THE SOCIAL BRAIN OF ZEBRAFISH

by

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DISSERTATION ABSTRACT

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Social behavior is arguably one of the most complex forms of behavior exhibited by animals. It requires active attention to dynamic multisnsory cues, recall of past experiences, and the generation of situationally appropriate responses. Given the swath of different cognitive systems required, it is unsurprising that social behavior is disrupted in many neurological disorders. Autism spectrum disorders (ASD) are particularly notable, as social impairment is a required diagnostic criteria. Efforts using animal models to both understand the etiology and improve behavioral outcomes for human ASD patients are complicated by the difficulty of replicating the genetic environmental causes. Similarly, measuring deficits in complex behaviors like social interaction is challenging and their neuroanatomical correlates are not yet fully described.

To address these issues, I utilized the highly social and genetically tractable zebrafish (*Danio rerio*) as a model system. I developed a novel assay that shows social engagement requires a behavioral visual stimulus provided by another socially-engaged fish. I demonstrated that both pharmacological manipulation of dopaminergic systems and ablation of a portion of the ventral telencephalon produce predictable deficits in social behavior. Our results also provide evidence that an as yet uncharacterized population of cholinergic neurons in the ventral forebrain are critical for social

interactions in zebrafish. This region corresponds to mammalian forebrain regions implicated in social behavior, suggesting an evolutionarily conserved population of cells may drive social orienting in zebrafish and mammals. Further, I identified the time points in early development when specific social behaviors are first observed, suggesting a progressive acquisition of increasingly complex social behaviors over a rapid timescale. This highly variable and early stage in development represents an opportunity to further understand how genetic and environmental factors affect the assembly of the neural circuits underlying complex behaviors.

This dissertation includes previously published and unpublished co-authored material.

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CHAPTER I

INTRODUCTION

The social brain of zebrafish

Social behavior is arguably one of the most complex forms of behavior exhibited by animals. It requires active attention to dynamic multisensory cues, recall of past experiences, and the generation of situationally appropriate responses. Social interactions are highly complex, multisensory, and challenging to quantify.

Social impairments are disadvantageous to individuals in a social species. For many animals, an individual's position in the social hierarchy is a powerful determinant of both their own survival and their odds of reproductive success (Alexander, 1974). Correspondingly, social impairments in humans can limit opportunities in academic, professional, and personal settings (Algozzine, Wang, & Violette, 2011; Travis & Sigman, 1998; Tsang, Lam, Ng, & Leung, 2000).

Effective social interactions are often considered a hallmark of humanity, and consequently perpetrators of social transgressions are viewed as 'less human' (Bastian & Haslam, 2010). Appropriate social interactions are considered so complex that mimicking human interactions is a perennial challenge for computer scientists developing artificial intelligence - only the best algorithms defeat the famed Turing test by deceiving a human conversation partner into thinking it is not artificial (Pinar Saygin, Cicekli, & Akman, 2000). Popular culture explorations of human identity similarly use social constructs - for

example, the human replicants in the 1982 film Blade Runner are deemed "perfect" because they are socially indistinguishable from biological humans. Our ability to understand the complexities of these behaviors not only promises to improve the treatment of social deficits, but it may pave the way for superior technologies (Breazeal, 2001).

Affiliative, non-reproductive social interactions are empirically valuable both to the individual animal, and to the social group at large. However, given the swath of different cognitive systems required, it is unsurprising that social behavior is disrupted in many neurological disorders. This includes conditions ranging from acute (brain injury, stroke), neurodevelopmental (autism spectrum disorders (ASD), schizophrenia) and chronic (depression, anxiety, neurodegeneration; (Aderka et al., 2012; Bellack, Morrison, Wixted, & Mueser, 1990; Dawson et al., 2004; Elamin, Pender, Hardiman, & Abrahams, 2012; Lezak, 1987; Robinson, Bolduc, Kubos, Starr, & Price, 1985). These disruptions reduce quality of life and represent a significant medical burden regardless of the cause. ASD is particularly notable, as social impairment is a required diagnostic criterion. While ASD represents many different conditions with varied genetic and environmental causes (Ratajczak, 2011), it is unified by early developmental disruptions that ultimately impair some aspect of complex sensorimotor processing that manifest as behavioral deficits.

Efforts utilizing animal models to both understand the etiology and improve behavioral outcomes for human ASD patients are complicated by several factors. The genetic and environmental causes of ASD and other neurodevelopmental disorders are often unclear and difficult to replicate in animal models. Social interactions can be highly

complex and challenging to objectively quantify. The anatomical regions that are important to generating social behavior are distributed widely throughout the vertebrate brain and their unique contributions are not fully described. Finally, the early social development of many model organisms is not yet completely characterized, despite the increasingly young diagnostic age of ASD in humans based on impairments of the first observable social behaviors.

Zebrafish are an excellent model to approach many of these challenges. By virtue of external fertilization, they are genetically tractable and amenable to environmental manipulation at the moment critical developmental processes begin. They are robustly social, and some of these interactions are visually dependent, simplifying the parameter space for high-throughput automated analysis. A large repertoire of genetic tools allows for manipulation of individual brain regions to better understand the relationship between distinct cell populations and specific behaviors. Finally, zebrafish social behavior develops early relative to the total lifespan, such that specific aspects of social development can be dissected rapidly in numbers sufficiently large to detect subtle effects that may contribute to significant deficits later in life.

In this dissertation, I present my efforts to develop the zebrafish as a model for neurodevelopmental disorders characterized by social impairments. I review the existing literature on early social development in zebrafish, characterize visually driven social behavior by developing a novel high-throughput assay, identify a region of the forebrain with homology to mammalian structures important for social interactions, and

characterize the progressive development of specific social behaviors over a rapid timespan in larval development.

The development of social behavior

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SUMMARY

Zebrafish are highly social animals that exhibit social behaviors early in their development. We review the literature on the developmental timelines and experience-dependent components of zebrafish social behavior, address the strengths and limitations of different assays used to examine the ontogeny of social behavior, and discuss how zebrafish development may be extrapolated to the developmental trajectories observed in other species. We propose a gradual development of social interaction in zebrafish, similar to humans and other animals, in which social behaviors of increasing complexity occur at distinct developmental stages.

Social development across species

Social interaction is a form of behavior that develops rapidly in juvenile animals. Most vertebrate species exhibit some form of social behavior, even in the case of solitary animals that encounter one another only for reproductive purposes. Social interactions can be highly complex, relying on the integration of elaborate multimodal sensory inputs and experience to produce adaptive responses (Chen & Hong, 2018).

The neuronal circuits that govern social behavior appear to be functionally conserved across vertebrates and share both molecular features and evolutionary origins (Chen & Hong, 2018; O'Connell & Hofmann, 2012), with behavioral outputs modified to suit unique environmental and species-specific selective pressures. Fundamentally, social behavior is evolutionarily advantageous by increasing access to mates, but also confers myriad survival advantages by reducing predation and enabling advanced cooperation.

Social ontogeny, or the process of becoming social (Mason, 1979), is highly variable across species and governed by both genetic and environmental influences throughout the lifetime of an animal. The relative contributions of these factors vary widely based on the life history of a given species, which allow them to reap different evolutionary benefits from sociality (Alexander, 1974). For example, mammals with a prolonged period of immaturity may rely more on learning from a parent-child social environment than a zebrafish, which is unlikely to ever encounter its parents or progeny in the wild or laboratory setting. However, experience-dependent plasticity is observed even in fish with no parental interaction in their lifetime, such as zebrafish, and consequently social learning occurs through interactions with conspecifics (Abril-de-Abreu, Cruz, & Oliveira, 2015; Brown & Laland, 2003; Webster & Laland, 2017).

Social behaviors increase in complexity over the course of development, as skills are acquired and implemented in increasingly elaborate behavioral repertoires (Carpenter, Nagell, & Tomasello, 1998). For example, human infants exhibit an immediate preference for human faces (Valenza, Simion, Cassia, & Umiltà, 1996), but joint attention and attending to the distress of others does not arise until 6-9 months of age

(Mundy & Jarrold, 2010; Valenza et al., 1996). Similarly, the ability of humans to mimic the behaviors of others, known as mirroring, is not fully established until 12 months (Woodward & Gerson, 2014). Importantly, deficits in these developmental milestones are diagnostic of neurodevelopmental disorders, like autism spectrum disorder (ASD), that affect social interaction (Bhat, Rajendra Acharya, Adeli, Muralidhar Bairy, & Adeli, 2014). Deficits in the most early forms of social attention in ASD are proposed to impair subsequent social development (Carpenter et al., 1998; Dawson et al., 2004), though it remains to be seen if this is true in genetic models of autism in zebrafish (Kozol, 2018).

The development of social behaviors can be experience-dependent, as animals can learn from both their parents and unrelated conspecifics how to interpret and react appropriately to social cues. Social deprivation in animals, including humans, during early critical periods when specific aspects of the nervous system mature is associated with impaired social behaviors later in life (Committee on Child Maltreatment Research, Policy, and Practice for the Next Decade: Phase II, Board on Children, Youth, and Families, Committee on Law and Justice, Institute of Medicine, & National Research Council, 2014; Fone & Veronica Porkess, 2008). The long-term repercussions of childhood neglect include deficits in cognitive and language abilities, difficulties in the processing of emotional facial expressions, and increased behavioral problems (Spratt et al., 2012). These abilities rely on learning to interpret social cues by exposure and feedback from conspecifics.

Early social experiences shape preferences for familiar social partners that can persist for the lifetime of an animal. For example, birds presented with inanimate objects as

parental surrogates will develop filial attachments, even to the extent that the appropriate "authentic" parent is rejected in favor of the familiar (Bolhuis, 1991), and similar imprinting for sexual partners has been observed in mammals (Kendrick, Hinton, Atkins, Haupt, & Skinner, 1998). Further, isolation during critical periods impairs myelination and disrupts the brain's ability to effectively communicate across distant anatomical regions, and this effect occurs in both human victims of neglect and in controlled rodent studies (Hanson et al., 2013; Makinodan, Rosen, Ito, & Corfas, 2012).

Established animal models such as zebrafish provide an excellent platform to manipulate developmental trajectories to better understand their role in the etiology of complex disorders like ASD (Kozol, 2018). Zebrafish are robustly social animals (Suriyampola et al., 2016) that are highly amenable to behavioral experiments due to the ease of eliciting social behavior in a laboratory setting. Due to their genetic tractability and external development, it is possible to manipulate both genes and environmental conditions, and subsequently observe the consequences on the nervous system, physiology, and behavior.

Zebrafish are proposed to have orthologues of 70% of human genes, and, critically, a large percentage (80%) of the genes disrupted in human disorders have orthologues in the zebrafish genome (Howe et al., 2013). Recent work in molecular genetics and functional neuroanatomy has illuminated evolutionarily conserved neuronal populations with functional homology between zebrafish and mammals (Ganz et al., 2014; Lal et al., 2018; Mueller, Dong, Berberoglu, & Guo, 2011; Nieuwenhuys, 2011; Stednitz et al., 2018). Further, both environmental and genetic perturbations to early development in zebrafish

can impair social behaviors later in life. Embryonic ethanol exposure is sufficient to impair shoaling in a model of fetal alcohol syndrome (Buske & Gerlai, 2011a), and early lead exposure alters aggressive behaviors in the adult (Weber & Ghorai, 2013). Similarly, zebrafish with mutations in genes related to autism in the human population causes social impairments (Liu et al., 2018b). These factors increase their potential merit in modeling human neurodevelopmental disorders with social phenotypes, despite the differences between the social environments of mammals and teleosts.

Defining social behavior in zebrafish

Social behavior is broadly composed of diverse interactions between two or more animals. Zebrafish are no exception, and multiple distinct social behaviors have been classified in the literature. For the purposes of this discussion, we will focus on affiliative behaviors that are observed before sexual maturity and are distinct from reproduction and dominance aggression. Importantly, these affiliative behaviors possess unique properties that emerge at different developmental stages and may allow for the dissection of specific components of the underlying neural circuits.

We propose four interrelated behaviors that are described in the literature:

1. Social attraction, the tendency to turn towards conspecifics
2. Shoaling, aggregative group swimming
3. Social cueing, using signals from conspecifics to guide social interactions
4. Social bias, a visually-driven preference for conspecifics of a given appearance

Individual social behaviors can be elicited using different assays in the laboratory setting. The open tank scenario in which live zebrafish interact using input from all sensory modalities is the most naturalistic assay. Nevertheless, reductionist assays that emphasize one aspect of behavior have merit for understanding specific processes or salient features of a social stimulus. We discuss the ontogeny of zebrafish social behavior by comparing findings across multiple experimental paradigms.

Classifying developmental stages

A major obstacle to interpreting the social ontogeny literature in zebrafish is that developmental staging beyond chronological age reported as days post fertilization (dpf) is rarely considered, leading to replicability challenges between laboratories when zebrafish develop at different rates due to strain differences or rearing conditions. While early developmental stages from fertilization through embryogenesis to freely swimming larvae is thoroughly characterized (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995), it is well established that the age at which anatomical features develop varies depending on temperature, age of initial feeding, quality and quantity of food, and strain (Carvalho, Araujo, & Santos, 2006; Maack, Segner, & Tyler, 2003). Unsurprisingly, these effects carry on into later developmental stages and exert considerable effects on an individual animal. Postembryonic classifications using morphological features of live fish are available, but are rarely employed in the behavioral literature (Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009; Singleman & Holtzman, 2014). Although size is the

greatest predictor of features like swim bladder and fin development and is a useful surrogate, temperature and population density exert significant effects on these features as well (Parichy et al., 2009). Further, there is notable variability in size for a given phenotypic class as defined by fin morphology, such that similar physical features may occur in zebrafish that range between 7 and 15mm standard length (Singleman & Holtzman, 2014).

Rather than an anatomically-defined developmental age, many authors of behavioral studies report chronological age in dpf (Dreosti, Lopes, Kampff, & Wilson, 2015; Hinz & de Polavieja, 2017; Larsch & Baier, 2018). Discrepancies in nutrition and other environmental factors during development could account for some of the variability reported by different research groups and between strains as discussed below. For the purposes of this chapter, we simplified the classification criteria from a number of sources in addition to our own data (Kimmel et al., 1995; Parichy et al., 2009; Singleman & Holtzman, 2014; Westerfield, 1997) and applied a naming system based on age, morphological features, and standard length range (Table 1, Figure 1.1). We delineate these stages by the presence of a yolk sac, notochord flexion (dorsal bending of the posterior notochord; Parichy et al., 2009), and fin morphology, terminating at a juvenile stage that is similar in appearance to a small adult. Zebrafish metamorphic remodeling from larval to adult phenotypes is a largely continuous process in contrast to other species of fish that may have considerable morphological (eg flatfish) or physiological (eg salmonids) changes in the transition between larval and juvenile stages (McMenamin & Parichy, 2013; Parichy et al., 2009); Kendall et al).

Ultimately, we suggest that absolute chronological age is unlikely to be a highly accurate predictor of the presence or absence of social behaviors due to the variability in development between strains and facilities. We include both approximate developmental stage and chronological ages to aid in interpretation throughout the text, but suggest that variability in behavioral onset within a given chronological window is expected.

Onset of specific social behaviors

Zebrafish larvae prior to one week of age are not known to exhibit attraction towards conspecifics, but it is worth noting that this is not due to a lack of visual acuity or motor abilities. Stereotyped behaviors emerge as early as 18 hours post fertilization (hpf), when larvae begin to exhibit spontaneous tail coiling (Brustein et al., 2003; Saint-Amant & Drapeau, 1998), and a startle response can be elicited as early as 3 dpf (Burgess & Granato, 2007). By 4 dpf the swim bladder may be fully inflated and coordinated swimming is observed, including avoidance of potential threats in the environment (Lindsey, Smith, & Croll, 2010), and larvae begin to perform visually demanding prey capture routines (Fero, Yokogawa, & Burgess, 2010; Marques, Lackner, Félix, & Orger, 2018).

