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Nuclear androgen and progestin receptors inversely affect aggression and social dominance in male zebrafish (*Danio rerio*)

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ABSTRACT

Aggression is a fundamental behavior displayed universally among animal species, but hyper- or hypo-aggressiveness can be maladaptive with negative consequences for individuals and group members. While the social and ecological significance of aggression is well understood, the specific neurobiological and hormonal mechanisms responsible for mediating aggression have not been fully elucidated. Previous studies have shown a relationship between aggressive acts and circulating gonadal steroids, but whether classical nuclear steroid receptors regulate aggression in animals is still uncertain. We examined whether the nuclear androgen receptor (Ar) and nuclear progestin receptor (Pgr) were necessary for aggressive behaviors and maintenance of a dominance relationship in male zebrafish (*Danio rerio*). Dyadic social interactions of Ar knockout (ArKO), Pgr knockout (PgrKO) and wildtype (WT) controls were observed for two weeks (2-weeks). ArKO zebrafish were significantly less aggressive and had a less defined dominance relationship, whereas PgrKO dominant zebrafish were significantly and persistently more aggressive with a robust dominance relationship. Our results demonstrate the importance of nuclear steroid hormone receptors in regulating aggression of adult male zebrafish and provide new models for understanding of the mechanisms of aggression.

1. Introduction

Aggression is fundamental for the survival and reproductive success of an individual and is conserved widely across animal taxa (Archer, 1991; Munley et al., 2018; O'Connell and Hofmann, 2012; Olivier and Young, 2002). In many species, aggressive behaviors are used instrumentally to secure food, mating opportunities or territories. However, with increased aggressive activities, animals may risk serious injury to acquire resources. Dominance hierarchies emerge among conspecifics within social groups to settle aggressive disputes without risking serious injury or energetic costs to either individual. Socially dominant individuals receive preferential access to food and mates by displacing weaker conspecifics, thereby increasing their own chances of social and reproductive success. Social dominance relationships are determined primarily by acts of physical aggression but are also influenced by prior social experience, stress coping style, body size of the animal and neuroendocrine signals (Archer, 1991; Lischinsky and Lin, 2020, Nelson and Trainor, 2007; Pavlidis et al., 2011; Rowland, 1989; Sloman and Armstrong, 2002; Weitekamp and Hofmann, 2017; Wright et al., 2019).

Aggressive behaviors have multiple underlying physiological mechanisms that are mediated by the integration of neuronal and endocrine signals. Experimental evidence correlates relative levels of neurotransmitters, pituitary hormones, gonadal steroids, and their respective receptors with incidences of aggression (Cunningham et al., 2012; Juntti et al., 2010; Rosell and Siever, 2015). The experimental evidence for the effects of gonadal steroids on aggression can be traced back to the classic experiment described by John Hunter in 1794 and Arnold Berthold in 1849 (Fores, 1947; Jorgensen, 1971; Sawin, 1996). In their experiments, removing testes of male chicken reduced secondary sex characteristics (comb, wattles) and male-typical reproductive and aggressive behaviors, while transplanting testes back to castrated roosters would restore normal aggressive behavior and secondary sex characteristics. Since

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then, a large body of evidence has been accumulated to support androgens, mainly testosterone (T), as main regulators for aggressive, territorial, and dominance behaviors in a variety of vertebrate species (Alward et al., 2020; Barfield et al., 1972; Collias, 1944; Geniole et al., 2020; Hunt et al., 2019; Kristensen et al., 2020; Moore et al., 2020; Nelson, 1995; Simon et al., 1996; Wingfield et al., 1999, 2006). Studies based on androgen injection, androgen implantation, androgen receptor (Ar) blockers, or Ar knockouts clearly demonstrated the cause and effects of androgens and Ars on agonistic behavior in various species (Alward et al., 2020; Barfield et al., 1972; Collias, 1944; Rodgers et al., 2013; Sato et al., 2004; Schwabl and Kriner, 1991; Searcy and Wingfield, 1980; Sperry et al., 2009; Wacker et al., 2016; Wingfield, 1984; Wingfield et al., 1999). Further studies suggest the involvement of androgens and Ars in aggression is complex, and results may vary depending on timing (e.g., breeding vs. non-breeding season), behavioral context (e.g., reproductive vs. non-reproductive associated aggression) or experimental conditions (Adkins-Regan, 2005; van Breukelen, 2013; Wingfield et al., 1999). Some studies reported no correlation between androgens and aggression (Alward et al., 2019; Cramer, 2012; McDonald et al., 2001; Wiley and Goldizen, 2003). It is argued that androgen administration does not affect aggressive behavior (O'Connor et al., 2002; Salas-Ramirez et al., 2008), or that androgen will be metabolized into estrogens in the brain to affect aggression, thus, androgen receptor blockers fail to inhibit aggression circuits in the brain (Clark and Nowell, 1980; Heilman et al., 1976; Huffman et al., 2013; Soma et al., 2000). Clearly, additional studies are needed to establish the roles of androgen and Ars in aggression, and to address the contradictions.

Zebrafish (*Danio rerio*) has one single *ar* locus, and the *ar* expresses ubiquitously in all adult tissues with sexually dimorphic expression in the gonad and muscle (Hossain et al., 2008). The expression of *ar* was also observed in telencephalon, preoptic area, and throughout the periventricular hypothalamus in the zebrafish (Gorelick et al., 2008), regions previously implicated in the regulation of sexually dimorphic behaviors in mammals (Arnold and Breedlove, 1985; Morris et al., 2004). Aggression in male African cichlid fish (*Astatotilapia burtoni*) could be manipulated via steroid receptors expressed in the preoptic area using agonists or antagonists (O'Connell and Hofmann, 2012). *A. burtoni* has two androgen receptor (Ar) paralogs, Arα and Arβ due to a linage specific duplication. Arα, but not Arβ, is required for aggressive displays. Moreover, Ar is sufficient in reducing female submissive behavior while interacting with males (Alward et al., 2020). Male territorial aggression was reduced in AR knockout mouse (Sato et al., 2004). Intriguingly, they also found that male aggression may be partially regulated through androgens acting via an AR-independent pathway distinct from the ER receptors (Sato et al., 2004). In contrast, evidence on the roles of progestins in animal aggression is very limited and still mixed. The subordinate male of African cichlid fish (*Astatotilapia burtoni*) is less likely to flee when treated with a progestin (17α-20β-P) (O'Connell and Hofmann, 2012). In rodents, progestin exposure inhibited male aggression in golden hamsters (Fraile et al., 1987) and female aggression in Syrian hamsters (*Mesocricetus auratus*, Meisel et al., 1989). However, administration of progesterone following castration upregulated aggression in male tree lizards (*Urosaurus ornatus*, Weiss and Moore, 2003). Aggression in male mice was found to be unaffected by progesterone, progesterone receptor (PGR) antagonism, or PGR knockout in mice (Schneider et al., 2003). Therefore, whether progestins and their receptors have roles in vertebrate aggression and social behavior are still uncertain.

Zebrafish (*Danio rerio*) is a useful model organism to study aggression because of the ease of genetic manipulation, structural organization of their social groups, and recent focus in neurobiological research (Loring et al., 2020; Oliveira et al., 2011). Zebrafish are a group living species in the wild, in which males compete with conspecifics for preferential access to spawning sites and females (Spence et al., 2008). The primary resource-accruing behavior in zebrafish is aggression (Sloman and Armstrong, 2002). Aggressive behavior in zebrafish is well characterized and can be measured repeatedly over time (Way et al., 2015). In the laboratory, male zebrafish in a dyadic pair will quickly establish a stable dominance relationship within one day (Miller et al., 2017; Clements et al., 2018; Orr et al., 2021). The social dominance phenomenon is well-characterized between domestically raised zebrafish (Jones and Norton, 2015). Dominant zebrafish attack the subordinate fish with a short bout of fast swimming toward its opponent in order to make contact. This contact may be only a lunge or may result in an actual bite. A subordinate fish's behavior is characterized by retracting the fins, freezing in place, or a quick retreat away from the attacking fish (Kalueff et al., 2013). The effect of the dominant's aggression on the subordinate is the formation and maintenance of a dominance relationship in which the dominant fish can occupy a larger territory and thus have a greater likelihood of acquiring resources (Miller et al., 2017).

The aim of the current study was to determine whether the androgen receptor (Ar) and progestin receptor (Pgr) influence the long-term agonistic interactions and stability of dominance formation between competing males by examining daily attacks and retreats and relative location of individual fish in the tank. We limited the study to male zebrafish due to the daily cyclical hormonal regulation of ovulation in females that may affect aggressive activity (O'Connell et al., 2013; Terranova et al., 2016). We report that knocking out the Ar and Pgr have dramatic and opposing effects on aggressive behavior and dominance formation.

2. Materials & methods

2.1. Fish care

WT AB strain and mutant zebrafish lines were housed in the zebrafish core facility with a 14-hour (h) light and 10 h dark photoperiod, at water temperature of 28.5 °C, pH of approximately 7.2, and salinity/conductivity ranging from 500 to 1200 μS in an automatically controlled zebrafish rearing system (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish were fed to satiation three times daily with a commercial food (Otohime B2, Reed Mariculture, CA, USA) containing high protein content, and supplemented with newly hatched brine shrimp *Artemia* (Brine Shrimp Direct, Utah, USA). The Institutional Animal Care and Use Committee (IACUC) at East Carolina University have approved all experimental protocols.

