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SHORT COMMUNICATION

Genetic editing of the androgen receptor contributes to impaired male courtship behavior in zebrafish

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ABSTRACT

Elucidating the genes that contribute to behavioral variation has become an important endeavor in behavioral studies. While advances in genomics have narrowed down the list of candidate genes, functional validation of them has lagged behind, partly because of challenges associated with rapid gene manipulations. Consequently, few studies have demonstrated causal genetic changes linked to behaviors. The 'gene editing revolution' has offered unprecedented opportunities to investigate candidate genes responsible for critical behaviors. Here, we edited the androgen receptor gene (*AR*), which is associated with male reproductive behavior in zebrafish, using TAL effector nucleases (TALENs), and tested whether modifications at the *AR* impacted courtship during mating trials. We reveal that males lacking AR courted females significantly less, showing reduced levels of stereotypic behaviors. Consistent with previous studies, disrupting androgen mechanisms can lead to behavioral changes with potential fitness consequences. Our study highlights the possibility of genetically altering a reproductive behavior, further solidifying the link between genotype and behavior.

KEY WORDS: Behavioral variation, Hormones, Gene knockout, Behavioral genetics, TALENs, *Danio rerio*

INTRODUCTION

Elucidating the genes or genetic loci that influence behavioral variation has been a longstanding research goal spanning many areas of biology (Robinson et al., 2008; Zuk and Balenger, 2014). While the environment plays a role, social behavior is heritable, albeit influenced by multiple genes and their interactions. Discoveries in behavioral genetics have indeed been expedited using genome-wide and candidate gene approaches, narrowing down genomic loci and putative causal genes responsible for social behavior (Robinson et al., 2008; Kitano et al., 2009; Greenwood et al., 2013; Rittschof et al., 2014). However, to date, most work has remained correlational, and warrants validation through experimental manipulation. Fortunately, the development of genetic editing tools, i.e. TALENs (transcription activator-like effector nucleases) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9), has opened opportunities to easily manipulate genes in unprecedented ways, affording the ability to test candidate genes linked to a behavior (Joung and Sander, 2013; Sander and Joung, 2014). While editing tools have become increasingly important for the genetic dissection of complex traits, their application for studying behavior has been limited (but see Juntti et al., 2016; Yabuki et al., 2016).

In many species, courtship represents an important premating ritual behavior, which can have significant fitness consequences (Andersson, 1994). Specifically, males perform elaborate behaviors to attract mates through a series of behaviors that combine physical, visual and acoustic displays (Foster, 1994; Borgia, 1995; Darrow and Harris, 2004; Schlinger et al., 2008). One critical mechanism by which such behaviors are controlled is through hormonal mediation, notably androgen mechanisms (Ball and Balthazart, 2004; Hau, 2007). Mechanistically, the binding of androgen ligands to cytosolic androgen receptors (ARs) influences behavioral changes via the transcriptional modulation of downstream genes and related neural pathways (Hau, 2007; Juntti et al., 2010). For example, receptors mediate male courtship display via their effects on both the brain and neuromusculature circuitry in birds (Fusani et al., 2014), where their elevated expression in the brain correlates with increased agonistic behaviors (Juntti et al., 2010; Rosvall et al., 2012). While the role of the AR in modulating male behavior is known, not all components of male-typical behavior, e.g. aggression, are necessarily removed when ARs are blocked (van Breukelen, 2013). Similarly, female sexual behaviors have been impaired through endocrine disruption of brain-specific prostaglandin receptors (prostaglandin F receptor, PTGFR) in *Astatotilapia burtoni* (Juntti et al., 2016), where females with altered PTGFR fail to complete courtship. Because the AR is critical for male-typical behavior, genetic changes in the androgen system could have similar negative effects.