Evidence suggests that social attraction emerges as early as 7 dpf, when the yolk sac is no longer present and larvae have entered an independent feeding stage (Hinz & de Polavieja, 2017). We refer to the stage between 7-13 dpf as early flexion, as the process of notochord flexion has initiated (Parichy et al., 2009). Prior to this, zebrafish larvae may avoid conspecifics to maximize the distance between them (Creton, 2009). The underlying basis of the switch between conspecifics acting as attractants rather than

neutral environmental stimuli over a very short timescale is an interesting open question in our understanding of larval neural circuits.

The onset of social behaviors occurs rapidly relative to the total lifespan of zebrafish, such that juvenile affiliative social behavior (at 30 dpf) has not yet been distinguished from sexually mature adults with a maximum described lifespan of 66 months (Gerhard et al., 2002). Sociality appears in conjunction with other complex behaviors such as the ability to learn operant tasks, as well as changes in dopamineric and serotoneric signaling (Mahabir, Chatterjee, Buske, & Gerlai, 2013; Valente, Huang, Portugues, & Engert, 2012). This temporal coincidence indicates a rapid increase in the function of neural circuits within the first month of life, rendering this period particularly interesting for exploring higher-order neurodevelopmental processes.

Social attraction

Social attraction, or the tendency to turn preferentially towards conspecifics during swimming, is a fundamental component to all social behaviors in the zebrafish. Preflexion larvae begin to turn towards one another preferentially by the onset of early flexion (7 dpf), and the frequency and strength of these interactions increases until postflexion (21 dpf) (Hinz & de Polavieja, 2017). Even when physically separated by a transparent divider, 7 dpf larvae exhibit a weak attraction to groups of other zebrafish (Dreosti et al., 2015). In experiments with a divider, attraction is not elicited by single conspecifics at 7 dpf, suggesting that groups may exert a stronger attractive power than individuals at this stage. These findings in physically separated conditions suggest that the initial development of social attraction is visually-mediated. Consistent with this

hypothesis, our group and others report that visual stimuli can elicit many social behaviors, including attraction, shoaling, and social cueing (Dreosti et al., 2015; Gerlai, 2017; Larsch & Baier, 2018; Stednitz et al., 2018).

Social attraction over time develops into shoaling and causes a reduction in interfish distance as preflexion individuals mature (Buske & Gerlai, 2011b). Wild-type early flexion zebrafish begin aggregating as early as 9 dpf, and this attraction increases such that by 12 dpf the mean distance between individuals is significantly reduced from a random distribution (Hinz & de Polavieja, 2017; Mahabir et al., 2013). Some strain differences are observed at this time period, and by 11 dpf inbred AB strain zebrafish have significantly reduced interfish distances (Hinz & de Polavieja, 2017) in contrast to the outbred wild-type strain described above. Minor developmental staging differences discussed previously might also account for this discrepancy, although they may also result from bona fide differences in development between strains.

Thus, social attraction begins in early flexion larvae and increases between the second and third week. By 14 dpf, zebrafish swim in groups and social attraction increases until postflexion (21 dpf), causing increased cohesion of groups. The local interactions governing social attraction behavior in larval zebrafish persist through adulthood, such that they are established early and may guide more complex social behaviors later on (Hinz & de Polavieja, 2017). similar to human models of cognitive development that rely on the gradual acquisition of skills (Fischer, 1980).

Shoaling & social preference

Shoaling is observed in wild zebrafish, but can also be elicited in reductionist settings such as when fish are separated by a transparent divider. In these experiments, by the late flexion stage (14 dpf) zebrafish are more likely to occupy physical spaces where both single and groups of conspecifics are visible (Dreosti et al., 2015), and this spatial preference is accompanied by a tendency to orient the head towards the stimulus fish at an angle of approximately 45*. Orienting behavior becomes very robust by postflexion (21 dpf) and persists for the lifetime of the animal (Stednitz et al., 2018).

It should be noted that individual variability is also present at these developmental stages, such that some zebrafish exhibit an above chance tendency to avoid conspecifics and this aversive phenomenon emerges in tandem with attractive behavior (Dreosti et al., 2015). The individual factors that determine the choice between approach and avoidance behavior remain unclear. Our work indicates similar individual variability in social approach and orienting in adults, which could be related to both stable individual differences and transient contextual cues, like nutritional state or the presence of a predator. Consistent individual differences such as speed are known to affect shoal cohesion in stickleback (Jolles, Laskowski, Boogert, & Manica, 2018). The factors that are most predictive of avoidant social responses in zebrafish remain an outstanding research question.

Virtual stimuli are capable of driving attractive behavior in late flexion (14 dpf) zebrafish (Larsch & Baier, 2018; Stowers et al., 2017). Stimuli were most effective when they moved in non-continuous bouts that imitate biological motion, regardless of the

trajectory of the stimulus. This also supports the previously reported attraction at early flexion that becomes increasingly robust up to 27 dpf in the postflexion stage (Dreosti et al., 2015).

The size of a social stimulus, whether a live conspecific or virtual, is important to driving social interaction. Yolk sac larvae (7 dpf) exhibit a minor aversive response when presented with larger postflexion (21 dpf) stimulus zebrafish (Dreosti et al., 2015). In contrast, when postflexion zebrafish are exposed to a larval stimulus, shoaling is still observed albeit reduced. Avoidance is recapitulated with a virtual stimulus, such that dots that are considerably larger than the animal elicit avoidance rather than attraction (Larsch & Baier, 2018). Presumably this phenomenon can be accounted for by a smaller fish not being perceived as a potential threat, while a larger fish might be. The optimal diameter for virtual stimuli increases with age, suggesting that relative size is one of the factors young zebrafish use to identify appropriate social partners (Larsch & Baier, 2018). This preference could provide a mechanism for zebrafish to aggregate with age and developmentally matched shoalmates.

Social cueing

Zebrafish utilize social cues from conspecifics to guide affiliative social behavior, but the developmental stage at which these cues become salient is relatively understudied. During courtship displays, zebrafish actively attend to the body movements and posture of their partner (Darrow & Harris, 2004). Similarly, when isolated fish are allowed to observe pairs of zebrafish, they are more likely to attend to interacting pairs than

non-interacting pairs (Abril-de-Abreu et al., 2015). The extent to which social cues drive social affiliative behaviors differ by which experimental paradigm is implemented.

Orienting behavior observed in assays in which zebrafish are separated by a transparent divider is guided by visual social cues. Synchronous orienting is reported as early as late flexion (14 dpf), and we find that pairs of juvenile (30 dpf) fish match the angle of their partner within one second (Dreosti et al., 2015; Stednitz et al., 2018). In our dyad assay, the greatest predictor of social orienting in the test fish is the orienting behavior of the stimulus fish. The stimulus fish no longer influences the test fish when normal social orienting is impaired due to pharmacological treatment or lesions of aspecific forebrain region (Stednitz et al., 2018). However, the reduced parameter space in these assays may elicit behaviors that do not occur frequently in a naturalistic setting, and the nature of these interactions need to be further pursued in open arenas.

Despite the caveats raised above, we observe that social orienting begins at the early flexion stage (as early as 12 dpf), and that this angle is refined over a short time scale such that by 16 dpf it is indistinguishable from juvenile (1 month) orienting. Similarly, reciprocal turning events as revealed by time lag cross-correlation analysis rapidly become more robust between 12 and 16 dpf, and this is reflected in an increased influence of the stimulus fish on the test fish's behavior (Stednitz and Washbourne, unpublished). This rapid refinement of social orienting suggests the early and late flexion stages is when cue-dependent orienting behaviors are first established.

The location and behavior of conspecifics also guides an individual's social behavior in a naturalistic context where zebrafish are not restricted to separate containers. In

contrast to orienting, shoaling in the open tank is dominated by parallel swimming of varying degrees of polarization and cohesion, both influenced strongly by habituation to the environment (Miller & Gerlai, 2012). Similar to dyad assays mentioned above, the presence of a single drug-treated zebrafish with impaired social behavior is sufficient to disrupt shoal cohesion (Maaswinkel, Zhu, & Weng, 2013). Zebrafish will also dynamically increase their exploratory behavior if paired with a more exploratory animal in a novel environment (Guayasamin, Couzin, & Miller, 2017). The developmental stage at which these complex open tank shoaling behaviors emerge is a promising avenue of further research.

Virtual stimulus experiments suggest that reciprocity may not be required to elicit following, though biological motion acts as a cue that drives social interactions (Larsch & Baier, 2018). Similarly, a photorealistic virtual social partner whose behavior is influenced by the test fish is more effective at eliciting following than a passive stimulus whose trajectory is predetermined or solely dictated by the test fish (Stowers et al., 2017) This difference may be due to the virtual stimulus failing to remain close enough to the test fish to elicit following. In contrast, in a dyad assay close proximity alone is insufficient to drive spatial preference or orienting when the stimulus fish does not reciprocate, suggesting that different social behaviors rely on distinct cues. The further development of virtual social stimuli that can account for all reductionist visual features that drive social attraction, shoaling, and reciprocity equivalent to live conspecifics is an exciting topic of further research.

Early experience

Zebrafish exhibit visually-mediated social preferences that are shaped by early life experiences, referred to here as social bias. In wild-type adult zebrafish, the thickness of stripes is an attractive cue that drives social bias, and related striped *Danio* species are a more effective social stimulus to wild-type adult males than *Danio rerio* pigment mutants that lack stripes (Engeszer, Wang, Ryan, & Parichy, 2008). A similar effect has been observed using simulated zebrafish as a stimulus (Saverino & Gerlai, 2008). The bias towards striped shoalmates can be subverted by raising zebrafish with pigment mutants that lack stripes, such as *nacre* (Engeszer, Da Barbiano, Ryan, & Parichy, 2007). The preferred pigmentation then becomes the mutant form, regardless of the test fish's own appearance or genetic background. The appearance of familiar conspecifics drives this phenomenon rather than an innate preference for stripes or zebrafish of similar genotypes. Interestingly, social bias does not occur until the juvenile stage (30 dpf), indicating that experience-dependent biases are not present until after social attraction, shoaling, and social cueing already occur. This effect is stable and not plastic later in life, suggesting a critical period wherein the appearance of an ideal attractive conspecific is established (Engeszer, Da Barbiano, et al., 2007).

Zebrafish are also vulnerable to the detrimental effects of early isolation. Preventing social interaction until 180 days disrupts normal social preference in adulthood, reinforcing the importance of social feedback early in life (Shams, Amlani, Buske, Chatterjee, & Gerlai, 2018; Shams, Chatterjee, & Gerlai, 2015), while short-term social isolation (0-7 dpf) is insufficient to drive similar effects. Similarly, isolating larvae until

postflexion (21 dpf) had no detectable effect on attraction towards virtual dot shoaling partners presented immediately after the isolation period (Larsch & Baier, 2018). However, long-term isolated animals display impaired shoal cohesion and reduced brain concentrations of dopamine and serotonin, behaviorally reinforcing neurotransmitters that are known to be involved in social interactions in zebrafish and other vertebrates (Gunaydin & Deisseroth, 2014; Kiser D, 2012). The neural mechanisms of these isolation-based behavioral changes warrant further investigation to understand their relationship to mammalian models of neglect.

Interestingly, a critical period for the establishment of circuitry required for normal social behavior in older larvae may occur well before even rudimentary social interactions are observed. Treatment with valproic acid (VPA) from 4 hpf to 5 dpf is sufficient to reduce social preference and interactions in postflexion larvae (21 dpf) (Dwivedi et al., 2019), and others report slight decreases in social preference in breeding adults (70 dpf) after 0-48 hpf VPA exposure (Zimmermann, Gaspary, Leite, De Paula Cognato, & Bonan, 2015). The behavioral consequences of this manipulation are not restricted to social deficits however, and further studies are necessary to narrow down the developmental window during which the specific circuits driving social behavior are affected.

CONCLUSIONS

Despite the methodological differences in the studies discussed above, they agree on key conclusions. Yolk sac stage larvae before the age of 7 dpf are essentially asocial and exhibit no preference for conspecifics. Between 7-13 dpf, early flexion larvae begin to exhibit social attraction and shoaling. By late flexion (14 dpf) larvae begin exhibiting group swimming, orienting, and limited social cueing. By postflexion (21 dpf), robust social behaviors that persist into adulthood appear to be fully established, including reciprocal orienting turns and coordinated group swimming. Juvenile (30 dpf) zebrafish are capable of establishing social biases for specific types of social partners that persist into adulthood. The noted discrepancies between studies underscore the importance of selecting the appropriate assay for the aspects of social behavior that are of interest. Similarly, morphologically-defined developmental stages may be more predictive of social development than chronological age and should be considered in experimental designs.

Altogether, this body of work suggests zebrafish exhibit a hierarchical development of social interaction similar to other animals, such that social behaviors of increasing complexity occur over an individual's development within an existing framework of sensorimotor and behavioral capabilities.

Table 1. Developmental staging based on age, morphological features, and approximate minimum standard length.

Developmental Stage	Age (dpf)	Standard Length (mm)	Morphological Features
Yolk Sac Larvae	3-6	3.7	continuous fin fold, no fin rays, presence of yolk sac
Early flexion	7-13	4.5	fin rays emerge, absence of yolk sac, notochord flexion begins
Late flexion	14-20	6.2	fin rays developed but not yet forked
Postflexion	21-29	7.8	flexion has completed, bilobate swim bladder
Juvenile	30-89	10	nearly complete adult fins and pigment
Adult	90+	14	breeding adult

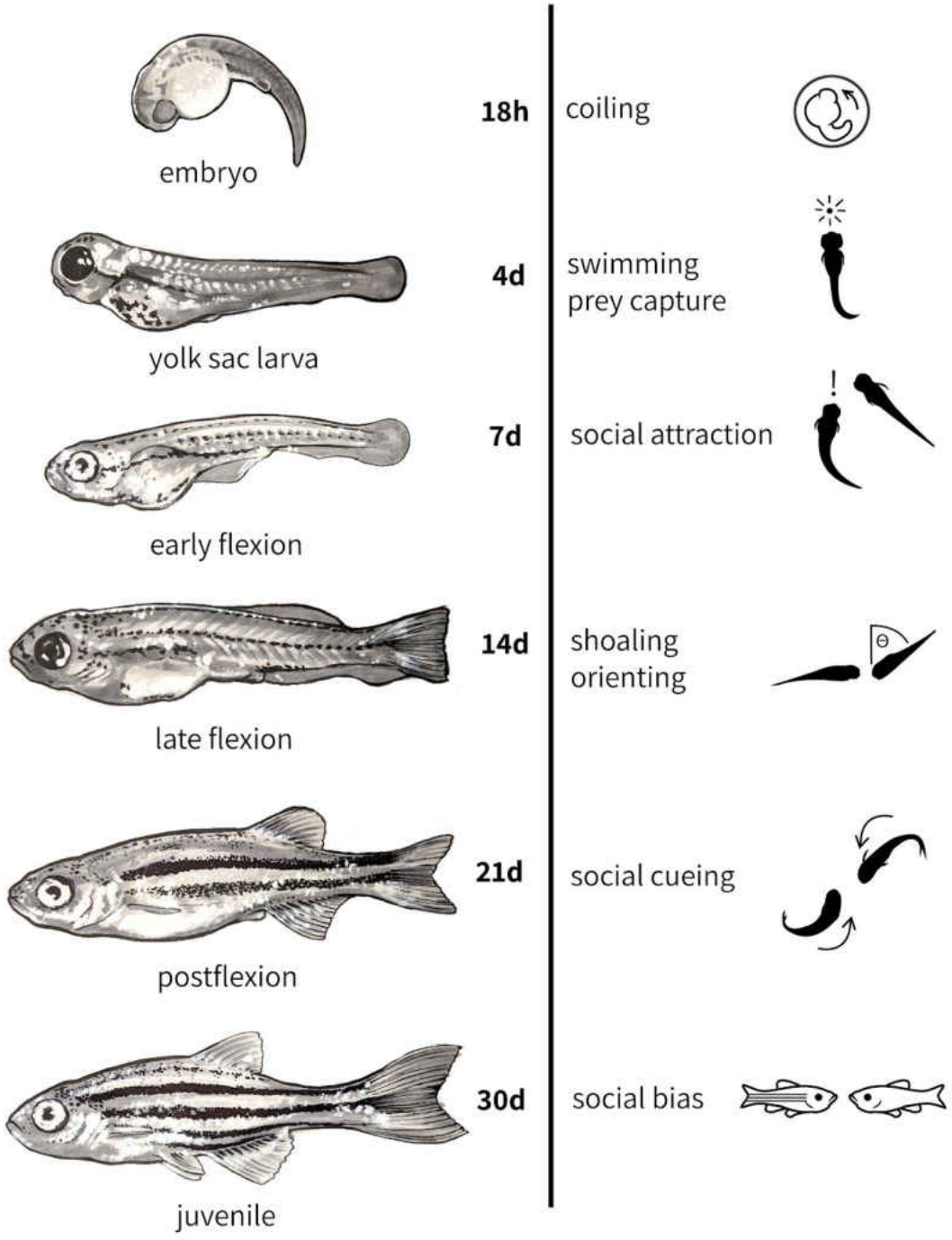

Fig 1.1 Morphological staging and approximate onset age of behaviors in the zebrafish.

