ABSTRACT

Title of Document: A COMPARISON OF PROGESTERONE RECEPTOR GENE EXPRESSION IN THE OLFACTORY EPITHELIUM OF REPRODUCTIVE AND NON-REPRODUCTIVE FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

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Fish have a specialized organ called the olfactory epithelium that enables them to detect chemical cues in water. Among these cues are progestogens excreted by females that function as pheromones and stimulate male reproductive behavior and spermiogenesis. The olfactory epithelium is hypothesized to contain receptors that are activated by these pheromones. In this study, I compared the expression of nuclear and membrane progesterone receptors in reproductive and non-reproductive fathead minnows and in male versus female fish. I found changes in *mPR* expression over time in both comparisons of reproductive adults to juveniles, and reproductive adults to non-reproductive adults, and no differences between sexes. This is the second study to examine gene expression in the olfactory epithelia of fish and is novel in the experimental approach taken. Results from this research will inform future studies aiming to make the functional linkage between pheromones and the regulation of teleost fish reproduction.

A COMPARISON OF PROGESTERONE RECEPTOR GENE EXPRESSION IN THE OLFACTORY EPITHELIUM OF REPRODUCTIVE AND NON-REPRODUCTIVE FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

By

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Dedication

Dedicated to my cat

Edison

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I am very grateful for the support and guidance of so many people throughout this journey. I would like to begin with giving thanks to Dr. Edward F. Orlando for initially giving me a chance in his lab as an undergraduate student. There he saw the potential in me to pursue a M.S. degree. Over the past two years, his mentorship has helped me not only survive, but thrive along this journey. I would also like to thank my other committee members Drs. Lisa A. Taneyhill and L. Curry Woods III for their assistance during my tenure as a M.S. student.

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Table of Contents

Chapter 1. Literature Review

Overview

Progestogens are endogenous ligands that bind and activate progesterone receptors and are best known for preparing and maintaining the body for pregnancy in mammalian species. These steroid hormones also play a large role in non-reproductive functions across a wide array of species. In mammals, progesterone (P4) is the physiological progestogen with 4,4-dimethyl-5α-cholesta-8, 14,24-trien-3β-ol being the progestogen to stimulate final oocyte maturation (Voronina & Wessel, 2003). However, in fish the known physiological progestogens are 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and 17,20β,21-trihydroxy-4-pregnen-3-one (20-βS) (Nagahama & Adachi, 1985; Trant *et al.,* 1986). DHP is obtained from the conversion of 17α-hydroxyprogesteone under the influence of gonadotropins from the pituitary (Habibi *et al.,* 1989).

In female mammals, the reproductive cycle induces at least one oocyte to mature in the ovary. The corpus luteum (CL) is formed once the oocyte is ovulated, and will begin producing P4 (Moyes & Schulte, 2008). If pregnancy does not occur, then the CL will degrade and the cycle will begin again. However, if pregnancy does occur the CL will continue to grow and secrete P4 until the placenta eventually takes over in the production of P4. The presence of the right circulating concentration of P4 promotes ovulation, implantation, decidualization, parturition, and mammary gland development (Gellersen *et al.*, 2009).

In female teleost fish, specific progestogens are the maturation inducing substance (MIS). MIS initiates oocyte maturation, which enables the oocyte to leave meiotic arrest (Figure 1). For the males in fish and mammalian species, progestogens aid in increasing spermiation and sperm motility. In certain cases, such as the Japanese eel

(*Anguilla japonica*), DHP supports the initiation of meiosis in the testis (Miura *et al.*, 2006; Hamdani & Doving, 2007). Progestogens also have responsibilities in other functions not related to reproduction that take place in the intestines, kidneys, and neural tissue. They are also an important intermediary for the synthesis of androgens and estrogens (Figure 2).

In this literature review, I will explain the mode of action that enables progestogens to cause biological effects, explore what is already known about progesterone receptors across species, and summarize what work still needs to be done to help further our understanding of progestogen function. Throughout this thesis I will refer to gestagens, the collective term for progestogen, endogenous ligands for progesterone receptors, and progestins, synthetic ligands for progesterone receptors (Orlando, 2014).

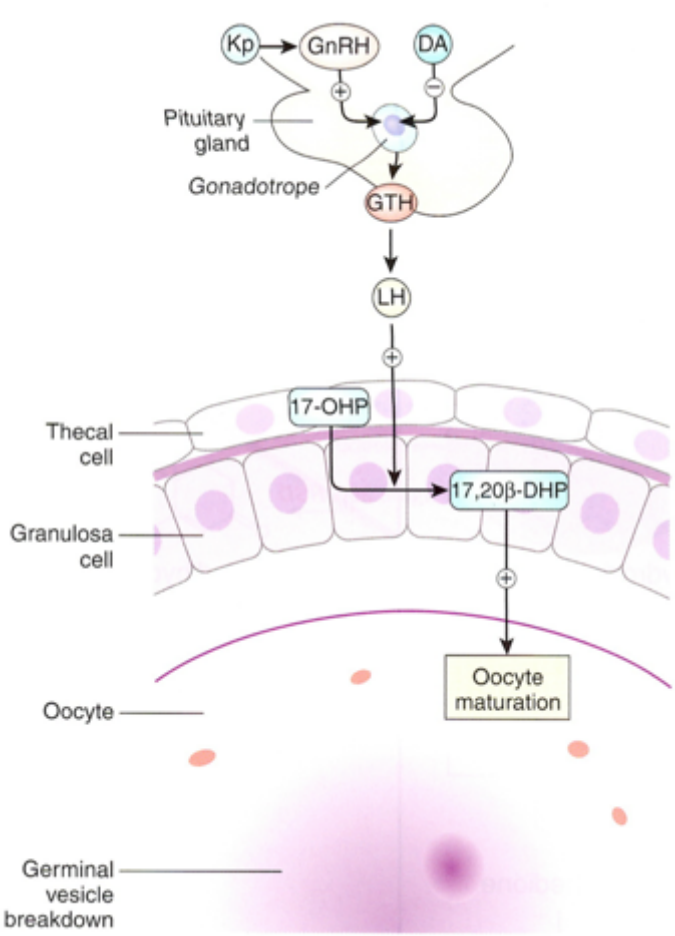


Figure 1: Final oocyte maturation in teleost. GnRH secretion from the hypothalamus stimulates LH release from the anterior pituitary. LH stimulates the production of DHP in follicular cells. This leads to the breakdown of the oocyte nucleus and the germinal vesicle. Finally the mature oocyte is ovulated (Norris & Carr, 2013).

