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

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SUMMARY STATEMENT

We used genetic editing tools to successfully modify a gene implicated in male reproductive behavior, resulting in the impairment of courtship behavior in a vertebrate model.

ABSTRACT

Elucidating the genes that contribute to behavioral variation has become an important endeavor in behavioral studies. While advances in genomics have narrowed down candidate genes, functionally validating them has been lagging, 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 interrogate candidate genes responsible for critical behaviors. Here, we edited the androgen receptor (AR), which is associated with male reproductive behavior in zebrafish using TAL effector nucleases (TALENs), and tested whether modifications at 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 to genetically alter a reproductive behavior, further solidifying the link between genotype and behavior.

Keywords: courtship, androgen receptor, genetic editing, behavioral genetics, TALENs, zebrafish

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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 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 warranted validation through experimental manipulation. Fortunately, the development of genetic editing tools, i.e. TALENs and CRISPR/Cas9, 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 important fitness consequences (Andersson, 1994). Specifically, males perform elaborate behaviors to attract mates through a series of behaviors that combine physical, visual, and accoustic displays (Foster, 1994; Borgia, 1995; Darrow and Harris, 2004; Schlinger and Fusani, 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 (AR) influence 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 its 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 AR's role in modulating male behavior is known, not all components of male-typical behavior, e.g. aggression, are necessarily removed when AR is blocked (van Breukelen, 2013). Similarly, female sexual behaviors have been impaired

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through similar endocrine disruption of brain specific prostaglandin receptors (PTGFR) in *Astatotilapia burtoni* (Juntti *et al.*, 2016), where females with altered PTGFR fail to complete courtship. Because AR is critical for male-typical behavior, genetic changes in the androgen system could have similar negative effects.

The zebrafish (*Danio renio*) 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 stereotypic 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 AR might affect male behaviors. However, it is worth noting that 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). While the behavior is controlled at different levels, our study does not address such detailed mechanisms, but tests whether disrupting AR has broad behavioral effects in males.

Here, we use TALENs to interrogate whether editing AR leads to variation in male courtship behavior in zebrafish. We: 1) generated genetic lines of zebrafish knockouts in which AR is fully altered through frameshift mutations, and 2) conducted mating trials to examine whether males with and without functional AR 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 at a gene influence behavior.

MATERIAL and METHODS

Animals

Zebrafish 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 at constant conditions (28°C, 14L:10D photoperiod, pH = 7.2) in a rearing system (Aquatic Habitats Z-Hab Duo systems, FL). Fish were fed three times per day with Otohime B2 feed (Reed Mariculture, CA), 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 (D#185d).

TALEN molecules design and assembly, validation of knockout lines

To determine TALEN targeting sequence sites, exon-intron boundaries, predicted transcriptional, and translational start sites for the only present zebrafish AR gene Accession# 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: CGGTGATACAGGCGGCG, reverse target: GATGAACTCTTGAGAA, and 16 nucleotide spacer harboring a *EcoRI* recognition site: CGGCGGCGAGCCGAATTCATTTTCT) were assembled according to Huang *et al.* (2011). All assembled molecules were verified using Sanger sequencing, linearized with *NotI*, gel extracted, and purified using QIAquick gel extraction kit (Qiagen, Maryland, USA). mRNAs were then transcribed using SP6 mMACHINE kit (Ambion, USA). Prior to microinjection, mRNA was diluted into workable concentrations (100 ng/µl) with nuclease-free water, and mixed with an equal volume of 0.5% phenol red solution (Sigma P0290). A mosaic mutant population (F0) was first generated, in which wildtype eggs (1-cell stage) were injected with approximately 1nL of the transcribed TALEN mRNA (100 ng/uL). Uninjected wildtype 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 wildtype or injected embryos (40-48 hours post fertilization or hpf) using the HotSHOT method (Meeker *et al.*,