ACKNOWLEDGEMENTS

We would like to thank Charles Kimmel, Judith Eisen, and Johannes Larsch for their extensive feedback and writing assistance.

CHAPTER II

ADULT BEHAVIOR & NEUROANATOMY

Forebrain control of behaviorally-driven social orienting in zebrafish

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SUMMARY

Deficits in social engagement are diagnostic of multiple neurodevelopmental disorders, including autism and schizophrenia [1]. Genetically tractable animal models like zebrafish (Danio rerio) could provide valuable insight into developmental factors underlying these social impairments, but this approach is predicated on the ability to accurately and reliably quantify subtle behavioral changes. Similarly, characterizing local molecular and morphological phenotypes requires knowledge of the neuroanatomical correlates of social behavior. We leveraged behavioral and genetic tools in zebrafish to both refine our understanding of social behavior and identify brain regions important for driving it. We characterized visual social interactions between pairs of adult zebrafish, and discovered that they perform a stereotyped orienting behavior that reflects social attention [2]. Furthermore, in pairs of fish, the orienting behavior of one individual is the

primary factor driving the same behavior in the other individual. We used manual and genetic lesions to investigate the forebrain contribution to this behavior and identified a population of neurons in the ventral telencephalon whose ablation suppresses social interactions, while sparing other locomotor and visual behaviors. These neurons are cholinergic and express the gene encoding the transcription factor Lhx8a, which is required for development of cholinergic neurons in the mouse forebrain [3]. The neuronal population identified in zebrafish lies in a region homologous to mammalian forebrain regions implicated in social behavior such as the lateral septum [4]. Our data suggest that an evolutionarily conserved population of neurons controls social orienting in zebrafish.

RESULTS & DISCUSSION

Social interactions in zebrafish

Adult zebrafish are highly motivated to aggregate, shoal, and school in both the wild and the laboratory [5]. Because of this natural sociability, zebrafish are an increasingly popular model for understanding the underlying genetic and developmental mechanisms that affect social behavior. When housed in pairs, zebrafish exhibit behaviors like parallel swimming and turning toward their partner [6]. Zebrafish are known to vary their behavior based on parameters of the social stimulus such as number, location, and velocity of conspecifics [7,8], but the extent to which these social interactions are driven by the behavior of the stimulus conspecific is largely undescribed. Existing metrics such as distance from conspecifics may be insensitive to disruptions in

more subtle components of social interactions, such as the importance of behavioral stimuli. To address this shortcoming, we designed a strictly visual assay to induce social behavior consisting of separate tanks divided by a panel of electrochromic film, which can be electronically switched from opaque to transparent (Figure 2.1A). We identified a stereotyped orienting pattern in adult fish, similar to behavior previously described in juveniles [9], that occurs when an individual fish is presented with a social stimulus, a fish in the neighboring tank (Figures 2.1B-C). We found that in these fish dyads, orienting between 45-90° relative to the electrochromic film divider transiently increases when the social stimulus is visible ($p < .001$, $n = 112$), returning to baseline within 5 min (Fig. 2.1D). These results suggest that zebrafish interact with a conspecific by orienting their body axis, and that in our assay they habituate to the social stimulus. Previous work suggests this behavior can reflect social attention in zebrafish, supporting the relevance of orienting in our assay [2,6]. Similarly, orienting metrics are highly correlated to average distance from the divider, a commonly used measure of social preference in other studies of fish social interactions (Fig 2.1E; $R^2 = .562$, $p < .001$, $n = 112$) [10].

To determine whether this behavior is explicitly social and driven specifically by conspecifics, we performed identical experiments using an empty tank or a novel object and found no similar transient increase in orienting behavior ($p = .367$, $n = 20$ and $p = .355$, $n = 20$ respectively, Fig 2.5A). Interestingly, when exposed to an empty tank stimulus zebrafish exhibit a statistically significant increase in preference for the side of the tank near the divider even in the absence of orienting ($p = .001$, $n = 20$, Fig 2.5B) such that there is an equal preference for both transparent sides, an effect not seen with a

novel object stimulus ($p = .948$, $n = 20$, Fig 2.5B). These results suggest that while zebrafish approach the divider in the absence of a social stimulus, orienting behavior requires an interaction between two fish. We conclude that orienting with an angle between 45-90° to the divider is a rigorous measure of social interaction that may reflect parallel swimming and orienting behavior in naturalistic settings [5,6,8,9].

We tested male-female pairs in two wild-type laboratory strains (ABxTU and WIK) and found they respond similarly to a social stimulus, suggesting that social orienting occurs across zebrafish strains (Fig 2.1F; $p = .997$, $n = 112$ and 16 respectively). We also found no differences in percentage of time in motion between strains (Figure 2.1G; $p = .393$). Measuring time spent between 45-90° allowed us to identify subtle differences between ABxTU dyads based on sex (Fig 2.5C), indicating that females are less likely to engage in social orienting than males regardless of the sex of the stimulus fish ($p = .014$, $n = 57$ and 55 respectively, Fig 2.5C). These findings replicate previous descriptions of sex differences in zebrafish social behavior [10,11]. There were no sex differences in the percentage of time spent in motion (Figure 2.5D; $p = .630$), suggesting that sex differences are specific to social engagement. In all subsequent experiments, we used ABxTU male-female dyads evenly distributed across experimental groups to equally represent both sexes in our dataset and to reduce potential confounds due to male-male aggression [11].

To determine if the orienting assay is sufficiently sensitive to detect impaired behaviors, we treated zebrafish with apomorphine (apo), a broad dopamine receptor agonist known to impair social interactions in mice [12]. Consistent with mammalian

studies, apo-treated fish had impaired social interactions and showed no significant increase in time spent at 45-90° when exposed to control fish (Fig 2.1F; $p = .260$, $n = 23$). We observed suppression in the percentage of time spent in motion for apo-treated fish, but this effect was not statistically significant (Fig 2.1G, $p = .635$). Interestingly, control fish paired with drug-treated stimulus fish (ctl > apo) also significantly reduced their orienting behavior and place preference, to the extent that they do not differ from the 'no stimulus' period. This effect was sustained over the 5 minute recording period (Figures 2.1C-D; Figure 2.1F; Figure 2.1E; $p = .516$, $n = 23$), suggesting that active social engagement of the partner is necessary for a fish to exhibit social orienting behavior. We observed suppression in the percentage of time spent in motion for apo-treated fish, but this effect was not statistically significant (Figure 2.1G, $p = .635$).

We probed whether social engagement is reduced because apo-treated fish spend more time distant from the divider. We placed apo-treated fish in a shortened tank that restricted their movement away from the divider, and found that test fish exposed to apo-treated stimulus fish still had suppressed social orienting relative to controls (Figures 2.2A-D; $p = .009$, $n = 26$). We conclude from these results that another socially-engaged fish is the stimulus required for social orienting, and that the presence of another fish that is not socially engaged is insufficient regardless of their proximity.

We further examined the role of behavior versus distance of the stimulus fish from the divider in driving social orienting. Orientation of the test fish and distance of the stimulus fish are highly correlated ($R^2 = .302$, $p < .001$), as is the orienting behavior of the stimulus fish ($R^2 = .458$, $p < .001$; Figures 2.2E-F). However, multiple linear

regression reveals that when both the orientation and distance of the stimulus fish are taken into account as predictive variables, only orienting behavior significantly accounts for variability in the test fish ($p < .001$ and $p = .178$ respectively, $n = 112$), suggesting that proximity of the stimulus fish exerts less influence than its orientation. There are no such relationships between the test and stimulus fish in the pre-stimulus period when they are not yet visible to one another (Figures 2.6A-B). We conclude that orienting behavior, and not proximity, of the stimulus fish is what drives orienting behavior in the test fish. We examined whether visual cueing between fish might account for the simultaneous orienting behavior of both stimulus and test fish. Time-lag cross correlation reveals that test fish mirror the stimulus fish by matching their angle, with a lag of about 1 second (Figure 2.6C).

To determine if this correlation was a spurious relationship and would occur regardless of the behavior of the stimulus fish, we performed a permutation analysis by shuffling data randomly such that results from each test fish were matched with results from a stimulus fish from a different dyad. We found that the correlation was lost and therefore directly reflects dynamic interactions between individuals orienting to one another (Figure 2.6C). These results suggest that zebrafish copy each other's motions, consistent with findings reported at earlier developmental stages [9]. In summary, our analysis of orienting behavior revealed that under normal conditions social interactions are reciprocated between fish, and that this effect is primarily driven by orienting behavior rather than absolute distance from the divider.

Neuroanatomical correlates of social behavior

The telencephalon, a region of the teleost brain proposed to be evolutionarily and functionally homologous to mammalian subcortical structures that regulate memory, emotion, and social behavior [13, 14], has been implicated in social behaviors in fish [15, 16, 17]. We investigated the role of the telencephalon in social orienting by manually lesioning it via insertion of a fluorescent dye-coated needle through the right nostril [18]. The needle pointed toward the brain midline, and was angled up or down to cause dorsal or ventral injury respectively (Figures 2.3A-B). Lesioned zebrafish were allowed to recover for one hour and then tested in our dyad assay against unlesioned controls. Injuries to the ventral telencephalon (vTel) significantly disrupted orienting behavior. In contrast, the behavior of dorsally lesioned animals was not significantly different from ABxTU controls (n = 16) from a separate experiment (Figures 2.3D-F; ventral: $p < .001$, n = 7; dorsal: $p = .076$, n = 9). To rule out the possibility that lesioned animals had locomotor deficits, we measured the percentage of time spent in motion and found that this parameter did not differ from controls (Figure 2.3G). We dissected a subset of brains (n = 7) and located the lesions using confocal microscopy (Figure 2.3B) [19].

We confirmed that severe social orienting deficits are most associated with injury to the ventral forebrain (Figure 2.3C). These deficits are accompanied by a disruption in the

correlation between distance from the divider and social orienting (Figure 2.3H; R^2 = .188, p = .243).

We further validated our finding that the vTel is important for teleost social behavior by using the GAL4/UAS system to chemo-genetically ablate different neuronal populations in the forebrain. We drove the expression of nitroreductase, a bacterial enzyme that is inert until exposed to its substrate metronidazole (MTZ), at which point it generates toxic metabolites. This paradigm provides a method for temporally-controlled ablation of discrete cell populations. We selected three transgenic lines with distinct expression patterns in the forebrain and elsewhere (Figure 2.4A-B), *y321* [ventromedial telencephalon and hindbrain, *Et(cfos:kGal4ff)y321;UAS:nfsB-mCherry*], *y299* [dorsal and anterior telencephalon, olfactory bulb, optic tectum, and hindbrain, *Et(cfos:kGal4ff)y299;UAS:nfsB-mCherry*], and *dlx* (ventrolateral telencephalon, optic tectum, ventral diencephalon and cerebellum, *dlx5a/6a:kalTA4;UAS:nfsB-mCherry*) [20]. We exposed each line to 10 mM MTZ by 24 hour bath application and subsequently tested behavior after washout. We confirmed the efficacy of this protocol by imaging cleared brains [19] (Figure 2.7A).

We found significant reductions in orienting behavior in y321 ablated zebrafish relative to drug-treated controls (Figures 2.4C-E, S3B; p = .006, n = 26), but not in y299 or dlx ablated animals (p = .995, n = 16 and p = .390, n = 16 respectively). Wild-type siblings exposed to MTZ did not differ from untreated transgenic or wild-type controls in any group (Figure 2.7B), ruling out drug effects. MTZ treatment significantly reduced percentage of time in motion relative to untreated controls in all conditions (Figure 2.4F;

$p < .001$), however no ablated animals differed from drug-treated control siblings during the stimulus period as determined by a post-hoc Tukey's test (*y321*: $p = .512$, *y299*: $p = .132$, *dlx*: $p = .299$). No changes in visually-mediated bias toward the transparent side of the tank during the no stimulus period were detected in any ablated or drug-treated animals (Figure 2.7C), indicating the behavioral deficits in y321 ablated zebrafish are specific to social orienting. We ruled out the possibility that the reduction in orienting is driven by the reduction in preference for the side of the tank adjacent to the divider in y321 ablated fish by analyzing only the percent time oriented when the animals occupy the 50% of the tank nearest the divider, and found y321 ablated fish still differed significantly from controls ($p = .004$). Similarly, the correlation between distance from the divider and orienting is disrupted in *y321* ablated fish such that there is no significant relationship between the two during the social stimulus period (Figure 2.7D; $R^2 = .131$, $p = .069$).

To further test how these cells might contribute to social orienting and how *y321* ablated animals respond to a non-social stimulus, we paired *y321* ablated zebrafish with apomorphine-treated stimulus fish. Ablated *y321* fish showed a nonsignificant reduction in orienting when presented with a suboptimal social stimulus, demonstrating the *y321* line may not capture the entire population of vTel cells downstream of visual, behavioral input (Figure 2.7E-F).

Although it is not possible in the current experiment to completely rule out the contribution of hindbrain neurons, we confirmed that there is a high degree of spatial

overlap between the hindbrain cells expressing in the *y321* and *y299* lines (Figure 2.7G), suggesting that vTel neurons likely play a more significant role.

To characterize the molecular identity of these vTel neurons, we performed immunohistochemistry and in situ hybridization on coronal sections of adult *y321:GAL4;UAS:GFP* zebrafish forebrains. The y321 enhancer trap insertion is located close to the lhx8a locus [20], which encodes a transcription factor associated with cholinergic neuron fate in the mouse forebrain [3]. In situ hybridization with probes to the gene encoding this transcription factor confirmed the transgenic population represents a subset of neurons expressing lhx8a (Figure 2.4G). We found that the overwhelming majority (97.6% +/- 2.58 on average, 625/640 total neurons across 4 brains) of GFP expressing cells were cholinergic (Figure 2.4H). Based on their anatomical location and gene expression patterns, these cells may be homologous to a population of lhx8-expressing, cholinergic basal forebrain neurons that is also found in mammals [3].

