSD is linked to intertrial (overnight) sensitization, a fascinating but not understood phenomenon that might involve sleep or active forgetting (Fig. 2C). Together, these findings suggest that the mechanisms that govern short-term (i.e., intratrial dynamics) and long-term (i.e., intertrial dynamics) learning are interwoven.

Filosa et al. (2016) described the influence of hunger on response toward aversive stimuli, which results in reduced avoidance in food-deprived zebrafish larvae. Provided that the state of hunger is the main modulator for changes of dark avoidance observed in our study, then both trials at 7 dpf would be expected to have decreased dark avoidance when compared with the trials at 6 dpf. However, comparisons of Trial 2 at 6 dpf and Trial 3 at 7 dpf (Figs. 2C, 3A) have uncovered an increase of dark avoidance after an overnight period, suggesting that dark avoidance behavioral changes are memory-modulated rather than hunger-driven. This notion is further supported by the observed diversity of individual behavioral dynamicity (Fig. 4).

Quantification of each larva's long-term learning index (LTLI) has revealed individual differences in their patterns of learning. In general, the population displays a considerable spread of distribution in long-term stimulus learning, which is also found in two recent studies (Randlett et al. 2019; Pantoja et al. 2020). However, our learning distribution shows both sensitization and habituation

while only habituation is reported in these previous studies. In addition, the distribution curve deviates from a gaussian curve with an exceptionally high frequency at extreme learning indices for dark avoidance (Fig. 4B–D), indicating a more divergent mechanism in shaping learning behaviors. A possible explanation for the relatively simple learning distribution in previous studies is that their learning performance is induced by intermittently delivered short-lived stimuli (Randlett et al. 2019; Pantoja et al. 2020). However, most stimuli in nature are stable over a longer period with more gradual transition of intensity. In addition to the mode of stimulus delivery, phenotyping paradigms used in previous studies usually restrain animals to a small well that partially immobilizes the animals. This potentially disrupts sensory feedback and alters neural responses (Stowers et al. 2017). In contrast, the

light/dark preference paradigm used in this study allows observation of free-swimming larvae in the presence of stable stimuli, thereby providing a better mimicry of natural environment that helps uncovering stimulus learning patterns likely adopted for optimal survival in natural settings.

Contrary to a stereotyped learning dynamicity observed at the population level, an individual learns stimuli with more stochasticity. By constructing transition diagrams that describe the likelihood for an individual to display a subsequent learning type, we have uncovered a prevalent homeostatic control of learning dynamicity at the individual level (Fig. 4L,M). Across successive trials, alternation between the two opposite learning types (sensitization and habituation) occurs with a probability about twice as high as that for the persistence of same learning types. Homeostatic processes control biological parameters via negative feedback to restore the variable to a preadapted value, also termed as a set point (Knobil 1999). Such a mechanism provides a plausible explanation for the alternating patterns between sensitization and habituation observed in our experiments.

While the majority of individuals display homeostatic patterns of learning in both dark and center avoidance, a small percentage of the population is noted to maintain a same learning type in successive trials (e.g., persistent habituation or persistent sensitization). These individuals might have a set point favored by an invariable learning style. Alternatively, this might be caused by a failure in homeostatic control, which is indicative of dysfunctionality in the underlying circuits. Further research focused on these “outlier” individuals could provide penetrating insights relevant to human disorders, which often represent extreme ends of a spectrum across the population.

Regarding individuality of behavioral phenotypes, it has been observed that acoustic startle response (ASR) habituation is stable over trials in larval zebrafish (Pantoja et al. 2016). This differs from our finding that avoidance behavior is subjected to homeostatic learning. A recent study has reported that another behavior, motor asymmetry, is stable over time in individual larval zebrafish (Horstick et al. 2020). In the case of variations in light/dark preference behavior, it has been observed that individuals with strong dark avoidance (sda) show stable dark avoidance over multiple trials. However, there are also individuals that show variable dark avoidance (vda) across trials. Here, vda is also considered an individualistic behavioral phenotype, even though it does not manifest as a stable response (Wagle et al. 2017; Dahlén et al. 2019).

