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# Investigating reproductive success and endocrine regulation of mating strategies in male medaka

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# Investigating reproductive success and endocrine regulation of mating strategies in male medaka

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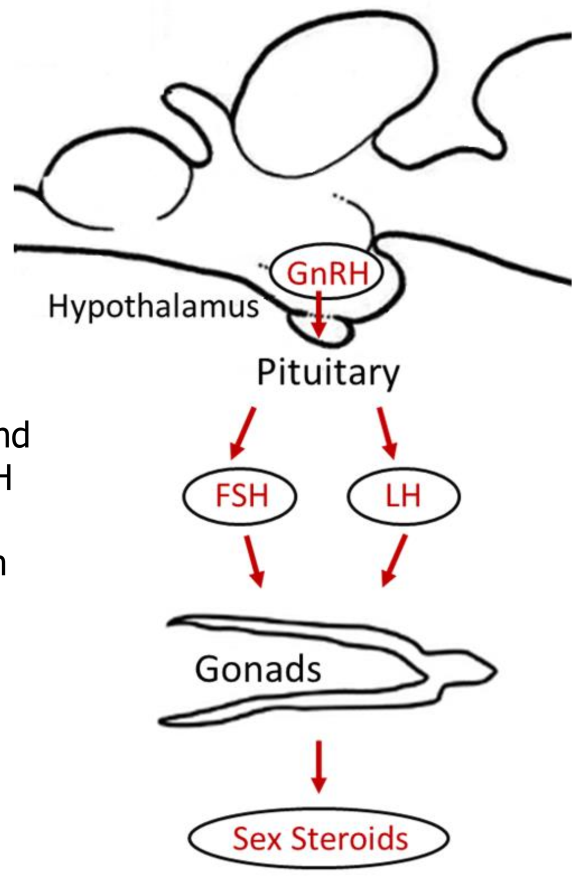
## INTRODUCTION

**Mate Guarding Behavior**

- Mate guarding is a reproductive strategy may increase individual male fitness by controlling access to females.
- The model teleost, *Oryzias latipes* (Japanese rice fish), exhibit mate-guarding in captivity (Fig.1).
- A previous study found that dominant male medaka fertilized over 93% of eggs, and that arginine vasotocin is required for mate guarding behavior in this species (Yokoi et al 2015).


**Fig. 1.** Medaka are a model organism that exhibit mate guarding behavior. In this image, the dominant male guards the female from the subordinate male.




**Reproductive Endocrine Axis**

**Fig. 2.** Diagram of the reproductive endocrine axis. In this system, the hypothalamus regulates the production and release of the gonadotrophins LH and FSH from the anterior pituitary gland. LH and FSH stimulate sex steroid production from the gonads. AVT is produced in neurons originating in the hypothalamus and terminating in the posterior pituitary.

**Medaka as a Model Organism**

**Fig. 3.** Japanese medaka are a teleost species and genetic model. They reproduce daily, producing transparent eggs that are fertilized externally. They exhibit observable mate guarding behavior in the lab (Yokoi et al 2015).

## OBJECTIVES

We investigated **(A)** the success of dominance and subordinance as reproductive strategies in male Japanese medaka, and **(B)** how these behaviors are linked to activity in the reproductive endocrine axis.

**Table 1.** Reproductive endocrine genes measured in this study.

| Gene | Protein Encoded | Protein Function | Location Measured |
|---|---|---|---|
| ***lhb*** | Luteinizing hormone | Stimulates gonadal steroid production and spermiation | Pituitary |
| ***fshb*** | Follicle stimulating hormone | Stimulates gonadal steroid production and gametogenesis | Pituitary |
| ***gnrh-1 gnrh-3*** | Gonadotropin releasing hormone | Hypothalamic stimulator of gonadotropin release | Brain |
| ***gnrhr1b gnrhr2a gnrhr2b*** | Gonadotropin releasing hormone receptors | Bind GnRH released from the hypothalamus | Pituitary |
| ***avt*** | Arginine vasotocin | Mediates aggression, linked to territorial behavior | Brain |
| ***v1a1 v1a2*** | Arginine vasotocin receptors | Bind AVT released from the hypothalamus | Brain |

## METHODS

**Behavior Assay**

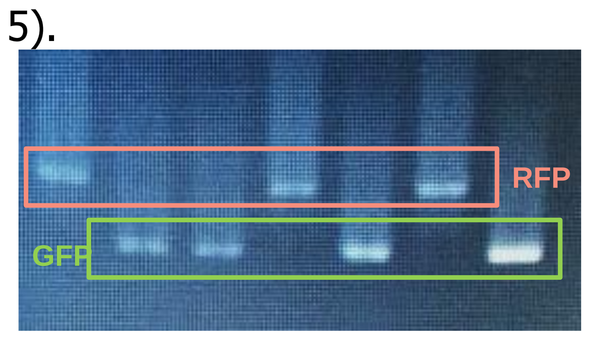
- Two male medaka were housed with one female (Fig. 4). Behaviors were observed at three time points between 9 am and 12 pm for at least 5 days. Males exhibiting dominant behavior at ≥80% of the last 10 time points were considered dominant.