2.2. Genetic manipulation

Ar knockouts (ArKO) and Pgr knockouts (PgrKO) were generated using Transcription Activator-Like Effector Nucleases (TALENs), and mutations were confirmed as previously described (Zhu et al., 2015; Yong et al., 2017). Briefly, zebrafish Ar (genomic sequence: CR396593; mRNA sequence: NM_001083123) consists of 868 amino acids and 8 coding exons. ArKO was generated by targeting the beginning of first exon, which encodes beginning part of A/B domain. TALENs generated three different frame shifts and premature stop codes, which led to truncated proteins with loss of major part of A/B domain, and all of C, D, E and F domains (Yong et al., 2017). Zebrafish Pgr (genomic sequence: CU459064; mRNA sequence: NM_001166335 & EF155644) consists of 618 amino acids and 8 coding exons. We used two sets of TALENS targeted two different parts of A/B domain, i.e., beginning or end of AB domain, which led to generation of three different Pgr mutant lines. Two Pgr mutants (Pgr15 and Pgr35) have a short-truncated protein with the loss of major part of A/B domain and all C, D, E, F domains, while the third Pgr mutant line (Pgr2d) retains major part of A/B domain, but lost all C, D, E and F domains (Zhu et al., 2015).

To distinguish ArKO and PgrKO mutants from their WT siblings, TALENs targeting regions were PCR amplified, verified with restriction endonuclease digestion and visualization on agarose gel. There were no active androgen or progestin receptors present in vivo due to premature stop codons that terminated the formation of the protein during gene translation. Out of several ArKO and PgrKO lines created, Ar9.1 (*ar*$^{ecu5/ecu5}$), Pgr15 (*pgr*$^{ecu1/ecu1}$) and Pgr2d (*pgr*$^{ecu3/ecu3}$) were used in the current study.

2.3. Observational period

Prior to social pairing, male fish of each respective genotype were socially isolated for one week to minimize the effects of pre-existing social experience formed during communal housing. Following social isolation, the fish were continuously paired for 2-weeks with a conspecific of similar size (3.5–4 cm) and age (7–12 months). The two males were introduced into a novel test tank, so as not to give either fish an advantage. There were three types of pair crossings: WT/WT, ArKO/ArKO, PgrKO/PgrKO. For all the pairs tested, daily observations of 5 min were conducted between 10 am and 12 pm during which the number of attacks and retreats were counted for each fish. Dominance was assessed based on the fraction of the number of attacks to retreats performed by each fish. Attacks were defined as chasing, lunging at, or biting of the conspecific, and retreats were defined as fleeing, or seeking refuge in the back, bottom corner of the tank. For a subset of the pairs tested, additional video recordings of the agonistic interactions were acquired during initial pairing (initial 24 h) and at 2-weeks marks. This was done for detailed behavioral analysis (see Fig. 2) and tracking of the fish swimming behavior and spatial distribution (see Fig. 3). A distance caliber was placed onto each tank to calibrate spatial distributions and motion analysis. Fish were fed only after each observation.

Two groups of students were assigned for genotyping, behavior scoring, and data analyses. Students who conducted behavior observation and scoring were not informed genotyping. The genotyping and social paring were conducted by a separate student who was not involved in behavior scoring. The first group of students used Ar9.1 and Pgr2d to conduct the experiments. To determine whether their experiments were repeatable, a second group of students used different offspring of Ar9.1 and a different PgrKO line (Pgr15) and repeated all the experiments in a different year.

2.4. Data analysis

2.4.1. Attack/retreat counts

The daily average number of attacks and retreats in a 5-minute period were graphed using GraphPad Prism. A social dominance relationship was determined by calculating the average number of daily attacks and retreats. Animals with the higher number of attacks during the observation period were considered dominant.

2.4.2. Spatial distribution and generation of heat-maps

According to our previous studies, dominant fish occupy a larger territory, typically in the upper and front part of a tank to increase its likelihood of acquiring food. In contrast, subordinate fish tend to occupy the bottom and back part of a tank (Miller et al., 2017). We used video clips and determined relative location of dominant and subordinate fish. Video recorded interactions during the initial 24 h and 2-weeks later were uploaded into ImageJ for processing. The Slice Remover add-on was used to remove frames containing interactions between the dominant and subordinate fish. Approximately 1 min of video, or approximately 1800 frames, were extracted at equal intervals from within the entire 5 minute recordings to eliminate sampling bias. The frames were then used for the Motion Tracking plugin analysis. The result of the motion tracking was to visually show the areas in which the fish were localized and to generate the XY coordinates of each fish in the testing chamber for each frame. The XY coordinates were then used in a custom program written for the statistical computing program R to create visual heat maps to show increasing probability of fish localization as described elsewhere (Miller et al., 2017). Two maps were made per pair, one red for the dominant and one blue for the subordinate fish. Measurement of overlapping territorial areas was done using ImageJ selection tool option. Imported images were calibrated to the actual tank size then overlapping territory was manually mapped and area tabulated in Excel for later analysis. Centroids were defined as the region of the heatmap where a dominant or subordinate swam most frequently illustrated graphically with the most intense heatmap color (darkest red for dominants or darkest blue for subordinates). To measure distance between centroids a straight line was drawn between the two darkest centroids for each animal pair. If an animal spent an equal amount of time at two different locations, thus, generating two centroids of equal color intensities, then an average distance was taken between these two centroids relative to the centroid generated by its animal partner.

2.4.3. Encounter analysis

All videos taken were viewed with a basic media player and the length of each encounter was recorded. An encounter was considered initiated when one fish causes a change in the swimming path of the other. The encounter ended when the fish were no longer reacting to each other's movements. The total number of encounters as well as the number of attacks observed during each encounter were recorded. With this data, the average encounter duration, average attacks per encounter, and encounter per minute for each pair could be obtained and compared across genotypes.

2.4.4. Statistical analysis

Statistical analyses and plots were conducted using IBM-SPSS and GraphPad Prism, respectively. Prior to statistical analysis, all data was tested for and passed Gaussian distribution. Unless specified otherwise, all comparisons were first subjected to one-way ANOVA or mixed-design (a mixture of between-group and repeated-measures variables) followed by Tukey's Multiple Comparison post hoc test for all multiple comparisons, and effect size numbers (η^2) and Cohen's (d) were calculated. All statistical tests were two-tailed with 95% confidence intervals. Results for average attack/retreats and encounter analysis are presented as mean ± SEM. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Time-course of aggressive behavior of WT, ArKO and PgrKO animals

Daily observations of aggressive activity showed clear differences in aggressive behavior among the three groups (Fig. 1). Most notable, ArKO pairs were significantly less aggressive compared to WT and PgrKO pairs. This is illustrated in the fewer daily number of attacks by the ArKO dominants toward subordinates than WT dominants (Fig. 1A, B; supplemental Videos 1 and 2). These differences in aggression led the WT pairs to form strong dominance relationships immediately after initial pairing that was strengthened with time as dominants increased their frequency of attacks during the subsequent two days. By comparison, the dominants of the ArKO pairs maintained consistently low level of aggression throughout the observation period (Fig. 1A, B). A second notable observation is that dominants of PgrKO pairs showed enhanced aggression and attacked more frequently than dominants of WT and PgrKO pairs (Fig. 1A–C). Cross correlation analysis of mixed design ANOVA of all dominants (within-subject factor days, between-subject factor group) further supports the notion that ArKO zebrafish are less aggressive relative to PgrKO and WT fish; while PgrKO zebrafish are more aggressive compared to WTs (Fig. 1G). There was a significant main effect of group ($F_{(2,45)} = 29.89$, $p < 0.0001$, effect size $\eta^2 = 0.57$; WT $n = 22$, ArKO $n = 23$, PgrKO $n = 12$), but there was no effect of days ($F_{(2,45)} = 0.434$, $p > 0.05$). Tukey's Multiple Comparison post hoc test showed that the average number of attacks by dominants among all groups differed significantly (ArKO vs WT, $p < 0.001$; WT vs PgrKO, $p = 0.007$; ArKO vs PgrKO, $p < 0.001$).