2007). The PCR reaction mixture included 4 µl 5X PCR buffer, 2µl 25mM $MgCl_2$, 0.4µl 10 mM dNTP, 0.3µl genomic DNA, 0.1µl (0.5U) *Taq* DNA polymerase (Promega #M8295), and 0.2µl forward or reverse primer (10 pmol/ul, forward: 5'-CCAGACGCAGTTTTTCACG-3', reverse: 5'-CACGGCTTTGCACAACTCTC-3'). Cycling conditions were: 94^0C for 2 min, 36 cycles of 95^0C 30 s, 56^0C 30 s, 72^0C 45 s, followed by 72^0C for 10 min. PCR product size (752 bp) was confirmed using gel electrophoresis. Leftover product was digested with *EcoRI* (5 U/µl) (NEB, Cambridge, MA) at 37°C, and then examined on a gel. Amplicons of TALEN injected embryos were undigested due to the loss *EcoRI* cutting site, suggesting that TALEN molecules acted efficiently (Figure 1B & C). Undigested bands were then cloned into a TA cloning vector and confirmed for genetic changes 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 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 embryo 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 3 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 wildtype (25%), heterozygous (50%), or complete homozygous knockouts (25%) at AR that were genotyped at 4 months old.

Testing for male courtship: No choice behavioral trials

To examine the effects of AR on courtship behavior, a male (n = 28; wildtype = 14 and knockout = 14) and a wildtype 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 wildtype males originated from 3 genetic lines (n = 8-9 per line, in which 4-5

were knockouts), 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 (16hrs prior), where a male was randomly selected from one of the mutant or wildtype tanks, and isolated with one female in a tank in a breeding tank with a plastic plant (21 x 11.6 x 10.8 cm). Though not visually isolated, they were kept separated by a clear plastic divider until the behavioral trial. The next morning (9am), the divider was removed, allowing the pair to physically interact. We recorded 50min behavioral trials using a Sony Handycam HDR-CX240 camera, in an isolated booth. Thereafter, males were lightly sedated with MS-222, and sperm was collected using 50uL capillary tubes to ensure that males were reproductively mature.

Courtship behaviors of both male and female were quantified according to Darrow and Harris (2004), and included the numbers of times males *chase* (swimming quick alongside the female), *tailed-nose* (touching the female body with nose or head), *encircle* (circling around and in front of the female), *quiver* (rapid tail oscillation against female's side), and *zigzag* (tail sweep and circle along female's body) at females. Females were scored for *approach* (abrupt swimming movement toward male independently of any male courtship behaviors), *escort* (swimming alongside male or remaining still while being courted), *present* (halting in front of the male exposing side or swimming in front of male), *lead* (returning at least three times to one location in the tank), and *egg lay* (release of eggs). All videos were analyzed on Windows Media Player (Microsoft). Latency (sec) to induce courtship was also quantified. All trials were scored by an observer (ZT) 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% 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, whereas male type as a fixed effect, and and female activity as a covariate.

Covariate and interaction terms (male type x female activity) were only reported when significant. Genetic line was also included as a covariate, but later removed as it was nonsignificant in all analyses. Statistics 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 *EcoRI* to cut at the restriction site within the first exon confirms that a biallelic deletion 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 AR contributed to impaired male courtship behaviors during the mating trials (Fig. 2). Homozygous knockout males were slower to initiate courtship upon the start of the trial ($F_{1,25} = 5.02$, $P = 0.034$). 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 zigzags ($F_{1,25} = 7.42$, $P = 0.01$; 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 for encircling behavior ($P = 0.17$), suggesting that encircling might not be unique to courtship or under androgenic control.

Taken together, our results provide functional support for the role of the androgen receptor in male reproductive behavior, corroborating findings in other species, and 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, where 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 AR on different behavioral components of courtship where changes at AR led to the reduction to a suite of courtship-related behaviors. Because 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) have been 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 to a reduced degree, this invokes the potential involvement of alternate mechanisms (Phelps *et al.*, 1998). For example, 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 lead males to become less receptive to females, thereby courting them less. Another hormone receptor system, progesterone, are 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 due to 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 AR is among those important genetic mechanisms.

Mechanisms by which a disrupted AR could have affected male courtship could include both organizational and activational (Arnold and Breedlove, 2005). 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). Though this remains to be tested, we predict that a nonfunctional 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 due to 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 organization 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. In any case, our genetic lines and resources open exciting research avenues and is 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 ethological-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 both genetic engineering and genomic-wide approaches will prove to be powerful for deepening our understanding of the causal genetic variants underlying behavior variation and evolution.

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Competing interests

We have no competing interests.