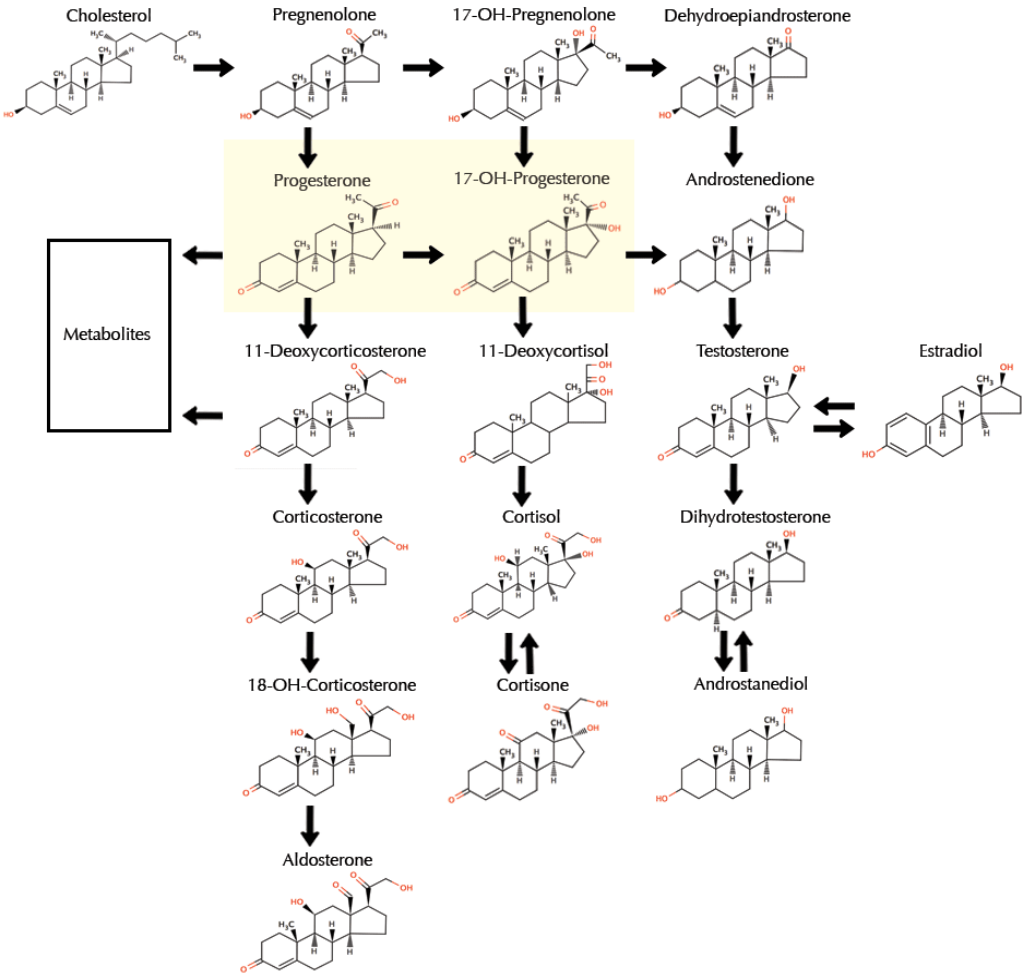


Figure 2: Cholesterol can be transformed into a variety of steroid hormones. Notice that progesterone is an intermediary for most other steroid hormones. (Tulane/Xavier Center for Bioenvironmental Research, http://e.hormone.tulane.edu/learning/progestins.html)

Progesterone Receptors

Certain functions regulated by progestogens follow the classical pathway and genomic actions of the well-studied nuclear progesterone receptors (nPRs) found in the cytosol (Tuohimaa *et al.,* 1996). However, there is evidence of nongenomic actions by progestogens. These actions have been explained by the presence of progesterone receptors in the cell membrane (mPRs). It is important to note that progesterone receptor membrane components (PGRMCs) also play a role in carrying out progestogen functions. PGMRCs have been found in heart, liver, placenta, and human sperm (Wendler *et al.*, 2012), and they may influence the acrosomal reaction in mammalian sperm and the regulation of anti-apoptoic actions of P4 in granulosa/luteal cells (Zhu *et al.,* 2008). A more in-depth role of PGMRCs will not be examined in this thesis; for further review, see (Thomas, 2008).

Genomic Actions of Nuclear Receptors

Nuclear receptors require the ligand to diffuse through the plasma membrane in order to bind to the receptor. The receptor-steroid hormone complex is then able to dimerize and enter the nucleus (Norman *et al.,* 2004). There it binds to the sex steroid response element on the gene and together with accessory transcriptional factors enable the appropriate gene to be expressed and protein to be made. For this reason, it may take hours or days to see a cellular response to the steroid hormone binding with its receptor (Figure 3).

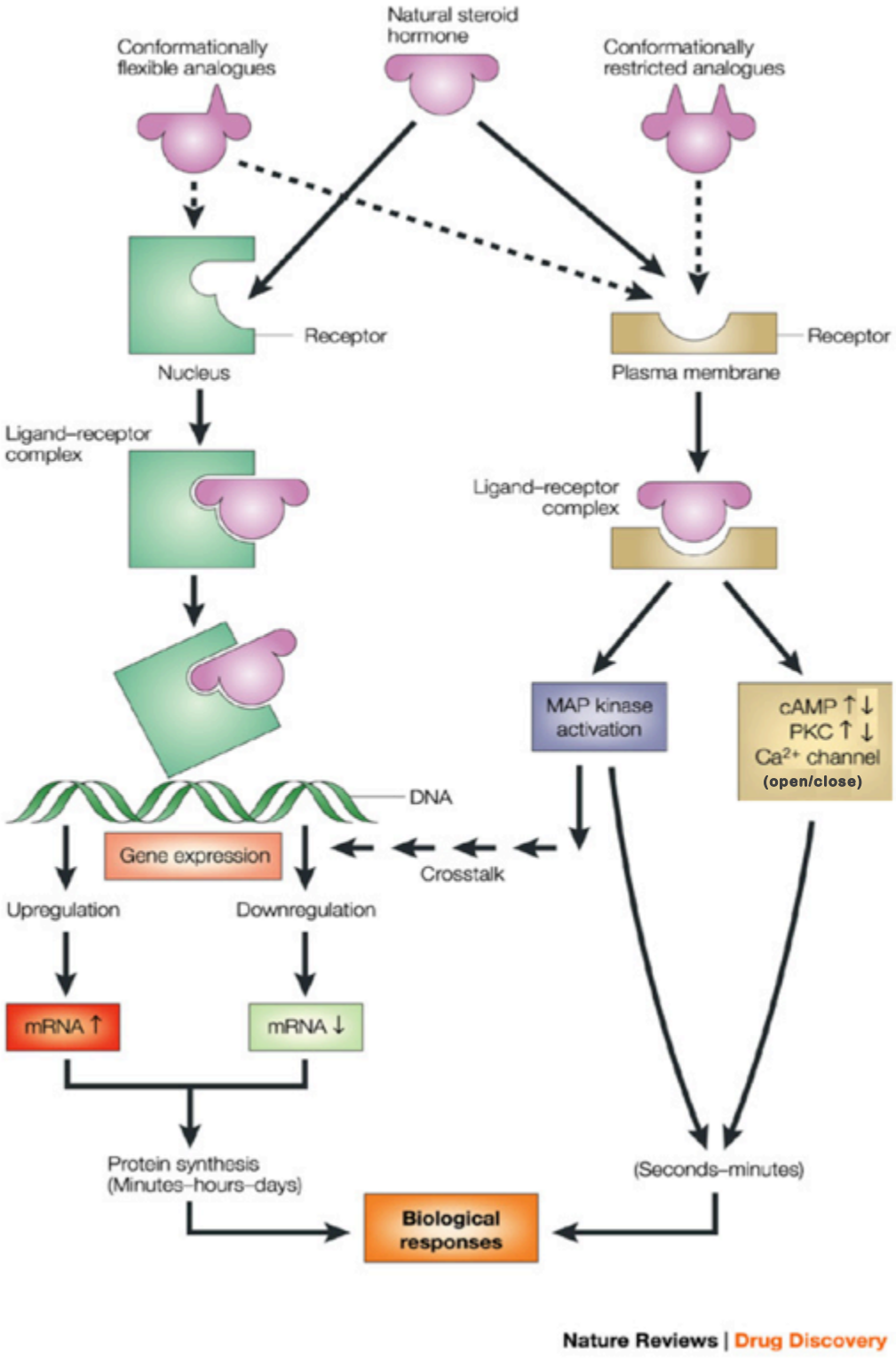


Figure 3: Comparison of the two pathways where the steroid hormone binds to either a nuclear receptor (left) or a membrane receptor (right) (modified from Norman *et al.*, 2004).

In mammals, nPRs are found to occur as two subtypes: nPR-A and nPR-B. nPR-A has been found to play a major role in mediating the actions of P4 in the uterus and ovary, whereas nPR-B is more important in mammary gland development (Stormshak & Bishop, 2008). nPR-A also induces the expression of genes responsible for structural processes. These processes included vascular development, tissue and cell morphology, lipid and carbohydrate metabolism, and skeletal and muscular development (Fair & Lonergan, 2012). nPR-B was found to induce genes associated with inflammatory cytokine networks such as IL-1 and TNF (Fair & Lonergan, 2012). Interestingly, in one study nPR-B acts as a strong activator of gene transcription, and that nPR-A is a ligand-dependent transrepressor of nPR-B (Chaudhary *et al.,* 2013).