These differences in aggressive activity were mirrored by corresponding differences in submissive behaviors by subordinates (Fig. 1D–F). ArKO subordinates retreated significantly less than WT and

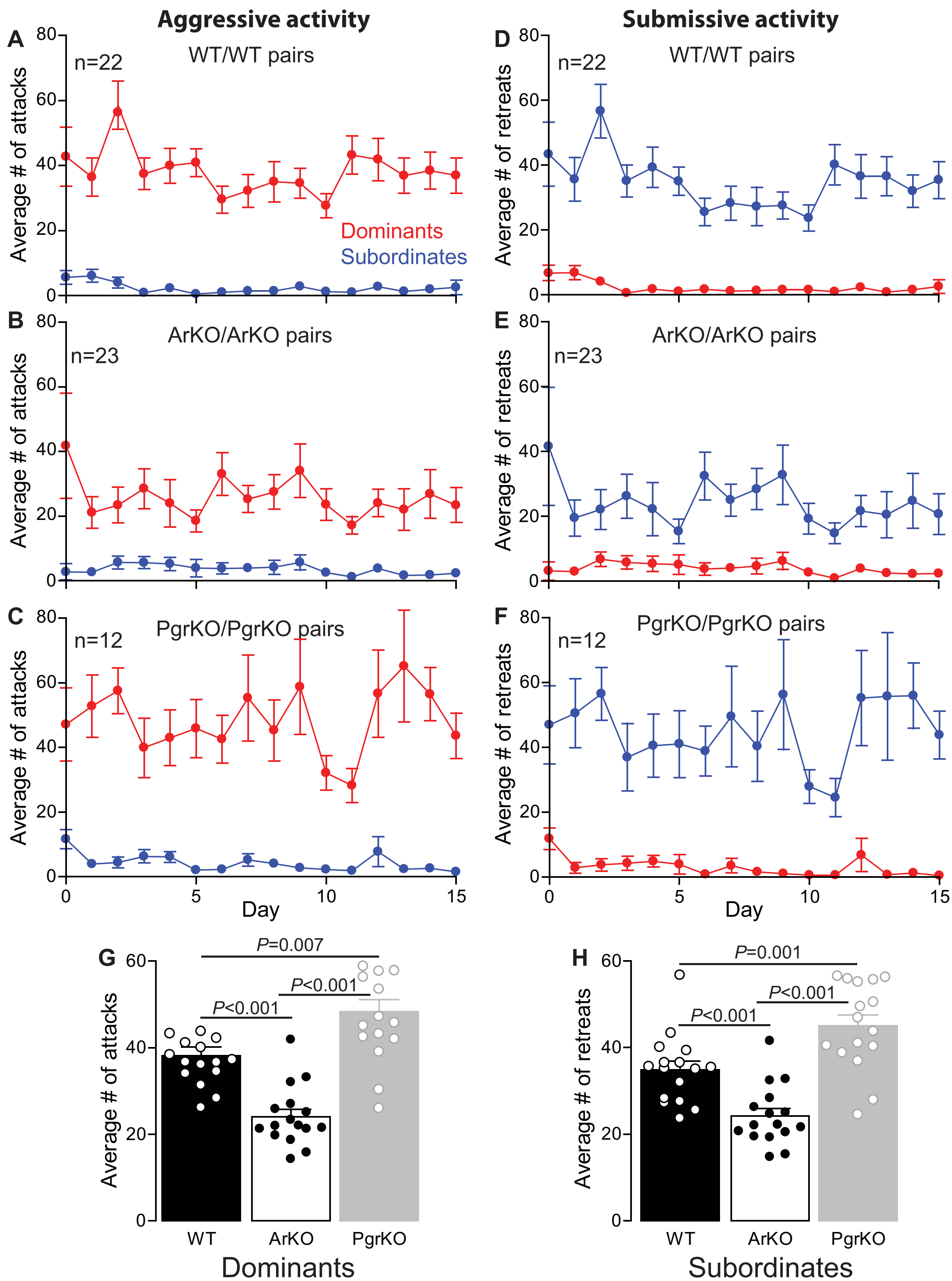

Fig. 1. Daily average numbers of attacks (left column) and retreats (right column) (Mean ± SEM) for WT/WT pairs (A, D) $n = 22$ pairs, ArKO/ArKO pairs (B, E) $n = 23$ pairs, PgrKO/PgrKO (C, F) $n = 12$ pairs. Dominants' data are illustrated in red, and subordinates' data are illustrated in blue. (G) Average number of attacks over two weeks period for WT, ArKO and PgrK dominants only. Bar graphs represent the average numbers of cumulative data points. Dots represent daily average number of attacks. (H) Average number of retreats over two weeks period for WT, ArKO and PgrK subordinates only. Bar graphs represent the average numbers of cumulative data points. Dots represent daily average number of retreats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PgrKO subordinates over the entire trial period, and subordinates of PgrKO pairs retreated significantly more frequently compared to the other two groups. We found a significant main effect of group ($F_{(2,45)} = 24$, $p < 0.001$, effect size $\eta^2 = 0.52$; WT n = 22, ArKO n = 23, PgrKO n = 12), and there was an effect of days ($F_{(2,45)} = 28.46$, $p < 0.05$). Post hoc test showed that the average number of retreats among subordinates of all three groups differed significantly (Fig. 1H; WT vs ArKO, $p < 0.001$; WT vs PgrKO, $p = 0.001$; ArKO vs PgrKO, $p < 0.001$). The reduction in submissive behavior of ArKO subordinates was likely due the decreased aggression by their dominant counterparts. Conversely, the increase in submissive behavior of PgrKO subordinates was likely due to the increase in aggressiveness of their dominant counterparts. Finally, we found that submissive behavior among dominants did not differ significantly among the three groups (no main effect of group ($F_{(2,45)} = 2.03$, $p > 0.05$, effect size $\eta^2 = 0.083$), and there was no effect of days ($F_{(2,45)} = 2.79$, $p > 0.05$)). Collectively, the results suggest that knocking out the *ar* and *pgr* genes has opposite effects on aggressive behavior of male zebrafish. ArKO male zebrafish are significantly less aggressive while PgrKO males are more aggressive than WT fish.

3.2. Comparison of aggressive levels among the WT, ArKO and PgrKO animals

Our results suggested that aggressive behavior of ArKO zebrafish is subdued compared to WT animals, while PgrKO fish displayed elevated aggressive behavior compared to WT fish. Moreover, examination of agonistic encounters of the PgrKO fish suggested that the intensity of those interactions maybe elevated compared to WT fish (supplemental Video 3). To quantitatively assess whether the social interactions of PgrKO fish are more aggressive compared to WT fish, we calculated the average number of encounters per minute, average duration of encounters, and number of attacks per encounter for the subset of pairs whose interactions were video recorded. The notion is that these parameters would provide more detailed information on the intensity of social interactions as animals fight for dominance.

We found that PgrKO pairs had significantly fewer encounters per minute compared to WT pairs during the first 24 h of observation (Fig. 2A). There was a significant main effect of group ($F_{(2,24)} = 3.59$, $p = 0.043$, effect size $\eta^2 = 0.23$; WT $n = 11$, ArKO $n = 7$, PgrKO $n = 9$). Tukey's Multiple Comparison post hoc test showed a significant difference between WT and PgrKO pairs ($p = 0.036$) but no difference between PgrKO and ArKO pairs ($p > 0.05$). The low number of encounters of PgrKO pairs was due to the fact that PgrKO pairs fought for a significantly longer time compared to WT pairs (Fig. 2B; $F_{(2,24)} = 4.54$, $p = 0.021$, effect size $\eta^2 = 0.274$; post hoc WT vs Pgr $p = 0.032$ with a marginal difference between PgrKO and ArKO pairs $p = 0.051$). This result suggested prolonged engagement in aggressive interactions of PgrKO pairs. However, this difference in the number of encounters per minute and duration of encounters dissipated among all three groups after 2-weeks of interactions (Encounters per minute: $F_{(2,24)} = 0.016$, $p = 0.984$, effect size $\eta^2 = 0.001$; Encounter duration: $F_{(2,24)} = 0.78$, $p = 0.470$, effect size $\eta^2 = 0.061$).

Dominants' attacks per encounter were also significantly higher in PgrKO pairs during the first 24 h compared to WT and ArKO pairs (Fig. 2C; $F_{(2,24)} = 5.84$, $p = 0.009$, effect size $\eta^2 = 0.33$; Tukey's Multiple Comparison post hoc Test, WT vs PgrKO $p = 0.017$, PgrKO vs ArKO $p =$

