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# Investigating Reproductive Success and Endocrine Regulation of Mating Strategies in Male Japanese Medaka

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# INVESTIGATING REPRODUCTIVE SUCCESS AND ENDOCRINE REGULATION OF MATING STRATEGIES IN MALE JAPANESE MEDAKA

By

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## ABSTRACT

Mate guarding, when two males compete for one female, is a reproductive strategy seen across a variety of vertebrate species. This often leads to hierarchical relationships, in which one male exerts dominance over other, subordinate males. However, the physiological mechanisms that promote dominance or subordinance in males remain largely unexplored. This study investigates the reproductive success and endocrine signals of these reproductive strategies in Japanese medaka (*Oryzias latipes*). To identify dominant and subordinate males, triads consisting of two males of different genotypes and one female were observed repeatedly for 5 days. Male reproductive success was determined by genotyping embryos from each female. We found that the number of eggs fertilized by dominants and subordinates did not differ ($p=0.29$), indicating that dominant behavior does not guarantee reproductive success and that subordinate males may successfully fertilize eggs using sneaker male tactics. We hypothesized that these behaviors are linked to activity in the reproductive endocrine axis. To test this hypothesis, we quantified pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) in dominant and subordinate males using ELISAs. While FSH did not differ between the groups, LH was unexpectedly higher in subordinate males ($p=0.047$). This indicates that either LH production is stimulated, or its pituitary release is inhibited in subordinates. To investigate these opposing explanations, we measured mRNA levels of LH, FSH, and GnRH receptors in the pituitary, and GnRH and AVT in the brain of dominant and subordinate males using qPCR. Mean differences between dominants and subordinates were not significant for any gene. Dominant fish expressed higher *lhb* in 8/12 tanks, indicating that LH production is not stimulated in subordinates, but as the transcripts for GnRH and its receptors also did not differ, further studies are needed to determine the mechanism by which LH release may be inhibited.

# TABLE OF CONTENTS

Committee Signatures……ii

Curriculum Vitae……iii

Acknowledgments……iv

Abstract……v

Thesis Proper

Introduction……1

Materials and Methods……7

Results……12

Discussion……17

Literature Cited……21

# INTRODUCTION

### *Mate guarding behavior*

Mate guarding is a male reproductive strategy that increases individual male fitness by reducing rival males' access to potential mating partners. This behavior is driven both by attraction to the female and competition among males (Yokoi et al 2015). Although mate guarding occurs across a variety of species from house sparrows to squirrels to humans, it has rarely been studied in a lab or in genetic model organisms (Hoi et al 2011, Sherman 1989, Yokoi et al 2015). In 2015, Yokoi et al established that male Japanese medaka, a teleost fish, prominently exhibit mate-guarding behavior in a triadic relationship (Yokoi et al 2015). When two males are housed together with one female, one of the males occupies a dominant position near the female and interferes with the subordinate male's access to the female. This distinct behavior appears to be a combination of courtship display directed toward the female and aggression toward the other male. As medaka reproduce every morning, mate guarding can be seen daily. Yokoi et al (2015) also found that dominant males had a significantly higher mating success rate, fertilizing over 93% of the eggs. Having established observable mate guarding behavior in a genetic model, Yokoi et al (2015) were also the first to investigate an underlying physiological mechanism for it. Using knockout mutants of arginine-vasotocin (*avt*), a homolog to mammalian arginine-vasopressin (AVP), or its receptors (*v1a1* and *v1a2*), they found that *avt* and *v1a2* are required for normal mate-guarding behavior. Furthermore, the impaired behavior in knockouts is due to loss of sexual motivation, not competitive motivation, as *avt* mutants exhibited fewer courtship displays but normal aggression in a non-mate guarding situation (Yokoi et al 2015). No other study has yet corroborated these results or attempted to identify additional physiological motivators of mate guarding behavior.

### *Medaka*

Medaka are a teleost fish native to the rice paddies of Japan, Korea, and eastern China. Already a popular model organism in Asia, they are becoming more commonly used in Europe and North America as molecular techniques and genetic tools continue to be developed (Wittbrodt 2002).

Medaka are used as a model for both fishes and vertebrates in general. Their small size (3-4 cm), resistance to common fish diseases, and short generation time of 2-3 months makes them an ideal lab animal. They tolerate a wide range of salinities and temperatures (4-40 °C) and can reproduce every day, year-round in a lab. They are oviparous, producing transparent eggs that are fertilized externally, which is beneficial for studying development and reproduction. The sequencing of their genome, development of transgenic lines, and characterization of mutant phenotypes have made medaka valuable for genetics research (Wittbrodt et al 2002, Kirchmaier et al 2015).

Medaka have many genetic and morphological similarities to another model fish species, the zebrafish, which are separated from their last common ancestor by 110 million years, making them ideal for comparative studies. Some differences between them include slower development, and unlike zebrafish, which do not have sex-linked genes, medaka have the same XX, XY sex determination system as mammals. These characteristics have also made medaka a popular model for sex determination and sexual dimorphism (Wittbrodt et al 2002).