The zebrafish (*Danio rerio*) exhibits a complex social repertoire, and is increasingly recognized as an important model in behavioral studies (Darrow and Harris, 2004; Engeszer et al., 2007; Parichy, 2015; Teles and Oliveira, 2016; Oliveira et al., 2016). Males engage in courtship displays to attract females, consisting of stereotypical behaviors (Darrow and Harris, 2004). As in many vertebrates, such male-typical behaviors are mediated through ARs (Gorelick et al., 2008), which are widely expressed in brain regions responsible for mating behaviors (Gorelick et al., 2008; Juntti et al., 2010). Also, the zebrafish is a powerful model for genomic editing manipulation, in which target genes can be rapidly edited via knockout and knock-in approaches (Huang et al., 2011; Zhu et al., 2015). Consequently, genetic manipulations at the *AR* might affect male behaviors. However, it is worth noting that the *AR* is expressed in a wide variety of tissues, making it challenging to pinpoint the exact tissues in which *AR* expression is most critical for behavior. For example, changes in brain-specific receptors could influence the initiation of male courtship, as behavior is centrally affected (Juntti et al., 2010; Yabuki et al., 2016). Alternatively, their effects could also be peripheral, where the lack of muscle-specific *AR* expression inhibits behavioral execution (Fuxjager et al., 2013). Although the behavior

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is controlled at different levels, our study does not address such detailed mechanisms, but instead tests whether disrupting the AR has broad behavioral effects in males.

Here, we used TALENs to interrogate whether editing the *AR* gene leads to variation in male courtship behavior in zebrafish. We: (1) generated genetic lines of zebrafish knockouts in which the *AR* is fully altered through frameshift mutations, and (2) conducted mating trials to examine whether males with and without functional ARs behave differently. To our knowledge, this is the first non-mammalian *AR* knockout vertebrate model. In addition to generating and establishing an important vertebrate genomic resource for functional genetic studies, our goal is to provide a proof-of-concept investigation that tests how changes in a gene influence behavior.

MATERIALS AND METHODS

Animals

Zebrafish, *D. rerio* (F. Hamilton 1822), were obtained and maintained according to Zhu et al. (2015). Briefly, fish originated from the Zebrafish International Resource Center, and were raised in the lab at East Carolina University. They were housed under constant conditions (28°C, 14 h light:10 h dark photoperiod, pH 7.2) in a rearing system (Aquatic Habitats Z-Hab Duo systems, Pentair, Apopka, FL, USA). Fish were fed three times per day with Otohime B2 feed (Reed Mariculture, Campbell, CA, USA), and fresh brined shrimp. All fertilized eggs used for microinjection were collected via natural spawning each morning. All procedures conformed with East Carolina University IACUC guidelines (D#185d).

TALEN molecule design and assembly, and validation of knockout lines

To determine TALEN target sequence sites, exon–intron boundaries and predicted transcriptional and translational start sites for the only zebrafish *AR* gene present (GenBank accession no. NM_001083123.1; Douard et al., 2008) were manually annotated, as described in Zhu et al. (2015). We selected the first exon (1568 bp) as it harbored the proper parameters for TALEN design (Fig. 1A). TALEN molecules (forward target: CGGTGATACAG-GCGGCG, reverse target: GATGAACTCTTGAGAA, and 16 nucleotide spacer harboring an *Eco*RI recognition site: CGGCG-GCGAGCCGAATTCATTTTCT) were assembled according to Huang et al. (2011). All assembled molecules were verified using Sanger sequencing, linearized with *Not*I, gel extracted, and purified using QIAquick gel extraction kit (Qiagen, Germantown, MD, USA). mRNAs were then transcribed using SP6 mMACHINE kit (Ambion, Austin, TX, USA). Prior to microinjection, mRNA was diluted into workable concentrations (100 ng μl^{-1}) with nuclease-free water, and mixed with an equal volume of 0.5% Phenol Red solution (P0290, Sigma, St Louis, MO, USA). A mosaic mutant population (F0) was first generated, in which wild-type eggs (one-cell stage) were injected with approximately 1 nl of the transcribed TALEN mRNA (100 ng μl^{-1}). Non-injected wild-type zygotes were also collected and incubated in parallel as controls.