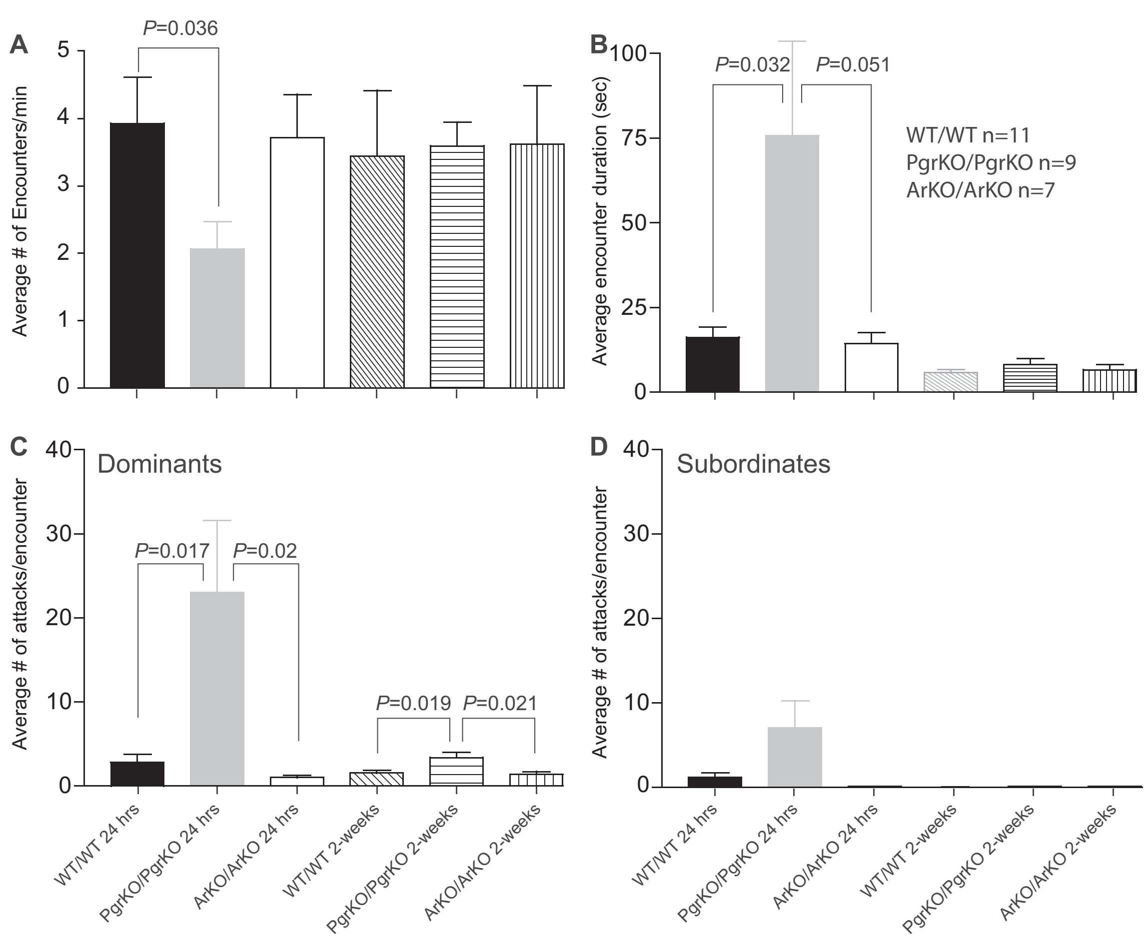

Fig. 2. Differences in aggressive activity among WT, ArKO and PgrKO pairs. Average number of encounters/min (A), average duration of encounters (B), and average number of attacks/encounter for dominants (C) and subordinates (D) during initial 24 h of pairing and 2-weeks between WT/WT, ArKO/ArKO, and PgrKO/PgrKO pairs. Results are presented as mean ± SEM. (WT/WT $n = 11$ pairs, PgrKO/PgrKO $n = 9$ pairs, and ArKO/ArKO $n = 7$ pairs).

0.02, WT $n = 11$, PgrKO n = 9, ArKO n = 7). Interestingly, even after 2-weeks of interactions and stable dominance relationship, the average number of attacks by dominants of PgrKO pairs persisted at significantly higher levels compared to dominants of WT and ArKO pairs (Fig. 2C; $F_{(2,24)} = 5.71$, $p = 0.009$, effect size $\eta^2 = 0.32$; with Tukey's Multiple Comparison post hoc Test, WT vs PgrKO $p = 0.019$, PgrKO vs ArKO $p = 0.021$, WT n = 11, PgrKO n = 9, ArKO n = 7). Moreover, significant group differences were observed in the average number of attacks per encounter among subordinates during the initial 24 h, but those differences did not persist after 2-weeks of observation (Fig. 2D; initial 24 h: $F_{(2,24)} = 3.86$, $p = 0.035$, effect size $\eta^2 = 0.243$; post hoc test, $p > 0.05$; 2-weeks: $F_{(2,24)} = 1.13$, $p > 0.05$, effect size $\eta^2 = 0.086$). Collectively, these results show that PgrKO fish are more aggressive than WT and ArKO fish, and their aggression is persistent.

3.3. Effect of ArKO and PgrKO on territorial behavior

To assess how the differences in aggression among the three different genotypes affect territorial behavior, we mapped the animals' spatial distribution during dominance formation (initial 24 h) and 2-weeks later (Fig. 3). As predicted from prior work (Miller et al., 2017), WT pairs displayed robust social status-dependent localization that appears within 24 h of pairing and becomes more robust as dominance solidifies. By 2-weeks, subordinates localized to the corners of the tank while dominants swam freely throughout with some overlap subordinates' territories (Fig. 3A). Measurement of the overlapping dominants and subordinates territories showed a significant decline between days 1 and 15 (Fig. 3D; Paired t-test, $p = 0.0472$, Cohen's $d = 0.727$; $n = 11$ WT pairs). This decrease in shared swimming area is mirrored by a significant increase in the distance between the two locations dominants and subordinates spent most of their swimming activity (centroids, darkest areas) (Fig. 3E; Paired t-test, $p = 0.0308$; Cohen's $d = 2.38$, n = 11 WT pairs). Conversely, differences in spatial distribution between ArKO dominants and subordinates were less distinct (Fig. 3B). Heat map analysis shows significant territorial overlap in swimming activity of dominants and subordinates during initial pairing. Although the overlapping territory decreased over time (Fig. 3D; Paired t-test, $p = 0.008$; Cohen's $d = 0.881$, $n = 14$ ArKO pairs), the distance between centroids where animals spent most of their swimming activity did not change significantly over the two weeks of observations (Fig. 3E; Paired t-test, $p = 0.1284$; n = 14 ArKO pairs). On the other hand, PgrKO pairs displayed significant differences in territorial activity as dominance matured. During the initial 24 h of observations, PgrKO pairs showed considerable overlap in territory as they fought aggressively for dominance. After two weeks of pairing and dominance formation, the animals localized to opposite corners of the tank with reduced territorial overlap (Fig. 3C). This is evident in the decrease in territorial overlap between day 1 and 15 (Fig. 3D; Paired t-test, $p = 0.027$; Cohen's $d = 0.696$, $n = 12$ PgrKO pairs) and increase in the distance between centroids where animals spent most of their swimming activity (Fig. 3E; Paired t-test, $p = 0.0057$; Cohen's $d = 1.35$, n = 12 PgrKO pairs). Comparative analysis of the distance between dominants and subordinates among WT, ArKO, PgrKO pairs shows that PgrKO pairs experienced the most change in their territorial activity ($F_{(2,29)} = 4.817$, $p = 0.0156$; Tukey's Multiple Comparison post hoc test: WT vs PgrKO $p > 0.05$; WT vs ArKO $p > 0.05$; PgrKO vs ArKO $p = 0.011$, WT n = 11 pairs, ARKO n = 14 pairs, PgrKO n = 12 pairs). Collectively, the results further support the conclusion that disruption of androgen and progestin signaling affects not only aggressive behavior, but also territorial activity. This is illustrated in ArKO pairs in which spatial swimming patterns overlapped extensively and persisted over the two weeks of observations. This was accompanied by low aggressive activities throughout the observation period. Conversely, PgrKO pairs showed elevated aggressive activity that led to strong dominance relationship evident by a strong spatial separation in swimming activity by 2-weeks between dominants and subordinates.

4. Discussion

Our results show that global knockout of Ar causes reduced levels of aggression compared to WT fish, while global loss of Pgr results in increased aggression in male zebrafish. These results are consistent with a large body of evidence that androgen and androgen receptors are key regulators in the aggression and suggest that Pgr is also an important part of signaling pathways in the regulation of aggression. However, we still do not know whether Ar and Pgr also play similar roles in the female aggression in the zebrafish due to limitation of current studies in the male zebrafish. Ar and Pgr express broadly not only in brain regions important for the regulation of aggression (Gorelick et al., 2008; Munchrath and Hofmann, 2010), but also in various peripheral tissues such as muscles (Chiang, 2021; Hossain et al., 2008; Sinha-Hikim et al., 2004). The relative contribution and effects of these receptors in different tissues in the regulation and coordination of agonistic behaviors are still unclear. Serum T and estradiol levels were found to be lower in ArKO males compared to those in wildtype males, while serum 11-ketotestosterone (11-KT) was higher in ArKO male zebrafish (Yu et al., 2018). These ArKO male zebrafish are infertile or sub-fertile due to impaired courtship behavior and lower sperm quality (Yong et al., 2017; Yu et al., 2018). In contrast, PgrKO male zebrafish has normal fertility (Zhu et al., 2015). Whether there is a difference in steroid synthesizing and secretion in PgrKO zebrafish is still unknown. Nevertheless, no difference in serum levels of testosterone and progesterone were found in PgrKO mice (Schneider et al., 2005). Interestingly, PgrKO mice had larger testes, greater sperm production, increased numbers of Sertoli and Leydig cells (Lue et al., 2013). Enhanced androgen receptor expression in the medial preoptic nucleus and bed nucleus of the stria terminalis were also found in PgrKO mice (Schneider et al., 2005). The disorder in gonadal steroidogenesis and difference in fertility also likely contributed to different aggression in ArKO or PgrKO male zebrafish. Future studies should address these limitations and unsolved questions.

