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Establishment of the miniature fish species *Danionella translucida* as a genetically and optically tractable neuroscience model

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ABSTRACT

The rapid adoption of the larval zebrafish as a systems neuroscience model has been driven largely by its small size and optical transparency, which enable the use of imaging and optogenetics techniques to record and manipulate activity throughout the brain. Unfortunately, larval fish lack the mature behavioral repertoire of adults and so a number of learning phenomena and social behaviors cannot be investigated in these animals. Here we establish the pedomorphic fish species *Danionella translucida* as a laboratory model that overcomes this limitation. Adult *Danionella* possess the size and optical transparency of late-larval zebrafish, which we exploit to image deep within the brains of a behaviorally mature animals using two-photon microscopy. The close phylogenetic relationship between *Danionella* and zebrafish enabled us to use existing reagents and techniques for transgenesis, and zebrafish-derived enhancer elements drove transgene expression in *Danionella* with their intended specificities. In a behavioral assay for socially-reinforced place preference, interactions between fish were found to be positively reinforcing and dependent on gender, demonstrating the power of this species for studies of learning and memory as well as social behavior. The establishment of *Danionella's* behavioral, molecular, and optical tractability provides a unique opportunity for researchers seeking to understand the relationship between circuits and adult-onset behaviors at a whole-brain level.

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INTRODUCTION

A central and challenging goal of systems neuroscience is to understand how the coordinated activities of many single neurons determine behavior. Recently, the creation, refinement, and widespread adoption of optical methods to manipulate and record neural activity has driven dramatic progress toward this goal by increasing the feasibility of whole-circuit investigations [1, 2]. The promise of these approaches has been most fully realized in small, genetically tractable model organisms such as *C. elegans* [3], *Drosophila* larvae [4, 5], and the larval zebrafish, *Danio rerio* [6, 7], where the ability to image and optogenetically control neurons throughout the brain enables truly comprehensive analyses of the relationship between circuits and behavior at single-cell resolution. As the only vertebrate model with these features, the larval zebrafish has seen rapid adoption as a neuroscience preparation and generated significant insight into neural dynamics at the whole-brain level [8, 9].

While the zebrafish will undoubtedly continue to contribute to our understanding of vertebrate brain function, the requirement for working with larval animals constrains the types of behavioral questions that can be addressed in this system. As a developmentally immature preparation, larval fish fail to exhibit a range of behaviors including courtship, mating, aggression, and other types of social interaction, as well as many forms of learning and memory [10-15]. Adult zebrafish do exhibit these behaviors [10, 11, 13-15] but due to their relatively large brain size and the presence of optically scattering and

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absorptive anatomical features such as scales, an ossified skull, and expanded pigmentation, they offer none of the optical advantages of younger fish, and require invasive surgical manipulations to enable imaging and optogenetics experiments that are ultimately restricted in scope and resolution [16].

Theoretically, the approaches that have revolutionized zebrafish neuroscience could be brought to bear on an expanded repertoire of behaviors in species that remain small and optically transparent throughout life. A fish species called *Danionella translucida* is one particularly appealing candidate. Originally discovered in 1986 in Myanmar [17], *Danionella translucida* is, like other members of its genus, pedomorphic [17-20], failing to develop the scales, pigmentation, and fully ossified skull that characterize adult fish of other teleost species [21], and at a nominal adult length of 1.2 cm it is among the smallest vertebrates yet identified. While little is known about the biology, behavior, and natural history of these animals, the close phylogenetic relationship between the *Danio* and *Danionella* genera [22] suggests that they will share many important biological features.

Here we establish several important prerequisites for the use of *Danionella* as a laboratory model. Using standard techniques for zebrafish husbandry and transgenesis, we have maintained a breeding colony of *Danionella* in our lab and expressed foreign transgenes in specific cells under the control of zebrafish-derived enhancer elements. We demonstrate the use of two-photon microscopy to image deep within the brains of living, adult fish. To illustrate the robust behavioral repertoire of this species, we show that

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Danionella exhibit an innate preference for social interaction that acts as a behavioral reinforcer in a conditioned place preference paradigm, and that these behaviors are differentially expressed depending on gender. The ability to interrogate adult behaviors using the tools and knowledge base drawn from larval zebrafish makes *Danionella* a powerful new option for systems neuroscience.

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RESULTS

Maintenance and Breeding of *Danionella translucida*

Because *Danionella* had not previously been studied in a laboratory context, we first determined whether its requirements for growth and breeding were compatible with the environmental conditions and husbandry techniques of an institutional zebrafish facility. These fish and other members of the *Danionella* genus are found in an environment that is grossly similar to that of *Danio* [17, 19, 20, 23, 24] and the anecdotal observation that they thrive in home aquaria and at least one public aquarium (personal communication; Paul Dixon and Pete Liptrot, Bolton Museum Aquarium, UK) gave hope that their maintenance would be straightforward. We found that colonies of 25-30 animals per 2.8 liter tank grew readily on the recirculating water system of our institutional zebrafish facility (Fig. 1A). To encourage breeding, each tank contained a number of silicon rubber tubes with an average length of 6 cm. Animals bred spontaneously, laying the majority of clutches within the tubes, and male fish appeared to perform courtship displays consisting of rapid darting movements into and out of the tubes in the presence of females. Each tank of sexually mature *Danionella* housed under these conditions produced an average of 2.5 +/-1.5 clutches per day (mean +/- sem; n=4 tanks monitored over a 2-month period), with an average of 19.25 +/- 3.5 embryos per clutch (Fig. 1B; n=224 clutches). Unlike zebrafish, embryos within each clutch remained tightly bound to one another until hatching, and premature mechanical separation of embryos from their

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clutch resulted in reduced viability. Fertilized embryos were similar in size to zebrafish, with an average yolk diameter of 0.64 +/- 0.023 mm (mean +/- S.E.M; range 0.5-0.8 mm; n=16).