CONCLUSIONS

Our data show that social engagement in zebrafish requires a behavioral visual stimulus provided by another socially-engaged fish. We demonstrated that both pharmacological manipulation of dopaminergic systems and ablation of a portion of the ventral telencephalon produce predictable deficits in social behavior. Our results also provide evidence that an as yet uncharacterized population of cholinergic neurons in the ventral telencephalon are critical for social interactions in zebrafish. The ventral telencephalic region corresponds to mammalian forebrain regions, such as the lateral

septum, that have been implicated in social behavior [21,22], suggesting an evolutionarily conserved population of cells may drive social orienting in zebrafish and mammals. Given that the inputs to these cells are undescribed, these findings are promising for future studies into the visual circuitry required to drive social behavior.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.J.S. & P.W.; Methodology: S.J.S., E.M.M. & D.N.; Investigation: S.J.S., E.M.M. & D.N.; Software and Formal Analysis: S.J.S.; Resources: A.T.; Writing – Original Draft: S.J.S.; Writing – Review & Editing: S.J.S, A.T., J.S.E. & P.W.; Funding Acquisition: J.S.E. & P.W.; Supervision: P.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURES

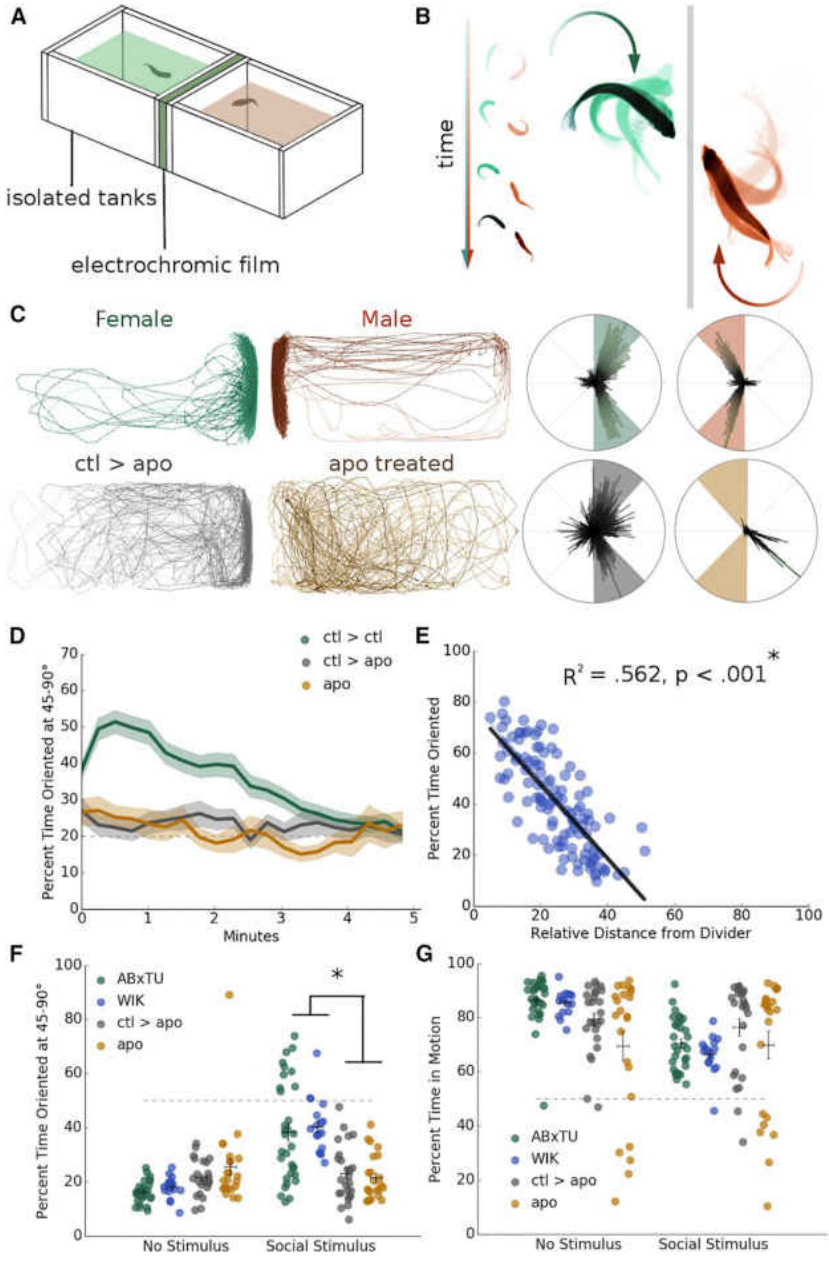

Fig 2.1 Automated analysis of social orienting in wild-type and drug-treated zebrafish.

A. Schematic of dyad assay apparatus, consisting of two isolated 7" (length) x 3.5" (width) x 2.5" (depth) tanks separated by a panel of electrochromic film.

B. Orienting behavior in a pair of isolated zebrafish.

C. Representative traces and polar histograms for a male and female ABxTU dyad and a control (ABxTU) animal (ctl>apo) paired with an impaired apomorphine-treated zebrafish (apo treated; apo). ABxTU animals paired with one another significantly increase their orienting behavior when exposed to another fish (*$p < .001$, $n = 112$) but not when paired with an apomorphine-treated fish that serves as a suboptimal social stimulus ($p = .516$, $n = 23$).

D. Percent time oriented over 5 minute period for zebrafish paired with a normal social stimulus (ctl > ctl), zebrafish paired with a suboptimal stimulus (ctl > apo), and zebrafish treated with apomorphine (apo).

E. Correlation between test fish's orienting behavior and relative distance from the divider. Relative distance is expressed in terms of minimum to maximum distance, 0-100%. Orienting behavior is significantly correlated with distance from the divider. $R^2 = .562$, *$p < .001$, $n = 112$ linear regression.

F. Average percent time oriented at 45-90° for male and female ABxTU, male and female WIK pairs, ctl>apo and apo zebrafish before and after presentation of a social stimulus. *$p < .05$, repeated measures mixed model ANOVA with post-hoc simple effects tests. Horizontal bars: mean, vertical bars: +/- s.e.m.

G. Percent time in motion for all groups before and during social stimulus presentation. Horizontal bars: mean, vertical bars: +/- s.e.m.

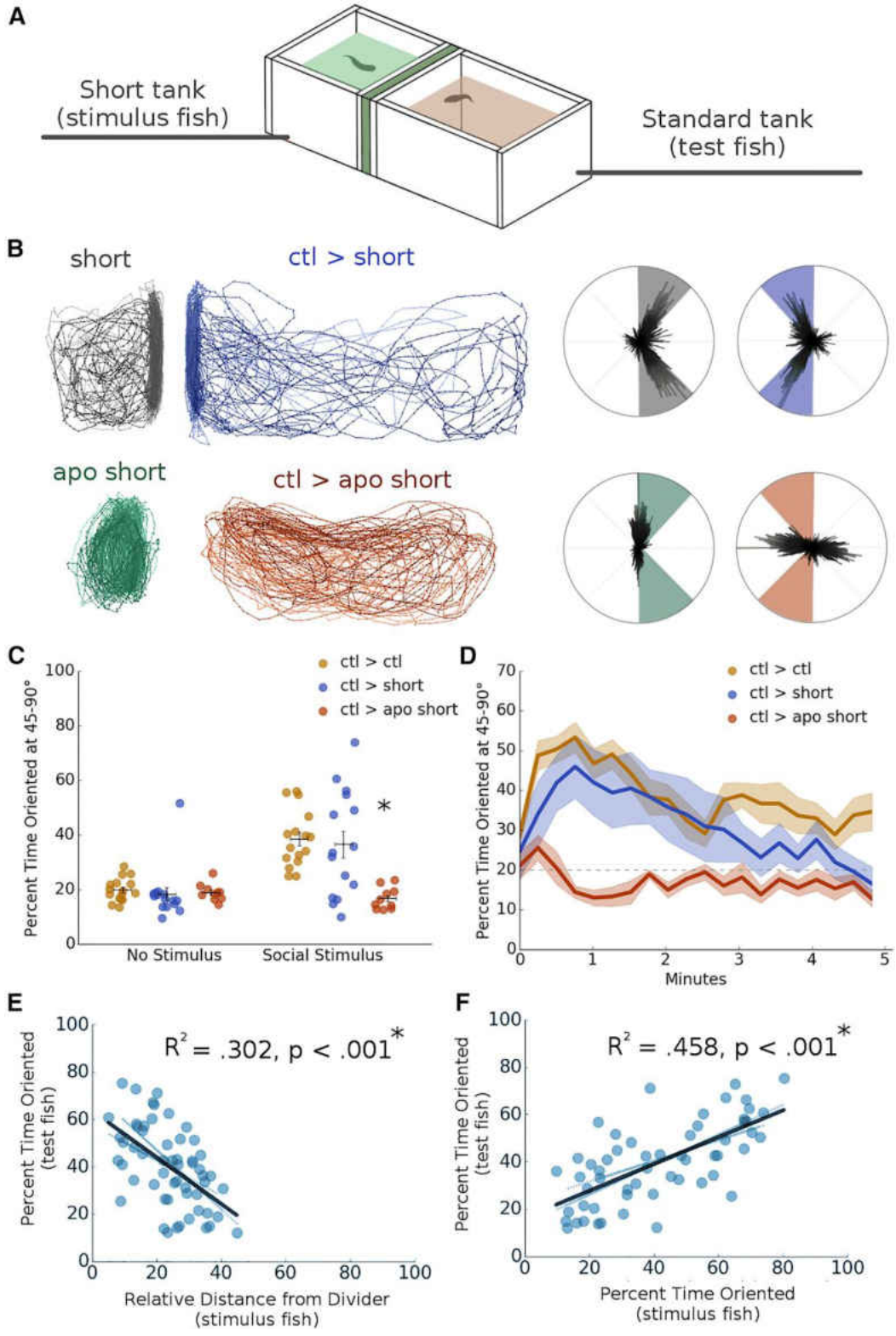

Fig 2.2. Behavioral feedback drives social orienting within a zebrafish dyad.

A. Schematic of short tank dyad assay apparatus, where the stimulus fish's tank is truncated to half size to restrict movement away from the divider.

B. Representative traces and polar histograms of control fish (ctl > short) and fish exposed to a sub-optimal social stimulus (ctl > apo short). Test fish exposed to a sub-optimal stimulus had significantly suppressed orienting behavior relative to controls (*p = .009, n = 26).

C. Average percent time oriented at 45-90° for short tank experiments before (no stimulus) and after (social stimulus) social stimulus presentation. *p < .05, repeated measures mixed model ANOVA with post-hoc simple effects tests. Horizontal bars: mean, vertical bars: +/- s.e.m.

D. Percent time oriented over 5 minute period for short tank experiments.

E. Correlation plot between the test fish's percent time oriented and the stimulus fish's relative distance from the divider (R^2 = .302, *p < .001, n = 112). *p < .05, linear regression.

F. Correlation plot between the test fish's percent time oriented and the stimulus fish's percent time oriented (R^2 = .458, p < .001, n = 112) *p < .05, linear regression.

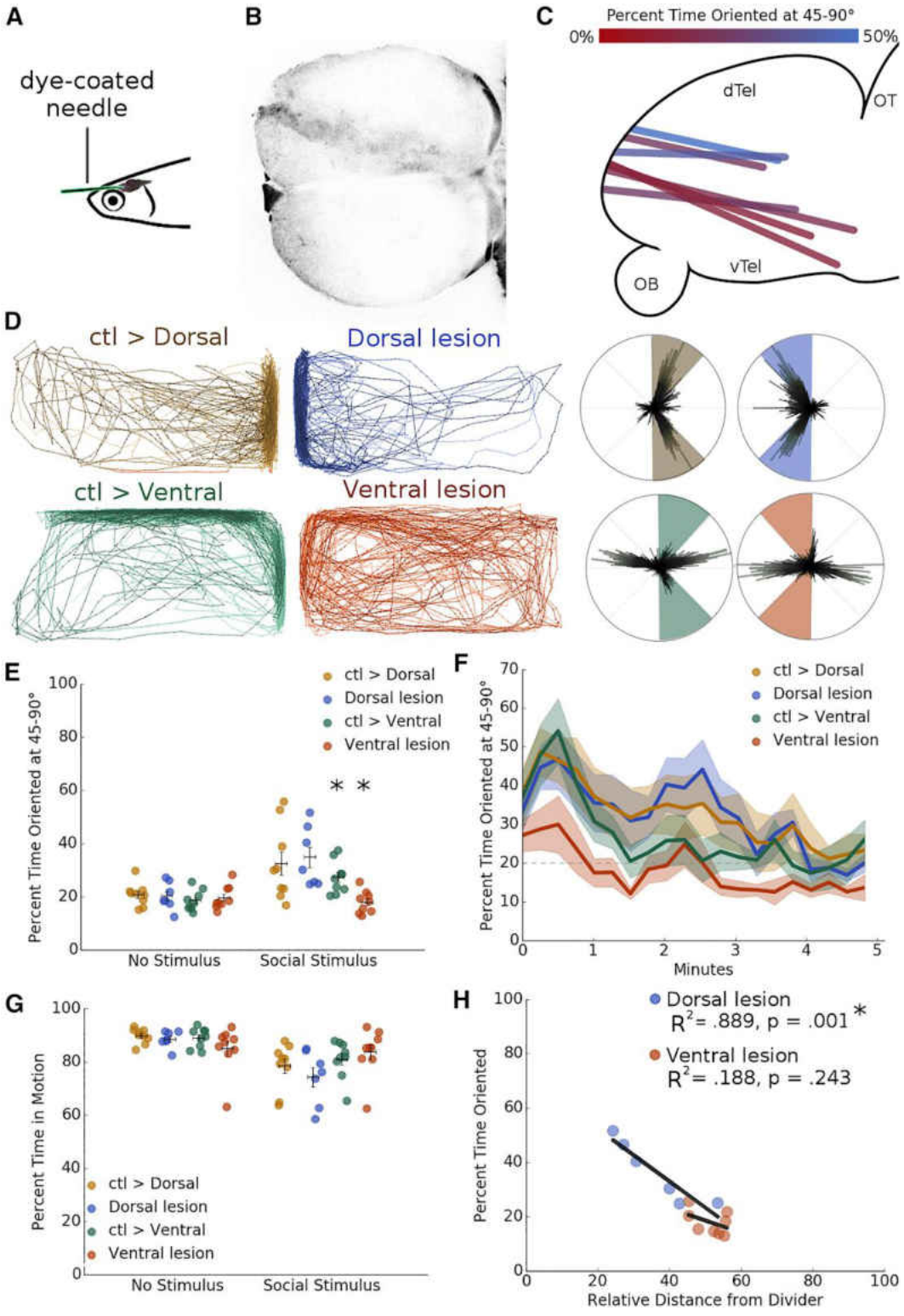

Fig 2.3. Ventral telencephalic lesions disrupt social orienting.

A. Schematic of lesion technique, where a dye-coated needle is inserted into the nostril of an anesthetized zebrafish to injure the forebrain.

B. Representative image of lesion track through forebrain (dorsal view).

C. Lesion tracks localized from a subset of zebrafish, color-coded to indicate severity of social deficit by location (sagittal view).

D. Representative traces of lesioned zebrafish and control stimulus fish. Dorsally lesioned zebrafish exhibit no social impairments relative to controls ($p = .974$, $n = 9$), however ventral injuries result in a severe reduction in both distance from the divider and orienting behavior (*$p = .007$, $n = 7$).