In domestic animals, nPRs are targeted in order to promote growth, synchronize estrus, and maintain pregnancy (Stormshak & Bishop, 2008). In the beef cattle industry, P4, both alone and combined with other hormones, is given to cattle to help promote rapid growth by increasing the conversion of feed to muscle mass. These supplements function by increasing body protein, metabolism of fat stores, and mineral uptake across the gut and by decreasing amino acid metabolism (Meyer, 2001). One progestin, melengestrol acetate (MGA), is FDA approved for use in beef cattle in the US, and it is used as an estrus suppresser (Preston, 1999). MGA functions by suppressing ovulation and allowing for continuous follicle development. By repressing the estrus cycle, the animal likely utilizes the conserved energy from reproduction to growth. Through suppression of estrus, facilities are also able to better manage herds containing maturing

steers and bulls that would otherwise be interested in females during estrus, making them aggressive towards male cattles (Preston, 1999).

In contrast to the multiple types of nPRs found in mammals, only a few teleost species express multiple subtypes of nPRs (Ikeuchi *et al.,* 2002). For most teleost fish, only mRNA expression of one nPR has been found. In zebrafish (*Danio rerio*), nPRs are expressed in the ovary, testis, and preoptic region of the hypothalamus (Hanna *et al.,* 2010). These findings support the concept that nPRs play a role in a variety of reproductive processes, including testicular germ cell proliferation in males and oocyte/follicle development and ovulation in females. The role of nPRs in development is critical for both early stage oocytes growth and development of follicles in later stages (Hanna *et al.,* 2010). In both mammalian and fish species, nPRs act as regulators of reproduction by influencing gene expression. However, these are not the only progesterone receptors responsible for these actions.

Nongenomic Actions of Membrane Progesterone Receptors

Unlike nPRs, mPRs do not require regulation of gene expression in order to obtain a response from the cell. Membrane bound progesterone receptors (mPRs) work via a nongenomic pathway (Norman *et al.,* 2004). Activation of the mPRs alters the synthesis of a secondary messenger, which causes a response in the cell (Figure 3). This response leads to an influx of ions, the activation of an enzyme, or even cause a change in gene expression. Due to the fact that this binding calls for activation of a secondary messenger that is able to immediately cause a response in the cell, this receptor has a much faster response time compared to nPRs. While nPRs take hours or days to initiate a response, mPRs takes seconds or minutes (Norman *et al.,* 2004).

mPRs are important to the cell for a variety of reasons, aside from them being able to respond quickly to the presence of a ligand. There are a few special circumstances where the responding cell cannot undergo genomic action (Revelli *et al.,* 1998). This is due to the cell not containing the appropriate machinery to undergo the synthesize mRNA or proteins, such as spermatozoa. Also if the steroid were conjugated to a substance with a high molecular weight, then it would be unable to cross the plasma membrane, and therefore would not be able to reach an nPR (Revelli *et al.,* 1998).

There are at least three mPR subtypes found in most mammals, amphibians, and teleost fish, *mPRα, mPRβ*, and *mPRγ* (with two variants *mPRγ -1* and *mPRγ -2*). In humans, there are two additional mPRs, *mPRδ* and *mPRε* (see Pang, 2013 for more information). Tissue distribution of the *mPRα, mPRβ*, and *mPRγ* has been performed in human tissue cultures, and shows tissue specific localization of these receptors at the mRNA level (Zhu *et al.,* 2003). *mPRα* is localized in reproductive tissues including ovary, testis, and placenta. There was also a small amount of *mPRα* mRNA detected in the uterus, bladder, kidney, and adrenal gland. *mPRβ* was exclusively localized to neural tissues and was detected throughout the brain and in the spinal cord but not in the pituitary. *mPR-γ* was present in the kidney, intestine, and the adrenal gland and lung (Zhu *et al.,* 2003).

mPRα is the most abundantly and widely expressed receptor in human tissues and is found in many reproductive tissues of fish (Labombarda *et al.,* 2010; Hanna & Zhu, 2009). In order to enable final maturation of oocytes, progesterone bound mPRα activates an inhibitory G protein, down-regulating adenylyl cyclase activity and inhibiting protein kinase A (Thomas, 2012). This will then lead to the release of the oocyte from meiotic

arrest, a crtical step in final maturation of the oocyte (Thomas, 2012). In Atlantic croaker (*Micropogonias undulatus*) spermatozoa, it was shown that mPRα is coupled to a stimulatory olfactory G protein. Once activated, this leads to an increase in cAMP production and will increase motility of sperm (Tubbs & Thomas, 2009). Although females and males have the same receptors, different pathways can be utilized to allow for different functions to occur.

In comparison to mPRα, there are fewer studies on mPRβ, and even less on mPRγs. *mPRβ* is often co-expressed with *mPRα*, and is coupled to inhibitory G proteins (Dosiou *et al.,* 2008). However, *mPRγs* are shown to be present in excretory tissues, such as the kidney, but their function remains unclear (Zhu *et al.,* 2003). In general, mammals, amphibians, and fish utilize mPRs in a similar fashion. However, there are some class and species differences, which will be discussed in the following sections.

Membrane Progesterone Receptors in Mammals

mPRs are widely distributed in mammalian tissues. This coincides with their functions in labor, neuroendocrine control of the reproductive cycle, sperm motility, the development of breast cancer, and immune responses (Sriraman *et al.,* 2010; Peluso, 2006; Blackmore *et al.,* 1990; Dressing *et al.,* 2012).

The existence of mPRs in mice became apparent when it was observed that for nPR-A null mice, the development of pre-ovulatory follicles was normal, but ovulation did not occur (Sriraman *et al.,* 2010). Further in the corpus luteum (CL) of hormonally primed mice, entrapped oocytes were present. It was also found that luteal cells isolated from mice and rats do not express nPR, and therefore a different receptor must be responsible for the P4 actions in these cells (Peluso, 2006). During pregnancy, prolactin

(the major luteotrophic hormone in mice) regulates the expression of *mPRs* in the CL. Prolactin is required to maintain CL structure and to produce the P4 necessary for maintenance of pregnancy. When *mPR* gene expression levels were measured throughout gestation in mice it was found that *mPRβ* levels remained almost constant; whereas, *mPRα* and *mPRγ* levels increased with advancing gestation. Near the end of pregnancy *mPRα* and *mPRβ* levels decreased while *mPRγ* levels remained constant (Cai & Stocco, 2005). The changes in these receptors during mammalian pregnancy suggest that they assume important physiological roles in the ovaries and that mPRα, mPRβ, *mPRγ* are the key regulators in mice (Charles *et al.,* 2010).

These results also compare to what is seen in humans during pregnancy. During pregnancy, P4 is vital to keep the myometrium quiescent and not contracting. mPRα and mPRβ proteins have been detected in pregnant female myometrial cells (Karteris *et al.,* 2006). Right before the end of pregnancy mPRα and mPRβ levels decreased, as seen in mice, implying that they contribute to progesterone withdrawal in the human myometrium during labor (Peluso, 2006). This change in *mPR* expression leads to (1) down-regulation of steroid receptor co-activator 2 (SRC2) mRNA and decreased *mPRβ:mPRα* ratio resulting in decrease mPRβ transactivational activity, and (2) inhibition of adenylyl cyclase and increased phosphorylation of the myosin light chain (Karteris *et al.,* 2006). These results provide further proof that mPRs are critical for maintaining a pregnancy, and for ensuring that labor is able to ensue at the right time.

Due to the expression and hormonal activation of *mPRs* throughout a reproductive cycle, progestins can be administered to inhibit ovulation and proliferation of the

endometrium, and therefore, are used as human contraception and in estrus regulation of beef cattle in the US and Canada (Zeilinger *et al.,* 2009; Mauleon, 1974).