Danionella developed at similar rates to zebrafish, with the first cell divisions occurring within an hour of fertilization (Fig. 1B). Animals were collected and held in zebrafish egg water (E3) until hatching, after which they were transferred to rotifer co-culture for rearing to adulthood. On average, 65 +/- 4% of embryos per fertilized clutch survived past 7 days post-fertilization (7 dpf; mean +/- SEM; n=35). Dramatically reduced pigmentation compared to zebrafish was evident from the earliest stages of development (Fig. 1C) and persisted throughout life (Fig 1D, E). While animals did grow to larger sizes if reared at low density for long periods of time, our observations are consistent with the published standard length for wild-caught animals of ~1.2 cm at adulthood (Fig. 1D), defined as the period during which female fish became visibly gravid and fertilized clutches began to appear in the home tanks. These findings show that *Danionella* can be readily cultivated in a laboratory setting, and confirm that they have pedomorphic anatomical traits that are potentially advantageous for imaging not just in adulthood, but at embryonic and larval stages as well.

Tissue-Specific Expression of Foreign Transgenes Using Zebrafish Enhancers

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Robust and reliable transgenesis methods are a prerequisite for any organism to achieve use as an experimental model. Since techniques for introducing foreign DNA into the genome are well-established in zebrafish, we asked whether these methods would be effective in *Danionella*. Clutches of fertilized eggs were collected from the home tanks within minutes of laying, prior to the first cell division, and placed onto an agarose injection mold. The clutches were immobilized against a wall of the mold and pressure-injected with plasmid DNA (Fig. 2A), then allowed to recover for 24 – 72 hrs before screening for transgene expression. On average, 39 +/- 3% of the injected embryos survived (mean +/- SEM; n=50 clutches).

Because of the close evolutionary relationship between *Danionella* and zebrafish [22, 24], we reasoned that zebrafish-derived enhancer sequences might suffice to drive tissue-specific expression without modification. After injection with a plasmid encoding GFP under the control of cardiomyocyte-specific promoter that is commonly used as a transgenesis marker in zebrafish (*Tg(cmlc2:egfp)*; [25]), 36 +/- 7% (n=8) of the surviving embryos had detectable GFP fluorescence in the heart by 24 hpf (Fig. 2B). Crucially, the utility of zebrafish enhancers applied to neuron-specific motifs as well. An average of 24 +/- 3% (n=10) of fish injected with the pan-neuronal marker *Tg(elavl3:egfp)* (aka *HuC:GFP*; [26]) were observed to have labeled neurons as early as 24 hpf, and expression persisted through the first week of development. Labeled cells were found throughout the brain and spinal cord, resembling expression patterns observed in larval zebrafish (Fig. 2C;

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Suppl. Movie 1). The expression of GFP under the control of a minimal enhancer sequence from the zebrafish *oxytocin* (*oxt*) gene; *Tg(oxt:egfp*), was found to be similarly faithful, with labeled cell bodies confined to the neurosecretory preoptic area (PO) and adjacent posterior tuberculum (Fig. 2D), and clear axonal projections into the neurohypophysis (not shown). We also observed specific expression of GFP linked to the zebrafish *mnx1* enhancer element (*Tg(mnx1:egfp)*; [27]**)** in primary motor neurons throughout proximal (Fig. 2F) and distal segments of the spinal cord. These results demonstrate that *Danionella* is labile to transient transgenesis using existing techniques, and that in many cases *Danionella*-derived regulatory sequences need not be identified to achieve the desired tissue specificity.

Next, we asked whether the transposase Tol2, a standard tool in making zebrafish germline transgenics [28], could be used to facilitate insertion of foreign DNA into the *Danionella* genome. Injecting *Tg(cmlc2:egfp),* which contains Tol2 transposable element sequences flanking the transgene, with and without 20 ng/μL of RNA encoding the transposase revealed that the inclusion of Tol2 caused a nearly two-fold increase in the percentage of injected embryos expressing GFP in the heart (Fig. 2G; +Tol2: 61.4 +/- 0.69%; n=9 clutches; -Tol2: 35.8 +/- 7.6% expression, n=9; p = 0.05), a hallmark of genome integration [25]. As in zebrafish, the presence of RNA caused a significant increase in mortality (+Tol2: 9.2 +/- 2.4% survival, n=9; -Tol2: 35.8 +/-3.4% survival, n=9; p = 0.0008).

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Last, we asked whether a common method for combinatorial transgene expression would be effective in *Danionella*. This scheme, based on trans-activation of the upstream activation sequence (UAS) regulatory element by the yeast transcription factor Gal4, has greatly simplified transgenesis efforts in a variety of species [25, 29] by reducing the number of required transgenic lines. Fish co-injected with the driver plasmid *Tg(elavl3:gal4-vp16)* and *Tg(uas:chrimson-tdTomato)* were observed to express the reporter in neurons throughout the CNS (Fig. 2F), suggesting that this approach will similarly facilitate the generation of *Danionella* transgenics.

Multiphoton Imaging in Adult Fish

The small size and optical transparency of adult *Danionella* make it a promising candidate for whole-brain imaging past its larval stages, a feat currently unattainable in any other vertebrate. To determine whether these qualitative features enabled imaging in living adults, we introduced a fluorescent fiducial marker by injecting Texas Red-conjugated dextran into the heart. The dye was rapidly transported throughout the animals' vasculature and was readily observed at low magnification on a fluorescence dissection microscope (Fig. 3A). Similar to larval zebrafish, eye pigmentation and the scattering nature of neural tissue made it difficult to discern vessels within the brain at this level (Fig. 3A), so live fish were immobilized in low-melt agarose and imaged by two-photon microscopy. Labeled vasculature was visible throughout the dorsoventral extent of the brain (Fig. 3B, C; Suppl. Movie 2), demonstrating that satisfactory images could be

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obtained even within the deepest structures of the CNS, without compensating for increased depth by varying the microscope settings. While sparse pigmentation within the skin partially obscured some structures near the surface of the brain, these "shadows" became less noticeable with increasing depth (Suppl. Movie 2). We next asked whether fluorescently labeled neurons could be similarly resolved. To accomplish this, the membrane-permeable dye OGB-1-AM was pressure injected into the tectal neuropil or hindbrain, and labeled cells were imaged in euthanized animals. Cell soma and processes were clearly visible in both of the injected brain regions (Fig. 3D, E). Together, these results show that *Danionella* offers a unique degree of optical access throughout the adult brain.