To validate TALEN efficiency in the injected embryos, we PCR amplified the targeted *AR* region using genomic DNA extracted from a pool of 30 wild-type or injected embryos (40–48 h post-fertilization, hpf) using the HotSHOT method (Meeker et al., 2007). The PCR reaction mixture included 4 µl of 5× PCR buffer, 2 µl of 25 mmol l^{-1} $MgCl_2$, 0.4 µl of 10 mmol l^{-1} dNTPs, 0.3 µl genomic DNA, 0.1 µl (0.5 U) *Taq* DNA polymerase (M8295, Promega, Madison, WI, USA) and 0.2 µl forward or reverse primer (10 pmol μl^{-1}; forward: 5′-CCAGACGCAGTTTTTCACG-3′, reverse: 5′-CACGGCTTTGCACAACTCTC-3′). Cycling conditions were: 94°C for 2 min, 36 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 45 s, followed by 72°C for 10 min. PCR product size (752 bp) was confirmed by gel electrophoresis of a sample. Remaining product was digested with *Eco*RI (5 U μl^{-1}; NEB, Cambridge, MA, USA) at 37°C, and then examined by gel electrophoresis. Amplicons of TALEN-injected embryos were undigested as a result of the loss of the *Eco*RI cutting site, suggesting that TALEN molecules acted efficiently (Fig. 1B,C). Undigested bands were then cloned into a TA cloning vector and genetic changes were confirmed via sequencing (Fig. 1B). Further validation was performed at the transcriptional level, where whole-brain tissues were collected from each family, and RNA was extracted using Trizol and reverse-transcribed. cDNA was then amplified, and the amplicon was gel purified, cloned and sequenced as stated above. The presence of the same deletions in the target region suggested that protein products would be truncated.

To generate stable knockout genetic lines, founders were outcrossed to establish non-mosaic F1 generations (Fig. 1D). Mutation transmission in F1 embryos was validated 40–48 hpf. The remaining F1 fish were raised to adulthood, and later individually genotyped to identify different mutations. Among the identified F1, we selected three F1 individuals with different frameshift mutations for generating stable lines, whereby each fish was used to generate an F2 generation of heterozygous offspring. Within each genetic line, heterozygous individuals were raised to adulthood and subsequently intercrossed, yielding F3 fish that were wild-type (25%), heterozygous (50%) or complete homozygous knockouts (25%) at *AR*, which were genotyped at 4 months of age.

Testing for male courtship: no-choice behavioral trials

To examine the effects of the AR on courtship behavior, a male (n=28 total: n=14 wild-type and n=14 knockout) and a wild-type gravid female (7–8 months) were allowed to engage in courtship in a no-choice mating trial. Females were unrelated to the tested males. Knockout or wild-type males originated from three genetic lines (n=8–9 per line, in which 4–5 were knockouts), and were approximately the same age, tested at sexual maturity (6 months old) and used once only.

A male–female pair was first isolated the day before the trial (16 h prior): a male was randomly selected from one of the mutant or wild-type tanks, and isolated with one female in a breeding tank with a plastic plant (21×11.6×10.8 cm). Though not visually isolated, the fish were kept apart by a clear plastic divider until the behavioral trial. The next morning (09:00 h), the divider was removed, allowing the pair to physically interact. We recorded 50 min behavioral trials using a Sony Handycam HDR-CX240 camera, in an isolated booth. Subsequently, males were lightly sedated with MS-222, and sperm was collected using 50 µl capillary tubes to ensure that males were reproductively mature.

Courtship behaviors of both males and females were quantified according to Darrow and Harris (2004), and included the number of times males chased (swimming quickly alongside the female), tail-nosed (touching the female's body with the nose or head), encircled (circling around and in front of the female), quivered (rapid tail oscillation against the female's side) and zig-zagged (tail sweeping and circling along the female's body). Females were scored for approach (abrupt swimming movement toward the male independently of any male courtship behaviors), escort (swimming alongside the male or remaining still while being courted), present (halting in front of the male exposing their side or swimming in front of the male), lead (returning at least three times to one location in the tank) and egg lay (release of eggs). All videos

