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Melanocortin system and puberty in zebrafish: a study model applied for aquaculture

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Precocious puberty results in side effects for aquaculture including slower growth rates and decreased flesh quality (Okuzawa, 2002). Regulation of pubertal timing remains unresolved in most species. In the platyfish (*Xiphophorus maculatus*) the onset of sexual maturity is regulated by the locus P (Kallman & Shreibman, 1973) which is filled by multiple copies of the melanocortin type 4 receptor (MC4R). The number of non-functional copies of this receptor seems to be correlated to the retardation of puberty onset (Lampert *et al*., 2010). Melanocortins are peptides derived of the precursor pro-opiomelanocortin (POMC). They include the melanocyte-stimulating hormones (MSHs) and the adrenocorticotropic hormone (ACTH). These peptides mediate their roles through five receptors (MC1R-MC5R). The endogenous antagonists, agouti-signaling protein (ASIP) and agouti-related protein (AGRP) compete with the melanocortin peptides for binding to melanocortin receptors. ASIP regulates skin pigmentation by inhibiting MC1R signaling but it also binds MC4R. AGRP works as an orexigenic factor, controlling food intake and growth by blocking MC4R hypothalamic activity (Cone, 2006). ASIP overexpressing zebrafish (*Danio rerio*) exhibit a disruption of dorso-ventral pigment pattern (Ceinos *et al*., 2015) and an enhanced growth that is explained by increased food intake and feeding efficiency (Guillot *et al*., 2016). However, no studies besides those in platyfish (Kallman & Shreibman, 1973; Lampert *et al*., 2010) have covered the involvement of the melanocortin system in fish reproduction. The present work will evaluate the relationship between the melanocortin system and the reproductive axis in the zebrafish.

Testis and ovaries (n=4/sex) were used to determine mc4r expression by quantitative PCR (qPCR). Total RNA was purified using TRI-reagent (Sigma) and treated with RQ1-DNAse

(Promega). RNA (0.3 µg) was retro-transcribed to cDNA using Superscript III TM reverse transcriptase (Invitrogen) that was subsequently used as template for qPCR. The expression of elongation factor 1a was used to normalize mc4r expression. qPCR reactions were as previously described (Cortés *et al.*, 2014).

The full-length sequence of the zebrafish mc4r was subcloned into the pGEM-T easy vector (Promega). A DIG labeled (Roche Diagnostics) antisense cRNA probe was in vitro transcribed using the SP6 polymerase (Promega). Adult zebrafish were euthanized by anesthetic overdose and brain, pituitary, testicles and ovaries were carefully dissected and fixed with 4% paraformaldehyde. Nucleic acid sequence was visualized in tissue preparations by in situ hybridization (ISH) procedure (Agulleiro *et al.*, 2013).

For morphological analysis of the gonads, same-aged embryos from different ASIP and wild-type TU breeding mates were used and reared under standardized conditions. Fifty fish were collected at different time points, including 30-34-38-42-46-60-75 days post fertilization (dpf). The fish were fixed in 1% glutaraldehyde and embedded in methacrylate resin. Sections of 2 µm were stained with toluidine blue. Determination of the developmental stage was according to Leal *et al.* (2009) and Chen & Ge (2013).

Our preliminary results show that ASIP transgenic fish exhibit higher gonadal mc4r levels than wild type (WT) fish suggesting a role in the gonadal development. ISH experiments (Fig.1) demonstrate the presence of mc4r in previtellogenic and vitellogenic follicles, spermatocytes, brain and pituitary, suggesting that melanocortin system is involved in the regulation of the hypothalamus-pituitary-gonad axis (HPG).

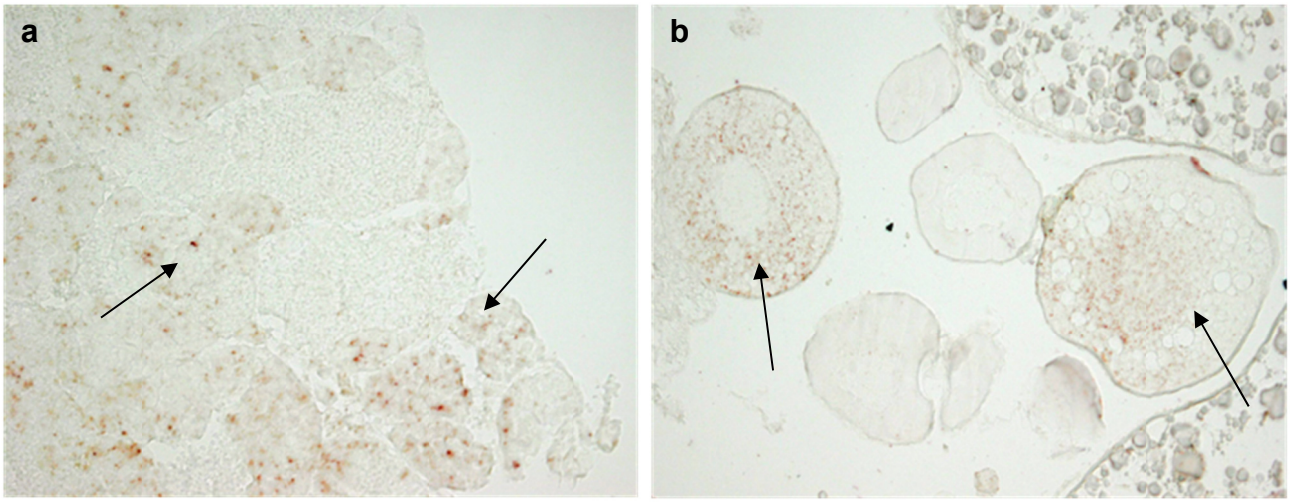

FIGURE 1. mc4r expression by ISH in testes (a) and ovary (b) at 10× magnification.

The presence of spermatogonia type B in male cysts (Leal *et al.*, 2009) and previtellogenic oocytes in females (Chen & Ge, 2013) is considered as the first step in the zebrafish puberty. Our histological analysis demonstrates that ASIP overexpression delays female puberty but accelerates the first sexual maturation in males suggesting that melanocortin system can modulate zebrafish gametogenesis. These results represent a study model to describe the relationship between melanocortin system and pubertal timing, especially in species with growth and reproductive problems in aquaculture.

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