Socially-Reinforced Learning

To validate the usefulness of *Danionella* as a systems neuroscience model, it was necessary to demonstrate its ability to perform quantifiable behaviors not observed in larval fish. Some behaviors, such as reproduction, are self-evident, but we endeavored to establish a quantitative paradigm to illustrate the power of these fish for studies of both social behavior and learning and memory. Zebrafish are known to use visual and olfactory cues to identify conspecifics [30, 31], and will seek out environments in which social interactions are possible. These interactions are positively reinforcing, as evidenced by their ability to support place conditioning to a previously neutral stimulus [32]. In order to determine whether these behaviors are exhibited by adult *Danionella*, we developed a

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behavioral paradigm modeled after previous work in zebrafish [12, 30, 32]. Briefly, an adult fish was placed in a custom-built U shaped arena in which two distinct visual patterns (conditioned stimulus (CS), and neutral stimulus (NS)) were projected evenly onto each arm, and which contained a glass barrier near each end (Fig 4A). The arena was evenly illuminated with infrared light, and the position of the experimental fish was monitored as it swam freely between arms. After measuring each animal's baseline distribution (Fig. 4B), a stimulus fish (US) from the same home tank was introduced behind the barrier marked by the CS, and the experimental fish's behavior was monitored to determine whether its side preference had changed in response to the conspecific during this "training phase". The stimulus fish was then removed and the experimental subject was evaluated for a learned place preference during the "testing phase". Place preference and stimulus-based changes were quantified using side preference indices (SPI) and difference scores as described in the Experimental Procedures. SPI scores ranged from +1, indicating an absolute preference for the social arm/CS, to -1, indicating an absolute aversion to the CS. Difference scores (ΔSPI) reflected each animal's change in SPI versus baseline during the training and testing periods. A positive ΔSPI indicates that the animal spent more time in the social arm of the arena when a US fish was present, or after training.

While some individual fish appeared to intrinsically prefer one visual cue (CS or NS) over the other (Fig. 4C, top), the mean SPI during the baseline phase was not significantly

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different from zero, presumably because the specific cues were paired with the social and non-social conditions at random ($SPI_{Baseline}$ = -0.16 +/- 0.10 [mean +/- sem], p=0.09, two-tailed t-test, n=35). Most fish exhibited a strong shift toward the social arm of the arena during the training phase ($\Delta SPI_{Training}$ = 0.69 +/- 0.11, p=0.0001 (two-tailed t-test), Fig. 4C, D, Suppl. Movie 3). When the US fish was removed, the preference for the social arm and its associated CS remained elevated relative to baseline throughout the testing period ($\Delta SPI_{Testing}$ = 0.73 +/- 0.20, p=0.008, Fig. 4C, D, Suppl. Movie 4). To determine whether learned place preference was conditioned to the CS, and not some other unidentified and uncontrolled environmental feature, we performed additional trials in which trained fish were tracked while the CS and NS were alternated from one side of the arena to the other for 10 minute periods. Four out of ten animals tested in this way showed a strong tendency to follow the CS, regardless of whether it was located in the original social arm of the arena (Fig. S1). In contrast, none of the nine fish tested before training exhibited similar tracking behavior. These results show that *Danionella* exhibit strong affiliative behaviors and appetitive learning, that social interactions are positively reinforcing, and that the fish can readily learn to associate arbitrary visual stimuli with appetitive experience.

We further characterized these behaviors by gauging the influence of US properties on their performance. First, to determine whether innate or learned place preference required a social stimulus, or if they could be driven by arbitrary, novel objects

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introduced into the arena after baseline, we evaluated behavior elicited by placement of a small marble into the "social" arm. Fish exposed to this stimulus showed no increase in occupancy of the social region during training or testing (Fig. 4E; $\Delta SPI_{Training}$ = -0.41 +/- 0.22, p=0.1036; $\Delta SPI_{Testing}$ = -0.35 +/- 0.33, p= 3226; n=8), demonstrating that the behaviors cannot be explained by a tendency to explore novel objects.

Second, we asked whether either behavior required exposure to conspecific, or if they might be generalized to other fish species, by simultaneously exposing the test animal to *Danionella* and a size-matched zebrafish (Fig. 4F). Given this choice, the fish retained a strong preference for the conspecific ($\Delta SPI_{Training}$ = 0.43 +/- 0.16, p=0.0167, n=16**)**, suggesting that they are able to identify members of their own species and prefer them to distinct but evolutionarily related fish. This preference for familiarity did not extend to members of the animal's social cohort; when given the choice between fish taken from the home tank or a foreign tank, there was no significant preference for either stimulus (Not shown; $\Delta SPI_{Training}$ = -0.13 +/- 0.14, p=0.3800, n=15).

Last, since social behaviors are strongly influenced by gender in other species, we asked whether social preference and reinforcement were different for male and female *Danionella* by performing experiments using all four possible pairwise combinations of test and stimulus fish (M:F, M:M, F:M, F:F). To our surprise, male animals were markedly more selective in their tendencies toward social preference than females. While male subjects exposed to another male exhibited strong place preference (Fig. 4G; $\Delta SPI_{Training_M:M}$

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= 0.75 +/- 0. 18, p=0.004, n=8), they showed no significant preference for the social environment when exposed to a female US ($\Delta SPI_{Training_M:F}$ = 0.36 +/- 0.22, p=0.1385, n=9). Males also exhibited no significant evidence of learning during the testing phase after exposure to a US of either gender, although M:M pairings showed a clear trend toward significance (Fig. 4H; $\Delta SPI_{Testing_M:M}$ = 0.42 +/- 0.19, p=0.0586, n=8, $\Delta SPI_{Testing_M:F}$ = 0.15 +/- 0.14, p=0.3015, n=9). In contrast, female animals exhibited strong place preference and behavioral reinforcement regardless of US gender ($\Delta SPI_{Training_F:F}$ = 0.62 +/- 0.22, p=0.0178, n=11; $\Delta SPI_{Training_F:M}$ = 0.50 +/- 0.20, p=0.035, n=10; $\Delta SPI_{Testing_F:F}$ = 0.86 +/- 0.24, p=0.0047, n=11; $\Delta SPI_{Testing_F:M}$ = 0.45 +/- 0.18, p=0.0351, n=10). These results suggest that male animals are selective in their social attention, and that social interactions – particularly with female fish – are less reinforcing for males in the context of this assay.