### *HPG axis*

The reproductive endocrine axis, also called the hypothalamus-pituitary-gonadal (HPG) axis, controls reproduction in vertebrates. In this system, the hypothalamus regulates the production and release of the gonadotrophins LH and FSH from the anterior pituitary gland into

the bloodstream. LH and FSH stimulate sex steroid production by the gonads by binding to their specific receptors, luteinizing hormone receptor (LHr) and follicle stimulating hormone receptor (FSHr) (Levavi-Sivan et al 2010).

LH and FSH are of central importance in the axis as they communicate between the brain and the rest of the body. Fish lack a hypothalamic-pituitary portal system. Instead, the pituitary is directly innervated by the hypothalamus. Gonadotrophin releasing hormone (GnRH) is the primary regulator of gonadotrophin release from the pituitary, but other factors also can play a role such as GABA, dopamine, and neuropeptide Y (Zohar et al 2010). Unlike in mammals, FSH and LH in teleost species are produced and released from separate pituitary cells. Therefore, in medaka, the production of these hormones can be studied in isolation. While the exact function of each hormone has not been clearly defined, research indicates that FSH is more important in gametogenesis and vitellogenesis, while LH is important in ovulation and spermiation (Levavi-Sivan et al 2010, Murozumi et al 2014, Takahashi et al 2016).

As the primary regulator of FSH and LH, studying GnRH communication with the pituitary provides insight into hypothalamic control of the HPG axis. In some species, larger GnRH cells have been associated with male alternative reproductive tactics. There are multiple GnRH isoforms that exert different functions via several classes of receptors. In cichlid fish, the presence of other males and the opportunity to increase social status caused an increase in one of three forms of GnRH (Knapp 2003). Medaka have three paralogous GnRH genes—*gnrh-1*, *gnrh-2*, and *gnrh-3*, although only *gnrh-1* and *gnrh-3* are expressed in the forebrain (Okubo et al 2000) and therefore more likely to play a role in gonadotropin regulation. GnRH neurons in the preoptic area express *gnrh-1*, while neurons in the terminal nerve express *gnrh-3* (Okubo et al 2002).

### *Arginine-Vasotocin*

Arginine-vasotocin (AVT) is a nonapeptide produced by neurons in the ventral hypothalamus and preoptic area (POA) in fish (Iwasaki et al. 2013). In medaka, AVT neurons originate in the ventral hypothalamus and the gigantocellular, magnocellular, and parvocellular POA and primarily project to the posterior pituitary. However, the AVT neurons in the three POA cell populations also send fibers into many other regions of the brain such as the telencephalon, mesencephalon and diencephalon (Kagawa et al 2016). Two AVT receptors (V1a1 and V1a2*)* are expressed in the brain, while a third (V2) is localized in the gills, heart, and kidney (Lema 2010). In addition to inducing antidiuretic effects in the kidney and regulating osmotic balance in teleosts, AVT mediates aggression and sociosexual behaviors in a variety of species (Knapp 2003, Lema 2010). It has also been linked to territorial behavior in a tropical damselfish and pair bonding in cichlid fish (Yokoi et al 2015). In some species, correlations between alternative male reproductive tactics and the size of AVT neurons in the preoptic area have been found, although the direction of relationship is species dependent (Knapp 2003). Yokoi et al (2015) found that *avt* and its receptor *v1a2* are required for mate guarding behavior in male medaka using knockout fish, while *v1a1* is not. Expression of the AVT system in wild-type dominant and subordinate males has not yet been investigated.

### *Transgenic Lines*

Transgenesis in medaka is achieved by injecting DNA into the cytoplasm of the 1-2 cell stage embryo. The embryos that stably integrate the DNA become transgenic founders (Wittbrodt et al 2002). At the Weltzien lab at the Norwegian University of Life Sciences (NMBU), several stable transgenic medaka lines have been developed. Two of these lines couple the expression of green florescent protein (*gfp*) to the *lhβ* promotor and red florescent protein

(*rfp*) to the *fshβ* promotor. So, LH-producing cells will also produce GFP, and FSH-producing cells will also produce RFP (Fontaine et al 2019). While transgenic lines can be useful for cell counting and visualizing locations of cells, in this study, they are used only as a marker for paternity testing.

***Objectives***

Although medaka have been established as an exciting new model for studying mate guarding behavior, the physiological mechanisms that promote dominance or subordinance in males remain largely unexplored. As a model for fishes and vertebrates in general, this research is useful for understanding mate guarding, a behavior widely seen across the animal kingdom. It also has practical applications in fish, providing information about important behavior and endocrine factors to improve reproduction in captivity, an action that would progress both aquaculture and restoration efforts. This project investigates the success of the two reproductive strategies in male medaka. We hypothesize that these behaviors are linked to activity in the HPG axis and will therefore examine the relationship between reproductive strategy and reproductive endocrine factors.