Membrane PRs are not only important in female reproductive physiology, but also in male fertility. When human spermatozoa are in the presence of P4, there is an influx of calcium (Blackmore *et al.,* 1990). It has been shown that sperm is transcriptionally inactive, therefore, this is not a genomic process, suggesting that mPRs are responsible for this function (Thomas *et al.,* 2004). Calcium influxes increase sperm hyperactivity. Hyperactivity is important for fertility because it allows the sperm to reach the oocyte within the oviduct and then penetrate the zona pellucida (Suarez, 2008). The role of P4 in increasing calcium concentrations at the time of fertilization also extends to the sperm undergoing the acrosome reaction. The acrosome reaction, release of enzymes from the sperm, is important for the sperm to penetrate the zona pellucida and fuse with the oocyte plasma membrane, thereby finalizing fertilization (Osman *et al.,* 1989).

Yet another role of mPRs is in the development of both breast and ovarian cancer. P4 has been shown to be important in breast tissue development and cause proliferation of immortalized breast cancer cell lines (Dressing *et al.,* 2012). Furthermore, P4 was able to inhibit apoptosis in both nPR-positive and nPR-negative breast cancer cell lines, suggesting that mPRs may be responsible for these actions. All *mPRs* are expressed in breast cancer cell lines, but *mPRα* is more abundantly expressed compared to *mPRβ*, *mPRγ-1,* and *mPRγ-2*. This indicates that mPRα likely contributes to the P4 inhibition of apoptosis in breast cancer cells, and may lead to the further development of breast cancer (Dressing *et al.,* 2012).

Progesterone plays a more protective function regarding ovarian cancer. It has been shown that women that have multiple pregnancies, twins, pregnancies later in life, or take estrogen-progesterone containing oral contraceptives all have lower incidence of ovarian cancer, possibly due to higher amounts of progesterone in their system (Charles *et al.,* 2010). This is especially apparent when compared to the increases rate of ovarian cancer in women who are post-menopausal or progesterone deficient. Studies have shown in HEY ovarian cancer cell lines that through mPRs, progestogens are able to increase cAMP levels, thereby decreasing proliferation of the cancer cells. Membrane PRs may also work by increasing the activation rate of apoptosis signal-regulating kinases. Both of these pathways together suggest a way to treat ovarian cancer (Charles *et al.,* 2010).

Membrane Progesterone Receptors in Amphibians

Membrane progesterone receptors were first identified in the African clawed frog (*Xenopus laevis*, here on called Xenopus*)*. When the maturation inducing substance (MIS) for Xenopus was injected directly into the oocyte, oocyte maturation did not occur (Godeau *et al.,* 1978). However, oocytes in the presence of MIS bound to a polymer, ensuring the MIS would not be able to cross the membrane, were able to undergo maturation. Furthermore, as receptor activity increased, so did progestogen-binding activity (Liu & Patino, 1993). Very little transcription occurred during the maturation process, and the addition of transcriptional inhibitors had no effect on steroid-mediated maturation *in vitro* (Deng *et al.,* 2009). The removal of the nuclei from oocytes also had no effect on inducing final oocyte maturation. These factors all indicate that no genomic actions were taking place during oocyte maturation. When Xenopus oocytes were injected with an antibody against the *mPRβ* isoforms, there was inhibition of

progesterone-mediated oocyte maturation (Deng *et al.,* 2009). These studies support the necessity for mPRs, and not nPRs, for final oocyte maturation in Xenopus.

Once confirming the presence of mPRs at the membrane of oocytes, focus turned towards determining the mechanism that controlled oocyte maturation. For frog and fish, maturation occurs through the regulation of adenylyl cyclases (Thomas, 2008). High intra-oocyte levels of cAMP are responsible for its meiotic arrest. Adenylyl cyclases catalyze the conversion of ATP to AMP. If progestogens were able to inhibit the activity of adenylyl cyclases, then the oocyte would leave its arrested state (Thomas, 2008). It is through the presence of mPRs that progestogens are able to perform this function.

Membrane Progesterone Receptors in Female Teleost Fish

Final Maturation of Gametes

The initial discovery of mPRs in spotted seatrout (*Cynoscion nebulosus*) was similar to that of Xenopus. In the spotted seatrout, induction of oocyte maturation occurred rapidly after exposure to 20β-S (Thomas *et al.,* 2001). Binding of 20β-S occurred at the cell surface, and when transcription was inhibited due to a lack of transcriptional machinery. From these results, researchers hypothesized that another receptor besides nPR was responsible for these actions (Thomas *et al.,* 2002; Stormshak & Bishop, 2008)

As in Xenopus, in teleost fish, like the spotted seatrout, mPRα is required for the MIS induction of oocyte maturation. Large amounts of *mPRα* mRNA exist in the oocyte before it became sensitive to MIS. In teleost fish, DHP and 20β-S have been identified as the major MIS (Thomas, 2012). With an increase in mPRs, oocytes increased their ability to respond to the presence of MIS and go through final meiotic maturation (Tokumoto *et*

al., 2006). Increases in mPRα protein levels induced by human chorionic gonadotropin coincided with the development of oocyte maturational competence, which allows for the oocyte to undergo successful fertilization. Membrane PRα involvement in this process was furthered by protein localization at the oocyte plasma membrane. The abundance of *mPRα* was also increased during the reproductive cycle when oocyte maturation was occurring; both naturally and when induced by gonadotropins (Thomas, 2008).

Abundant expression of *mPRα* and *mPRβ* mRNA in testis and ovaries of the zebrafish was consistent with their role in nongenomic reproductive processes, with mPRα being the key player in the process (Hanna & Zhu, 2009). mPRα interprets extracellular signaling of progestogen, and initiates meiosis resumption in zebrafish. By injecting oocytes with *mPRα*, *mPRβ*, and *nPR* transcripts and then exposing the oocytes to DHP the rate of DHP induced oocyte maturation was significantly accelerated for those oocytes injected with *mPRα* compared to those injected with *mPRβ, nPR*, and control injected oocytes (Hanna & Zhu, 2011).

Although evidence supports mPRα inducing the maturational process of oocytes, it is still unclear as to how this occurs. In follicle-enclosed oocytes when *mPRα* expression is increased more than normal, the oocyte was able to go through maturation even in the absence of exogenous progestogen, due to the increased MAPK activation followed by increased cyclin B production within the enclosed oocyte (Hanna & Zhu, 2011). The author has hypothesized that a signal transduction repressor is present normally, but it can be dissociated by an increased amount of receptor (Hanna Zhu, 2011). Another hypothesis is that a secondary messenger is always being produced by the *mPRα* present in low quantities. When *mPRα* is activated or up-regulated, it is then able

to produce more of this secondary messenger and reach the necessary threshold to allow for final oocyte maturation to occur (Hanna & Zhu, 2011).

Further support of mPRα as the mPR responsible for oocyte maturation in zebrafish is that DHP induced oocyte maturation was almost completely blocked by the microinjections of *mPRα* antisense oligonucleotides into the oocytes (Thomas *et al.,* 2004). When using *mPRβ* antisense oligonucleotides, the same result was reported, showing that mPRβ also plays a role in oocyte maturation. In fact, greater inhibition of oocytes completing maturation was seen when the experiment was performed with the *mPRβ* subtype. Zebrafish antisense oligonucleotides to *mPRα* and *mPRβ* together also blocked the response to the MIS in inducing maturation suggesting that both subtypes may be required to initiate this progestogen response in oocytes (Thomas *et al.,* 2004).

When similar studies were performed in goldfish (*Carassius auratus*), *mPRβ, mPRγ-1*, and *mPRγ-2* antisense oligonucleotides reduced the protein levels of all three mPRs (Tokumoto *et al.,* 2012). However, there was no significant difference in the number of oocytes that underwent oocyte maturation when *mPRγ-1* and *mPRγ-2* antisense oligonucleotides were used. *mPRβ* antisense oligonucleotides decrease oocyte maturation from to 50% from 85% in the control group (Tokumoto *et al.,* 2012).

This same group also performed binding assays with the endocrine disruptor diethylstilbestrol, DES. This synthetic hormone has been shown to induce oocyte maturation via mPRα (Tokumoto *et al.,* 2006), and the binding studies performed with *mPRβ, mPRγ-1*, and *mPRγ-2* showed that DES had a high affinity for *mPRβ* as opposed to *mPRγ-1* and *mPRγ-2* (Tokumoto *et al.,* 2012). These results once again suggest that mPRβ may play a role together with mPRα in initiating final oocyte maturation.