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DISCUSSION

We report the first experimental use of the pedomorphic zebrafish relative *Danionella translucida* in a laboratory setting. Our establishment of imaging, genetic, and behavioral methodologies in these fish paves the way for their broad adoption as a neuroscience model, enabling one to explore a range of behavioral phenomena from a whole-brain perspective. Here, we discuss the likely challenges and benefits of adopting this new species as a model for systems neuroscience in light of our data.

The tendency to avoid publishing negative results makes it difficult to estimate the true limits of the larval zebrafish's behavioral repertoire, but a number of studies have shown that behavioral complexity increases with age. While simple forms of aversive conditioning have been demonstrated at larval stages [33], these phenomena are not robust, being exhibited by only a small subset of individual fish, and disappear completely under modestly different experimental conditions [14]. Studies that have carefully measured behavioral ontogeny show that aversive conditioning becomes much more reliable with age [14], a finding that is borne out by work on this and other forms of learning in mature fish [16, 34-36]. Similarly, larval zebrafish fail to exhibit social behavior in most published reports. One exception is a tendency to form loose shoals that has been seen in some studies as early as 7 dpf [13], but it is nonetheless clear that even this simple form of social interaction is not mature until much later [30]. Since zebrafish do not become gendered until metamorphosis occurs at approximately 21 – 30

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dpf [24], it is unsurprising that courtship behaviors, aggression, and the formation of social hierarchies only occur at adulthood [10, 37, 38]. Other social phenomena, such as the behavioral response to an alarm pheromone given off by injured conspecifics, have similarly been shown to appear only around sexual maturity [15].

Our findings unambiguously establishment the feasibility of studying two adult-onset behaviors in *Danionella*: Social preference and appetitive learning, and it is clear that many more will be experimentally tractable. Preliminary measurements of aversive conditioning and pheromone-triggered social alarm suggest that it will be similarly feasible to explore these phenomena in *Danionella*. While it remains to be quantified, we also observed frequent and seemingly elaborate male-female courtship behaviors and aggressive interactions between male fish related to mating. Presumably owing to their relatively small clutch size and unique ecological niche, *Danionella* appear to take a quite different breeding strategy compared to zebrafish, being more selective depositors of their eggs in hidden locations. Quantification of these courtship interactions and related phenomena such as dominance hierarchies, social alarm, and social buffering will establish entirely new avenues of research. Similarly, we expect other learning phenomena to be tractable, allowing *Danionella* to extend the behavioral toolbox far beyond what is currently possible in larval zebrafish.

Our observations of social affiliation and socially-reinforced place preference open the door to investigating a number of specific questions about the neural mechanisms of

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these phenomena in *Danionella*. While our behavioral results show that social interactions are mediated at least in part by visual cues, as has been observed in zebrafish [12, 30, 31, 39], other sensory modalities are likely to be equally important. Olfaction is a common mediator of social interactions in teleosts [39-41] and its potential contribution to the affiliative and learning behaviors described here will need to be explored. The fact that *Danionella* communicate vocally also presents an extremely interesting avenue for investigating the sensory bases of these social phenomena.

Our observation that learning and social preference are dramatically influenced by gender raises additional questions. Taken at face value, the fact that the behaviors of male fish are less strongly reinforced by females would seem maladaptive for encouraging reproduction. However, it is likely that the context of the behavioral assay strongly modulates the nature of gender interactions in these fish, making male-male aggression [37, 42] and social buffering of stress [39, 43] more relevant than the drive toward courtship and breeding. Systematically varying assay conditions to better understand the influence of gender in this and other contexts is a major immediate goal of our work.

Homology to zebrafish and other vertebrates suggests several neural systems that could be important for social behavior and learning. Among these, the peptide oxytocin is well known to mediate several aspects of social affiliation and reproduction across a range of species from nematodes to mammals [44, 45], including fish [46], and is a strong

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candidate for further study in *Danionella*. We have observed that OXT neuroanatomy is highly conserved between *Danio* and *Danionella,* which sets the stage for a systematic examination of how these neurons shape affiliative behaviors by building on the existing body of zebrafish data [47].

Our finding that *Danionella* are amenable to transient transgenesis and express foreign genes under the control of zebrafish-derived enhancers with a high degree of target fidelity suggests that it will be straightforward to create the transgenic lines needed to perform comprehensive imaging and optogenetics experiments. Techniques relying on Tol2 transposon-mediated insertion of transgenes into the genome are simple, robust, and revolutionized transgenesis in *Danio* and other fish [28, 48-50]. Our observation that these techniques similarly increase transgene expression, presumably through genome insertion, is strong evidence for their promise in *Danionella*.

The findings presented here establish *Danionella* as a powerful new model organism for systems neuroscience. While the combination of optical transparency and behavioral maturity in these fish makes them uniquely suited for exploring particular questions about neural circuits, there are many areas in which zebrafish will rightfully remain the standard in the field. The enormous array of existing mutant and transgenic zebrafish lines will continue to make questions that can be adequately addressed in larvae more practical to pursue in *Danio*. The relatively small size of a *Danionella* clutch will also make it less suitable for experiments that require large numbers, including genetic screens.

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Accordingly, we expect *Danionella* to become a complementary – but essential – option for researchers seeking to address specific, largely unexplored, aspects of neuronal function.

AUTHOR CONTRIBUTIONS

A.D.D. conceived the project, A.P. and A.D.D. designed the experiments and wrote the manuscript, A.P., J.B., E.S.B.C., E.P.L.B., J.P.B., and A.D.D. performed the experiments, and A.P., E.S.B.C., J.P.B. and A.D.D. analyzed the data.

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METHODS

Fish Husbandry

All work with live fish was performed in accordance with the policies of the University of Utah Institutional Animal Care and Use Committee (IACUC), protocol number 15-09006. *Danionella translucida* stocks were bred from two sources. The first was a captive population maintained by Paul Dixon and Peter Liptrot of the Bolton Museum Aquarium (Bolton, UK). The second was a wild-caught sample purchased from The Wet Spot Tropical Fish (Portland, OR). The mature colony arose from spontaneous matings between these groups. Adult fish were fed twice a day with finely crushed flake food (TetraMix) and freshly-hatched *Artemia* nauplii. Upon hatching, fry were raised in co-culture with saltwater rotifers (*Brachonius plicatilis*) until 10 dpf, then moved onto a recirculating water system and fed rotifers three times per day until 30 days post-fertilization (dpf). All fish were maintained on a 14:10–hour light/dark cycle at an average temperature of 28°C.