The first specific objective was to identify dominant and subordinate fish using a behavioral assay, and then genotype embryos to determine paternity, and therefore male reproductive success. The second objective was to identify underlying mechanisms that drive the different mating behaviors. As central elements of the reproductive axis, LH and FSH are likely candidates for involvement in reproductive strategy determination. To determine their involvement, we compared LH and FSH gene expression and protein levels in the pituitaries of dominant and subordinate males using qPCR and ELISAs. Based on the known function of LH and FSH, we predicted that gonadotropin levels and transcription would be higher in the

dominant fish. We also used qPCR to analyze pituitary expression of three gonadotrophin releasing hormone receptors (*gnrhr1b, gnrhr2a, gnrhr2b)* and brain expression of *gnrh-1* and *gnrh-3* which stimulate the release of LH and FSH. After considering the pituitary gonadotropin level results, we predicted that gene expression of GnRH or its receptors would be downregulated in subordinate fish, preventing the release of LH from the pituitary. AVT and its receptors play a role in aggression and territorial behavior in some teleost species. To verify the results of a previous knockout study which suggests their involvement in medaka mate guarding behavior (Yokoi et al 2015), I analyzed expression of *avt*, *vlal*, and *vla2* in the brain of dominant and subordinate males using qPCR. We predicted that expression of *avt* and *v1a2* would be higher in dominant males.

# MATERIALS AND METHODS

### *Animals*

Medaka were kept at the Weltzien lab at NMBU in Oslo, Norway. Adult fish were maintained in a recirculating system at 28°C on a 14-h light, 10-h dark cycle. They were fed three times daily with a combination of dry feed and live *Artemia salina*.

### *Behavior Assay*

The behavior assay was completed at the Weltzien lab to determine dominant and subordinate males. Triads were formed with two male medaka and one female medaka co-housed in a 3-L tank. The males were distinguished by clipping the top or bottom corner of the tail fin of lightly anesthetized fish. We observed triads for 1-3 minutes each at three time points between 9 am and 12 pm for at least 5 days. The male exhibiting the dominant behavior, characterized as guarding the female and aggression toward the other male, was recorded. All observations were blind, without knowledge of the records from the previous time points. Males that exhibited dominant behavior at ≥80% of the last 10 time points were considered dominant, while males that exhibited dominant behavior ≤20% of the 10 time points were labeled subordinate.

### *Measuring Reproductive Success*

Reproductive success of dominant and subordinate males was measured using one male from each transgenic line (*lhβ:gfp* and *fshβ:rfp*) paired with wild type females to form triads (n=10). Only fertile males homozygous for the respective transgene were used. Following the behavior assay and determination of dominance and subordinance, we collected 20-21 embryos from 2-4 clutches and incubated them 1-5 days in petri dishes at 26°C. Embryo DNA was

amplified directly from the tissue using the Thermo Scientific Phire Animal Tissue Direct PCR kit. The embryos were genotyped individually for the transgenes using gel electrophoresis with 2% agarose gel to determine paternity. Sequences for the primers used in PCR are displayed in Table 1. Reproductive success was determined by comparing the percentage of eggs fertilized by dominant males verses subordinate males, and difference in fertilization rate was assessed using a two-sample t-test.

Table 1. Primer sequences for PCR genotyping.

| Target Gene | Forward Sequence (5'- 3') | Reverse Sequence (5'- 3') |
|---|---|---|
| *rfp* | GTGTAGTCCTCGTTGTGGGA | AGTTCATGCGCTTCAAGGTG |
| *gfp* | TGGTGGAGATCCGCAGCGACAT | ATGGCGGTCTCGTGCTGCTCTA |

***ELISAs***

Enzyme-linked immunosorbent assays (ELISAs) were used to quantify FSH and LH protein levels in the pituitaries of dominant and subordinate fish instead of plasma levels, as the size of the fish prevents adequate blood collection. Following the behavior assay using wild-type males, we collected the brains and pituitaries as a unit from the dominants and subordinates (n=34). Tissue samples were homogenized in PBST with 0.1% BSA. We performed competitive ELISAs developed and validated by Burow et al. for LH and FSH in medaka according to the protocol described by the developer to calculate the amount of hormone in ng/pituitary (Burow et al. 2019). The results were analyzed using a two-way ANOVA where reproductive strategy is one factor and the separate ELISA plates are another factor.

***RNA extraction and cDNA synthesis***

Wild-type dominant and subordinate males (n=20) were dissected and brains and pituitaries were collected separately and stored in Trizol. Tissue was homogenized with lysing beads three times for 20 seconds at 4.0 m/s in an MP Biomedicals FastPrep-24 Instrument, and frozen at -80 °C. The samples were then shipped on dry ice from NMBU to the University of Mary Washington. We extracted RNA from the samples using the Zymo Research Direct-zol RNA Microprep kit for the pituitaries and the Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA) for the brains according to the manufacturer protocol. The RNA concentration was calculated from $OD_{260}$ and purity was assessed by 260/280 and 260/ 230 ratios, measured using a Thermo Scientific Nanodrop 2000 spectrophotometer. We used a Thermo Scientific Maxima H Minus First Strand cDNA synthesis kit (Thermo Fisher, Waltham, MA) to reverse transcribe 60 ng of RNA per sample into cDNA according to the manufacturer protocol.