**Fig. 4.** Triads were prepared with two male medaka and one female medaka housed together as pictured. Males were distinguished by clipping the top or bottom corner of the tail fin.

**1 Measuring Reproductive Success**

- Behavior assays were conducted on ten triads with two males from different transgenic lines and one wild type female. After identification, 20-21 embryos were collected from 2-4 clutches per female and genotyped (Fig. 5).

**Fig. 5.** Embryos were genotyped individually for the paternal transgenes using PCR and gel electrophoresis (pictured) to determine paternity.




**2 Measuring Hormone Levels**

- Behavior assays were conducted on 34 wild-type triads. Intact brain-pituitary tissue was collected from males and homogenized.
- Pituitary LH and FSH were measured using competitive ELISAs for medaka LH and FSH (Burow et al. 2019).

**3 Measuring Gene Expression**

- Behavior assays were conducted on 12 wild-type triads. Brains and pituitaries were collected individually from males.
- RNA was isolated (Zymo Research Direct-zol RNA kits) and reverse transcribed into cDNA (Thermo Scientific Maxima H Minus First Strand kit).
- Relative transcript levels were measured in triplicate used SYBR green qPCR. Target genes (Table 1) were normalized to the housekeeping genes *rpl7* in the pituitary and *gapdh* in the brain.

**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 were compared using a two-way ANOVA with behavior and ELISA plate as factors.
- Difference in expression was calculated as normalized subordinate CT values minus normalized dominant CT values for each pair. T-tests were used to assess differences.

## RESULTS

**1 Reproductive Success**

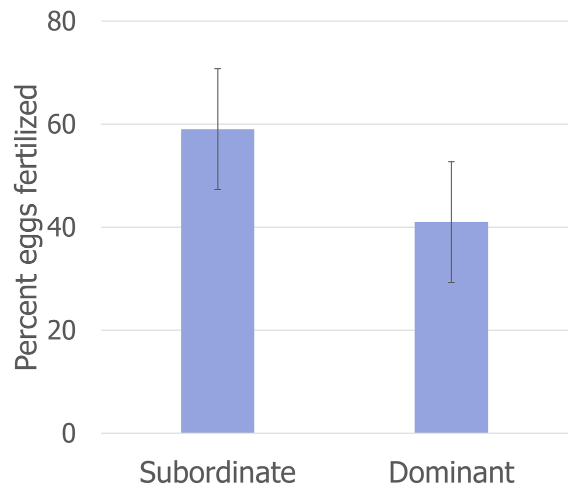



**Fig. 6.** Fertilization rates of dominant and subordinate males (n=10) did not differ significantly (p=0.29). The results are indicated as mean ± SEM.

Mean body mass and length did not differ between dominants and subordinates. Fertilization rate did not differ according to transgenic line or position of fin clip.