To encourage breeding, the bottom of each tank contained at least two lengths of opaque silicon rubber tubing (35-A durometer, 8-mm outside diameter, 6-mm inside diameter) with an average length of 6 cm. Recently fertilized embryonic clutches were typically found and collected from within these tubes, primarily within 3 hrs of light onset. Tubes were tipped upwards to allow the clutch to sink to the bottom of the tank. The embryos

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were collected with a 50 mL transfer pipette, released into a petri dish filled with E3 medium, and held in a 28°C incubator prior to propagation or use in an experiment.

Expression Constructs

To construct *Tg(UAS:chrimson-tdTomato)*, the transgene was amplified from *pCAG-flex-fwd(chrimson-tdTomato)* (Addgene) by PCR to introduce flanking SpeI and SacII sites, and then ligated into the corresponding sites of *pME-MCS* [25]. The resulting entry clone was LR-reacted with the Tol2kit (http://tol2kit.genetics.utah.edu/index.php/Main_Page) plasmids *p5E-UAS*, *p3E-pA*, and *pTol2CG3*. *Tg(elavl3:EGFP)*, *Tg(elavl3:gal4)*, *Tg(cmlc2:egfp)*, *Tg(mnx1:egfp)*, and *Tg(oxt:egfp)* are described elsewhere [25-27, 51].

Transgene Injections

Embryonic clutches prior to the first cell division were collected as described above, then placed into a 4% agarose/E3 injection mold with six beveled troughs. Clutches were positioned within a trough with the aid of a glass pipette and gentle shaking of the injection tray, taking care not to separate the attached embryos from one another. One-cell embryos were microinjected with a small volume (1-2 nL) of plasmid DNA at 30 ng/μl and 1% phenol red (Sigma-Aldrich). Injections were made into the yolk or directly into the cell body. Injected embryos were placed into E3-filled Petri dishes and held at 28°C to allow for recovery and development, and were screened for fluorescence expression at 24 and 48 hours post-injection.

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Two-Photon Microscopy of Adult *Danionella*

To label the vasculature, adult fish (2–4 months old) were anesthetized with 1% tricaine methanesulfonate (Sigma) and placed on a sylgard petri dish (Dow Corning). The heart was pressure injected with 10% w/v Texas Red-conjugated dextran (5000 MW; Molecular Probes) through a micropipette injection needle using a microinjector (Picospritzer). Animals were allowed to recover for 1 hour to ensure diffusion of the dye throughout the vasculature. Labelled fish were embedded in 1.5% low-melt agarose and immediately imaged with a custom-built two-photon microscope (920 nm excitation @ 5 mW). Z-stacks were acquired at intervals of 1-2 μm.

To image neurons, adult *Danionella* were prepared for injection as above. A micropipette was back-filled with the synthetic calcium indicator Oregon Green Bapta-AM (Molecular Probes) and used to inject the dye directly into the tectal neuropil and adjacent regions. Fish were allowed to recover in system water for 1 hour, then were euthanized and imaged as above.

Behavioral Assay

Experiments were performed in a U-shaped arena 3D-printed in ABS plastic. A 1-mm slit near the end of each arm held a 35x50 mm glass coverslip to isolate the stimulus fish from the rest of the arena. The arena was evenly illuminated from below using an array of near-IR LEDs (810 nm) diffused through several layers of photographic diffuser paper

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and imaged through an IR bandpass filter onto a Flea3 USB camera (FLIR) at 30 frames per second. Each side of the arena was evenly illuminated with a unique color pattern using an LED Pico Projector (AAXA Technologies). The CS and neutral stimulus were randomized for each fish. For each trial, the arena was filled with system water obtained from the home tank and maintained at 28°C throughout the experiment.

All fish used in these experiments were adults of at least two months of age. The experimental animal was first placed in the horizontal arm of the arena and allowed to acclimate for 15 minutes, then tracked for a 15 minute baseline period. Next, a randomly selected stimulus fish (US) from the same home tank was introduced into one end of the U-shaped arena, behind the glass coverslip. The experimental fish was allowed to swim freely for a total of 45 minutes and tracked during the last 15 minutes of this training period. Afterwards, the US was carefully removed from the arena, and the behavior of the experimental fish was immediately recorded for a 15 minute testing period.

Fish were tracked in real time using a custom-written workflow in the Bonsai programming language [52]. All subsequent analysis was performed in MATLAB. For each phase of the experiment, a social preference index (SPI) was calculated by subtracting the number of frames in which the fish was detected within the non-social arm (NSA) from the number spent in the social arm (SA). The difference was divided by the total number of frames recorded during the experiment. Possible SPI values ranged from -1 (complete NSA preference) to 1 (complete SA preference).

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To account for fish-to-fish variability in baseline preferences, behavioral changes during the training and testing phases were expressed relative to baseline using a "training difference" or "testing difference" score, e.g. Training_Difference = $SPI_{Training}$-$SPI_{Baseline}$. Difference values above zero indicate an increased preference for the social arm of the arena during the corresponding phase, and the potential range is from -2 (complete aversion to conspecific) and +2 (complete attraction). Statistical significance was determined using a student's two-tailed t-test at a threshold of p=0.05. The summary panel in Figure 4 was created using the MATLAB "notBoxPlot" functions (Rob Campbell).

Data and Code Availability

Data supporting the central findings of this study, and the Bonsai code used for behavioral data acquisition and analysis are available from A. Douglass upon request.