Thus, both stability and variability can be considered a phenotypic trait. It is possible that regulation of individual learning in different contexts (e.g., ASR habituation vs. avoidance learning) is subject to distinct underlying processing mechanisms.

In contrast to dark avoidance, center avoidance displays marginal changes within a trial (Fig. 2D) but low and steady long-term habituation across trials without the phenomenon of overnight sensitization (Fig. 3B). In addition, individual learning performance of center avoidance display a near normal distribution without significant deviations as is shown in the learning distribution of dark avoidance (Fig. 4B–D). Despite these distinctions, individual long-term learning of center avoidance also displays alternating patterns of sensitization and habituation that is subjected to homeostatic control (Fig. 4M). Thus, although both dark avoidance and center avoidance are anxiety-related and controlled by a homeostatic process, distinct patterns of learning appear to be involved, the underlying mechanisms of which warrant further investigation. Intriguingly, a recent study reported enhanced thigmotaxis (another term describing center avoidance) as a sensitization phenomenon elicited by a chemical stimulus (Roberts et al. 2020), suggesting that center avoidance can be sensitized, in this case, by a different sensory modality.

Two previous studies using the same light/dark preference paradigm fail to observe consistent behavioral changes within and across trials (Wagle et al. 2017; Dahlén et al. 2019). This is likely due to the fact that stimulus learning results in small but significant changes while displaying individual differences, making it difficult to detect with a small sample size. By subsampling the entire data set, we have shown that a population of 200 is sufficient to detect short-term learning with initial sensitization followed by habituation. We have further demonstrated an exponential growth of the power for detecting long-term habituation when the sample size increases to 500. However, in order to uncover the overnight sensitization phenomenon, larger sample size is needed, as the power is <60% even with the maximized sample size of 1680 individuals. Thus, sufficient sample size is an important factor in studies of complex behaviors such as learning. Additionally, the higher power for trial pairs with a 2-h interval suggests a more robust detection compared with trial pairs with an overnight interval. Ongoing development in paradigm design and analytical tools will allow us to further increase throughput, which in turn should facilitate future mechanistic studies of genes and pathways involved in regulating the observed dynamic learning patterns.

In conclusion, our current study probes how larval zebrafish learn upon continuous exposure to a stable surrounding, mimicking possible encounters in nature. We uncover comprehensive patterns of stimulus learning, both short-term and long-term, and with stereotyped temporal dynamics and stimulus specificity at the population level. On an individual basis, learning exhibits stochasticity but is remarkably subjected to homeostatic control across stimuli. Some of these aspects require a sufficient sample size to unravel while others are tractable in smaller cohorts. These findings are well-suited for future investigation of underlying cellular and molecular mechanisms owing to the accessibility of larval zebrafish for in vivo functional imaging (Ahrens et al. 2013; Muto et al. 2013; Stewart et al. 2014) and high-throughput molecular genetic studies (Wolman et al. 2015; Chiu et al. 2016)

## Materials and Methods

### Animals

Adult zebrafish (*Danio rerio*) used for larval production were bred in our facility at the University of California and treated in accordance with IACUC regulations. After crossing, fertilized embryos

were collected into petri dishes with blue egg water (0.12 g of CaSO<sub>4</sub>, 0.2 g of instant ocean salts from aquatic ecosystems, 30 µL of methylene blue in 1 L of H<sub>2</sub>O) and raised for 2 d in a 28°C incubator (Fig. 1A). At the third day postfertilization (3 dpf), dishes were transferred outside onto a light-blue-colored surgical pad (VWR underpad 82020-845) and exposed to a normal circadian cycle with 14-h light and 10-h dark period. On the day prior to behavior test (5 dpf), larvae were individualized into 12-well plates filled with 7 mL of egg water. All plates were kept under the same conditions.