However, it is important to note that mPRγ-1 had high affinity for androgens, which have also been shown to induce oocyte maturation in goldfish.

While many studies suggest that mPRs are responsible for oocyte maturation, there are still varying pathways by which these receptors could potentially elicit a response. A common mechanism is the decrease of cAMP levels that leads to germinal vesicle breakdown. In the Atlantic croaker, the naturally occurring MIS, 20β-S, did indeed reduce cAMP levels and induced germinal vesicle breakdown. However, exposure of follicles to cAMP-dependent protein kinase inhibitors in the absence of 20β-S was not able to promote germinal vesicle breakdown (Pace & Thomas, 2005). Through the use of inhibitors of phosphatidylinositol-3-kinase (Pl3K)/Akt signaling pathways, 20β-S was blocked from promoting germinal vesicle breakdown. Thus, 20β-S and the use of Pl3K/Akt signaling pathways in the Atlantic croaker is important for germinal vesicle breakdown, an important component of final oocyte maturation. Further studies are required to see if this is the same in all teleost fish, or if more than one mechanism occurs.

Membrane Progesterone Receptor Role in Apoptosis

Besides final oocyte maturation, membrane progesterone receptors also seem to play a role in the apoptotic/antiapoptotic process, specifically mPRα. In the Atlantic croaker, mPRα is present on plasma membrane of granulosa and theca cells. In these granulosa and theca cells it was once again shown that 20β-S caused a down-regulation of cAMP (Dressing *et al.,* 2010). In this same study, these cells were placed in a serum-starvation culture to induce cell death. When the cells were exposed to 20β-S, or the progestin Org OD 0-02, the percentage of cell death was significantly decreased,

compared to cell death in the control group and the group expose to the nPR agonist R5020. The finding that 20β-S and Org OD 0-02, and not R5020, was able to decrease the incident of cell death helps supports the hypothesis that an mPR, most likely mPRα, is present at the plasma membrane of granulosa and theca cells and prevents apoptosis (Dressing *et al.,* 2010). This was further supported when a siRNA was used against *mPRα* and caused a loss of this antiapoptotic effect. The use of an *nPR* siRNA did not alter the ability to avoid starvation induced cell death. It is also most likely that this response occurs via an Erk/Akt pathway due to the increase of both Erk phosphorylation and Akt phosphorylation when the cells were exposed to 20β-S (Dressing *et al.,* 2010).

Membrane Progesterone Receptors and Regulation of GnRH

Other studies on the Atlantic croaker have observed the effect of 20β-S on gonadotropin regulation in both sexes. When preoptic anterior hypothalamus tissue is exposed to 20β-S there is an immediate down-regulation in the amount of gonadotropin-releasing hormone (GnRH) secreted (Mathews *et al.,* 2002). This rapid action indicates that the response is due to the binding of 20β-S to a mPR, hypothesized to be mPRα, implying that progesterone receptors play a role in the negative feedback action of 20βS on neuroendocrine function (Thomas *et al.,* 2004). Once the largest, most MIS-responsive oocytes have gone through final maturation, gonadotropin secretion is inhibited in order to prevent smaller, less developed oocytes from maturing. This neuroendocrine function suggests that mPRs may be present on GnRH secreting neuronal membranes.

Membrane Progesterone Receptors in Male Teleost Fish

While the binding of 20β-S to mPRs decreases gonadotropins, thereby regulating oocyte maturation in females, in males the up-regulation of gonadotropins increases the abundance of sperm progestogen receptors (Thomas *et al.,* 2006). Studies examining the types of *mPR* expressed along the sperm membrane have shown that there was a 33% higher *mPRα* expression in Atlantic croaker sperm with high motility, as compared to sperm with low motility (Tubbs *et al.,* 2010). This suggests a relationship with *mPR* gene expression and motility in sperm (Thomas, 2008). Sperm motility is not the only factor affected by 20β-S. When Atlantic croaker sperm was treated with 20β-S for five minutes, there was a significant increase in sperm motility, as well as sperm velocity (Thomas *et al.,* 2004). The same study showed that other steroids were ineffective at influencing change in sperm motility and velocity. Since sperm is considered transcriptionally inactive, and these actions occur too quickly to be mediated by nPRs genomic action, these findings suggest that these responses are likely regulated through mPRs in the spermatozoa membrane. These findings suggest that mPR is critical for fertility in males.

Research done on salmonids conflicts with results found in Atlantic croaker. In masu salmon (*Oncorhynchus masou*), it was shown that MIS increases sperm motility via a genomic action acting at the sperm duct to increase the pH of the seminal fluid; thereby, increasing cAMP levels (Miura *et al.,* 1992). However, with Atlantic croaker MIS has been shown to act directly on the plasma membrane in order to cause rapid changes in intracellular cAMP concentrations (Thomas *et al.,* 2004). Although this nongenomic mechanism is the same mode of action that has been seen in Southern flounder

(*Paralichthys lethostigma*), and seems to be widespread among fish species (Tubbs *et al.,* 2011), it is still possible that there are exceptions where genomic actions occur in sperm.

Membrane Progesterone Receptors and Pheromones

The olfactory system allows for response to stimuli in the water responsible for homing, schooling, locating food, predator avoidance, social behaviors, and reproduction. In some fish, progestogens, and other hormones, secreted by one sex function as pheromones for the opposite sex. mPRs present in the specialized sensory organ, the olfactory epithelium (OE), coordinate reproductive behaviors. When progestogens bind to mPRs in the OE, they activate the $G_{\alpha i}$ (pertussis toxin sensitive) proteins to reduce cAMP levels (Sorenson & Sato, 2005). Both *mPRγ-1* and *mPRγ-2* are commonly found in epithelial tissues, making them candidates for the receptors responsible for actions within the OE (Kolmakov *et al.,* 2008). Transcriptome evidence was found for *mPRγ-1* and *mPRγ-2* in both goldfish and zebrafish OE. *mPRβ* was also detected in both species, and *mPRα* was not detected. These receptors have a high affinity for progestogens but not other steroid hormones, further indicating their specific role in detecting pheromonal progestogens in fish (Kolmakov *et al.,* 2008). Although mPRγ-1 and mPRγ-2 seem like the strong candidate for phermonal regulation in teleost fish, mPRα has been detected to be expressed in the olfactory epithelium of Atlantic croaker, and may also be the potential mediator for the phermonal effect of progestogens (Tubbs *et al.,* 2010).

Electro-olfactograms performed in the OE of goldfish showed that neurons responding to sex pheromones differed from those responding to amino acids and the neurons responding to reproductive pheromones activate cAMP regulated pathways (Sorenson & Sato, 2005). The importance of pheromones is that they allow for

conspecifics to synchronize reproduction. At the time prior to ovulation, there is an LH surge that, in goldfish, causes the females to release a mixture of androstenedione and DHP (Kobayashi *et al.,* 1987). Once the male perceives this signal via receptors in the OE, they too will have an LH surge that causes testicular production of DHP in order to increase sperm motility (Defraipont & Sorensen, 1993). Altogether, progestogens and their receptors allow for reproduction to occur on multiple levels. Not only does it prime the male and female so they have fully mature gametes, but it also helps in the coordination of reproduction so that once the female is ready for ovulation, the male will be ready as well.

Other Functions of Progestogens

Progestogens also have roles independent of reproductive physiology and behavior. In humans, progesterone has been shown to have a neuroprotective function, therefore leading to its use to treat traumatic brain injuries (Wright *et al.* 2007). It functions by blocking voltage-gated calcium channels and inhibiting depolarization-induced cell death. This protects the brain from inflammation, edema, demyelination, and excitotoxicity (Luoma *et al.,* 2011).