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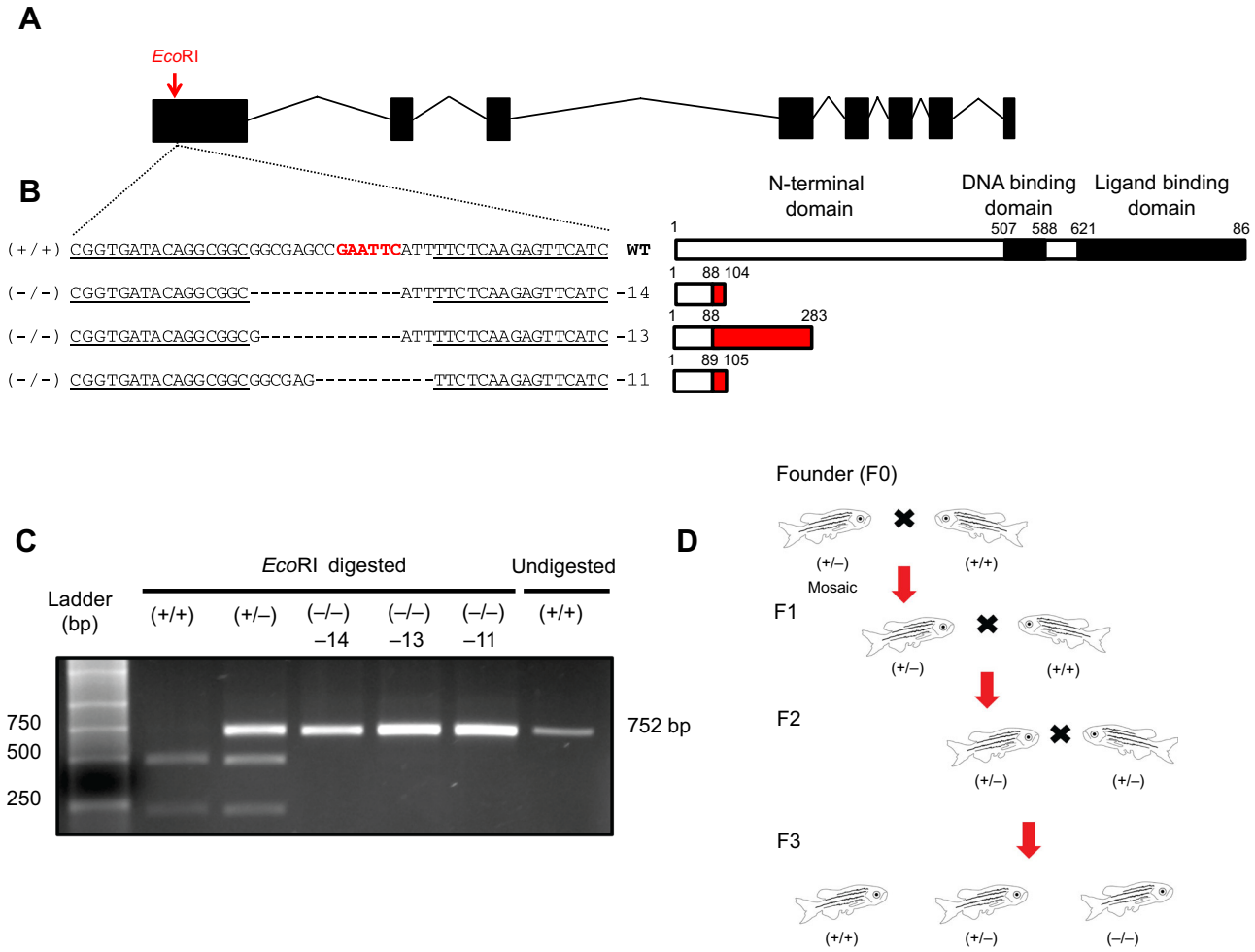

Fig. 1. Targeted and heritable genetic modification of zebrafish androgen receptor (*AR*) gene. (A) Location of the exon harboring an *Eco*RI cutting site (in red). (B) Left, DNA sequence of wild-type and confirmed knockout lines, showing the TALEN binding sites (underlined) and generated deletions (–). Right, predicted truncation of the AR protein (in red) resulting from genetic editing, which deletes DNA- and ligand-binding domains. (C) Gel confirming the mutations in the *AR* gene in three stable lines (–/–) by *Eco*RI digestion. (+/+), wild-type fish; (+/–), heterozygous fish; (–/–), homozygous knockout fish. (D) Breeding design for obtaining full *AR* knockout zebrafish.

were analyzed on Windows Media Player (Microsoft). Latency (in s) to induce courtship was also quantified. All trials were scored by an observer (Z.T.) with no knowledge of the genetic identity of males.

Statistical analyses

Calculated behavioral rates were +1 log-transformed to improve normality. To quantify female activity, all female behaviors were summarized into major axes of variation using principal component (PC) analysis, where the first PC accounted for 48% of female behavioral variation. General linear models were then used to test for differences in courtship behaviors between male types. Each male behavior was treated as a response variable, with male type as a fixed effect, and female activity as a covariate. Covariate and interaction terms (male type×female activity) were only reported when significant. Genetic line was also included as a covariate, but later removed as it was non-significant in all analyses. Statistical analyses were conducted in R (v.3.1).