***qPCR***

We performed qPCR using 1× Thermo Fisher SYBR Select Master Mix. Each cDNA sample was measured in triplicate with 1 μL of cDNA per 10 μL reaction in a Thermo Fisher Quantstudio 3 real-time PCR system. Standard curves made with pooled cDNA were included on each plate to determine efficiency. Efficiencies above 85 percent were accepted. The following genes were measured in the brain: *gnrh-1, gnrh-3, avt, v1a1, v1a2*. These genes were measured in the pituitary: *lhb, fshb, gnrhr1b, gnrhr2a, gnrhr2b*. The housekeeping genes *rpl7*, *18s*, and *gapdh* were measured and assessed for stability in each tissue using RefFinder (Burow et al 2019). Primer sequences for each gene used are displayed in Table 2. Data was normalized to *rpl7* in the pituitary and *gapdh* in the brain by subtracting the CT value for the housekeeping gene from the CT value for the target gene for each sample. Differences in gene expression were

calculated as normalized subordinate CT values minus normalized dominant CT values for each pair. Mean differences were evaluated using t-tests.

Table 2. Primer sequences for qPCR analysis.

| Target Gene | Forward Sequence (5'- 3') | Reverse Sequence (5'- 3') |
|---|---|---|
| *lhb* | CCACTGCCTTACCAAGGACC | AGGAAGCTCAAATGTCTTGTAG |
| *fshb* | GACGGTGCTACCATGAGGAT | TCCCCACTGCAGATCTTTTC |
| *gnrh-1* | GTGTCGCAGCTCTGTGTTC | AGTATTTCAGTTCTCGCTTCCC |
| *gnrh-3* | GATGATGGGCACAGGAAGAGTG | GGGCACTTGCATCTTCAGGA |
| *gnrhr1b* | TCCTGCTACACATCCACCAG | GCCTTTGGGATGATGTCTGT |
| *gnrhr2a* | GGGCGATGAGTGTGATCCTC | CCCGAGTGGCACATTGAGT |
| *gnrhr2b* | TTGAGATATCAAGCCGCATC | GAGTCCTCATCCGAGCTTTG |
| *avt* | CCGCCTGTTACATCCAGAACT | GGGCCACAAGACATGCACT |
| *v1a1* | GTGGGACCAGACCTTCTCC | TGTAGATCCAGGGGTTGCAG |
| *v1a2* | TGTGGTCTGTGTGGGATGAA | TGTAAATCCACGGGTTGCAG |
| *rpl7* | TGCTTTGGTGGAGAAAGCTC | TGGCAGGCTTGAAGTTCTTT |
| *18s* | CCTGCGGCTTAATTTGACTC | AACTAAGAACGGCCATGCAC |
| *gapdh* | GCAAAGTCATCCCTGCTCTC | CCACAGACACATCAGCCACT |

***Data analysis***

Fertilization rates, body masses, and lengths of dominant and subordinate males were compared using two sample t-tests. Effects of transgenic line and position of fin clip on fertilization rate were also tested by t-tests. Pituitary FSH and LH levels were compared using a two-way ANOVA with behavior and ELISA plate as factors. Difference in gene expression within each pair was calculated as normalized subordinate CT value minus normalized dominant

CT value. T-tests were used to assess mean differences. The differences were considered significant if $p \leq 0.05$.

# RESULTS

### *Reproductive success*

Eggs from 10 triads composed of a wild-type female and males from different transgenic lines (*lhβ:gfp* and *fshβ:rfp*) were genotyped for the transgenes to determine paternity. The percentage of eggs fertilized by the subordinate and dominant fish was calculated as a measure of reproductive success (Figure 1). The subordinate fish fertilized on average 59% ± 11.7 (mean ±SEM) of eggs while dominants fertilized 41% ± 11.7. The percentage of eggs fertilized by dominant and subordinate males did not significantly differ ($t(18) = 2.1$, $p = 0.29$).