Author's contribution

LY and YZ conceived the experiment and generated the zebrafish lines. LY and ZT coordinated the behavioral trials. LY and YZ wrote the manuscript.

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Data availability

Behavioral data are provided in the supplementary file.

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Figures

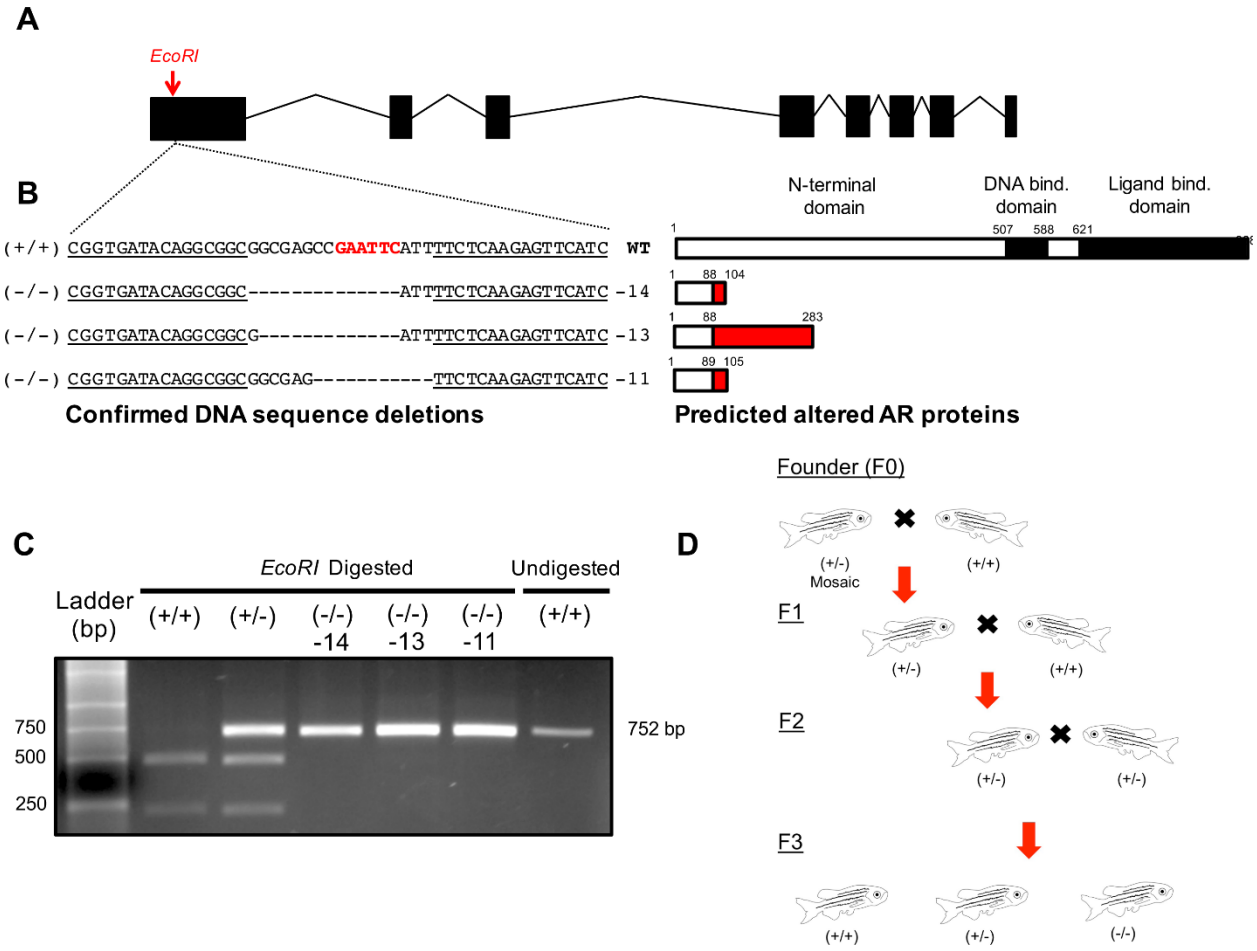

Figure 1. Targeted and heritable genetic modification of zebrafish *AR* gene. (A) Location of the exon, harboring an *EcoRI* cutting site (in red). (B) DNA sequence of wildtype and confirmed knockout lines, showing the TALEN binding sites (underlined) and generated deletions (-). Predicted truncation of the protein (in red) from the genetic editing, deleting DNA and ligand binding domains. (C) Gel confirms mutations at *AR* in 3 stable lines (-/-) by *EcoRI* digestion. (+/+) = wildtype; (+/-) = heterozygote; (-/-) = homozygote knockout. (D) Breeding design for obtaining full AR knockout zebrafish.