RESULTS AND DISCUSSION

We first targeted and successfully edited the *AR* gene, which was confirmed at both genomic and transcriptomic levels using PCR and RT-PCR, respectively. The inability for *Eco*RI to cut at the restriction site within the first exon confirmed that a biallelic deletion had occurred. Mutations were confirmed by DNA sequencing. Further, the deletions within the first exon led to a premature stop codon and putative truncated proteins (Fig. 1B,C).

As predicted, the editing of the *AR* gene contributed to impaired male courtship behaviors during the mating trials (Fig. 2). Homozygous knockout males were slower to initiate courtship when the trial began ($F_{1,25}$=5.02, P=0.034). Even after controlling for female behavior, males continued to exhibit significantly less courtship-related behaviors, such as chases ($F_{1,24}$=8.02, P=0.009; female activity: P=0.012; interaction: P=0.013) and zig-zags ($F_{1,25}$=7.42, P=0.01; female activity: P=0.008). Also, males engaged less in tactile behaviors related to spawning stimulation toward females, e.g. tail-nose ($F_{1,24}$=9.76, P=0.004; female activity: P=0.01; interaction: P=0.016) and quiver ($F_{1,24}$=21.5, P=0.0001; female activity: P<0.0001; interaction: P<0.0001). Interestingly, males did not differ in encircling behavior (P=0.17), suggesting that encircling might not be unique to courtship or under androgenic control. Behavioral data are provided in Table S1.

Taken together, our results provide functional support for the role of the AR in male reproductive behavior, corroborating findings in

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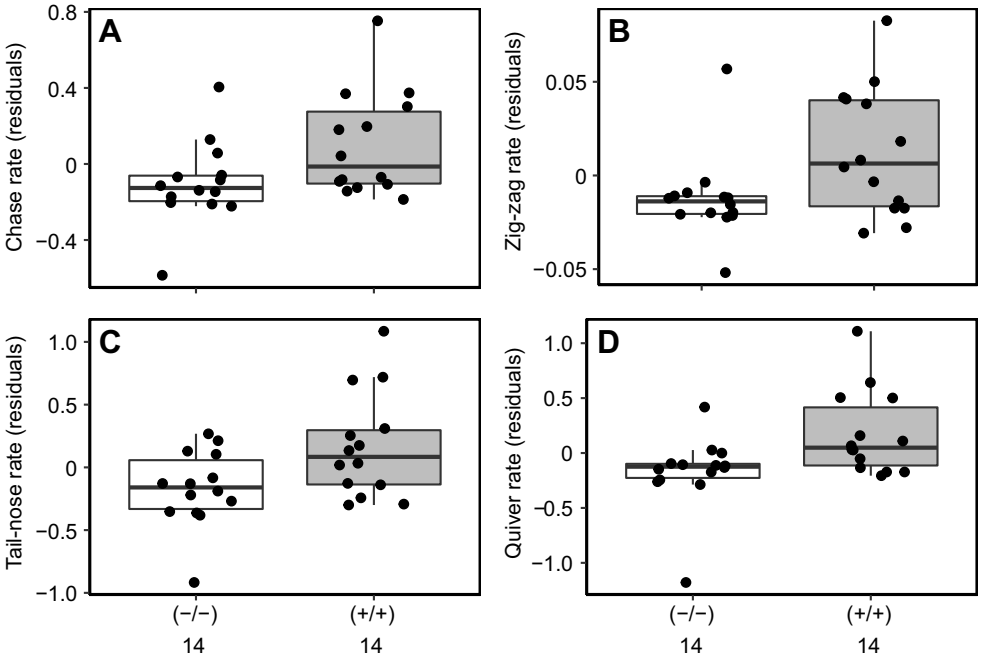

Fig. 2. Boxplots showing differences in behavioral components of courtship between *AR* knockout and wild-type males. Open bars, *AR* knockout; filled bars, wild-type. Residual scores (controlling for female activity) are plotted. Numbers on the *x*-axis represent the sample size. Plots show interquartile range with median and 25th–75th percentiles.