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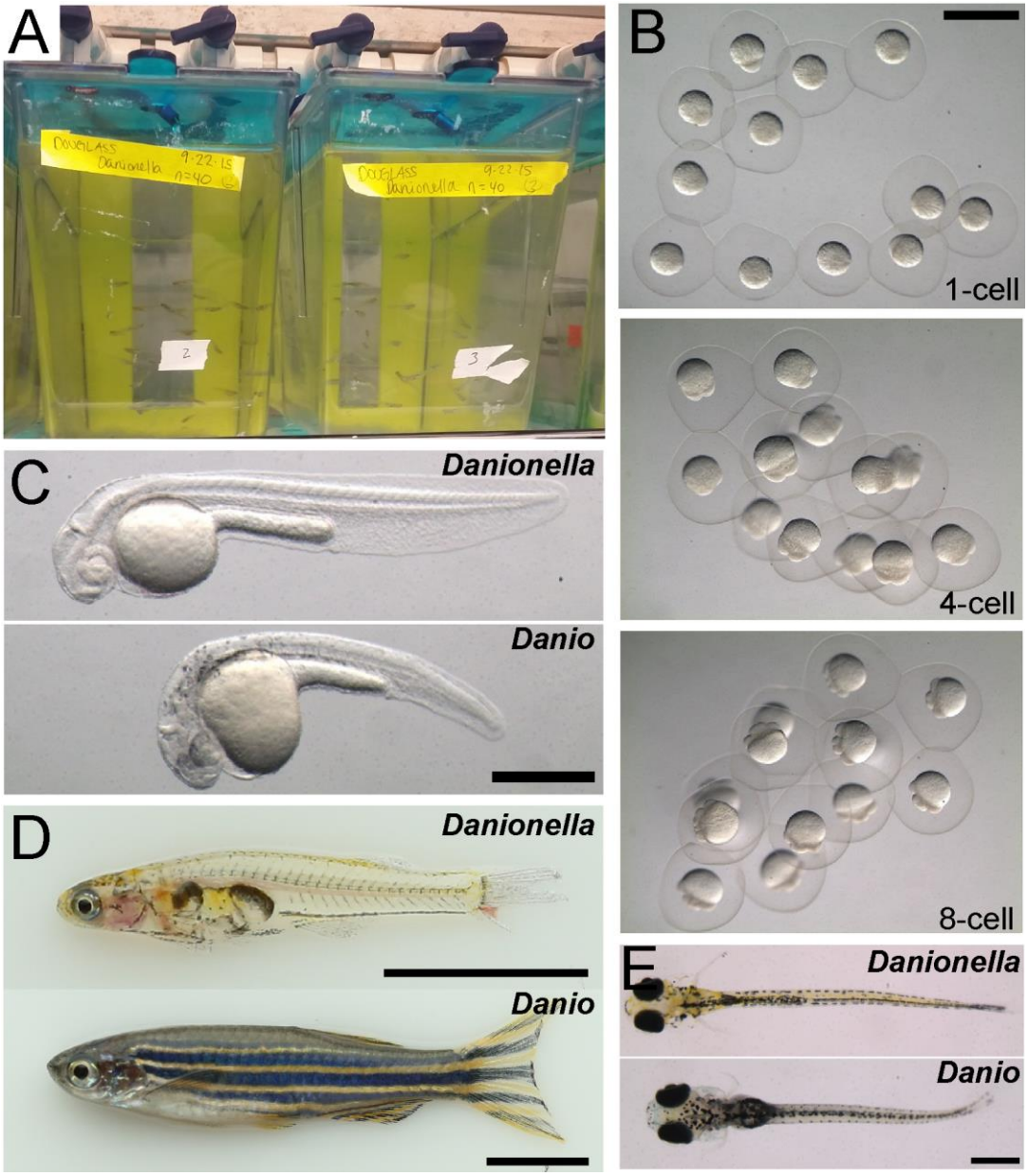

Figure 1. Laboratory maintenance and breeding of *Danionella translucida*. (A) Adult *Danionella* housed under standard conditions in an institutional zebrafish facility. (B) Typical clutch of fertilized embryos at 1-, 4-, and 8-cell stages. Scale bar = 1 mm. (C) - (D) *Danionella* retain immature anatomical characteristics throughout life. Images show fish at 24 hpf (C) and 3 months (D), with zebrafish at equivalent developmental stages for

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comparison. Scale bars = 0.5 (embryonic) and 5 mm (adult). (E) Dorsal view of 7 dpf *Danionella* (top) and zebrafish (bottom) showing reduced melanophore pigmentation above the brain. Scale bar = 0.5 mm.

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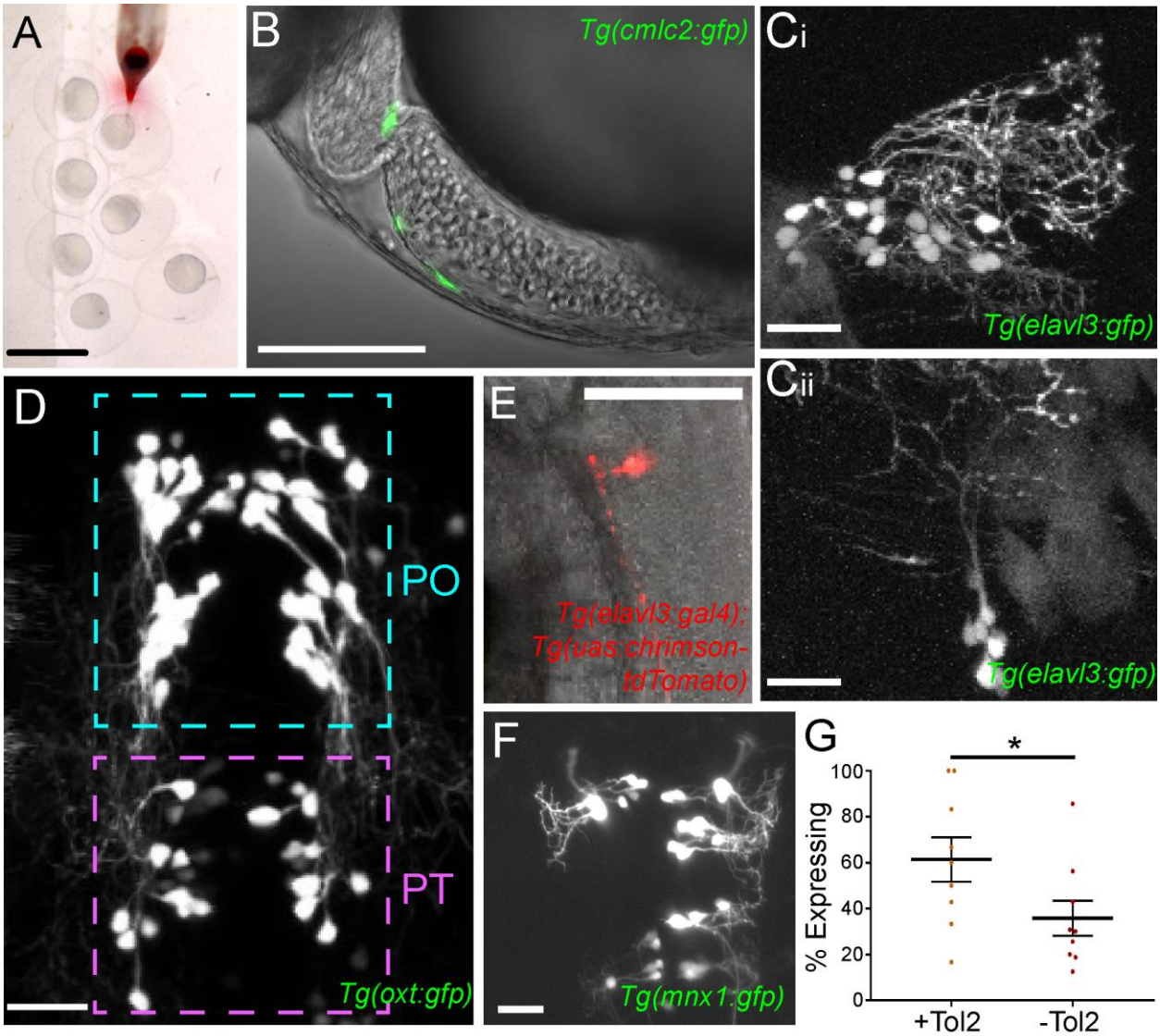