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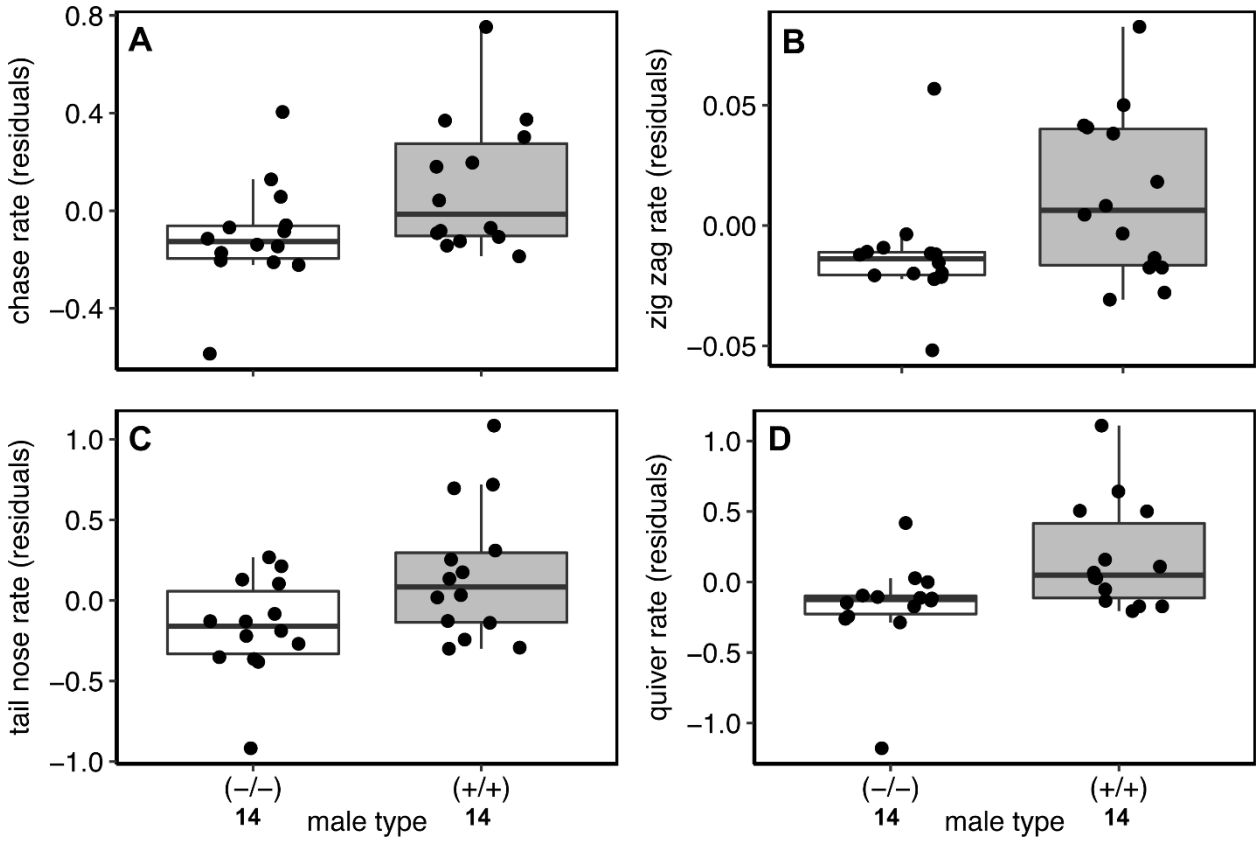

Figure 2. Boxplots showing differences in behavioral components of courtship between AR knockout (open bar) and wildtype (shaded bar) males. Residual scores (controlling for female activity) were plotted. Numbers on x-axis represent the sample size. Plots show interquartile range with median and 25th–75th percentiles.

Supplemental data - LY_AR_data_deposit

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Supplemental data - Table S1

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