In the current study, ArKO dominant zebrafish attacked significantly less than their WT dominant counterparts, and ArKO dominant fish also retreated significantly more than the WT dominants. Furthermore, although ArKO dominants attacked less frequently than WT dominants, the frequency of attacks by ArKO subordinate in ArKO pairs was increased. This suggests that the subordinate fish are not being subdued by their ArKO dominant counterparts. ArKO pairs also failed to form a clear localization pattern within the test tank, indicative of a weakly defined dominance relationship.

These results suggest that the androgen receptor is necessary for normal levels of aggressive behavior in zebrafish. In some species, plasma concentrations of T are positively correlated with aggressive behavior in males (O'Connell and Hofmann, 2012; Weitekamp and Hofmann, 2017; Munley et al., 2018), while in other species T concentrations did not correlate with aggressive activity (Alward et al., 2019; Maruska, 2015). The discrepancies found in these studies may not reflect species differences but due to different experimental conditions. One possible explanation for the variability in the relationship between androgens and aggression is the expression of the androgen receptor, which has been supported by findings from several studies. Zebrafish and other members of the *Cyprinidae* family carry only one functional *ar* gene, which binds preferentially with 11-KT, T, and dihydrotestosterone (DHT) and is expressed strongly in the preoptic area and hypothalamus, which are implicated in social aggression (Greenwood et al., 2008; Hossain et al., 2008; Gorelick et al., 2008). In the California mouse, winning territorial disputes increases AR expression in the nucleus accumbens and ventral tegmental area (Fuxjager et al., 2010). In dark-eyed juncos (*Junco hyemalis*), *ar*, *erα*, and aromatase expression levels in the ventromedial telencephalon are positively correlated with increased aggression, while serum T levels are correlated with aggression only in males (Rosvall et al., 2012). In humans, men with more sensitive AR proteins displayed increased aggression after exposure to T while men with less sensitive ARs (more GAC repeats) did not show an

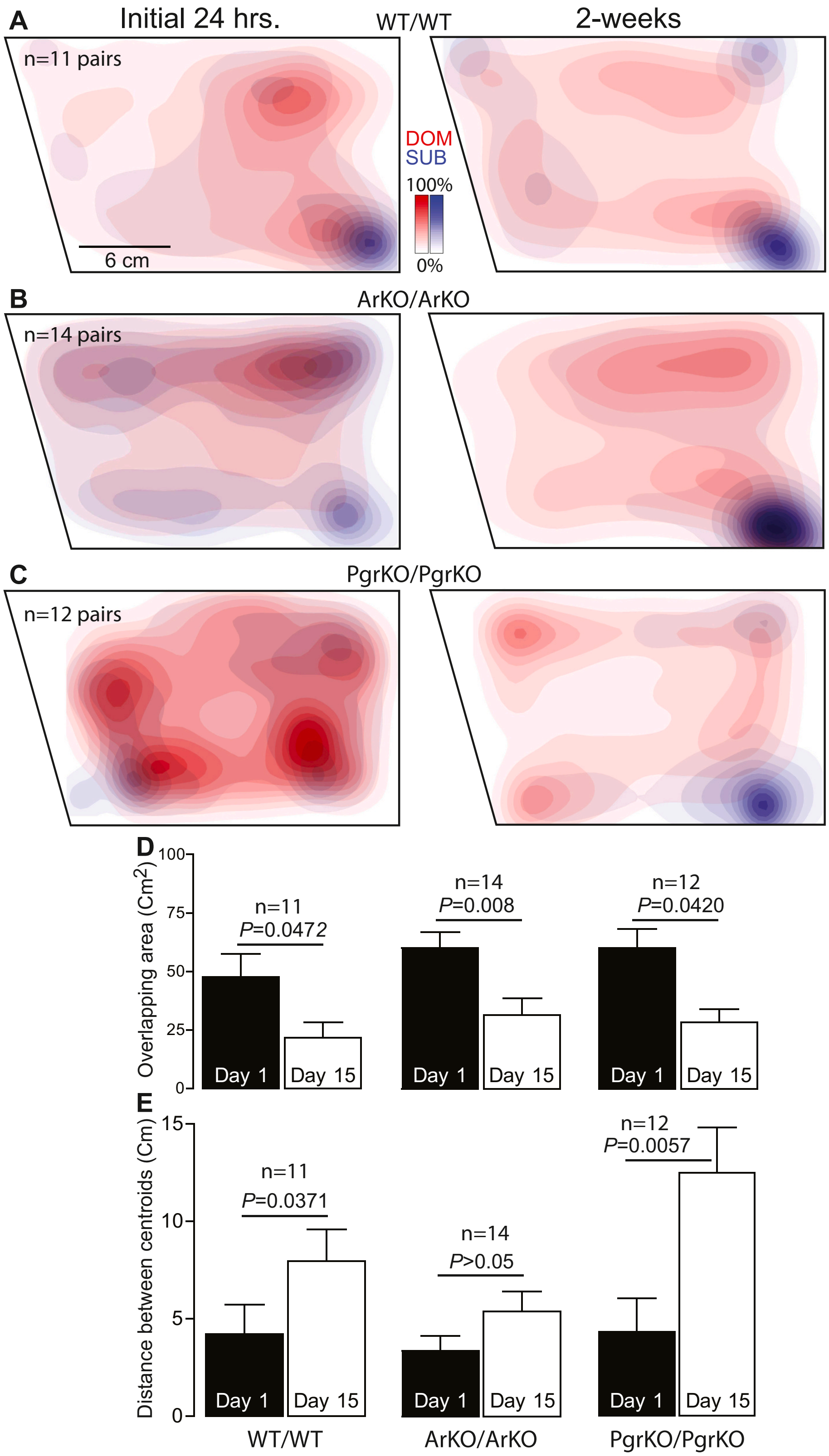

Fig. 3. Localization heat maps indicating average spatial distribution of all dominants (red) and all subordinates (blue) zebrafish during non-interacting periods at the initial 24 h and 2-weeks post pairing for WT/WT n = 11 pairs; ArKO/ArKO *n* = 14 pairs; PgrKO/PgrKO n = 12 pairs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increase in aggression (Geniole et al., 2019). In zebrafish, Filby et al. analyzed transcriptome profiles of dominant and subordinate fish and found that *ar* and *erβ* transcripts are upregulated in dominant fish hypothalamus (Filby et al., 2010). After an aggressive encounter, the concentration of 11-KT is elevated in both dominant and subordinate zebrafish but T concentration is elevated only in subordinate fish (Teles and Oliveira, 2016). Based on our results, and evidence from other groups (Cunningham et al., 2012; Dugger et al., 2007; O'Connell and Hofmann, 2012; Sato et al., 2004), the expression of functional Ar in the brains of male zebrafish is necessary for increased expression of aggressive behaviors. We have previously shown that male ArKO zebrafish exhibited decreased courtship behavior when paired with a female conspecific (Yong et al., 2017). Additionally, research in rats and mice demonstrates that AR functionality is required for full brain masculinization in mammals (Dugger et al., 2007; Sato et al., 2004). Collectively, this raises the possibility that in zebrafish, Ar is necessary for full masculinization of the zebrafish brain and manifestation of normal mating and social behavior. Certainly, the importance of brain Ar in aggression does not necessarily exclude Ar's roles in peripheral tissues/organs such as muscles in coordinating and executing aggressive behavior.

Our results showed that not only were dominants of PgrKO pairs significantly more aggressive compared with dominants of WT pairs (Fig. 1), but the intensity of aggressive interactions was also elevated (Fig. 2). Additionally, subordinates of PgrKO pairs retreated significantly more than WT zebrafish due in part to the persistently elevated attacks by dominants. Closer analysis of their interactions showed that the encounters of PgrKO pairs lasted much longer than WTs, and dominant PgrKO fish attacked more frequently than WT dominants. Social interactions between PgrKO animals were continuous bouts of dominants pursuing and attacking their submissive counterparts, while the subordinates mostly retreated from those interactions. Those interactions were prolonged, allowing for less overall encounters to be recorded per minute (Fig. 3B). These results suggest that PgrKO animals are more aggressive compared to their WT counterparts. This heightened aggression of PgrKO animals culminated with significant differences in territorial activity (Fig. 3C).

Although brain androgens are known to be important in regulating territoriality in many animal species including fish, birds, and mammal species (Heimovics et al., 2015; Munley et al., 2018; Silva et al., 2020); results in male song sparrows (*Melospiza melodia morphna*) show that T does not activate territorial aggression; rather it increases the intensity of aggressive to territorial intrusions (Wingfield, 1994). Similarly, nonbreeding seasonal territorial aggression in male lizards (*Sceloporus jarrovi*) was minimally affected with supplemented T (Moore and Marler, 1987). These findings along with our results suggest that territorial behavior may be controlled by different mechanisms, perhaps via the action of nuclear androgenic receptors that act as transcription factors to activate different regulatory pathways, and brain regions involved in social aggression and territorial activity. For instance, Pgr expression extensively overlaps with the mesocortical dopaminergic pathway of ventral tegmental area (VTA), whose projection and regulation of the prefrontal cortex is critical in regulating executive functions, attention, memory, social behavior and cognition (Quadros et al., 2007; Lopez and Wagner, 2009). Furthermore, inhibition of Pgr activity decreases dopaminergic innervation in the VTA and impairs cognitive flexibility and behavioral inhibition (Willing and Wagner, 2015). Collectively, these findings suggest that Ar and Pgr can induce functional and structural reconfigure of neuromodulatory brain circuits implicated in social behavior by independently influencing the manifestation of aggressive and territorial behaviors. For instance, our results show that despite the significant decrease in aggressiveness in ArKO fish, territorial roaming by dominants was unimpeded.