E. Average percent time oriented at 45-90° for lesion experiments before (no stimulus) and after (social stimulus) social stimulus presentation. *$p < .05$, repeated measures mixed model ANOVA with post-hoc simple effects tests. Horizontal bars: mean, vertical bars: +/- s.e.m.

F. Percent time oriented over 5 minute period for lesion experiments.

G. Percent time in motion for all lesion groups before and after social stimulus presentation. Horizontal bars: mean, vertical bars: +/- s.e.m.

H. Correlation between orienting behavior and relative distance from divider in dorsally and ventrally lesioned zebrafish. Dorsally lesioned fish retain a significant correlation between orienting and distance from the divider ($R^2 = .889$, *$p < .001$), but ventrally lesioned fish lose this relationship and more closely resemble the no stimulus period ($R^2 = .188$, $p = .243$). *$p < .05$, linear regression.

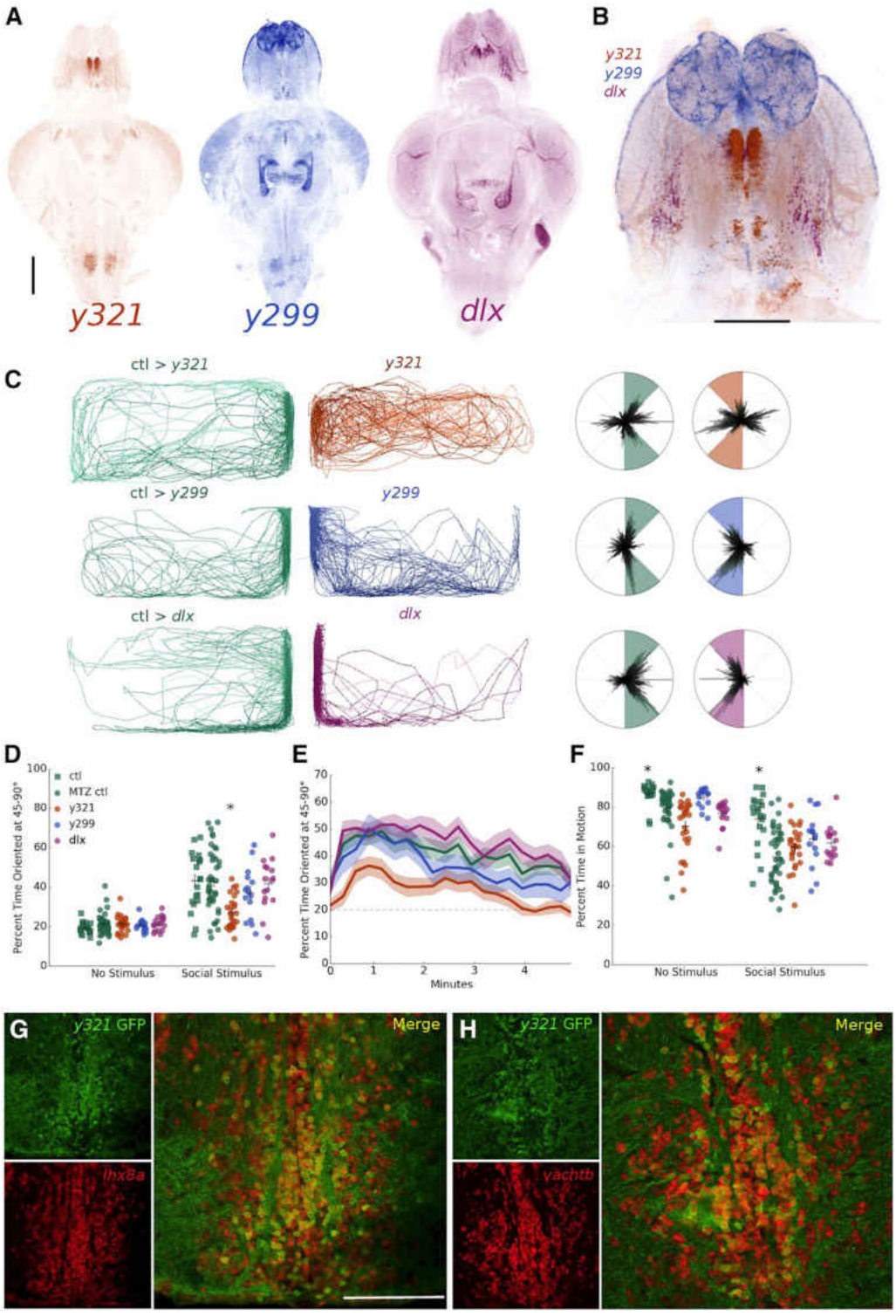

Fig 2.4. Chemo-genetic ablation of cholinergic neurons in the ventral telencephalon disrupts social orienting.

A. Whole-brain z projections of transgenic expression in y321, y299, and dlx gal4 lines. Scale bar: 200 μm.

B. Z-projection overlay of registered brains showing expression overlap and differences in the telencephalon. Scale bar: 200 μm.

C. Representative traces and polar histograms of y321, y299, and dlx lines following nitroreductase ablation of transgene-expressing cell populations.

D. Average percent time oriented at 45-90° for chemo-genetic ablation experiments before and after social stimulus presentation. *p < .05, repeated measures mixed model ANOVA with post-hoc simple effects tests. Horizontal bars: mean, vertical bars: +/- s.e.m.

E. Percent time oriented over 5 minute period for chemo-genetic ablation experiments.

F. Percent time in motion for all ablation groups before (no stimulus) and after (social stimulus) social stimulus presentation. *p < .05, repeated measures mixed model ANOVA with post-hoc simple effects tests. Horizontal bars: mean, vertical bars: +/- s.e.m.

G. *In situ* hybridization images of *y321:gal4;UAS:GFP* neurons labeled for lhx8a transcripts. Scale bar: 100 μm.

H. *In situ* hybridization images of *y321:gal4;UAS:GFP* neurons labeled for vachtb transcripts.

STAR METHODS

Contact for reagent & resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Philip Washbourne (pwash@uoregon.edu).

Experimental model and subject details

Zebrafish (ABxTU and WIK) were maintained according to standard protocols [24] at 28°C with a 14/10 light/dark cycle. All procedures were performed according to a protocol approved by the University of Oregon Animal Care and Use Committee (#15-33). Tg(dlx5a/6a:kalTA4) fish were generated by inserting the optimized Gal4 element kalTA4 (Distel et al., 2009) into a plasmid containing the dlx5a/6a intergenic regulatory elements (Yu et al., 2011; generous gift of Marc Ekker). The DNA construct was injected into 1-cell stage Tg(UAS:GFP) embryos. Transgenic fish recapitulate the expression pattern of the line ot1 (Tg[1.4dlx5a-dlx6a:GFP]).

Unless otherwise specified, all experiments consisted of male and female pairs of zebrafish from the ABxTU background evenly distributed across conditions. Animals were between 2-12 months for all experiments and test and stimulus fish were age-matched. Transgenic animals were screened for fluorescent protein expression at 2-5 days post fertilization.

Method details

Social behavior assay

Two custom acrylic tanks composed of ¼” panels measuring 3.5” (width) x 7” (length) x 2.5” (depth) separated by a divider (a panel of opaque electrochromic film encased in ⅛” thick acrylic sheets) were used. One long (7”) side of the tank was opaque white acrylic, while all other sides were transparent panels, allowing for an additional measure of visually-mediated bias to the transparent side of the tank within the same experiment. The behavior apparatus was illuminated from above using a daylight white LED panel measuring 18” x 24” (Environmental Lights, UTLP-18-24), and contrast enhanced using a panel of light-diffusing plastic as the tank lid. The behavior apparatus was located in a room heated to 28°C. Tanks were placed on a 1” thick sheet of clear acrylic and imaged from below with a Logitech HD Webcam C310 at 640x480 resolution. Zebrafish were placed individually into tanks with clean fish water at a depth of 2” and recorded for 5 minutes at 10fps (no stimulus stage). The electrochromic film was then switched on to become transparent and allow the individuals to view one another. In the case of non-social stimulus experiments, the adjacent tank was either empty or contained a novel object placed near the divider (a plastic yellow object approximately the same size as an adult zebrafish). Recordings were performed for an additional 5 minutes (social or control stimulus stage). Both average relative distance from the divider (0-100%) and percentage of time oriented between 45-90° were used as dependent measures. Percentage of time spent in motion (as defined by moving a

minimum of ⅓ the fish's body length from one frame to another) and x,y location in the tank was also computed for each frame.

Electronics control

Data acquisition and electronics control were achieved with a combination of Python 2.7 and an Arduino Uno R3 microcontroller. A Python script captured individual frames from a Logitech HD Webcam C310 camera for all behavior experiments. Electrochromic film (Justin Cary, CaryShop) was switched on by a Python script communicating via USB with an Arduino Uno R3 microcontroller, using a DC 12V relay module (SainSmart) and a 12-60V inverter.

Apomorphine treatment

ABxTU zebrafish (ages 2-12 months) were incubated in a 20 μM solution of apomorphine (R-(-)-Apomorphine hydrochloride hemihydrate, CAS 41372-20-7, Sigma) prepared in fish water for ten minutes. They were then netted to a rinse solution of clean fish water, allowed to recover for approximately one minute, and transferred again into fresh fish water and tested in our social behavior assay.

Manual lesions

Adult ABxTU zebrafish (6-12 months) were anesthetized in 4 mg/mL MS-222, then placed into a slit cut in a sponge soaked in MS-222. A 27½ G needle coated in DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Invitrogen) was inserted 1 mm into the right nostril and pointed toward the brain midline. The needle was angled upward or downward to cause dorsal or ventral injury respectively. Following injury, zebrafish were returned to fresh fish water and allowed to recover for 1 hour

before behavioral analysis. Animals were monitored for signs of discomfort or distress as per our IACUC protocol. A subset of zebrafish were euthanized by hypothermal shock and processed using our modified CUBIC protocol [20].

Chemo-genetic ablation

Transgenic zebrafish (ages 2-3 months) expressing UAS:nfsB-mCherry were incubated in 10mM metronidazole (MTZ) prepared in fish water in groups of 20 overnight in the dark. They were then transferred to fresh fish water and allowed to recover for 1 hour before behavioral testing. Zebrafish with the genotypes *y321:GAL4;UAS:nfsB-mCherry, y299:GAL4;UAS:nfsB-mCherry and dlx5/6:KaltA;UAS:nfsB-mCherry* maintained in an ABxTU background were chemo-genetically ablated. For all experiments, three control groups consisting of clutchmates were included to control for effects of genotype and drug exposure: wild-type MTZ-, wild-type MTZ+, and transgenic MTZ-. No chemogenetically ablated animals exhibited signs of discomfort or distress as described by our IACUC protocol.

Brain clearing & imaging

We utilized a modified (one-step) CUBIC protocol [20] to clear transgenic and lesioned brains for imaging. Zebrafish were anesthetized and euthanized in ice water, immediately decapitated, and their heads fixed in 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS) at room temperature. Brains were then dissected and fixed overnight in 4% PFA at room temperature. After rinsing in 1X PBS, brains were transferred to CUBIC 1 solution (25 wt% of 80 wt% Quadrol, 25 wt% urea, 15 wt% Triton X-100 in dH2O) [20] and kept in a 37°C water bath for 2-3 days until transparent.

Brains were removed from CUBIC 1 reagent and immediately mounted in either 1.5% low-melt agarose (lesions) or ProLong Gold antifade mounting medium (Invitrogen, transgenics).

Cleared brains were imaged by fluorescence confocal microscopy with a Leica DMI8-CS and a 10x objective by tiling multiple z stacks using Leica LAS X 2.0.0.14332.2 software. Overlays of multiple GAL4 lines were generated via nonrigid registration using the Computational Morphometry Toolkit (CMTK) [21,25].

***In-situ* hybridization**

Adult *y321:GAL4;UAS:GFP* zebrafish (age 2-12 months) were anesthetized and euthanized in ice water, then decapitated and placed into 4% PFA for 1-1.5 hours before brains were dissected and fixed overnight in 4% PFA at room temperature. After fixation, brains were rinsed 3x in PBS and dehydrated in 20% sucrose in PBS for 24 hours, followed by cryosection after mounting in agarose.

RNA *in situ* hybridization on 16 μm brain sections was carried out according to the protocol by J. Talbot [26] using digoxigenin labeled probes for either *lhx8* or *vachtb*. Sections were incubated overnight at 65°C with 200ng of probe per section. The following day, sections were washed in a graded concentration series of 5X saline sodium citrate (SCC) with 50% formamide/2X SSC ending in an incubation step with anti-digoxygenin Fab fragments (Roche) overnight at 4°C. Sections were washed 8x in 1M Tris HCl pH 7.5, 5M NaCl and 0.25% Tween 20 (TNT) and incubated for 5 minutes in the dark with amplification diluent, then incubated for 1 hour at room temperature in the dark with cyanine 3 (Cy3) for subsequent visualization. Endogenous peroxidase

activity was quenched by incubation in 2% hydrogen peroxide in TNT for 1 hour. Sections were first incubated in primary antibody (chicken anti-GFP, 1:500, Aves Laboratories) and then secondary antibody (goat anti-chicken IgY-488, 1:500, Molecular Probes) overnight in 0.25% PBSTx. Sections were imaged on a Leica DMI8-CS confocal fluorescence microscope using a 40x objective.

Quantification and statistical analysis

Video analysis

Behavioral data were analyzed using bespoke software written in Python 2.7 (DaniOPEN, https://github.com/stednitzs/daniopen). This software tracks the center of mass of fish and calculates their orientation for each frame, generating text files of these data for further analysis. Where necessary, images were pre-processed using ImageJ (NIH) [27] to subtract the background and enhance detection accuracy.

Cell quantification

Co-expression of GFP and lhx8 or vachtb was quantified using ImageJ (NIH) [27]. GFP expressing cells were manually identified and the number of GFP+ neurons that also expressed lhx8 or vachtb were counted.

Statistics

For all experiments, data were screened for normality using descriptive statistics of skewness and kurtosis. A p value $< .05$ was considered significant. Outliers were not removed from any experimental groups. Control zebrafish that exhibited excessive freezing behavior (as determined by spending less than 10% of the duration of the experiment in motion) were removed prior to analysis (6 out of 430 fish across all

experiments, 2 female ABxTU dyads and 1 male ABxTU dyad). No lesioned, ablated, or drug-treated animals were excluded from our analyses.

For social behavior experiments, analysis was performed in IBM SPSS Statistics 24 via repeated measures mixed-model ANOVA, comparing the no stimulus and post-stimulus period by group. In the event of a significant time by group interaction effect, main effects between groups were analyzed by post-hoc simple effects tests using the Bonferroni correction for multiple comparisons. Linear regression analyses were also performed in SPSS 24.

Cross-correlations were computed in Python using a time lag window of +/- 5 seconds (50 frames).

Data and software availability

Behavioral data were analyzed using bespoke software (DaniOPEN) written in Python 2.7, available from github (https://github.com/stednitzs/daniopen).