Figure 2. Tissue-specific expression of foreign DNA under the control of zebrafish-derived enhancers. (A) Delivery of plasmid DNA by injection at the one-cell stage. Scale bar = 1 mm. (B) Transient expression of an injected *cmlc2:egfp* transgene (green) in the heart of a 24 hpf *Danionella* embryo. Scale bar = 50 µm. (C) Stochastic neuronal expression of *elavl3:egfp* imaged by two-photon microscopy in a living fish at 5 dpf. Images are gamma-adjusted maximum-intensity z-projections of neurons in the hindbrain (top) and

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optic tectum (bottom). Scale bar = 20 μm. (D) Two-photon image of *oxt:egfp*-expressing neurons in the preoptic area (PO) and adjacent posterior tuberculum (PT). Scale bar = 20μm (E) Gal4/*UAS* mediated expression of *uas:chrimson-tdTomato* driven by *elavl3:gal4-vp16* in a *Danionella* spinal neuron (red). Scale bar = 100 μm. (F) Two-photon image of primary motoneurons expressing *mnx1:egfp* in the proximal spinal cord. Scale bar = 50μm (G) Fraction of injected embryos expressing *cmlc2:egfp* with and without Tol2 mRNA, per clutch. *, $p < 0.05$, t-test.

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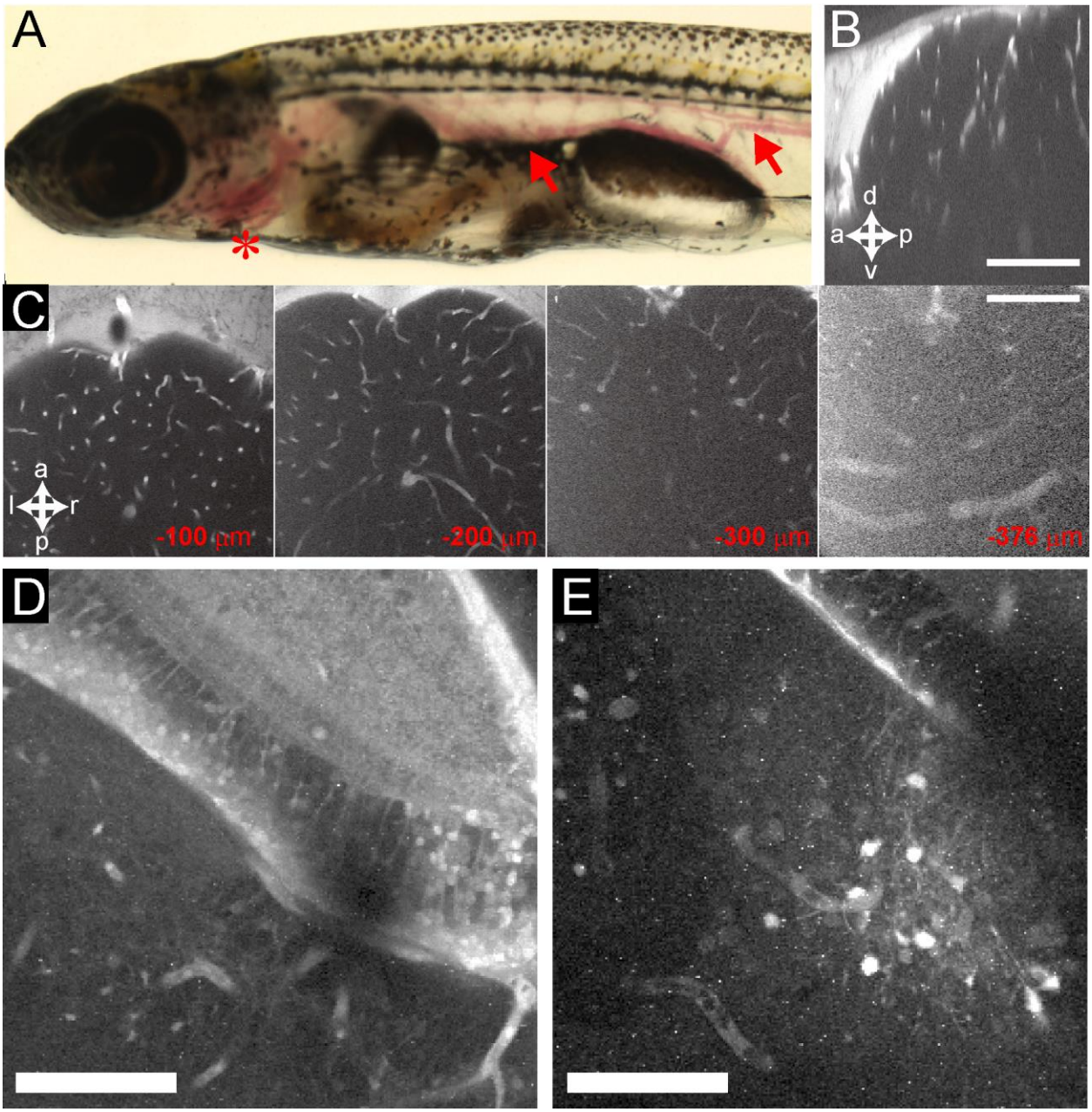