**2 Pituitary LH Levels** **Pituitary FSH Levels**

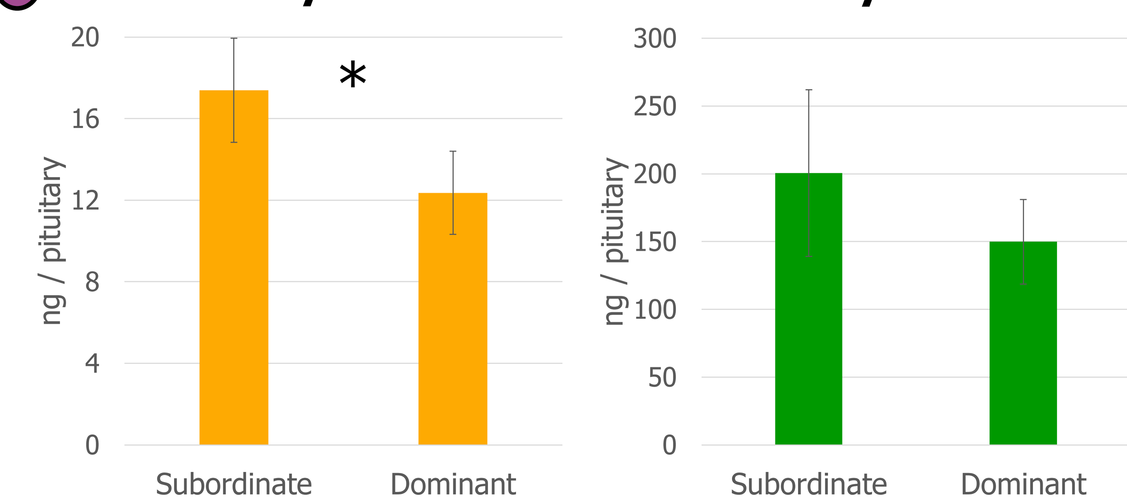
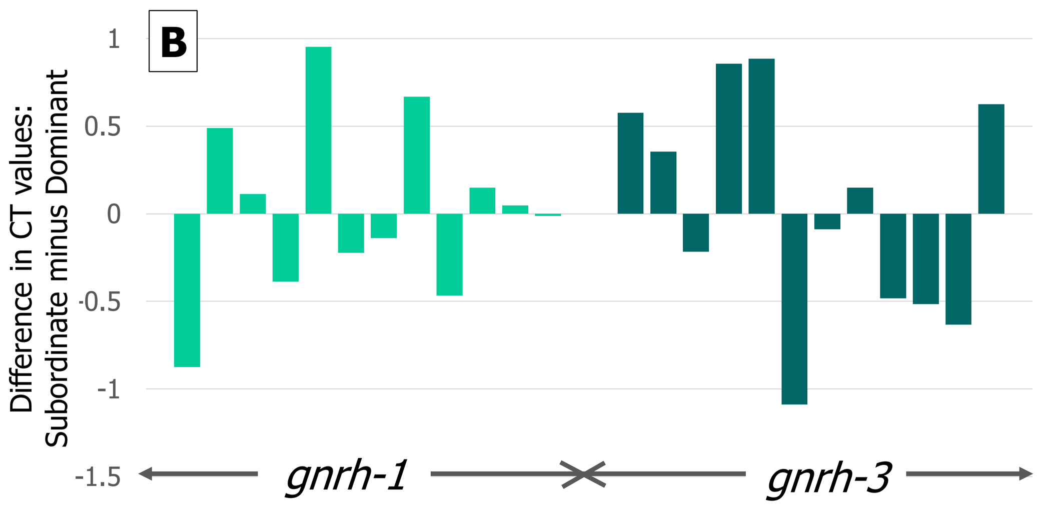
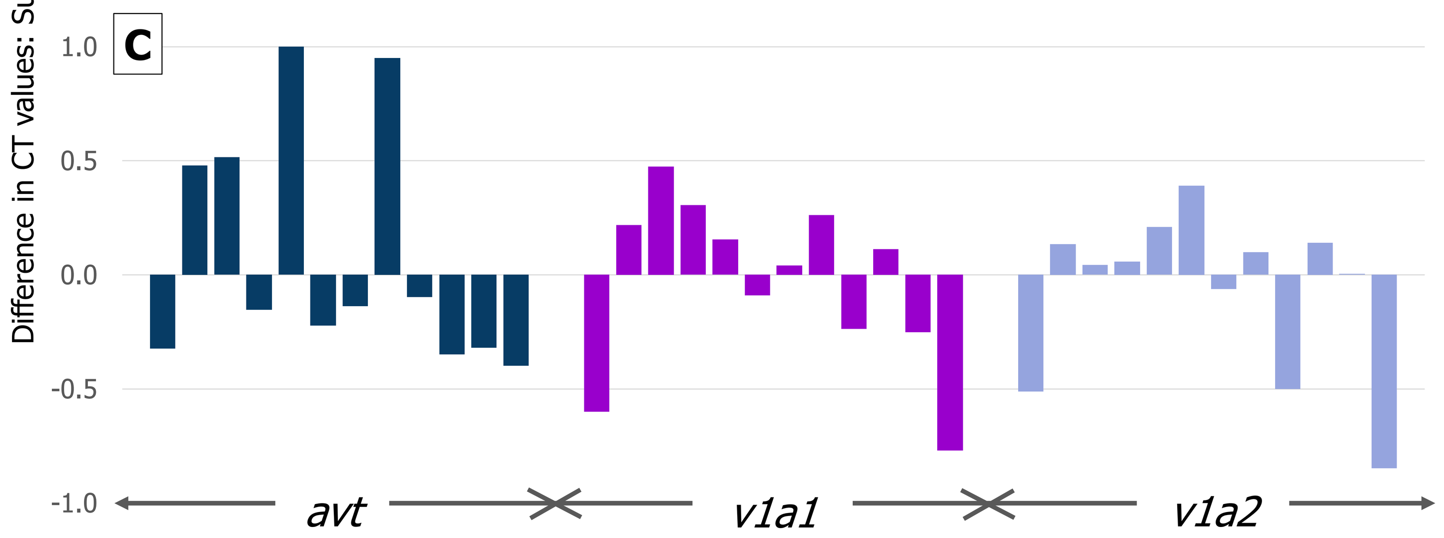