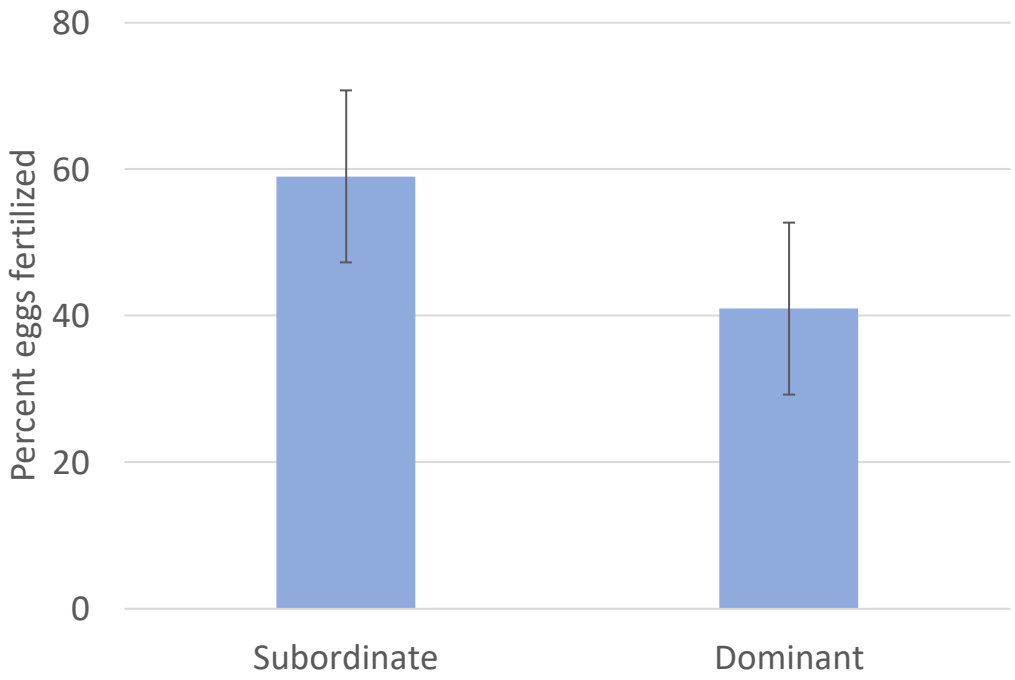


Figure 1. Effect of strategy on reproductive success. Fertilization rates of dominant and subordinate males (n =10) did not differ (p =0.29). The results are indicated as mean ± SEM.

Fertilization rate did not differ according to transgenic line (t(18) =2.1, p=0.56) or position of fin clip (t(18) =2.1, p=0.64), as determined by t-tests. Dominant and subordinate males also did not differ in mean body mass (t(57) =2.0, p =0.09) or length (t(57) =2.0, p =0.58).

***Pituitary gonadotropin levels***

Wild-type males from 34 triads were used for measurement of FSH (n=15) and LH (n=19) by ELISA (Figure 2). Mean LH levels were significantly higher in subordinate males (17.4 ±2.55 ng/pituitary; mean ± SEM) than in dominants (12.4 ±2.04 ng/pituitary). Mean FSH levels did not differ between dominant males (150 ±31.2 ng/pituitary) and subordinates (201 ±61.4 ng/pituitary). Comparison of gonadotropin levels within pairs shows that LH levels were higher in the subordinate in 14/19 triads, whereas FSH levels were higher in the subordinate in 9/15 triads and the males in two triads had equivalent FSH levels.

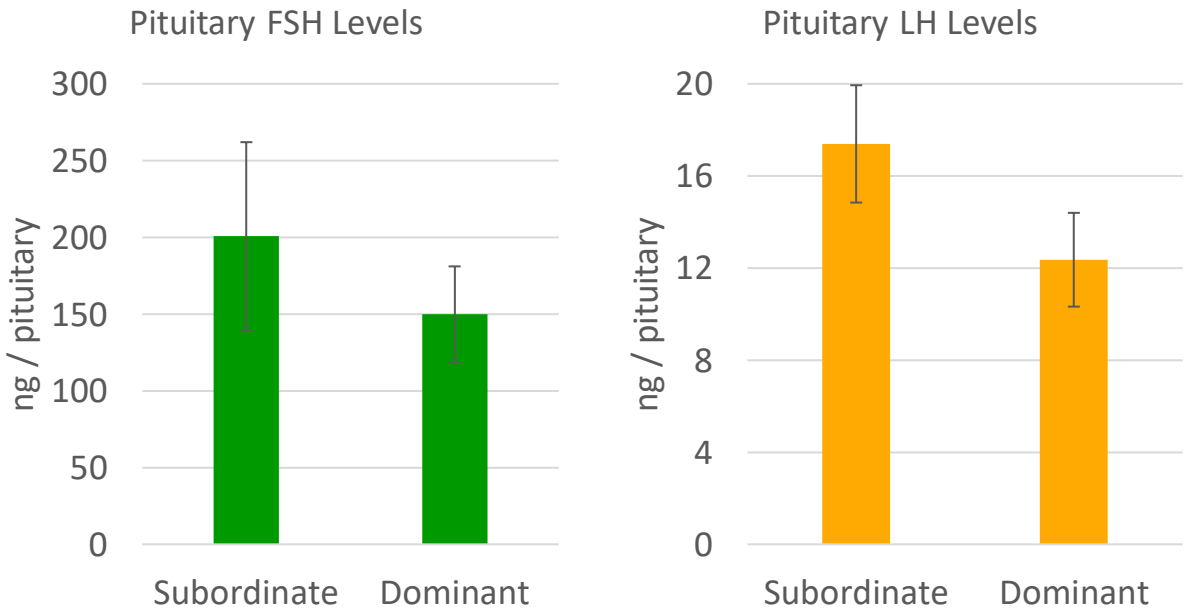


Figure 2. Pituitary gonadotropin levels (mean ± SEM) measured by ELISA. FSH levels (n=15) did not differ, but LH (n=19) was significantly higher in subordinate males (ANOVA, p=0.047).