other species, and suggesting that its effects can be pleiotropic (Hau, 2007; Juntti et al., 2010; Fuxjager et al., 2013). Previous work has demonstrated similar behavioral effects by blocking ligand access to receptors using antagonists (van Breukelen, 2013). However, antagonists can often have limitations, including producing off-target effects (Rissman et al., 1997; Adkins-Regan, 2005). Our experimental approaches circumvent such issues, as the androgen signaling system is completely disrupted by deleting the only *AR* present in the zebrafish genome (Douard et al., 2008), thereby preventing ligand binding. Our results also highlight the pleiotropic effects of the *AR* on different behavioral components of courtship where changes in the *AR* led to the reduction of a suite of courtship-related behaviors. Because the AR is widely known to control various physiological and behavioral functions, it is likely the case that other associated behavioral (aggression) and morphological traits (secondary sexual characters) were affected (Hau, 2007). Further, our model system will be useful for investigating the extent to which suites of correlated traits, be they morphological, physiological or behavioral, are hormonally mediated.

It is worth noting that not all behavioral components related to courtship were completely disrupted in some knockout individuals. Because some mutants could still court, albeit to a reduced degree, this invokes the potential involvement of alternative mechanisms (Phelps et al., 1998). For example, the male courtship response has been shown to be mediated via sensory mechanisms, specifically olfactory receptors that are sensitive to female prostaglandin F2α (Yabuki et al., 2016). Editing of the olfactory genes leads males to become less receptive to females, such that they court them less. Another hormone receptor system, progesterone, is recognized to compensate for male sexual behaviors (Phelps et al., 1998). Also, while it is possible that courtship might be initiated via other genetic mechanisms centrally, it becomes inhibited to some degree because of the lack of *AR* expression in the periphery and muscles (Juntti et al., 2010; Fuxjager et al., 2013). Clearly, courtship behavior is the product of multiple genes responsible for the production, reception and interpretation of behavioral signals, and our results show that the *AR* is among these important genetic mechanisms.

Mechanisms by which a disrupted *AR* gene could have affected male courtship could include both organizational and activational processes (Arnold and Breedlove, 1985). Early on during ontogeny, androgens are recognized to organize neural systems, which can have long-term consequences on behavioral development (Adkins-Regan, 2005; Hau, 2007; Partecke and Schwabl, 2008). Although this remains to be tested, we predict that a non-functional AR led knockout males to be less sensitive to circulating androgens during neural development, thereby partly influencing reduction in courtship behavior among knockout males. At the same time, the knockout effects of AR might be activational, where behavioral execution was limited as a result of the lack of *AR* expression. As *AR* is expressed in many tissues, it is often presumed that the lack of brain-specific *AR* expression is the primary contributor to behavior. Yet, modified ARs in muscle tissues could equally limit the fine motor control of courtship (Fuxjager et al., 2013). This might explain why some individuals were not able to perform elaborate movements, e.g. zig-zag, but could encircle, for instance. It is likely that both brain- and muscle-specific ARs synergistically contribute; however, variation in *AR* expression among tissues might also exist, such that muscle-specific ARs could be more important. Although our study cannot discriminate such variation in organizational or activational effects, either central or peripheral (Regnier and Herrera, 1993; Fuxjager et al., 2013), measuring *AR* expression across tissue types coupled with conducting behavioral assays unrelated to courtship, e.g. a spinning test or swimming performance (Blazina et al., 2013; Conradsen and McGuigan, 2015), might prove insightful. Regardless, our genetic lines and resources open exciting research avenues and are a fruitful start for tackling detailed signaling and physiological pathways of social behaviors across biological levels.

In sum, we demonstrate that a behavior can be genetically altered in a vertebrate. Novel functional approaches, such as genetic editing, will become indispensable for testing genes that contribute to behavioral variation and dissecting ethologically relevant behaviors beyond traditional gene-association methods. As the technology is increasingly adapted for studies in evolution and behavioral ecology (Chen et al., 2014), the integration of genetic

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engineering and genome-wide approaches should prove to be powerful for deepening our understanding of the causal genetic variants underlying behavior variation and evolution.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.Y., Y.Z.; Methodology: L.Y.; Validation: L.Y.; Formal analysis: L.Y.; Investigation: L.Y., Z.T.; Resources: Y.Z.; Writing - original draft: L.Y.; Writing - review & editing: L.Y., Y.Z.; Supervision: Y.Z.; Project administration: L.Y., Y.Z.; Funding acquisition: Y.Z.

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Supplementary information
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Supplemental data - Table S1

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