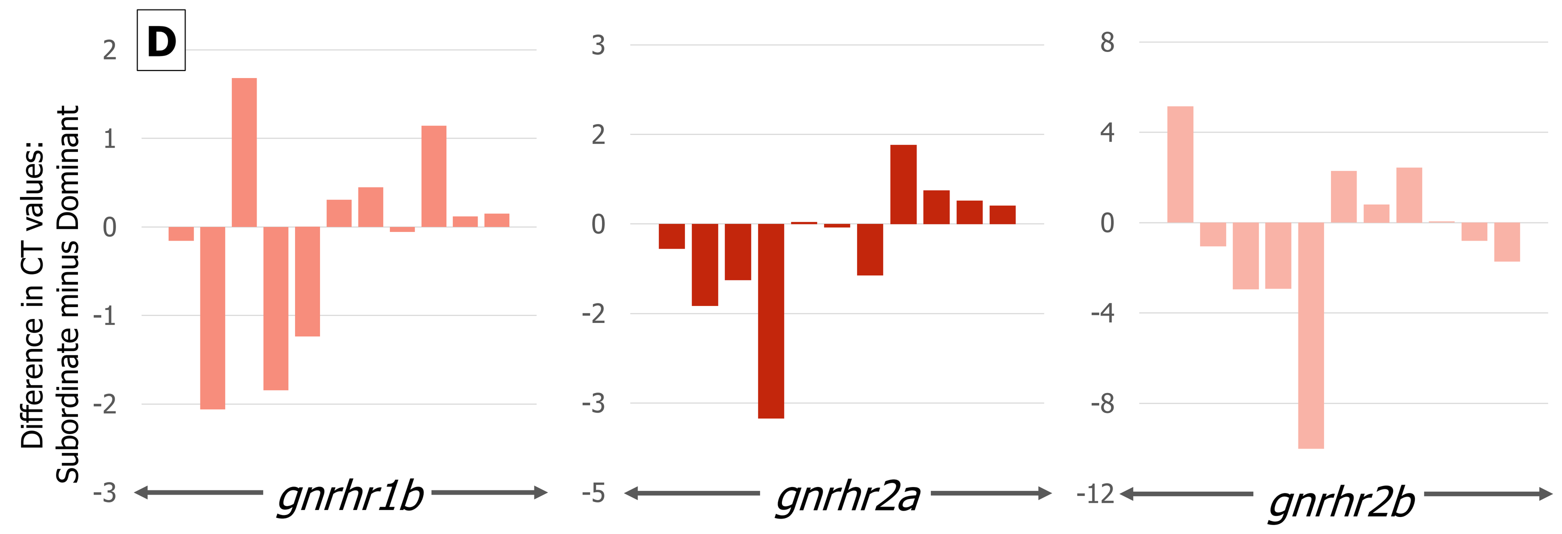

**Fig. 7.** Pituitary gonadotropin levels measured by ELISA. FSH levels (n=15) did not differ but LH (n=19) was significantly higher in subordinate males (ANOVA, p=0.0468). The results are indicated as mean ± SEM. The subordinates had higher LH levels in 14/19 triads.

**3 Difference in Transcript Levels by Dominant/Subordinate Pairs**


**Fig. 8.** Differences in normalized CT values between dominants and subordinates. Positive values represent higher transcript levels in the dominant male. Dominants had higher *lhb* expression in 8/12 triads and higher *fshb* expression in 3/12 triads (**A**). *Gnrh-1* and *gnrh-3* were higher in the dominant male in 6/12 triads (**B**). Dominants had higher *gnrhr1b* levels in 6/11 triads and greater *gnrhr2a* and *gnrhr2b* in 5/11 triads (**D**). *Avt* levels were higher in the dominant fish in 4/12 triads, and dominants had higher *v1a1* in 7/12 triads and higher *v1a2* in 8/12 triads. Mean differences between dominants and subordinates were not significant for any gene (p>0.05).




## CONCLUSIONS

- Dominant and subordinate male medaka fertilization rates did not differ, indicating that subordinance is a viable reproductive strategy in medaka. Subordinate males may be using sneaker male tactics to fertilize eggs.
- Subordinate males have higher pituitary LH levels but do not transcribe more *lhb*, indicating that LH release may be is inhibited. However, as GnRH and GnRH receptor transcripts are not significantly different between dominants and subordinates, other regulators of LH release may play a role in LH differences. Further research is needed to determine this mechanism.
- Dominant and subordinate medaka did not clearly differ in whole brain *avt, v1a1,* or *v1a2* expression, although there may still be relevant differences in specific regions.

## ACKNOWLEDGMENTS

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