### *Gene expression*

Males from 12 triads were used for qPCR analysis in the brain and pituitary, although one pair was excluded for the genes encoding pituitary receptors due to undetermined values that did not cross the CT threshold. Differences in gene expression between dominants and subordinates are expressed according to dominant/subordinate pair in Figure 3 and Figure 4. Mean differences between dominants and subordinates were not significant for any gene ($p>0.05$). However, dominant fish had higher *lhb* expression in 8/12 triads, *v1a1* expression in 7/12 triads, and *v1a2* expression in 8/12 triads. Subordinate fish had higher *fshb* expression in 9/12 triads and *avt* expression in 8/12 triads. They also had higher *gnrh-1* and *gnrh-3* expression in 6/12 triads, higher *gnrhr1b* expression in 5/11 triads, and *gnrhr2a* and *gnrhr2b* expression in 6/11 triads.

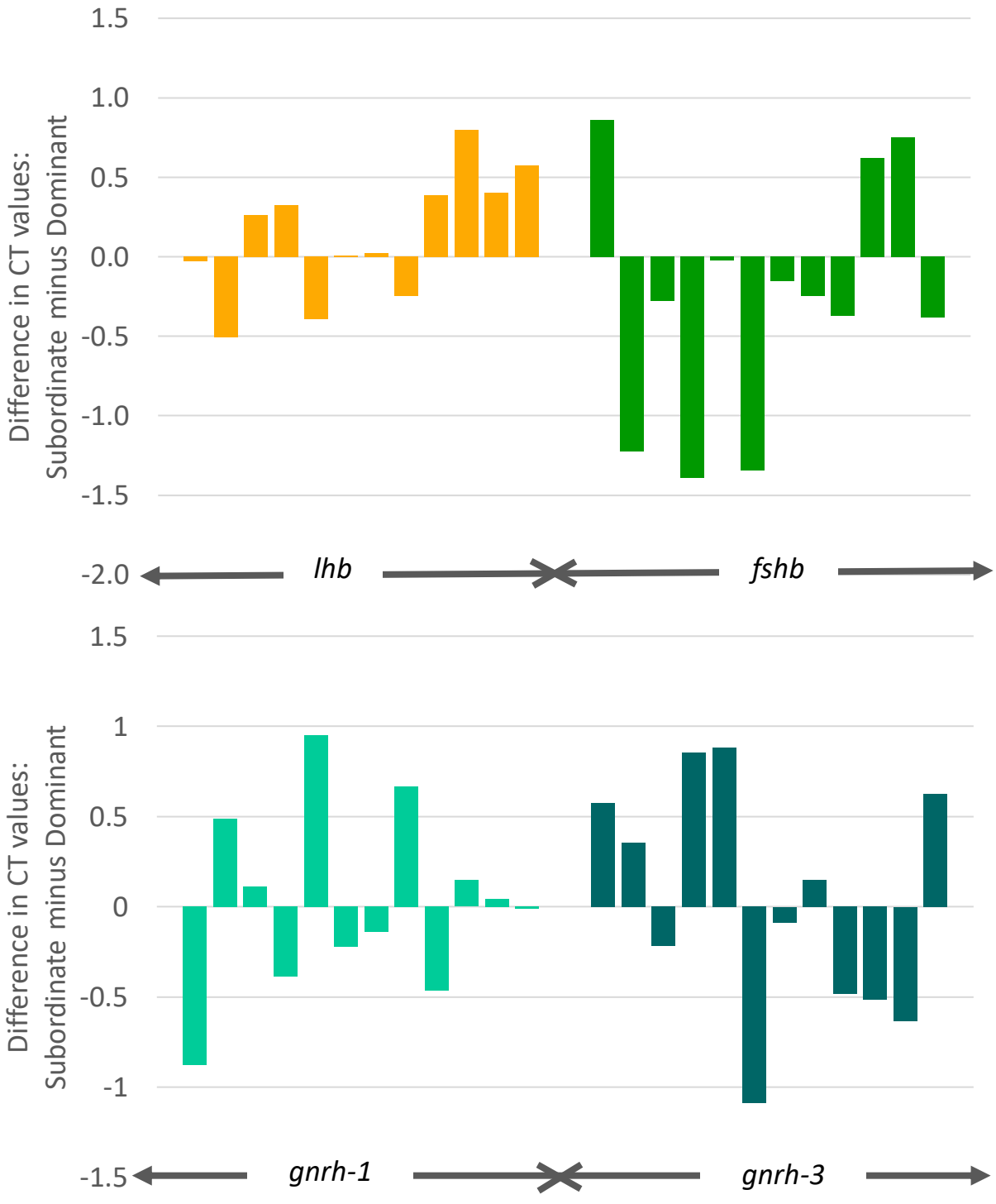


Figure 3. Differences in normalized CT values between dominants and subordinates. Positive values represent higher transcript levels in the dominant male. Negative values represent higher transcript levels in the subordinate male. Dominant fish had higher *lhb* expression in 8/12 triads while subordinate fish had higher *fshb* expression in 9/12 triads. *Gnrh-1* and *gnrh-3* were higher in dominant and subordinate fish each in 6/12 triads.