In T cell lines, progesterone has the ability to aid in the differentiation of lymphocytes into one of three subsets of cluster of differentiation 4^+ T helper cells. Progesterone promotes T cell differentiation into the T helper cells 2 type which is responsible for producing interleukin-4 and interleukin-5 (Piccinni *et al.,* 1995). While neither of these progestogen functions shows a direct use of mPRs, mPRα and mPRβ are expressed in human peripheral blood cells, isolated T cells, and Jurkat T cells. Also, the exact pathway in which progestogens exert this immune function is still unknown, but

one possibility is that T cells activate inhibitory G protein pathways via these receptors (Piccinni *et al.,* 1995).

Progestogens also affect calcium currents in smooth muscle and renal cells via nongenomic actions, which leads to potassium concentration alterations (Steidl *et al.,* 1991). This affects Na^+ and Cl^- reabsorption across the nephron, which will alter the electrolyte balance of the body. Smooth muscle contractions are also important for reproduction in order to help expel the ovulated oocyte or milt. This shows that even the seemingly unrelated functions of progestogens can still be important for reproduction.

Conclusions

Progestogens and their receptors are vital for both reproductive and non-reproductive functions across all species. The information provided in this literature review has shown that in mammalian, amphibian, and fish species progestogens work via nongenomic pathways to induce final oocyte maturation in females, sperm motility in males, neuroprotective functions, and much more. Progestogens work by coupling to G proteins and regulating synthesis of cAMP, MAPK and many other enzymes. However, there are many questions left unanswered, for example, what are the roles of mPRγ-1 and mPRγ-2? While mRNA expression patterns have been studied, their functional role has been left unexamined. Are they required for the pheromone detection as suggested by Kolmakov et al.? Are these receptors responsible for initiating reproductive behaviors in teleosts such as goldfish and fathead minnow (*Pimephales promelas)?* Could they have other functions in the olfactory epithelium? How do they exert these actions? Are there other receptors expressed in fathead minnow olfactory epithelia? Does their expression change over their life cycle from juveniles to reproductive adults to non-reproductive

adults? Does the expression differ between male and females? The following chapter contains my thesis research in which I have begun the journey to explore some of these questions further and with the broad goal to increase our understanding of PRs in the olfactory epithelium of teleost fish**.**

Chapter 2. A Comparison of Progesterone Receptor Gene Expression in the Olfactory Epithelium of Reproductive and Non-Reproductive Fathead Minnows, *Pimephales promelas*

Abstract

Fish species have a specialized organ called the olfactory epithelium that enables them to detect chemical cues in water. Among these cues are progestogens excreted by females that function as reproductive pheromones and stimulate male reproductive behavior and spermiogenesis. The olfactory epithelium is hypothesized to contain receptors that are activated by these pheromones. Here, I have begun to test this hypothesis by comparing the expression of nuclear and membrane progesterone receptors in reproductive and non-reproductive fathead minnows and in male versus female fish. I expected to find a lower expression of progesterone receptors in the non-reproductive compared to reproductive fish, and higher expression in males compared to females. This is the second study to examine gene expression in the olfactory epithelia of fish and is novel in the experimental approach taken. Results from this research will inform future studies aiming to make the functional linkage between pheromones and the regulation of teleost fish reproduction.

Introduction

Structure of the Olfactory Epithelium

The olfactory epithelium (OE) is a chemosensory organ required for the detection of social, food, and reproductive pheromonal cues in fish and other animals (Hansen, 2007). Pheromonal cues help alert fish to changes in the surrounding environment and allow them to respond accordingly. The OE is a paired structure located on the dorso-anterio aspect of the head (Burne, 1909) (Figure 4A). This structure is rosette shaped with multiple lamellae (Figure 4B, C) and has two nares. Water enters through the anterior nare and exits through the posterior nare. Through this passage, chemicals in the water bind to receptors on the apical surface of the OE and induce a behavior response.

Sensory Neurons of the Olfactory Epithelium

Within the OE there are three types of olfactory response neurons that project to the olfactory bulb (Figure 4D) (Bazáes *et al.,* 2013). These include the ciliated neurons (cORNs), microvillus neurons (mORNs), and the crypt cells (CCs) (Figure 4E). The cORNs are characterized by long dendrites and a few cilia (Kermen *et al.*, 2013). The cORNs project to the dorsal medial olfactory bulb via the olfactory-specific G-protein, $G_{\alpha olf}$, and an adenylyl cyclase-based transduction pathway. Due to sequence analysis, localization of mRNA, and the similarity in the mechanism of action, these receptors are said to be homologous to the mammalian olfactory receptors (Kermen *et al.*, 2013). These projections go to the dorso-medial olfactory bulb along the medial bundle of the medial olfactory tract (mMOT) (Figure 4F, blue). The mMOT has been shown to respond

to alarm substances in the crucian carp (*Carassius carassius*) (Hamdani *et al.*, 2000; Weltzein *et al.,* 2003). However, another receptor type may also be present in the cORNs called trace amine associated receptors (TAARs). These TAARs are known to function as either a social or pheromonal detector, but their roles are not completely understood (Liberles, 2009).

The mORNs contain shorter dendrite microvilli and are said to be homologous to mammalian V2R-type receptors (Kermen *et al.,* 2013). Although, the transduction pathway is not well understood, the goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) express transient receptor potential C2 ion channel (TrRPC2). The similarities in transduction pathways between fish and mammals indicate that teleost mORNs may be similar to the group of mammalian mORNs that act via a phospholipase C-dependent transduction pathway (Bazáes *et al.*, 2013). The mORNs respond to amino acids and nucleotides. Their signals project to the lateral olfactory bulb by the lateral olfactory tract (Figure 4F, green) (Hamdani *et al.*, 2000; Weltzein *et al.,* 2003). These two pieces of evidence suggest that mORNs are important for responding to food odorant stimuli.

The CCs are a unique cell type for teleost fish, for there appears to be no homologous structure in any other vertebrate (Bazáes *et al.,* 2013). Crypt cells are also in lower abundance compared to the other ORNs. CCs are caused by an apical invagination that opens towards the pit. They are oval shaped, have no dendrites, few cilia, multiple microvilli, and are surrounded by one or two supporting cells. Many studies have been performed to identify the ligands that activate the wide variety of G-protein coupled receptors that are expressed among teleost CCs. Such examples include channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), and round goby (*Neogobius*

melanostomus) whose CCs express $G_{\alpha o}$ proteins. Goldfish CCs express both $G_{\alpha o}$ and $G_{\alpha q}$ G-proteins, and zebrafish CCs express Gi_{1b} (Belnager *et al.,* 2003; Hansen *et al.,* 2003; Oka & Korschung, 2011; Bazáes & Schmachtenberg, 2012).

Crypt cell neurons project to the ventral olfactory bulb via the lateral bundle of the medial olfactory tract (Figure 4F, red) (Bazáes & Schmachtenberg, 2012; Kermen *et al.*, 2013). In crucian carp (*Carrasius carrasius*) and goldfish, this pathway transmits reproductive information to both males and females by responding to reproductive hormones released by conspecifics. Pheromones bind to mPRs and enable the coordination of behavior and gamete release in both sexes (Hamdani *et al.,* 2000; Sorensen *et al.,* 1991).

Particularly relevant to my research, is that CCs have been shown to change in localization and number depending on which season during the year samples were collected. In crucian carp, there were a higher abundance of CCs present and more localized in the apical layer of the OE during spawning seasons than in non-spawning seasons (Hamdani *et al.,* 2008). This same result was not seen in zebrafish, but this may be due to the fact that although zebrafish and other tropical species have a seasonal aspect to their reproduction, the period of active reproduction is broad and not as sharply defined as temperate zone species. In temperate zone species, photoperiod and temperature provide strongly demarcated environmental cues (Munro, 1990; Hansen & Finger, 2000; Spence *et al.,* 2008).