### Behavior test

At 6 dpf, dishes with individualized larvae were first moved to a same blue pad (luminance = 298 lux) in the behavior room 1 h before test (preadaptation). Each larva was characterized for its light/dark preference and periphery/center preference (also known as thigmotaxis) with a previously established paradigm (Lau et al. 2011; Bai et al. 2016; Wagle et al. 2017; Dahlén et al. 2019). Briefly, an individual was transferred to a behavior chamber (4 cm [L] × 4 cm [W] × 1.5 cm [H]) that was evenly divided into a light and a dark compartment by an opaque tape applied to the outside wall (Fig. 1A, top panel). The border between the two side-wall compartments was aligned with clear and opaque black acrylic stripes placed underneath the transparent chamber; together, they were placed on a trans-illuminator so that light only pass through the clear (luminance = 576 lux) but not the opaque black acrylic stripes (luminance = 0 lux). For behavior recording, a camera (Panasonic) with infrared filters (Acrylite IR acrylic 11460) was fixed over the chamber and was connected to the Noldus MPEG recorder 2.1. Groups of 16 individuals at a time were recorded for an 8-min trial and up to 192 individuals were tested in a 2 h session (Fig. 1A, middle panel). Individuals were positioned in the center of the arena to avoid bias of initial placement. An early trial was performed from 9 a.m. to 11 p.m. followed by a later trial from 11 a.m. to 1 p.m. at 6 dpf, leaving a 2-h interval between the two trials of a same individual. Same procedure was repeated at 7 dpf for a total of four trials. This experiment assay was weekly conducted to test a total of 1680 larvae. Behavior recordings were digitized by EthoVision XT 13 to produce raw data output for further analysis (Fig. 1A, bottom panel).

### Data analysis

All data analysis was performed with custom code written in Python (Supplemental Code). The light/dark choice index (LD-CI) and periphery–center choice index (PC-CI) were calculated using the equations described in Dahlén et al. (2019) (Fig. 1B). An increase of each index indicates an increased time an animal spent in the area to which it was initially aversive (i.e., dark zone for LD-CI and inner zone for PC-CI). These two indices were calculated for each trial as well as for 16 nonoverlapped 30-sec periods within a trial, producing a trial index and a bin index for each individual (Supplemental Tables S1, S2).

Population mean of bin indices was calculated and plotted chronologically to illustrate within-trial kinetics (Fig. 2A,D). The resulted graph indicated two sequentially occurred periods within each trial: sensitization and habituation. To characterize these two periods, we randomly subsampled 200 individuals and found the bin with lowest mean LD-CI as the one that divides the period of sensitization from that of habituation. We defined the period(s) from the beginning to the middle point of the divider bin as the short-term sensitization duration (STSD) and the remaining time of the trial as the habituation duration. Using an equation (Eq. 1.1) derived from Raymond et al. (2012), a short-term sensitization index (STSI) was calculated for each subsampled data consisting of 200 individuals to indicate a percentage reduction of bin LD-CI over the sensitized period. Similarly, a short-term habituation index (STHI) was computed by comparing percentage change of bin LD-CI across the habituated period (Eq. 1.2). By iterating the subsampling process for 1000 runs, we generated vectors of STSD (s), STSI (percentage), and STHI (percentage) of each trial followed



by intertrial comparisons using paired two-tailed *t*-test (Fig. 2B,C):

$$\text{STSI}(\%) = \left(1 - \frac{\text{LD} - \text{CI}_{\text{initial}}}{\text{LD} - \text{CI}_{\text{sensitized}}}\right) \times 100\% \quad \text{and} \quad (1.1)$$

$$\text{STHI}(\%) = \left(1 - \frac{\text{LD} - \text{CI}_{\text{habituated}}}{\text{LD} - \text{CI}_{\text{sensitized}}}\right) \times 100\%. \quad (1.2)$$