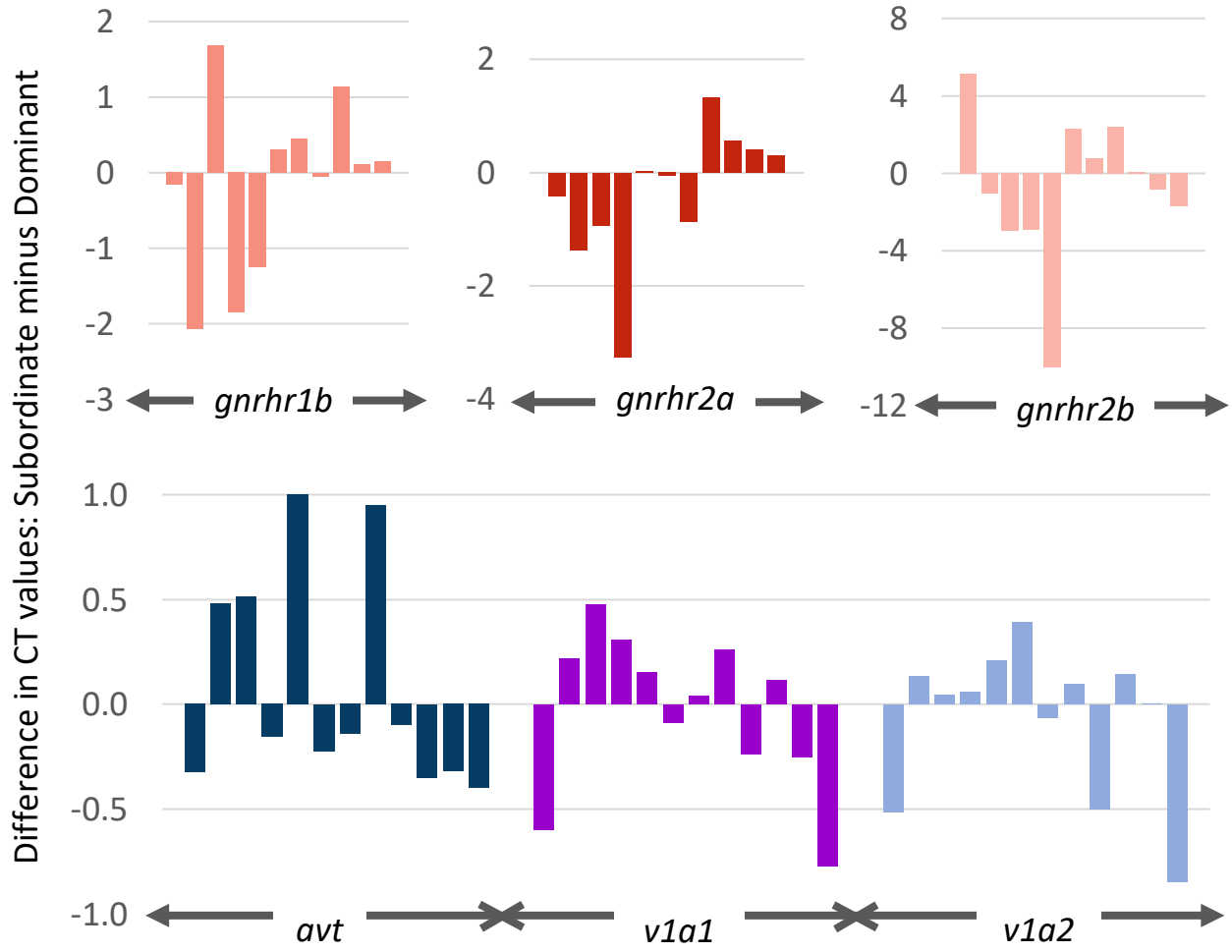


Figure 4. Differences in normalized CT values between dominants and subordinates. Positive values represent higher transcript levels in the dominant male. Negative values represent higher transcript levels in the subordinate male. Dominant fish had greater *gnrhr1b* expression in 6/11 triads and greater *gnrhr2a* and *gnrhr2b* expression in 5/11 triads. Subordinate fish had greater *avt* expression in 8/12 triads. Dominants has greater *v1a1* expression in 7/12 triads and greater *v1a2* expression in 8/12 triads.

## DISCUSSION

The similar fertilization rate between dominant and subordinate males (Figure 1) indicates that subordinate fish are finding a way to fertilize eggs, not with only moderate success, but with equal success as the dominant fish. This is surprising, given that dominant fish appear to use more energy to chase the female and fight off the opposing male. These results also contradict prior findings by Yokoi et al (2015) who reported that dominant males fertilize over 93% of eggs. Our results also imply that subordinate fish may be using sneaker tactics to bypass dominant males. Sneaker males in other species are often smaller and may be perceived as less of a threat or facilitate escape from the dominant male's notice (Aubin-Horth and Dodson 2007). However, any sneaker male strategy potentially used by these subordinate fish does not appear to involve size since neither mean body mass nor length significantly differed between the dominant and subordinate fish.

