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ペヘレイの性決定機構期における脳の性分化に関する神経解剖学・分子生物学的研究

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博士学位論文要約  
Summary of Dissertation

専攻 Major	<b>Doctoral Course of Applied Marine Biosciences</b>	氏名 Name	<b>TORRES MARTINEZ AARON ALEJANDRO</b>
論文題目 Title	<b>Neuroanatomical and molecular studies on brain sex differentiation in relation to gonadal sex determination of Pejerrey <i>Odontesthes bonariensis</i></b> (ペヘレイの性決定機構における脳の性分化に関する 神経解剖学・分子生物学的研究)		

The pejerrey *Odontesthes bonariensis*, a fish native to South America, has been introduced in Japan and has become a model for studying environmental sex determination. This species is unique as it exhibits a combination of genetic and environmental sex determination. Exposure of larvae to high (Male Promoting Temperature, MPT) and low (Female Promoting Temperature, FPT) temperatures during the critical period of sex determination (CPSD) induce masculinization (testis formation) and feminization (ovary formation), respectively. At intermediate temperatures the sex is determined by the Y-linked antimüllerian hormone gene, so XY individuals develop as males and XX as females. In fish, recent research has shown that not only the gonads are bipotential but also the brain is sexually labile and able to adjust its sex with those of the gonads in order to avoid functional mismatches. Moreover, recent evidence in mammals has shown that the sexual fate of the brain is determined by an interaction between steroid hormones and the genotype of the cells. In fish, this issue is even more complex because environmental factors such as temperature can also influence the sexualization of the brain during early life stages. In pejerrey, some studies have provided evidence that the brain can influence the sex differentiation of the gonads by inducing the activation of the reproductive axis and the secretion of cortisol. However, the mechanisms by which these regulatory differences are established during early and larval development remain unknown. Therefore, the study of brain sexualization can provide valuable information about sex determination and its role during sex reversal. This thesis explored the relationship between stress response and temperature-induced sex reversal, as well as the impact of heat stress on the brain transcriptome during the onset of sex differentiation. These topics are discussed in Chapter 1 and Chapter 2, respectively.

Chapter 1 examined the impact of genotype and temperature, as well as their interactions, on the activation of the CRH system and the hypothalamus-pituitary-interrenal axis (HPI) in the brain during temperature-induced sexualization in relation to gonadal sex differentiation. An experiment was conducted to expose larvae aged 2-6 weeks after hatching (wah) to high (29°C, MPT) and low (17°C, FPT) temperatures, as temperature plays a crucial role in the sex differentiation of pejerrey. The heads and the trunks were sampled to quantify gene expression in brains and gonads, respectively. The heads of larvae were also sampled for gene transcript localization by *in situ* hybridization. The remaining fish were sampled at the end of the experiment (20 wah) to determine the phenotypic sex ratios through histology. Then, gene expression analyses (RT-qPCR) of the Crh (Corticotropin Releasing Hormone) family and their associated carrier protein, receptors, and other stress-related genes in response to temperature during the CPSD were conducted to explore the potential roles of the CRH system in central nervous system sexualization and the HPI axis in sex determination in this species. The Crh family genes *crhb*, *uts1*, *ucn3*, the receptor *crhr1* and the stress-related genes *gr1*, *gr2*, *nr3c2* were transiently upregulated in the heads of pejerrey larvae during the CPSD by high temperature alone or in combination with other factors. Only *crhr2* transcript abundance was not influenced by temperature but was upregulated independently by time and genotype. In most cases, mRNA abundance was higher in the XX heads compared to that of XY individuals. To

determine the functions of the upregulated genes, their mRNA localization was assessed using *in situ* hybridization. Some of the genes were identified to be expressed in the tuberal hypothalamus, located near the pituitary gland, indicating their significance in neuroendocrine processes related to sex determination. Additionally, whole larvae cortisol levels were measured using EIA analysis to investigate genotype variations at MPT. Interestingly XX larvae also showed higher whole-body cortisol titers than the XY, downregulation of *cyp19a1a* and upregulation of the testis-related genes *amhy/amha* in trunks (gonads) and were 100% masculinized by high temperature. In contrast, at low temperature, *crhbp* and *avt* were upregulated in the heads, particularly the former in XY larvae. *cyp19a1a* and *amhy/amha* were up- and downregulated, respectively, in the gonads, and fish were 100% feminized. Signaling through the HPI axis was observed concurrently with the initial molecular indicators of sex determination and differentiation in the gonads. These findings strongly indicate a genotype-specific, temperature-dependent regulatory role of the brain, particularly involving the Crh family and stress-related genes, in the environmental sex determination of pejerrey.

Chapter 2 analyzed the brain transcriptome response to different temperature regimes. Building on the findings from the initial experiment, the study further investigated the impact of temperature and genotype on global gene expression in the brains of 2 wah pejerrey larvae during the sex determination period. This was done using DNA nanoball Next Generation Sequencing technology (DNBSeq) to identify transcriptomic changes during sex reversal of XX and XY larvae at high (29°C, MPT) and low (17°C, FPT) temperatures, respectively. The aim was to discover potential candidate genes involved in brain sexualization and their potential role in sex determination. A De Novo Assembly of the brain transcriptome and a differential expression analysis with volcano plots were conducted to identify differentially expressed genes. Gene Ontology and Reactome analyses were used to enrich the genes found to be differentially expressed. Thirty-two genes were upregulated and 37 genes were downregulated in the brains of XX larvae undergoing sex reversal at MPT, while 60 genes were upregulated and 44 genes were downregulated in the brains of larvae undergoing sex reversal at the FPT. The differentially expressed genes were validated using RT-qPCR, and their functions in the brain were investigated through literature mining. Furthermore, the up- and downregulated genes were enriched with Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, and Reactome databases to analyze their functions in the context of sex determination processes.

In summary, the studies presented in this thesis demonstrate the involvement of the brain in sex determination in pejerrey. The data shows that thermal stress can influence gonadal development and also affect the brain through the Crh system (*crhb*, *uts1*, *ucn3*) and glucocorticoid and mineralocorticoid receptors (*gr1*, *gr2*, *nr3c2*), potentially playing a role in brain reprogramming during sex reversal in XX pejerrey larvae at MPT. Additionally, arginine vasotocin (*avt*) and corticotropin releasing hormone-binding protein (*crhbp*) may be necessary for brain changes during low temperature-induced feminization. Furthermore, a genotype-specific response to thermal stress was observed for the first time in fish, supported by transcriptome analysis using DNBSeq. This study also identified new genes that go beyond known pathways related to sex determination and differentiation, suggesting novel insights into the neurological processes involved in sex determination in fish. The studies also highlight the sensitivity of intercommunication between developing brain and developing gonads in response to environmental factors such as temperature. They underscore the importance of a deeper understanding of brain and gonad sex differentiation to identify potential reproductive disruptions caused by increasing temperatures in fishes with thermolabile sex determination.