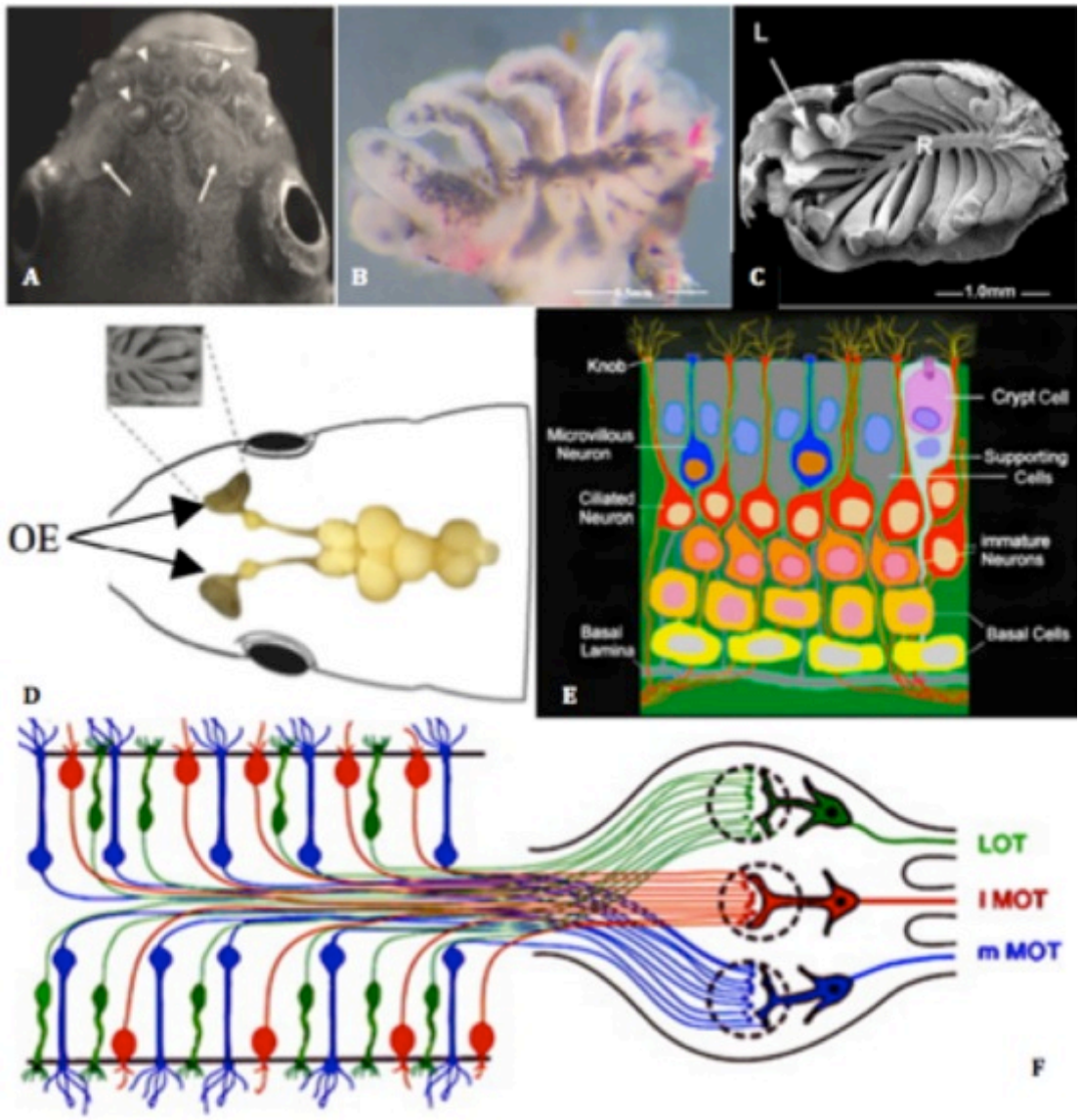


Figure 4: A. Dorsal view of adult fathead minnow (FHM) head. Triangles indicate nuptial tubercles and arrows point to olfactory nares that contain olfactory epithelium (OE). B. Photograph of dissected adult female FHM OE collected from our lab that shows lamellae epithelial structure. C. Scanning electron microscope of the olfactory epithelium of *Triplopjysa dalaica* (Waryani *et al.*, 2013). D. Schematic diagram of the paired OE connected to the olfactory bulb via the olfactory nerves. E. Cell types within the OE. Abundant presence of ciliated olfactory receptor neurons (ORNs) and microvillus ORNs with crypt cells scarce in comparison (Bazáes *et al.*, 2013). F. Diagram of the three pathways responses travel from the OE to the olfactory bulb (Hamdani & Doving, 2007) Lateral olfactory tract (LOT), lateral bundle of the medial olfactory tract (l MOT), medial bundle of the medial olfactory tract (m MOT).

Another study examined the differences of CCs in juvenile and adult rainbow trout (Bazáes & Schmachtenberg, 2012). It was found that the percentage of apical CCs was higher in both sexes of adults and the adults had larger CCs than juveniles. However, there was no significant difference in either of these characteristics between adult males and females. The CCs were then exposed to various odorants, andjuveniles responded to all presented odors with no specificity for any particular class. The CCs had no response to the female sex hormones estrogen, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), or 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in adult females. Female CCs did response strongly to testosterone, seminal fluid, and testicular extracts. In contrast, adult male CCs have elevated responses to female ovarian extracts, and to the progestogen, DHP. Both mature females and males had a low-to-no response rate to all other odors. One characteristic that remained constant among all three groups was that most of the CCs responded to only one odorant, with only a small percentage responding to two or more. Results from this study show that CCs begin as generally tuned responders in juvenile rainbow trout and become more reproductive-based stimuli sensors as rainbow trout mature (Bazáes & Schmachtenberg, 2012).

Two other cell types found in the OE are ciliated non-sensory cells and basal cells. Ciliated non-sensory cells are scattered throughout the OE and help to distribute mucus over the surface of the OE (Kermen *et al.*, 2013). The basal cells are a layer of undifferentiated cells that are crucial for the life-long regeneration of sensory and accessory neurons (Bazáes *et al.*, 2013)

Physiology of the Olfactory Epithelium

Olfactory sensory neurons are responsible for sensing social, feeding, or reproductive cues and mediating a response. The social response is one that is neither related to feeding nor reproduction. One of these behaviors is the alarm and fear response. In species such as zebrafish, catfish, and rainbow trout, specialized cells in the epidermis release alarm substances due to trauma or disease (Speedie & Gerlai, 2008). Other fish are able to detect these substances and react by rapid swimming, grouping, hiding, darting, freezing, and general excitation (Bazáes *et al.,* 2013; Kermen *et al.,* 2013). It has been hypothesized that two types of alarm substances are responsible for these actions. First, it was thought that hypoxanthine-3-N-oxide was responsible, but it was found that it does not trigger a true alarm response on its own (Whitlock, 2006). Later, it was found that in zebrafish, mucus containing chondroitin fragments caused a true alarm response (Mathuru *et al.,* 2012). Whether this is the same across fish species, or if there are other substances to create this response, is not fully known. Finally, homing is a social behavior seen in diadromous species such as salmonids. This is another response that uses amino acids and possibly bile salts to elicit a social interaction. Bile salts, such as taurocholic acid and glycocholic acid, are steroid acids that emulsify fats. When these compounds are released into the water through feces and urine, salmonids are able to detect the initial territory where they were hatched after being away in the ocean for years (Bazáes *et al.,* 2013).

The next response, feeding, is especially crucial for fish when finding food late at night or in other low-visibility waters where the animal may not be able to rely on sight to find food (Bazáes *et al.,* 2013). As a result, these receptors are highly sensitive to food-

related compounds. Neurons that are responsible for sensing food activate in the presence of amino acids, polyamines (i.e., spermine and putrescine), and nucleotides. While amino acids make up most food compounds, polyamines indicate the presence of decaying tissue. In comparison, adenosine triphosphate (ATP) and inosine triphosphate (ITP) relay information on the freshness of food (Kermen *et al.,* 2013). Fish exposed to these signals have been shown to increase the number of turns and swimming speed in the direction of stimuli (Kermen *et al.,* 2013).