Behavioral data are archived via Mendeley (http://dx.doi.org/10.17632/d79p9sttkn.1)

SUPPLEMENTAL INFORMATION

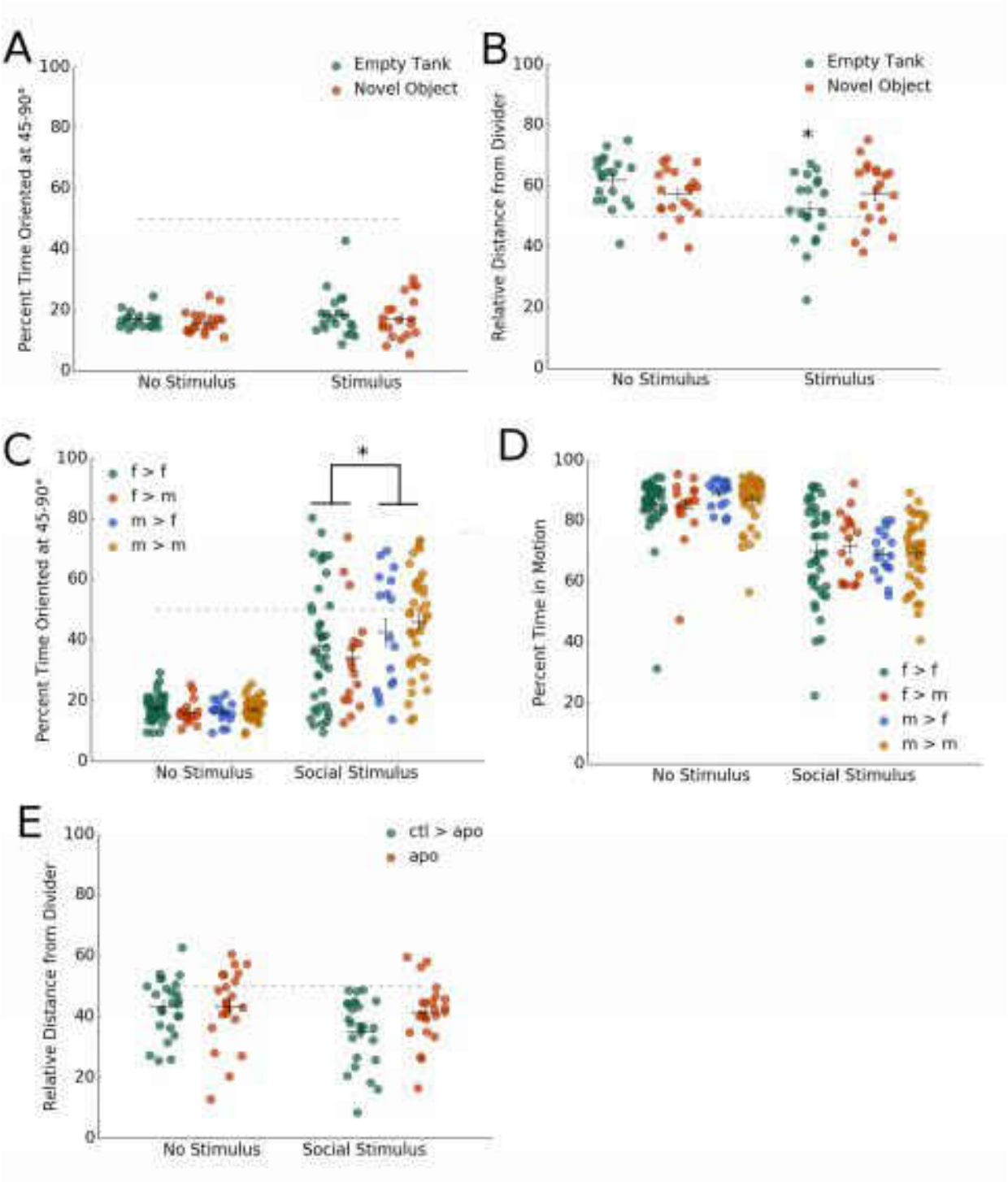

Figure 2.5. Non-social controls and sex effects on social orienting.

Related to Figure 2.1.

A. Percent time oriented at 45-90° over 5 minutes for zebrafish exposed to either an empty tank or a novel object. Horizontal bars: mean, vertical bars: +/- s.e.m.

B. Average relative distance from the divider over 5 minutes for zebrafish exposed to either an empty tank or a novel object. Zebrafish exposed to an empty tank stimulus significantly decrease their distance to the divider when the empty tank is visible, *p = .001. *p < .05, repeated measures mixed model ANOVA, n = 20 per group.

C. Percent time oriented at 45-90° over 5 minutes for all possible sex pairings of ABxTU zebrafish. *p < .05, mixed model repeated measures ANOVA with post-hoc simple effects test.

D. Percent time in motion over 5 minutes for all possible sex pairings of ABxTU zebrafish. *p < .05, mixed model repeated measures ANOVA with post-hoc simple effects test.

E. Average relative distance from the divider over 5 minutes for apomorphine (apo) teated zebrafish and control fish exposed to apo treated partner.

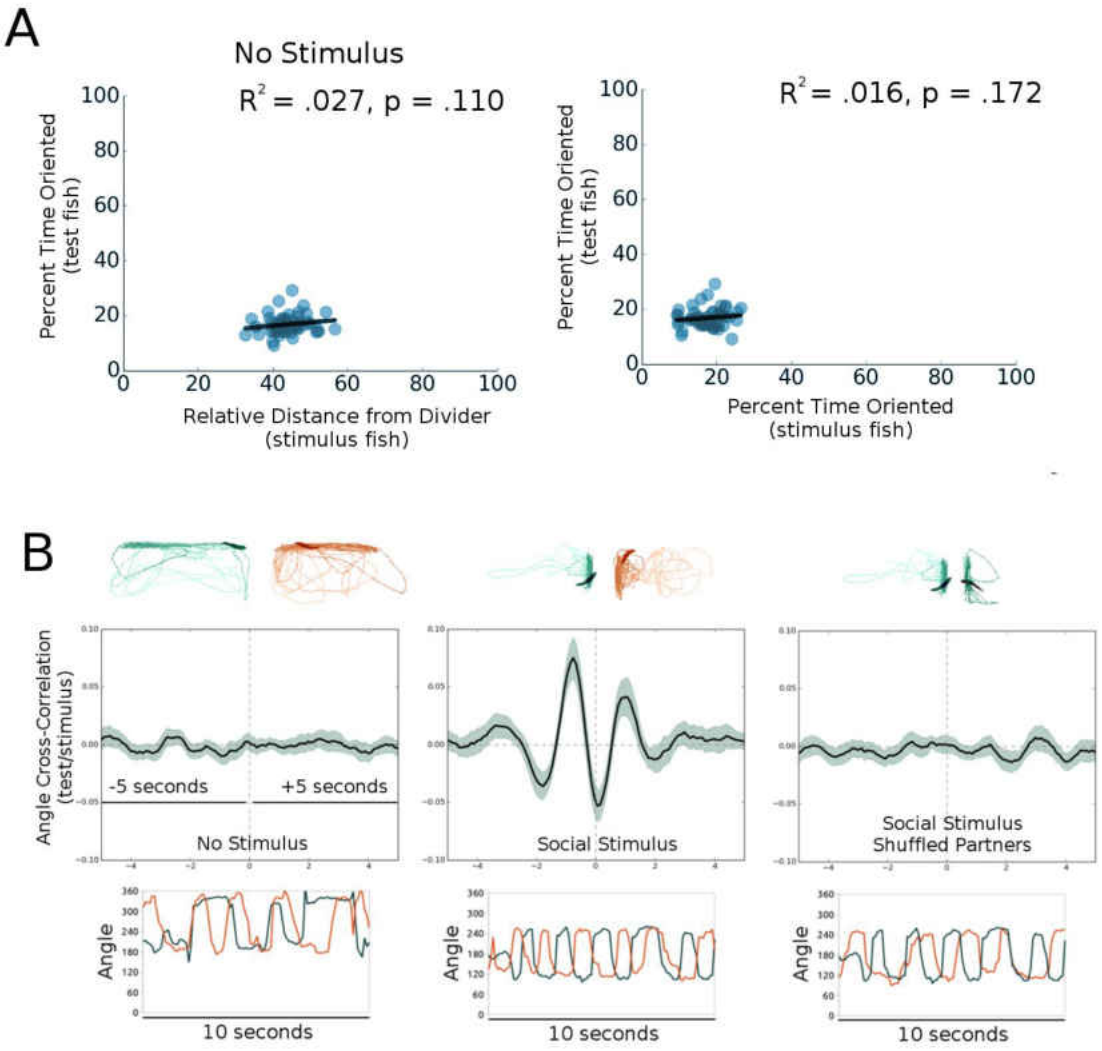

Fig 2.6. The effects of mirroring and distance during zebrafish social orienting.

Related to Figure 2.2.

A. Correlation plots showing no relationship between orienting behavior of the test fish and relative distance/orienting behavior of the stimulus fish in the pre-stimulus period. *p < .05, linear regression, n = 112.

B. Representative traces, time-lag cross correlations, and sample angle plots over time showing high correlation only in the social stimulus condition where zebrafish are matched to the correct partner, n = 112.

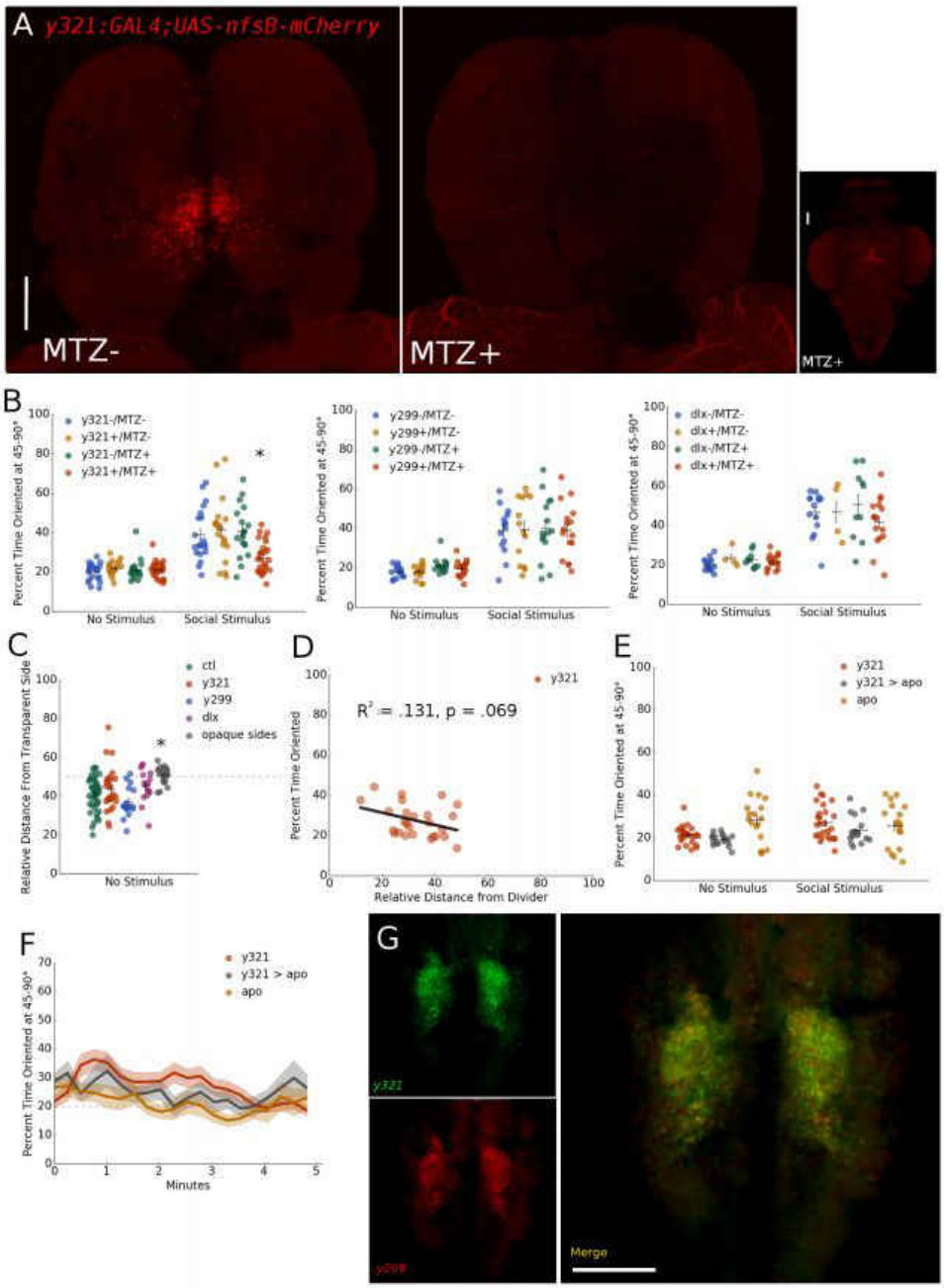

Figure 2.7. Extended data on chemo-genetic ablation screen for social behavior deficits. Related to Figure 2.4.

A. Cleared brain showing the effects of MTZ exposure on nfsB-mCherry expressing neurons in *y321:GAL4;UAS-nfsB-mCherry* transgenic zebrafish. Right: whole brain of *y321* ablated zebrafish showing loss of entire expression pattern. Scale bars: 250 µm.

B. Control data for percent time orienting for all fish in the genetic ablation screen with all four possible transgene/drug exposure combinations within clutchmates.
C. Relative visually-mediated bias toward the transparent wall of the tank in the pre-stimulus condition for all groups. Also included are data obtained in the same apparatus with opaque sides where zebrafish have no side preference. *$p < .05$, ANOVA with post-hoc Tukey's-b test for homogenous subsets.
D. Correlation between orienting behavior and relative distance from divider in y321 ablated zebrafish, illustrating a loss of the relationship between place preference and orienting behavior ($R^2 = .131$, $p = .069$), linear regression.
E. Orienting behavior of *y321* ablated fish paired with control animals (*y321*), *y321* ablated fish paired with apomorphine treated animals (y321 > apo), and apomorphine treated animals paired with *y321* ablated fish (apo).
F. Orienting behavior of *y321, y321* > apo, and apo zebrafish over time.
G. Comparison of transgene expression in the hindbrain of *y321* and *y299* lines, demonstrating spatial overlap. Scale bar: 250 μm.

CHAPTER III

DEVELOPMENT

Rapid, Progressive Development of Zebrafish Social Behavior is Predicted by Length

Sarah J Stednitz & Philip Washbourne. Manuscript in preparation.

INTRODUCTION

Zebrafish (*Danio rerio*) are highly social animals that engage in a diverse variety of non-reproductive social behaviors. An increasingly extensive literature describes many of these behaviors in depth (Engeszer, Patterson, Rao, & Parichy, 2007; Orger & de Polavieja, 2017; Suriyampola et al., 2016). The experimental tractability and the genetic similarity between zebrafish and humans renders zebrafish an attractive model for neurodevelopmental disorders that affect social interactions (Howe et al., 2013). Social behaviors in the zebrafish share many similarities with other vertebrates, including mammals, and some of the brain regions driving these behaviors may be evolutionarily conserved (Mueller, Dong, Berberoglu, & Guo, 2011; Nieuwenhuys, 2011; O'Connell & Hofmann, 2012; Shinozuka & Watanabe, 2004; Teles, Cardoso, & Oliveira, 2016). Attraction towards conspecifics is proposed to begin as early as 7 days post fertilization (dpf) and interactions rapidly increase in complexity, however how they eventually develop into orienting routines, reciprocal interactions driven by social cues, and

experience-dependent preferences for shoalmates with specific visual characteristics had not yet been investigated (Abril-de-Abreu, Cruz, & Oliveira, 2015; Dreosti, Lopes, Kampff, & Wilson, 2015; Engeszer, Da Barbiano, Ryan, & Parichy, 2007; Hinz & de Polavieja, 2017; Larsch & Baier, 2018; Stednitz et al., 2018)

A full description of social development in zebrafish will be useful for understanding how early genetic and environmental perturbations affect behavioral outcomes (Buske & Gerlai, 2011a). Several groups have studied social ontogeny, or the developmental stages at which zebrafish begin to reliably engage in these behaviors. By 14 dpf, zebrafish begin to exhibit features of adult social interactions and these are robust by approximately 21 dpf. These findings are consistent across different experimental paradigms, including open field contexts where fish are able to interact freely, physically separated animals where the social stimulus is purely visual, and virtual stimuli that mimic biological motion (Buske & Gerlai, 2011b; Dreosti et al., 2015; Hinz & de Polavieja, 2017; Larsch & Baier, 2018).