A repeated measure ANOVA was performed as a preliminary test, which indicated that there is a significant mean difference between trials for each parameter (Supplemental Table S3). The means of trial indices across all individuals were then compared with a paired two-tailed *t*-test among the four trials to reveal intertrial changes (Fig. 3A,B). For every pair of two successive trials, a significantly higher index of the subsequent trial compared with that of the previous trial indicates a habituation to the aversiveness of the stimulus at the population level. On the other hand, a later trial with a lower index suggests a sensitization to the stimulus. Three other LD-CI related components including number of dark zone entries, average duration of dark zone entry, and the latency to the first dark zone entry were also calculated for intertrial comparisons (Fig. 3C–E).

The long-term learning index (LTLI) was calculated for individual larva to indicate a change of aversiveness in the later trial relative to early trial using Equation 2. To make the distribution comparable across the different behavior components, a min-max normalization (Juszczak et al. 2002) was used before plugging values into the equation, so that the values for each parameter are between 0 and 1. For instance, LDCI was converted from (−1, 1) to (0, 1). When calculating latency, value of early trial instead of later trial is used as the numerator in Equation 2 so that habituation of all the behavior components is always indicated by a value >1. In contrast, a LTLI <1 suggests an individual sensitized to the stimuli. Distribution of LTLI for each behavioral component is presented using a kernel density curve implemented by Scott's rule (Fig. 4B–D; Scott 2015):

$$\text{LTLI} = \frac{\text{Later Trial}_{\text{adj}}}{0.5 \times (\text{Early Trial}_{\text{adj}} + \text{Later Trial}_{\text{adj}})}. \quad (2)$$

As indicated by Equation 2, LTLI ranges from 0 to 2 and the two limits are reached when a minimum LDCI value −1 is detected in one of two successive trials, resulting in either Later Trial<sub>adj</sub> or Early Trial<sub>adj</sub> being 0. Individuals in such a scenario would have a fixed LTLI of either 0 or 2 regardless of their true learning performance. Therefore, these LTLIs cannot properly reflect the learning behavior and were therefore excluded for plotting the distribution curve. However, these individuals are still categorized into the group of habituation or sensitization based on calculating their intertrial differences. Moreover, individuals with a minimum LDCI value of −1 in both trials are categorized as nonlearners (LTLI=1). Portion of each group is presented as a pie chart for learning of dark avoidance (Fig. 4E–G) and center avoidance (Fig. 4H–J).

Based on the differences of LD-CI in two successive trials, each individual was assigned a certain learning type, resulting in three learning types for each individual across four trials (Fig. 4K). We then calculated the probability of learning type switching to analyze learning dynamics. For larvae with a given learning type (e.g., S), we computed the portion with the same learning type (e.g., S) after a transition to derive the probability of maintaining learning consistency. We also computed the portion with a different learning type (e.g., H) after a transition to indicate the probability of learning type switching. The resulting two sets of probabilities were summarized in a transition diagram to illustrate learning dynamics at each transition: Solid arrows indicate majority while dashed arrows indicate minority, and numbers in black indicate transition 1 while numbers in blue denote transition 2 (Fig. 4L,M).

For the impact of population size on the detection of long-term habituation, we subsampled data of different sizes ranging from 100 to 1600. For a given size *n*, we randomly selected a subset of *n* individuals from the entire data and performed paired two-tailed *t*-test between the trials to compute a power value using “power\_ttest” function in a Python package “pingouin” (Vallat 2018). The process was iterated 100 times to produce a mean value as the ultimate estimate of the power corresponding to the sample size *n*. In this way, we recorded power changes across 16 different sample sizes (Fig. 5).

## Competing interests statement

The authors declare that they have no competing interests.

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*Author contributions:* J.X. conceived the study, curated the animals, managed the data, and performed the formal analysis and investigation. R.C. curated the animals and recorded the behavior. S.G. conceived and supervised the study and performed the formal analysis

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