Figure 3. Multiphoton imaging in living adult fish. (A) Texas Red dextran injections into the tail vasculature labeled blood vessels throughout the animal. Arrows indicate labeled vessels, asterisk indicates the heart. (B) Sagittal and (C) horizontal optical sections acquired from a living *Danionella* adult. Panels depict single multiphoton planes imaged in the brain of a living, vascular-filled animal. Image planes in (C) were acquired at the

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indicated depths; -376 μm corresponds to the ventral surface of the brain. Scale bar = 100 μm. (D) Tectal and (E) cerebellar neurons labeled with the synthetic calcium indicator OGB-AM, imaged as for (C). Shown are single planes located approximately 150 μm below the dorsal surface. Scale bar = 50 μm.

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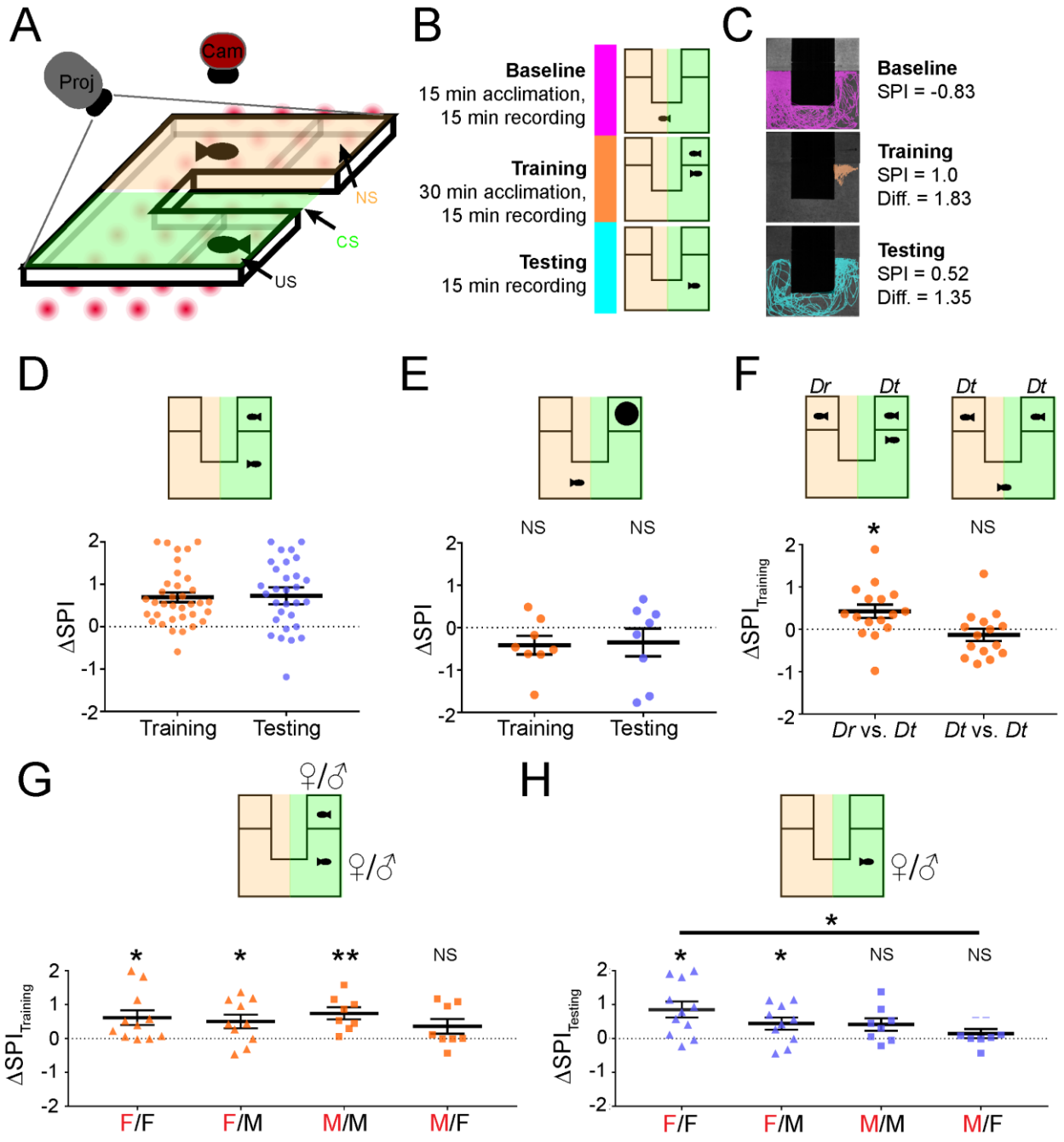

Figure 4. ***Danionella*** **exhibit gender-specific social preference and socially-reinforced learning.** (A) Experimental setup. During training, a test fish (top) is placed in a U-shaped arena and allowed to swim freely between regions in which a stimulus fish (lower right) is or is not visible. Visual cues indicating the presence (orange, NS) and absence

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(green, CS) of a conspecific are delivered via a projector (Proj) while the test animal is illuminated with an array of infrared LEDs and imaged using a camera (Cam). (B) Experimental timecourse. Test fish are tracked for 15 minutes during baseline, training, and testing phases. (C) Trajectories of a single test fish during baseline (top), training (middle), and testing (bottom). Background images are composited single frames of the arena, with and without the US. (D) Fish exhibit a preference for the "social" arm of the arena that reinforces associations with neutral visual cues. SPI difference scores during training (left), and testing phases (right), where a value of zero indicates no change from baseline, and positive values indicate a shift toward the social arm. (E) An arbitrary novel object (marble, top right) paired with the visual CS failed to support place preference or behavioral reinforcement. (F) Test fish exhibited a social preference for conspecifics (*Dt*) over size-matched *Danio rerio* (*Dr*). (G) Pairwise gender combinations revealed a reduced social preference and (H) reinforcement for male test animals, particularly in the presence of a female US. Red letters indicate the test animal in each pairing. * indicates $p < 0.05$; **, $p < 0.005$. All bars indicate mean +/- SEM.