The current results support the role of progestins and Pgr in inhibiting aggression in zebrafish males, since loss of the progestin receptor caused increased aggressiveness in socially dominant fish. The zebrafish Pgr is activated by several progestins, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), 17,20β,21-Trihydroxy-4-pregnen-3-one (20β-S), progesterone (P4), and 17α-hydroxyprogesterone (Tokarz et al., 2013). The roles of progestins in regulating aggression in other animals is more ambiguous than the androgens, since some species show increases in aggressive behavior in response to progesterone exposure (Weiss and Moore, 2003) while other species show decreases in aggression in response to progesterone (Fraile et al., 1987; Kohlert and Meisel, 2001). The different results may not be due to species difference but could be difference in the experimental conditions. In male horneros (Aves, *Furnarius rufus*), simulated territory intrusions caused progesterone levels to rise after an encounter during the mating period, but not during the parental care period (Adreani et al., 2018). Progesterone treatment increased infanticidal aggression in male mice (Schneider et al., 2009) and progestin receptor knockouts virtually eliminated infant-directed aggression (Schneider et al., 2003).

In female Syrian hamsters (*Mesocricetus auratus*), progestin implants in the ventromedial hypothalamus reduced aggression (Meisel et al., 1989). The zebrafish Pgr is present in the hypothalamus as well and may be the site of progestin action in inhibiting zebrafish aggression (Hanna et al., 2010). Another alternative hypothesis is that developmental changes may have led to increased aggression, as progestins are important in the brain for neuronal survival and proliferation. During developmental, the Pgr is expressed widely across the zebrafish brain and expressed particularly strongly in radial glia cells (Diotel et al., 2011).

Our results coupled with previous findings suggest that the Ar and Pgr are necessary for maintaining normal expression of aggression required for social dominance. Maladaptive aggression levels are a comorbid symptom of a variety of psychiatric illnesses, and a greater understanding of the molecular mechanisms regulating these behaviors is important. With the zebrafish becoming an increasingly popular model for biomedical, neuroscience, and pharmacological research, these findings can aid future studies in delineating the neurohormonal underpinning of aggression. The current study identified the importance of the Ar and Pgr in regulating zebrafish aggression, but the molecular regulatory mechanisms are still unknown. It is possible that other steroid hormones and receptors such as membrane receptors may also play roles in regulating aggression as well. Further, due to the nature of global genetic knockouts, it is also unknown if the changes in behavior were caused by changed levels of active transcription in adult fish, or due to alternation of brain organization during early development. Answering these questions will help uncover the hormonal regulation of aggression in vertebrate species.

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CRediT authorship contribution statement

FI and YZ conceived the project. FI designed experiments. YZ and LY generated knockouts. JC, MC, JB, and SC performed the experiments, analyzed the data, and wrote the draft. YZ, FI and LY revised the manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Adkins-Regan, E., 2005. Hormones and Animal Social Behavior. Princeton University Press, Princeton.

Adreani, N.M., Goymann, W., Mentesana, L., 2018. Not one hormone or another: aggression differentially affects progesterone and testosterone in a South American ovenbird. Horm. Behav. 105, 104–109. https://doi.org/10.1016/j.yhbeh.2006.10.001.

Alward, B.A., Hilliard, A.T., York, R.A., Fernald, R.D., 2019. Hormonal regulation of social ascent and temporal patterns of behavior in an African cichlid. Horm. Behav. 107, 83–95.

Alward, B.A., Laud, V.A., Skalnik, C.J., York, R.A., Juntti, S.A., Fernald, R.D., 2020. Modular genetic control of social status in a cichlid fish. PNAS 117, 28167–28174. https://doi.org/10.1073/pnas.2008925117.

Archer, J., 1991. The influence of testosterone on human aggression. Br. J. Psychol. 82, 1–28. https://doi.org/10.1111/j.2044-8295.1991.tb02379.x.

Arnold, A.P., Breedlove, S.M., 1985. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm. Behav. 19, 469–498.

Barfield, R.J., Busch, D.E., Wallen, K., 1972. Gonadal influence on agonistic behavior in the male domestic rat. Horm. Behav. 3, 247–259. https://doi.org/10.1016/0018-506x(72)90038-4.

van Breukelen, N.A., 2013. Androgen receptor antagonist impairs courtship but not aggressive behavior in the monogamous cichlid, *Amatitlania nigrofasciata*. Horm. Behav. 63, 527–532. https://doi.org/10.1016/j.yhbeh.2013.01.008.

Chiang, S., 2021. Recent advances in smooth muscle tumors with PGR and PLAG1 gene fusions and myofibroblastic uterine neoplasms. Genes Chromosom. Cancer 60, 138–146. https://doi.org/10.1002/gcc.22920.

Clark, C.R., Nowell, N.W., 1980. The effect of the non-steroidal antiandrogen flutamide on neural receptor binding of testosterone and intermale aggressive behavior in mice. Psychoneuroendocrinology 5, 39–45. https://doi.org/10.1016/0306-4530(80)90007-4.

Clements, K.N., Miller, T.H., Keever, J., Hall, A.M., Issa, F.A., 2018. Social status related differences in motor activity between wild type and mutant zebrafish. Biol. Bull. 235 (2), 71–82.

Collias, N.E., 1944. Aggressive behavior among vertebrate animals. Physiol. Zool. 17, 83–123.

Cramer, E.R.A., 2012. Are androgens related to aggression in house wrens? Ethology 118, 975–983.

Cunningham, R.L., Lumia, A.R., McGinnis, M.Y., 2012. Androgen receptors, sex behavior, and aggression. Neuroendocrinology 96, 131–140. https://doi.org/10.1159/000337663.

Diotel, N., Servili, A., Gueguen, M.-M., Mironov, S., Pellegrini, E., Vaillant, C., Zhu, Y., Kah, O., Anglade, I., 2011. Nuclear progesterone receptors are up-regulated by estrogens in neurons and radial glial progenitors in the brain of zebrafish. PLoS One 6, e28375. https://doi.org/10.1371/journal.pone.0028375.

Dugger, B.N., Morris, J.A., Jordan, C.L., Breedlove, S.M., 2007. Androgen receptors are required for full masculinization of the ventromedial hypothalamus (VMH) in rats. Horm. Behav. 51, 195–201.

Filby, A.L., Paull, G.C., Hickmore, T.F.A., Tyler, C.R., 2010. Unravelling the neurophysiological basis of aggression in a fish model. BMC Genomics 11, 498. https://doi.org/10.1186/1471-2164-11-498.

Fores, T.R., 1947. Testis transplantations performed by John Hunter. Endocrinology 41, 329–331.

Fraile, I.G., McEwen, B.S., Pfaff, D.W., 1987. Comparative effects of progesterone and alphaxalone on aggressive reproductive and locomotor behaviors. Pharmacol. Biochem. Behav. 30, 729–735. https://doi.org/10.1016/0091-3057(88)90091-3.

Fuxjager, M.J., Forbes-Lorman, R.M., Coss, D.J., Auger, C.J., Auger, A.P., Marler, C.A., 2010. Winning territorial disputes selectively enhances androgen sensitivity in neural pathways related to motivation and social aggression. PNAS 107, 12393–12398. https://doi.org/10.1073/pnas.1001394107.

Geniole, S.N., Procyshyn, T.L., Marley, N., Ortiz, T.L., Bird, B.M., Marcellus, A.L., Carré, J.M., 2019. Using a psychopharmacogenetic approach to identify the pathways through which—and the people for whom—testosterone promotes aggression. Psychol. Sci. 30, 481–494. https://doi.org/10.1177/0956797619826970.

Geniole, S.N., Bird, B.M., McVittie, J.S., Purcell, R.B., Archer, J., Carré, J.M., 2020. Is testosterone linked to human aggression? A meta-analytic examination of the relationship between baseline, dynamic, and manipulated testosterone on human aggression. Horm. Behav. 123, 104644 https://doi.org/10.1016/j.yhbeh.2019.104644.

Gorelick, D.A., Watson, W., Halpern, M.E., 2008. Androgen receptor gene expression in the developing and adult zebrafish brain. Dev. Dyn. 237, 2987–2995. https://doi.org/10.1002/dvdy.21700.

Greenwood, A.K., Wark, A.R., Fernald, R.D., Hofmann, H.A., 2008. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. Proc. Biol. Sci. 275 (1649), 2393–2402. https://doi.org/10.1098/rspb.2008.0622.