Considerable behavioral variability is observed at these early stages. Further, comparisons across these studies are potentially complicated by inconsistencies in developmental staging criteria, as chronological age may not be a reliable reflection of true developmental state - are morphological features more predictive of individual behavior than age? Similarly, there are often considerable chronological gaps between when these measurements are made - is social ontogeny a continuous process, or one that occurs very rapidly over developmental time?

To address these questions, we adapted our assay for social orienting and cueing in the zebrafish and used it to probe a narrow chronological window that we expected to be relevant given the findings of other groups. Additionally, we performed measurements of body length to understand if this morphological feature is predictive of individual sociality (Parichy, Elizondo, Mills, Gordon, & Engeszer, 2009). Finally, we investigated the effect of early nutrition by comparing social ontogeny in zebrafish reared with different feeding practices (Carvalho, Araujo, & Santos, 2006).

MATERIALS & METHODS

FISH HUSBANDRY

ABxTU strain zebrafish were maintained in standard conditions as described in the Zebrafish Book, on a 14 hour light cycle (Westerfield, 2000). Unless otherwise noted, zebrafish were introduced to food at 4 dpf and fed rotifers 3 times daily. All procedures carried out in this study were approved by the University of Oregon Institutional Animal Care and Use Committee.

SOCIAL BEHAVIOR

Socially-motivated place preference and orienting behavior of larval zebrafish was measured using a modified version of our dyad assay for juveniles and adults (Stednitz et al., 2018). Zebrafish are placed in isolated tanks (50mm length x 20mm width x 20mm depth) separated by an opaque divider and allowed to habituate for 5

minutes, then the divider is removed and the animals are allowed to interact for an additional 5 minutes. In a subset of animals pre-stimulus periods were recorded in addition the social stimulus period to determine the baseline exploratory behavior in the tanks. Recordings were obtained from below at 10 fps using a Mightex SME-B050-U camera and illuminated by an overhead white LED panel (Environmental Lights). Fish that spent less than 10% of the experiment in motion (as defined by moving at least ⅓ of their total body length per frame) were not included in subsequent analyses. Frames where the animal was not effectively segregated from the background were also discarded. Altogether, 33 experiments were not included out of 407 total.

Shoaling is parameterized as the average relative distance from the divider and the percentage of time spent at 45-90° using our previously described software written in Python (Fig 3.1; available from https://github.com/stednitzs/daniopen). Refinement of orienting behavior was measured using vector strength in a subset of animals, where no frames were discarded due to detection errors. Polar histogram plots were collapsed about the 180° axis to generate “calzone” plots, and vector strength calculated relative to 45° for each animal using Python (Fig 3.2A-C). Social cueing was quantified using time-lag cross correlation in Python for a subset of zebrafish dyads where no frames were discarded due to detection errors in either fish. Latency was calculated for each dyad by measuring the distance from time 0 to the peak correlation (Fig 3.2D-F).

MORPHOLOGICAL FEATURES

Following behavioral experiments, individual zebrafish were anesthetized in MS-222 and imaged on a stereomicroscope (Leica M205 FA). Animals were imaged alive, as the fixation process was expected to alter the length of the animal. We measured the standard length as described by (Parichy et al., 2009), from the tip of the nose to the most posterior end of the body, excluding the fins (Fig 3.3A).

NUTRITION

We investigated the role of nutrition, a major factor known to influence larval development. We measured social behavior and standard length as previously described, but instead reared zebrafish on GemmaMicro dry feed, feeding three times daily as performed in Carvalho et al., 2006. All other conditions (feeding schedule, light cycle, water quality, temperature) remained constant.

STATISTICS

Statistical analyses were performed in Python or SPSS version 24. A one-way Analysis of Variance was used to compare different ages with Tukey's B post hoc tests to correct for multiple comparisons. Correlation analyses were performed using linear regression.

RESULTS

SOCIAL BEHAVIOR

We measured a number of distinct parameters of social behavior in zebrafish between 10-30 dpf, which encompasses stages prior to the flexion (or dorsal bending) of the notochord through to the juvenile stage (Fig 3.4). Spatial preference refers to an animal's tendency to prefer the side of the tank where conspecifics are visible. Orienting behavior was measured by calculating the head angle for every frame. Finally, we measured social cueing by measuring the extent to which orienting turns in one fish influenced orienting turns in the partner fish using cross-correlation analysis.

We observed no differences in orienting behavior by age in the pre-stimulus period, before the divider was removed and conspecifics were not visible (Fig 3.5C). In contrast, spatial preferences differed among ages such that younger larvae were somewhat more likely to be located adjacent to the opaque divider, suggesting that orienting and spatial preference are distinct (Fig 3.5B).

At 10 dpf, we found that preflexion larvae were largely asocial and did not have increased spatial preference or orienting behavior in the social stimulus phase of the experiment relative to the pre-stimulus period ($p = .581$). Spatial preference for the side of the tank adjacent to conspecifics increased at 12 dpf relative to 10 dpf, such that 12 dpf zebrafish began to exhibit interest in conspecifics and were more likely to be found adjacent to the divider when a social stimulus was visible compared to 10 dpf larvae ($p =$

.021; Fig 3.1A). Spatial preference plateaued at 12 dpf and remained constant through the juvenile stage (Fig 3.1B).

Orienting behavior was weakly, although significantly, correlated to chronological age ($R2 = .047$, $p < .001$), and at 14 dpf larvae began to more reliably exhibit orienting compared to 12 dpf (Fig 3.1C). By 16 dpf the percentage of time spent at 45-90° was statistically indistinguishable from that of 30 dpf postflexion larvae ($p = .709$, Fig 3.1C). The progression of orienting behavior was gradual, such that two homogenous subsets can be identified through post-hoc tests: pre- and early flexion (10, 12 and 14 dpf) and late flexion/juvenile stages (14, 16, and 30 dpf; Fig 3.1C). These results indicate that the social behaviors measured by our dyad assay develop rapidly.

Interestingly, we observed a refinement of the stereotyped 45-90° orienting behavior we previously described in adults over this time scale (Stednitz et al., 2018). While the preferred angle was consistently within this window, variability decreased over time (Fig 3.2A). We quantified the refinement of social orienting by calculating the vector strength at this characteristic angle across ages (Fig 3.2B). We found that vector strength increased significantly at each time point measured until 14 dpf, and remained constant thereafter (Fig 3.2C). These results suggest that orienting behavior observed in juveniles and breeding adults can be fully established as early as 14 dpf.

Next, we probed social cueing across chronological age by measuring time lag cross-correlation of orientation between social partners. Juvenile and adult zebrafish mirror one another's orienting behavior such that the turn of one animal elicits a corresponding turn in the other in less than one second (average 0.73s; Fig 3.2D). We

applied the same analysis across developmental time and found that the latency to peak correlation decreased and the time lag between turns decreased (Fig 3.2E). Notably, these turning events had a greater latency in 14 dpf larvae relative to 16 dpf (p=.067) and 30 dpf juveniles (p=.005), but by 16 dpf zebrafish exhibit a similar average latency to juveniles (p=.284; Fig 3.2F). While the speed of animals increased such that average distance traveled per frame increased by age ($p < .001$), speed was not significantly correlated to latency and did not account for the decreased response time ($R2 = .055$, $p = .123$). These results suggest that zebrafish larvae actively attend to the behavior of conspecifics by 16 dpf.

MORPHOLOGICAL FEATURES

Chronological age influenced overall standard body length (as measured from the front of the face to the end of the tail, excluding fins; Fig 3.3A), and we report an increase in standard body length from 10-20 dpf ($R2 = .229$, $p < .001$; Fig 3.3B). Standard length was weakly predictive of orienting behavior ($R2 = .067$, $p <.001$; Fig 3.3C). However, when each age group was considered separately, length was only predictive at 14 and 16 dpf ($R2 = .050$ and .134, $p < .012$ and 009, respectively). The effect is greatest at 16 dpf, indicating that size is most related to sociality at the late flexion stage. When these data were analyzed using multiple regression with both age and standard length as predictors of orienting behavior, length was still significantly related but age is not ($p = .002$ and $p = .113$ respectively), suggesting that size (and therefore

actual developmental stage) is at least partially responsible for driving the effect of chronological age.

Considerable variability in developmental features and behavior between labs could be due to differences in rearing practices. To further explore the relationship between standard length, age, and social behavior, we raised a cohort of larvae to 14 dpf using a dry food regimen that impairs the rate of growth relative to live food (n = 22). We observed social deficits in the dry food cohort relative to our larvae reared on live food ($p = .002$; Fig 3.3D), and reduced standard lengths ($p < .001$; Fig 3.3E). Considering the entire 14 dpf dataset, length remained significantly correlated to orienting behavior ($R2 = .131$, $p < .001$). These findings suggest that standard length has superior predictive power than chronological age, especially when considered across multiple nutrition regimens

DISCUSSION

Zebrafish rapidly acquire complex social behaviors between 10-16 dpf. Further, this is related to standard length on an individual basis beginning at 14 dpf. This highly variable and early stage in development represents an opportunity to further understand how genetic and environmental factors affect the assembly of the neural circuits underlying complex behaviors.

Spatial preference for conspecifics is the first social behavior observed in our assay, occurring at 12 dpf. Orienting behavior is exhibited at 14 dpf, which gradually increases in precision. Zebrafish begin attending to cues from conspecifics by 14 dpf, and they respond more quickly to these cues by 16 dpf. Altogether, these findings suggest a

sequential acquisition of progressively more complex social behaviors over a rapid timescale (Fig 3.4).

Standard length of larval zebrafish is predictive of individual variability in social orienting behaviors, concurring with previous work showing other developmental features like fin morphology and pigment formation is predicted by length (Parichy et al., 2009). Interestingly, this effect of standard length only occurs at 14 dpf and beyond, suggesting a critical period before which orienting is unlikely to occur. Similarly, when zebrafish were developmentally delayed by a nutritionally restricted diet, both their size and social behavior were impaired. In light of these results, we propose that standard length should be reported in conjunction with chronological age in behavioral studies of early and late flexion larvae.

The neuronal mechanisms behind these rapid behavioral changes remain an outstanding question. While zebrafish can detect and pursue small prey by 4 dpf (Patterson, Abraham, MacIver, & McLean, 2013), preflexion larvae do not respond to biological motion, a complex and salient visual feature of social behavior (Larsch & Baier, 2018). The receptive field size in the optic tectum is reported to decrease between 14-20 dpf, providing a potential neuronal mechanism for refined visual behaviors during this time period (Bergmann et al., 2018). Specific visual social cues influence social preferences in the adult, such as local features of the head, direction of motion, and pigmentation patterns (Engeszer, Ryan, & Parichy, 2004; Neri, 2012), but the developmental progression of these effects are as yet unexplored.

Previous work shows that developmental perturbations through chemical insults, social isolation, or genetic mutations can have profound effects on the social behavior of animals later in life, including zebrafish (Baronio et al., 2018; Buske & Gerlai, 2011a; Liu et al., 2018; Shams, Amlani, Buske, Chatterjee, & Gerlai, 2018; Weber & Ghorai, 2013; Zimmermann, Gaspary, Leite, De Paula Cognato, & Bonan, 2015). The refinement of sensorimotor processing during critical periods may be governing the rapid changes in social behavior. Given our increasing knowledge about the neuroanatomical correlates of social behavior in zebrafish, 12-16 dpf is a promising time period to investigate how these circuits may be affected by developmental disturbances known to influence neurodevelopmental disorders in humans.

ACKNOWLEDGMENTS

The authors would like to thank Kip Keller and Avinash Singh Bala for advice on data analysis. We would also like to thank Adam Christensen, John Dowd, and the University of Oregon Aquatic Animal Care Services for fish husbandry.

FIGURES

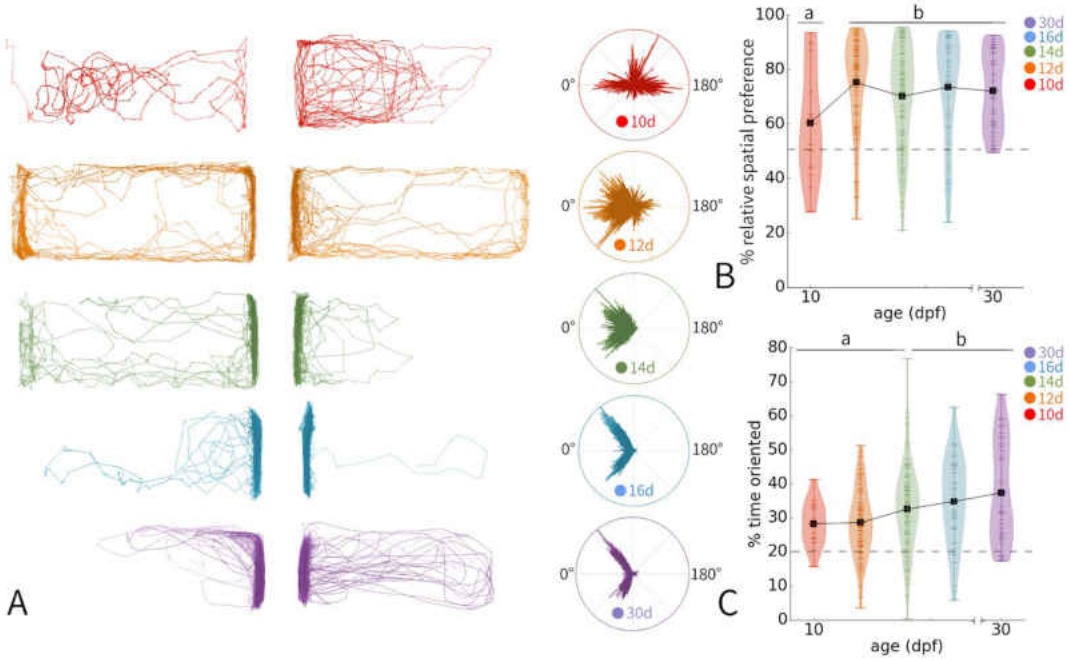

Fig 3.1. Progression of spatial preference and orienting behavior by age.

A. Representative dyad traces and polar histograms of body orientation at each age assayed.

B. Relative spatial preference arises and plateaus at 12 dpf. Preference is calculated as the inverse of the average relative distance from the divider throughout the recording period. The dotted line represents chance as determined by the grand mean in the pre-stimulus period (49.83%).