To investigate how these behaviors are linked to activity in the reproductive endocrine axis, we quantified pituitary levels of LH and FSH in dominant and subordinate males. These gonadotropins are central to the HPG axis function, acting as the signal between the brain and the gonads. Since medaka do not have enough blood to accurately measure circulating levels, we measured levels in the pituitary, where LH and FSH are synthesized, as an indicator of gonadotropins in the system. While FSH did not differ between the groups, LH was unexpectedly higher in subordinate males. It makes sense that LH would vary in these adult males since FSH is thought to be more important for earlier processes like gametogenesis, while LH is more important in spermiation (Levavi-Sivan et al 2010, Murozumi et al 2014, Takahashi et al 2016). However, we did not expect the subordinate males to have higher LH. This indicates that either LH production was stimulated in subordinate fish, or alternatively that its pituitary

release was inhibited, in which case measuring pituitary levels is not a good indicator of circulating levels.

To investigate these opposing explanations, we measured expression of genes encoding LH and FSH in the pituitary to determine whether increased pituitary LH could be attributed to increased transcription. We also measured transcription of the genes encoding GnRH and its receptors, as it is the primary player controlling gonadotropin release, and AVT and its receptors, since *avt* and *v1a2* were previously found to be essential for mate guarding behavior in knockout fish (Yokoi et al 2015). Mean differences between dominant and subordinate males were not significant for any gene, so we examined pairs for emerging trends. Dominant fish expressed higher *lhb* in 8/12 tanks (Figure 3), indicating that LH production is not stimulated in subordinates, and instead, the higher pituitary LH seen in subordinate males may be due to an inhibition of its release from the pituitary.

Dominant fish had lower *avt* expression than subordinates in 8/12 tanks, but higher *v1a1* expression in 7/12 tanks and *v1a2* expression in 8/12 tanks (Figure 4). While these results are not consistent with the findings of Yokoi et al (2015), it is still possible that AVT in a specific area of the brain is important for mate guarding behavior. We measured expression of the whole brain and as AVT neurons are located in both the ventral hypothalamus and preoptic area and project to receptors throughout the brain (Kagawa et al 2016), differences in expression in one essential area may not be evident when measuring expression in the brain as a whole.

In addition to nonsignificant mean differences, relative *gnrh-1* and *gnrh-3* expression was also evenly split according to pairs, both with higher expression in the dominant fish in 6/12 tanks (Figure 3). Expression of the three GnRH receptors was nearly as evenly split (Figure 4), indicating that the possible inhibition of LH release is not due to a downregulation of expression

of GnRH or its receptors in the pituitary. Further studies are needed to determine the mechanism by which LH release may be inhibited. Although GnRH expression was also measured using whole brains, this has not changed our interpretation since *gnrh-1* is only expressed in the preoptic area, *gnrh-3* is only expressed in the terminal nerve (Okubo et al 2002), and the GnRH receptors were measured only in the pituitary.

Another player in the HPG axis that could explain the inhibition of LH is sex steroids. While in mammals, sex steroids generally exert negative feedback on both the pituitary and the hypothalamus (Sheckter 1989, Michopoulos et al 2009), in fish, their effect varies according to species. Estrogens have negative effects on the pituitary and hypothalamus in goldfish, as treatment with anti-estrogens increased serum gonadotropin levels (Billard and Peter 1977). Testosterone treatment had a positive effect on the HPG axis in rainbow trout, increasing pituitary and plasma gonadotropin levels. This effect was augmented when combined with a GnRH analogue (Crim and Evans 1983). Estradiol (E2) treatment has both positive and negative effects in female tilapia depending on the dose. Low doses of E2 increased FSH release and transcription of the genes encoding GnRH receptors. High doses of E2 decreased transcription of gonadotropin genes as well as gonadotropin release (Levavi-Sivan 2006). In medaka, estradiol and testosterone treatment increase the number of LH and FSH cells in males. Treatment with the non-aromatizable 11-ketotestosterone did not have this effect, suggesting that testosterone is aromatizatized into estradiol to promote proliferation of both gonadotrope cell types in male medaka (Fontaine et al 2020). The direct feedback effect of sex steroids on LH and FSH levels as well as any effect via GnRH and gonadotrope cell proliferation remains to be investigated in medaka.

The similar fertilization rate of dominant and subordinate males is most important finding of this research, as it indicates that subordinance is a viable reproductive strategy. Future studies should investigate potential sneaker tactics and the role of female choice to determine how subordinate males fertilize eggs with equal success as the dominant males. Subordinates having higher pituitary LH levels is also an important finding. Future studies should measure the sex steroids in dominant and subordinate males to determine what role they may play in mate guarding behavior, as well as in feedback on the pituitary.

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