Hanna, R.N., Daly, S.C.J., Pang, Y., Anglade, I., Kah, O., Thomas, P., Zhu, Y., 2010. Characterization and expression of the nuclear progestin receptor in zebrafish gonads and brain. Biol. Reprod. 82, 112–122. https://doi.org/10.1095/biolreprod.109.078527.

Heilman, R.D., Brugmans, M., Greenslade, F.C., DaVanzo, J.P., 1976. Resistance of androgen-mediated aggressive behavior in mice to flutamide, an antiandrogen. Psychopharmacology 47, 75–80. https://doi.org/10.1007/BF00428705.

Heimovics, S.A., Trainor, B.C., Soma, K.K., 2015. Rapid effects of estradiol on aggression in birds and mice: the fast and the furious. Integr. Comp. Biol. 55, 281–293. https://doi.org/10.1093/icb/icv048.

Hossain, M.S., Larsson, A., Scherbak, N., Olsson, Per-E., Orban, L., 2008. Zebrafish androgen receptor: isolation, molecular, and biochemical characterization. Biol. Reprod. 78, 361–369. https://doi.org/10.1095/biolreprod.107.062018.

Huffman, L.S., O'Connell, L.A., Hofmann, H.A., 2013. Aromatase regulates aggression in the African cichlid fish *Astatotilapia burtoni*. Physiol. Behav. 112-113, 77–83. https://doi.org/10.1016/j.yhbeh.2018.12.010.

Hunt, K.E., Hahn, T.P., Buck, C.L., Wingfield, J.C., 2019. Effect of testosterone blockers on male aggression, song and parental care in an arctic passerine, the Lapland longspur (*Calcarius lapponicus*). Horm. Behav. 110, 10–18. https://doi.org/10.1016/j.yhbeh.2019.02.001.

Jones, L.J., Norton, W.H.J., 2015. Using zebrafish to uncover the genetic and neural basis of aggression, a frequent comorbid symptom of psychiatric disorders. Behav. Brain Res. 276, 171–180. https://doi.org/10.1016/j.bbr.2014.05.055.

Jorgensen, C.B., 1971. John Hunter, A.A. Berthold, and the Origins of Endocrinology. Odense University Press, Odense, Denmark, p. 1971.

Juntti, S.A., Tollkuhn, J., Wu, M.V., Fraser, E.J., Soderborg, T., Tan, S., Shin-Ichiro Honda, S.I., Harada, N., Shah, N.M., 2010. The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors. Neuron 66, 260–272. https://doi.org/10.1016/j.neuron.2010.03.024.

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C.F., Weng, W., Bally-Cuif, L., Schneider, H., ZNRC, 2013. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10, 70–86.

Kohlert, J.G., Meisel, R.L., 2001. Inhibition of aggression by progesterone and its metabolites in female Syrian hamsters. Aggress. Behav. 27, 372–381.

Kristensen, T.T., Christensen, L.L., Frystyk, J., Glintborg, D., T'Sjoen, G., Roessler, K.K., Andersen, M.S., 2020. Effects of testosterone therapy on constructs related to aggression in transgender men: a systematic review. Horm. Behav. 128, 104912. https://doi.org/10.1016/j.yhbeh.2020.104912.

Lischinsky, J.E., Lin, D., 2020. Neural mechanisms of aggression across species. Nat. Neurosci. 23, 1317–1328.

Lopez, V., Wagner, C.K., 2009. Progestin receptor is transiently expressed perinatally in neurons of the rat isocortex. J. Comp. Neurol. 512, 124–139.

Loring, M.D., Thomson, E.E., Naumann, E.A., 2020. Whole-brain interactions underlying zebrafish behavior. Curr. Opin. Neurobiol. 65, 88–99. https://doi.org/10.1016/j.conb.2020.09.011.

Lue, Y., Wang, C., Lydon, J.P., Leung, A., Li, J., Swerdloff, R.S., 2013. Functional role of progestin and the progesterone receptor in the suppression of spermatogenesis in rodents. Andrology 1, 308–317. https://doi.org/10.1111/j.2047-2927.2012.00047.x.

Maruska, Karen, 2015. Social Transitions Cause Rapid Behavioral and Neuroendocrine Changes. Integrative & Comparative Biology 55 (2), 294–306. https://doi.org/10.1093/icb/icv057.

McDonald, P.G., Buttemer, W.A., Astheimer, L.B., 2001. The influence of testosterone on territorial defence and parental behavior in male free-living rufous whistlers, *Pachycephala rufiventris*. Horm. Behav. 39, 185–194.

Meisel, R.L., Fraile, I.G., Pfaff, D.W., 1989. Hypothalamic sites of progestin action on aggression and sexual behavior in female Syrian hamsters. Physiol. Behav. 47, 219–223.

Miller, T.H., Clements, K., Ahn, S., Park, C., Ji, E.H., Issa, F.A., 2017. Social status-dependent shift in neural circuit activation affects decision-making. J. Neurosci. 7 (8), 2137–2148. https://doi.org/10.1523/JNEUROSCI.1548-16.2017.

Moore, M.C., Marler, C.A., 1987. Effects of testosterone manipulations on nonbreeding season territorial aggression in free-living male lizards, Sceloporus jarrovi. Gen. Comp. Endocrinol. 65, 225–232. https://doi.org/10.1016/0016-6480(87)90170-5.

Moore, I.T., Hernandez, J., Goymann, W., 2020. Who rises to the challenge? Testing the challenge hypothesis in fish, amphibians, reptiles, and mammals. Horm. Behav. 123, 104537 https://doi.org/10.1016/j.yhbeh.2019.06.001.

Morris, J.A., Jordan, C.L., Breedlove, S.M., 2004. Sexual differentiation of the vertebrate nervous system. Nat. Neurosci. 7, 1034–1039.

Munchrath, L.A., Hofmann, H.A., 2010. Distribution of sex steroid hormone receptors in the brain of an African cichlid fish, *Astatotilapia burtoni*. J. Comp. Neurol. 518, 3302–3326. https://doi.org/10.1002/cne.22401.

Munley, K.M., Rendon, N.M., Demas, G.E., 2018. Neural androgen synthesis and aggression: insights from a seasonally breeding rodent. Front. Endocrinol. 9, 136. https://doi.org/10.3389/fendo.2018.00136.

Nelson, R.J., 1995. An Introduction to Behavioral Endocrinology. Sinauer Assoc., Inc., Sunderland, MA.

Nelson, R.J., Trainor, B.C., 2007. Neural mechanisms of aggression. Nat. Rev. Neurosci. 8, 536–546. https://doi.org/10.1038/nrn2174.

O'Connell, L.A., Hofmann, H.A., 2012. Social status predicts how sex steroid receptors regulate complex behavior across levels of biological organization. Endocrinology 153, 1341–1351. https://doi.org/10.1210/en.2011-1663.

O'Connell, L.A., Ding, J.H., Hofmann, H.A., 2013. Sex differences and similarities in the neuroendocrine regulation of social behavior in an African cichlid fish. Horm. Behav. 64, 468–476. https://doi.org/10.1016/j.yhbeh.2013.07.003.

O'Connor, D.B., Archer, J., Hair, W.M., Wu, F.C., 2002. Exogenous testosterone, aggression, and mood in eugonadal and hypogonadal men. Physiol. Behav. 75, 557–566. https://doi.org/10.1016/S0031-9384(02)00647-9.

Oliveira, R.F., Silva, J.F., Simões, J.M., 2011. Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. Zebrafish 8 (2), 73–81. https://doi.org/10.1089/zeb.2011.0690.

Olivier, B., Young, L.J., 2002. Animal models of aggression. In: Neuropsychopharmacology: The Fifth Generation of Progress, vol. 118, pp. 1699–1708.

Orr A, Stephen, Ahn, Sungwoo, Park, Choongseok, Miller H, Thomas, Kassai, Miki, Issa A, Fadi, 2021. Social Experience Regulates Endocannabinoids Modulation of Zebrafish Motor Behaviors. Frontiers in Behavioral Neuroscience 15 (668589), 1–17. https://doi.org/10.3389/fnbeh.2021.668589.

Pavlidis, M., Sundvik, M., Chen, Yu-C., Panula, P., 2011. Adaptive changes in zebrafish brain in dominant-subordinate behavioral context. Behav. Brain Res. 225, 529–537. https://doi.org/10.1016/j.bbr.2011.08.022.

Quadros, P.S., Pfau, J.L., Wagner, C.K., 2007. Distribution of progesterone receptor immunoreactivity in the fetal and neonatal rat forebrain. J. Comp. Neurol. 504, 42–56.

Rodgers, C.M., Neff, B.D., Knapp, R., 2013. Androgen-mediated nurturing and aggressive behaviors during paternal care in bluegill sunfish (*Lepomis macrochirus*). Horm. Behav. 63, 454–461. https://doi.org/10.1016/j.yhbeh.2012.12.008.

Rosell, D.R., Siever, L.J., 2015. The neurobiology of aggression and violence. CNS Spectr. 20, 254–279.

Rosvall, K.A., Bergeon Burns, C.M., Barske, J., Goodson, J.L., Schlinger, B.A., Sengelaub, D.R., Ketterson, E.D., 2012. Neural sensitivity to sex steroids predicts individual differences in aggression: implications for behavioural evolution. Proc. Biol. Sci. 279, 3547–3555. https://doi.org/10.1098/rspb.2012.0442.