C. Percent of time oriented between 45-90° increases gradually over time. The dotted line represents chance as determined by the grand mean in the pre-stimulus period (19.88%).Lowercase letters indicate homogenous subsets as determined by Tukey's b post hoc tests. Violin plots show individual data points as horizontal tick marks, and the width of each plot represents the density of data points along the distribution. The group mean is indicated by a black square. Dotted lines represent chance values, as determined by the grand mean in the pre-stimulus period. Sample sizes for each age are the following: 10d: 16, 12d: 86, 14d: 126, 16d: 50, 30d: 36

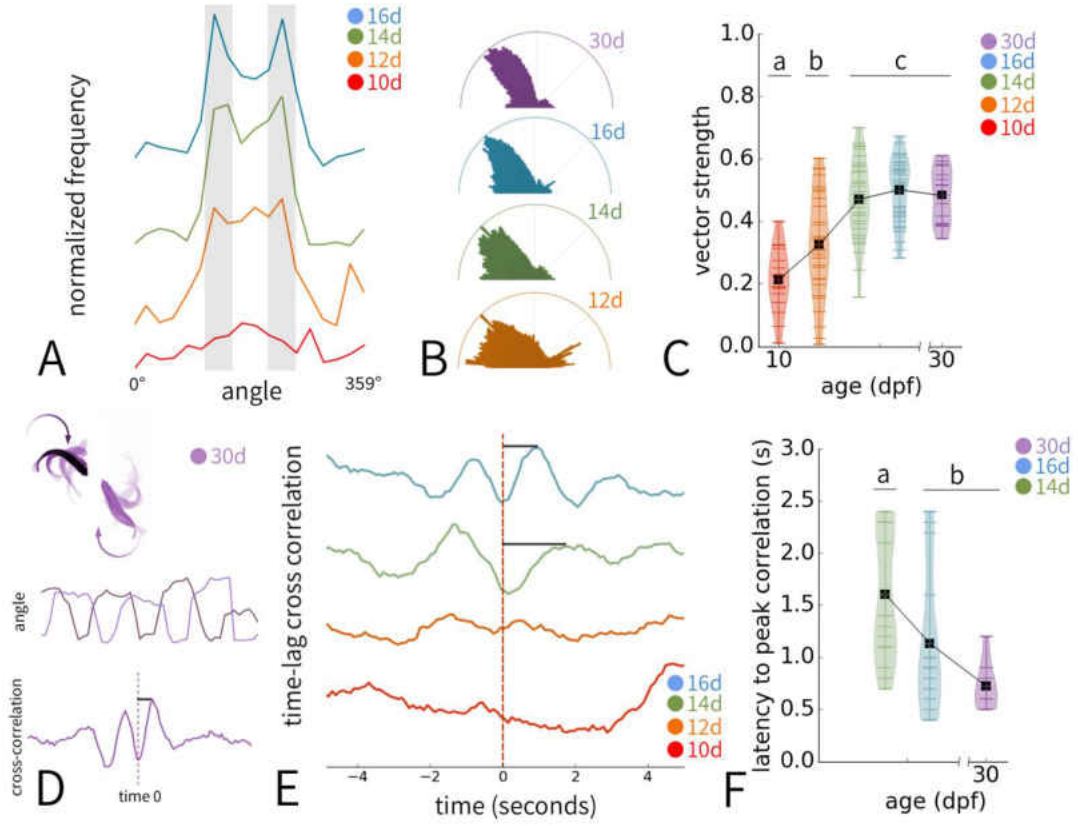

Fig 3.2. Refinement of orienting behavior and social cueing.

A. Normalized frequency of orienting behavior at 10-16 dpf. Portions highlighted in grey indicate the 45-90° region.

B. Average polar plots across each age group sampled collapsed about the 180° axis to generate "calzone" plots. Vector strength was calculated from these data based on 45° relative to the divider.

C. Vector strength increases across time and plateaus at 14 dpf.

D. Example of frame-by-frame orienting behavior and time lag cross-correlation structure for 30 dpf zebrafish, showing that angles are highly correlated between dyads with a slight latency, n = 8 pairs. Black bar indicates latency from time 0 to peak.

E. Developmental timeline of time-lag cross correlation, indicating that correlated structures begin to occur at 14 dpf and decrease in latency by 16 dpf. Black bars indicate latency from time 0 to peak.

F. Quantification of latency to peak correlation in 14, 16, and 30 dpf zebrafish dyads. Lowercase letters indicate homogenous subsets as determined by Tukey's b post hoc tests. Violin plots show individual data points as horizontal tick marks, and the width of each plot represents the density of data points along the distribution. The group mean is indicated by a black square. Sample sizes for each age are the following unless otherwise noted: 10d: 16, 12d: 31, 14d: 38, 16d: 34, 30d: 16. Only dyads where no frames were dropped due to detection errors are included.

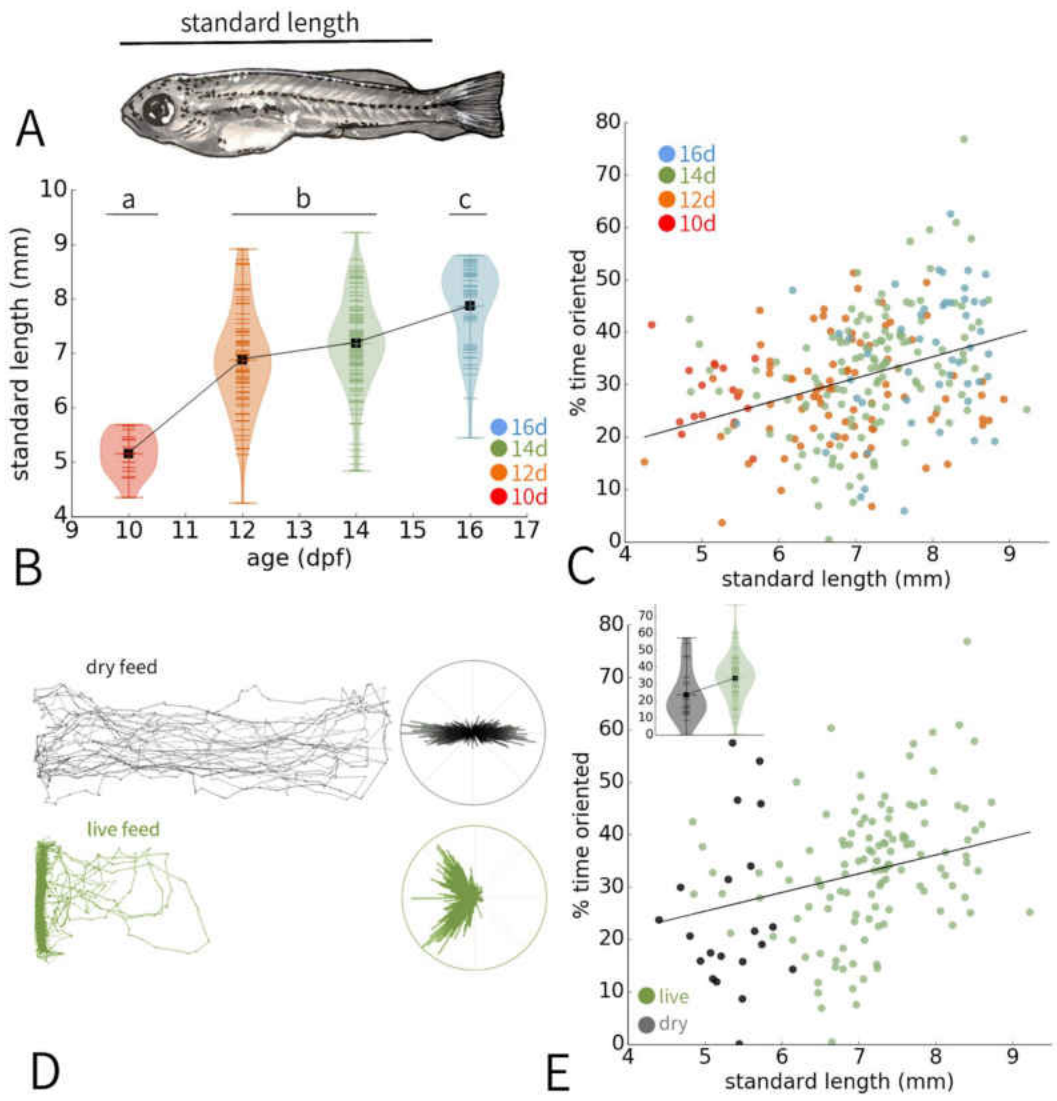

Fig 3.3. Length is predictive of individual social behavior.

A. Diagram of standard length measurements, from the tip of the nose to the end of the tail, excluding the tail fins.

B. Standard length plotted by age.

C. Orienting behavior plotted by standard length for individual animals, showing an increase in orienting behavior with increasing standard length.

D. Representative traces of 14 dpf zebrafish reared on dry vs live food (n = 22 and 126 respectively).

E. Orienting behavior plotted by standard length for 14 dpf zebrafish reared on dry vs live food. Inset shows violin plot by condition of the same dataset, not considering standard length.

Lowercase letters indicate homogenous subsets as determined by Tukey's b post hoc tests. Violin plots show individual data points as horizontal tick marks, and the width of each plot represents the density of data points along the distribution. The group mean is indicated by a black square. Sample sizes for each age are the following unless otherwise noted: 10d: 16d, 12d: 85, 14d: 126, 16d: 50.

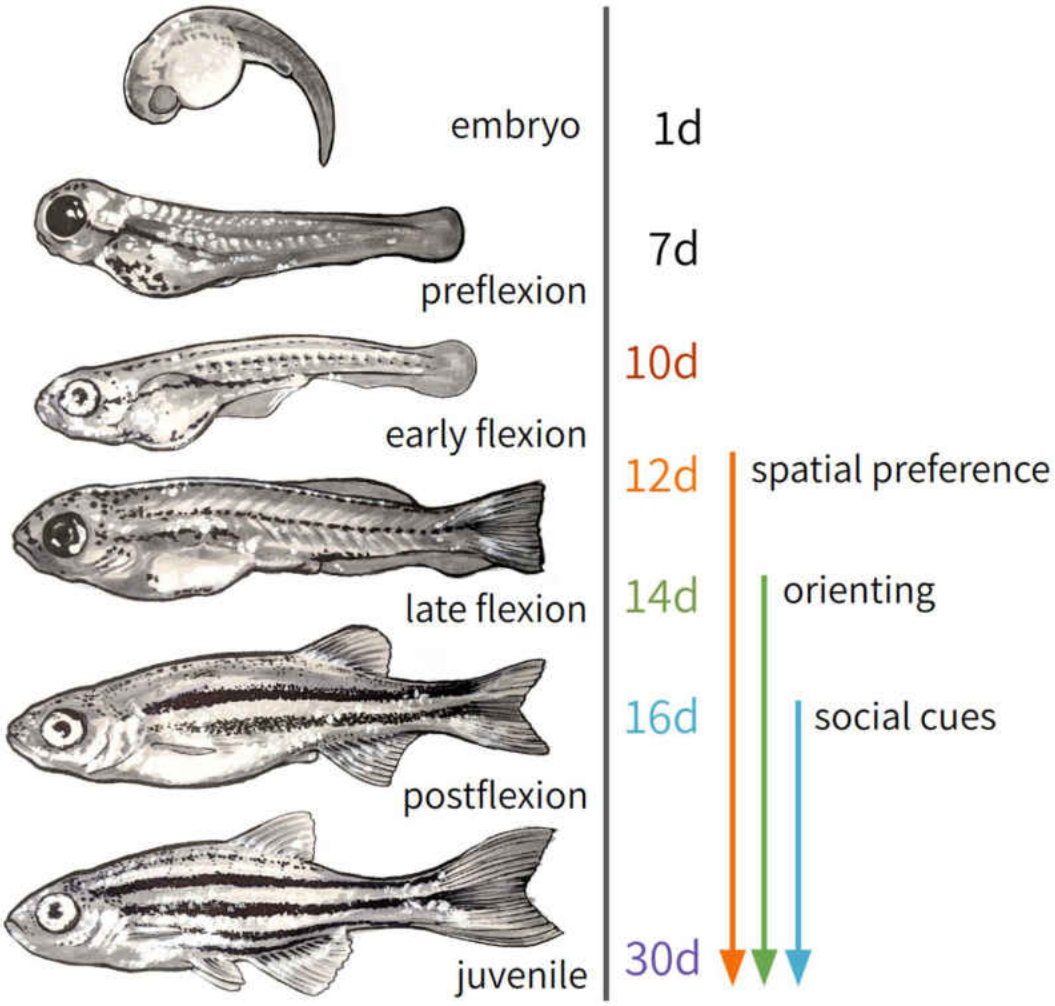

Fig 3.4. Behavioral-developmental timeline.

Approximate developmental stages of zebrafish, and the ages at which specific social behaviors are first detectable as described in this manuscript.

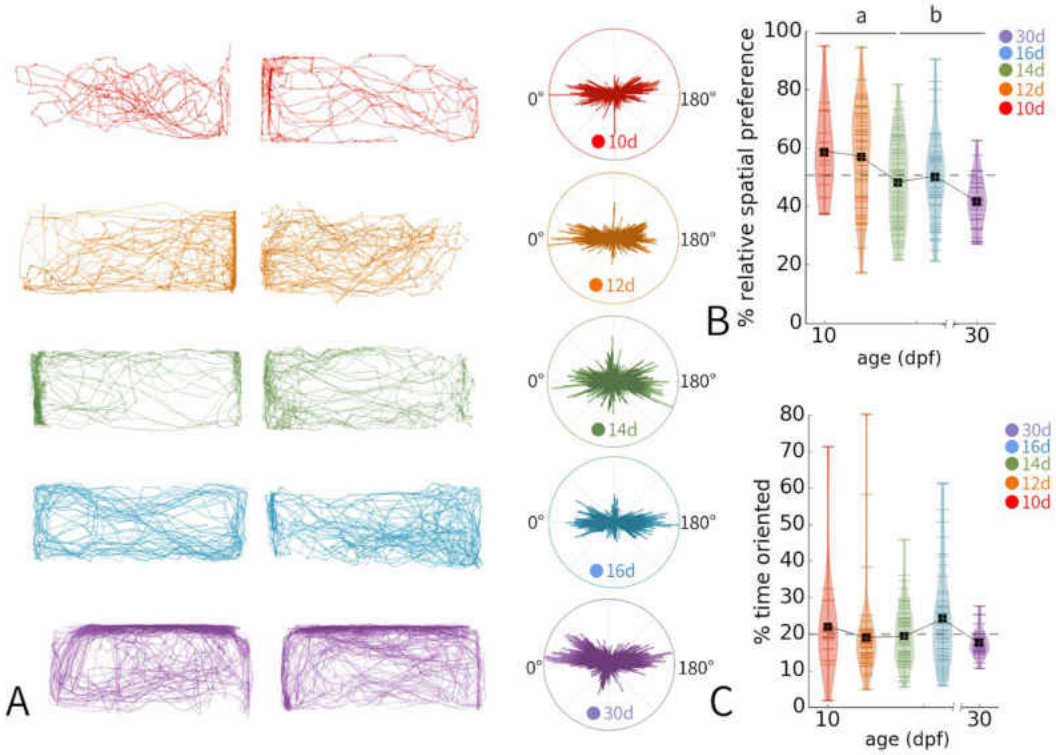

Fig 3.5. Pre-stimulus data on spatial preference and orienting behavior by age.

A. Representative dyad traces and polar histograms of body orientation at each age assayed in the absence of social stimulus.

B. Relative spatial preference for all ages in the absence of a social stimulus. Preference is calculated as the inverse of the average relative distance from the divider throughout the recording period. The dotted line represents chance as determined by the grand mean in the pre-stimulus period (49.83%).

C. Baseline percent of time oriented between 45-90° does not increase in the absence of a social stimulus. The dotted line represents chance as determined by the grand mean in the pre-stimulus period (19.88%).

Lowercase letters indicate homogenous subsets as determined by Tukey's b post hoc tests. Violin plots show individual data points as horizontal tick marks, and the width of each plot represents the density of data points along the distribution. The group mean is indicated by a black square. Dotted lines represent chance values, as determined by the grand mean in the pre-stimulus period. Sample sizes for each age are the following: 10d: 16, 12d: 49, 14d: 73, 16d: 50, 30d: 36

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