Rowland, W.J., 1989. The effects of body size, aggression and nuptial coloration on competition in male threespine sticklebacks, *Gasterosteus aculeatus*. Anim. Behav. 37, 282–289.

Salas-Ramirez, K.Y., Montalto, P.R., Sisk, C.L., 2008. Anabolic androgenic steroids differentially affect social behaviors in adolescent and adult male Syrian hamsters. Horm. Behav. 53, 378–385.

Sato, T., Matsumoto, T., Kawano, H., Watanabe, T., Uematsu, Y., Sekine, K., Fukuda, T., Aihara, K., Krust, A., Yamada, T., Nakamichi, Y., Yamamoto, Y., Nakamura, T., Yoshimura, K., Yoshizawa, T., Metzger, D., Chambon, P., Kato, S., 2004. Brain masculinization requires androgen receptor function. PNAS 101, 1673–1678.

Sawin, C.T., 1996. Arnold Adolph Berthold and the transplantation of testes. Endocrinologist 6, 164–168. https://doi.org/10.1097/00019616-199605000-00002.

Schneider, J.S., Stone, M.K., Wynne-Edwards, K.E., Horton, T.H., Lydon, J., Omalley, B., Levine, J.E., 2003. Progesterone receptors mediate male aggression toward infants. PNAS 100, 2951–2956. https://doi.org/10.1073/pnas.013010010010.1073/pnas.0130100100.

Schneider, J.S., Burgess, C., Sleiter, N.C., DonCarlos, L.L., Lydon, J.P., O'Malley, B., Levine, J.E., 2005. Enhanced sexual behaviors and androgen receptor immunoreactivity in the male progesterone receptor knockout mouse. Endocrinology 146, 4340–4348. https://doi.org/10.1210/en.2005-0490.

Schneider, J.S., Burgess, C., Horton, T.H., Levine, J.E., 2009. Effects of progesterone on male-mediate infant-directed aggression. Behav. Brain Res. 199, 340–344. https://doi.org/10.1016/j.bbr.2008.12.019.

Schwabl, H., Kriner, E., 1991. Territorial aggression and song of male European robins (*Erithacus rubecula*) in autumn and spring: effects of antiandrogen treatment. Horm. Behav. 25, 180–194.

Searcy, W.A., Wingfield, J.C., 1980. The effects of androgen and antiandrogen on dominance and aggressiveness in male red-winged blackbirds. Horm. Behav. 14, 126–135.

Silva, A.C., Zubizarreta, L., Quintana, L., 2020. A teleost fish model to understand hormonal mechanisms of non-breeding territorial behavior. Front. Endocrinol. 11, 468. https://doi.org/10.3389/fendo.2020.00468.

Simon, N.G., McKenna, S.E., Lu, S.-F., Cologer-Clifford, A., 1996. Development and expression of hormonal systems regulating aggression. Ann. N. Y. Acad. Sci. 794, 8–17.

Sinha-Hikim, I., Taylor, W.E., Gonzalez-Cadavid, N.F., Zheng, W., Bhasin, S., 2004. Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. J. Clin. Endocrinol. Metab. 89, 5245–5255. https://doi.org/10.1210/jc.2004-0084.

Sloman, K.A., Armstrong, J.D., 2002. Physiological effects of dominance hierarchies: laboratory artefacts or natural phenomena? J. Fish Biol. 61, 1–23.

Soma, K.K., Sullivan, K.A., Tramontin, A.D., Saldanha, C.J., Schlinger, B.A., Wingfield, J. C., 2000. Acute and chronic effects of an aromatase inhibitor on territorial aggression in breeding and nonbreeding male song sparrows. J. Comp. Physiol. A. 186, 759–769. https://doi.org/10.1007/s003590000129.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behavior and ecology of the zebrafish, Danio rerio. Biol. Rev. Camb. Philos. Soc. 83, 13–34. https://doi.org/10.1111/j.1469-185X.2007.00030.x.

Sperry, T.S., Wacker, D.W., Wingfield, J.C., 2009. The role of androgen receptors in regulating territorial aggression in male song sparrows. Horm. Behav. 57, 86–95. https://doi.org/10.1016/j.yhbeh.2009.09.015.

Teles, M.C., Oliveira, R.F., 2016. Androgen response to social competition in a shoaling fish. Horm. Behav. 78, 8–12. https://doi.org/10.1016/j.yhbeh.2015.10.009.

Terranova, J.I., Song, Z., Larkin II, T.E., Hardcastle, N., Norvelle, A., Riaz, A., Albers, H. E., 2016. Serotonin and arginine-vasopressin mediate sex differences in the regulation of dominance and aggression by the social brain. PNAS 113, 13233–13238. https://doi.org/10.1073/pnas.1610446113.

Tokarz, J., Möller, G., de Angelis, M.H., Adamski, J., 2013. Zebrafish and steroids: what do we known and what do we need to know? J. Steroid Biochem. Mol. Biol. 137, 165–173. https://doi.org/10.1016/j.jsbmb.2013.01.003.

Wacker, D.W., Khalaj, S., Jones, L.J., Champion, T.L., Davis, J.E., Meddle, S.L., Wingfield, J.C., 2016. Dehydroepiandrosterone heightens aggression and increases androgen receptor and aromatase mRNA expression in the brain of a male songbird. J. Neuroendocrinol. 28 https://doi.org/10.1111/jne.12443 n/a.

Way, G.P., Ruhl, N., Snekser, J.L., Alexis, K.L., McRobert, S.P., 2015. A comparison of methodologies to test aggression in zebrafish. Zebrafish 12, 144–151. https://doi.org/10.1089/zeb.2014.1025.

Weiss, S.L., Moore, M.C., 2003. Activation of aggressive behavior by progesterone and testosterone in male tree lizards, *Urosaurus ornatus*. Gen. Comp. Endocrinol. 136, 282–288.

Weitekamp, C.A., Hofmann, H.A., 2017. Neuromolecular correlates of cooperation and conflict during territory defense in a cichlid fish. Horm. Behav. 89, 145–156. https://doi.org/10.1016/j.yhbeh.2017.01.001.

Wiley, C.J., Goldizen, A.W., 2003. Testosterone is correlated with courtship but not aggression in the tropical buff-banded rail, *Gallirallus philippensis*. Horm. Behav. 43, 554–560.

Willing, J., Wagner, C., 2015. Progesterone receptor expression in the developing mesocortical dopamine pathway: importance for complex cognitive behavior in adulthood. Neuroendocrinology 157 (1), 77–82. https://doi.org/10.1210/en.2015-1775.

Wingfield, J.C., 1984. Environmental and endocrine control of reproduction in the song sparrow, *Melospiza melodia*. II. Agonistic interactions as environmental information stimulating secretion of testosterone. Gen. Comp. Endocrinol. 56, 417–424. https://doi.org/10.1016/0016-6480(84)90084-4.

Wingfield, J.C., 1994. Regulation of territorial behavior in the sedentary song sparrow, Melospiza melodia morphna. Horm. Behav. 28, 1–15. https://doi.org/10.1006/hbeh.1994.1001.

Wingfield, J.C., Jacobs, J.D., Soma, K., Maney, D.L., Hunt, K., Wisti-Peterson, D., Meddle, S., Ramenofsky, M., Sullivan, K., 1999. Testosterone, aggression, and communication: ecological bases of endocrine phenomena. In: Konishi, M., Hauser, M. (Eds.), The Biology of Communication. MIT Press, Cambridge, MA, pp. 255–283.

Wingfield, J.C., Moore, I.T., Goymann, W., Wacker, D.W., Sperry, T.S., 2006. Contexts and ethology of vertebrate aggression: implications for the evolution of hormone-behavior interactions. In: Nelson, R.J. (Ed.), Biology of Aggression. Oxford University Press, New York, pp. 179–210.

Wright, E., Galbany, J., McFarlin, S.C., Ndayishimiye, E., Stoinski, T.S., Robbins, M.M., 2019. Male body size, dominance rank and strategic use of aggression in a group-living mammal. Anim. Behav. 151, 87–102.

Yong, L., Thet, Z., Zhu, Y., 2017. Genetic editing of the androgen receptor contributes to impaired male courtship behavior in zebrafish. J. Exp. Biol. 220, 3017–3021. https://doi.org/10.1242/jeb.161596.

Yu, G., Zhang, D., Liu, W., Wang, J., Liu, X., Zhou, C., Gui, J., Xiao, W., 2018. Zebrafish androgen receptor is required for spermatogenesis and maintenance of ovarian function. Oncotarget 9, 24320–24334. https://doi.org/10.18632/oncotarget.24407.

Zhu, Y., Liu, D., Shaner, Z.C., Chen, S., Hong, W., Stellwag, E.J., 2015. Nuclear progestin receptor (Pgr) knockouts in zebrafish demonstrate role for Pgr in ovulation but not in rapid non-genomic steroid mediated meiosis resumption. Front. Endocrinol. 6, 37. https://doi.org/10.3389/fendo.2015.00037.