The ability to detect and locate members of the opposite sex in the surrounding water is of utmost importance for aquatic species. It has been shown that amino acids may be involved in pheromonal signaling and reproductive behaviors. One example is in masu salmon (*Oncorhynchus masou*), in which females excrete a tryptophan metabolite to attract males and trigger male reproductive behaviors (Yambe *et al.,* 2007). Bile salts have been shown to be a species-specific tracer, guiding orientation, and territorial marker (Bazáes *et al.,* 2013). However, PGF2α and steroids, such as androstenedione and DHP, are the critical players in pheromonal communication in the cyprinid species, including goldfish, crucian carp, common carp (*Cyprinus carpio*), common roach (*Rutilus rutilus*), and tench (*Tinca tinca*) (Lastein *et al.* 2006; Chung-Davidson *et al.* 2008; Scott *et al.,* 2010). These hormones are produced by the gonads of fish and are able to cause an endocrinological and/or behavioral response in the opposite sex (Stacey *et al.*, 2003). For example, the release of certain pheromones, such as androstenedione and testosterone by male goldfish, has been shown to regulate oocyte maturation and ovulation timing in conspecific females (Sorensen *et al.,* 2005). When a female is ready to ovulate a specific mixture of compounds, such as PGF2α, DHP and sulphated DHP,

are released into the water (Bayunova *et al.,* 2011). When detected by conspecific males, DHP causes an increase in luteinizing hormone, which in turn causes an increase in sperm and milt production and reproductive behavior (Defraipont & Sorensen, 1993). Overall, the particular composition of secreted steroid hormones is crucial for a species to specifically target a member of the opposite sex for mating (Bazáes *et al.,* 2013).

Progesterone Receptors in the Olfactory Epithelium

Although the OE is a sense organ that plays a role in the regulation of many behaviors in teleost fish, the focus of this study will be on progesterone receptors in the OE. Progesterone and other progestogens play a key role in the regulation of reproductive functions of both female and male vertebrates. In teleost fish, the endogenous progestogens that are responsible for reproductive actions are DHP and 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S). In females, DHP is important for initiating oocyte final maturation, when the oocyte leaves its state of meiotic arrest and reenters the cell cycle making the egg fertilizable (Thomas, 2008). In male teleosts, progestogens play a role stimulating spermiation, sperm motility, and behavior. Atlantic croaker (*Micropogonias undulatus*) and Southern flounder (*Paralichthys lethostigma*) sperm increased motility, velocity, and turning rate when exposed to 20β–S, and in the Japanese eel (*Anguilla japonica*), there was a study done that supports a role for DHP in the initiation of meiosis during early spermatogenesis within the testis (Miura *et al.*, 2006; Hamdani & Doving, 2007; Thomas, 2012). Progestogens can also affect courtship behaviors in some fish. For example, when goldfish were exposed to DHP or its sulfated form, DHP-S, there was an increase in courtship behaviors by the males. This included increased pursuit nudging of females (Poling *et al.,* 2001).

The classical pathway and genomic actions of the well-studied nuclear progesterone receptors (nPRs) explain slower progestogen actions such as gene transcription and the translation of protein. In mammalian reproduction, progesterone (P4) plays a critical role in ovulation and maintaining pregnancy through feedback loops of the hypothalamic pituitary gonadal axis (Moyes & Schulte, 2008). After ovulation, increased circulating P4 levels are synthesized by the corpus luteum. This helps to prepare and maintain the uterus for implantation of the embryo. P4 does so by promoting endometrium maturation as well as reducing muscle contractions of the uterus. Eventually, the corpus luteum will begin to degenerate, and P4 will be secreted from the newly formed placenta in eutheria mammals. These increased levels continue to repress smooth muscle contractions in the uterus and prevent ovulation (Moyes & Schulte, 2008).

Progesterone also helps in vascular development, tissue and cell morphology, lipid and carbohydrate metabolism, skeletal and muscular development, testicular germ cell proliferation, and neuroprotection. Nuclear receptors require the binding ligand to diffuse through the plasma membrane in order to be able to bind to the receptor (Norman *et al.,* 2004). The receptor is then able to activate transcription factors that allow gene(s) to be expressed or repressed (Figure 3, left side). For this reason, it may take hours or even days to see a cellular response to a progestogen binding to the nuclear receptor.

Membrane progesterone receptors (mPRs), on the other hand, cause a relatively immediate biological change within the cell. This was first observed when maturation-inducing steroids were able to quickly induce an oocyte to leave meiotic arrest and re-enter the cell cycle (Hanna & Zhu, 2011). After that initial discovery, these fast actions have been explained by the nongenomic pathway of both membrane-bound progesterone

receptors (mPRs) and progesterone receptor membrane components (PGRMCs) (Figure 3 above) (Thomas, 2012). This means that these receptors allow for a substrate to have an effect on the cell without first initiating transcription and translation. These actions are able to occur rapidly due to the ligand being able to bind directly to a receptor in the plasma membrane and activate a secondary messenger, which will then produce a response in the cell (Norman *et al.,* 2004) (Figure 3, right side). This action allows for a response time of seconds to minutes as compared to these minutes or hours of the nuclear receptor response. In spotted seatrout (*Cynoscion nebulosus*), activation of *mPRα* has been shown to couple to an inhibitory G-protein and cause down regulation of adenylyl cyclase activity (Thomas *et al.*, 2007). This leads to inhibition of protein kinase A and induces the release of the oocyte from meiotic arrest and for oocyte maturation to occur. In Atlantic croaker, binding of 20β-S to this receptor couples to stimulatory olfactory G-protein at the sperm membrane and causes an increase in intracellular cAMP and Ca^{+2} production (Moussatche & Lyons, 2012; Tubbs & Thomas, 2009). As for mPRβ, limited studies have been done to determine its function. What has been done shows that mPRβ works with mPRα to induce oocyte maturation, and it may also have a role in neuroprotection (Thomas, 2012; Thomas & Pang, 2012). While there are currently no studies to determine the exact role of mPRγ1 and mPRγ2, they have been detected at the mRNA level in the OE of goldfish and zebrafish, along with *mPRβ*, and, therefore, they may have a role in pheromonal detection (Kolmakov *et al.,* 2008).

Ontogeny of Progesterone Receptors and other Steroid Receptors

Many studies have focused on the ontogeny of steroid receptor gene expression. One of the more commonly studied receptors are the estrogen receptors (ERs). In fathead

minnows (FHMs), studies have shown the changes of *Er* mRNA expression during early development. One study, looking from 0 days post fertilization (dpf) to 28 dpf shows that *Er2b* increases and then returns to prehatch values (Johns *et al.,* 2009). A more recent study continued this to 45 dph and also found that *Er1* increased in expression, but that the pattern was more erratic with some decreasing and increasing over this time period (Leet *et al.,* 2013).

Another comprehensive study examined the mRNA expression of all three *Ers*, 1, 2a, and 2b, in FHMs (Filby & Tyler, 2005). All three were detected at all stages of development and their expression varied between 5 and 20 days post fertilization (dpf). Their expression patterns also varied in different tissue types including liver, gonads, and brain, with sexual dimorphism observed as well. During early development in other fish species, *Ers* are undergoing vast changes in transcription levels. In rainbow trout, all four *Er* subtypes (a1, a2, b1, and b2) initially begin at low levels and then increase until each reach a peak, and then begin to decline (Boyce-Derricott *et al.*, 2010). In medaka (*Oryzias latipes*), *Era, Erb1*, and *Erb2* are low during embryonic development, but there is an increase of *Erb2* at 6 dph and then a gradual decrease after 7 dpf (Chakraborty *et al.,* 2011).

Studies examining the developmental expression of *PRs* are limited, but a study was conducted on female rat brains suggests that there is a change in the expression of *PRs* at the transcript level over time. This study showed two isoforms of *PR* (A and B) and looked at the change of both in two portions of the brain throughout early development, 2 days before birth to 8 weeks of age (Kato *et al.,* 1993). In both the cerebral cortex and hypothalamus preoptic area, there was an increase in *PR-A* over time.

In both of these structures, there was also an increase in *PR-B*. However, in the cerebral cortex there was an initial increase followed by a decrease in the mRNA levels of *PR-B*. In the hypothalamus preoptic area, there was a much slower increase in *PR-B* overtime (Kato *et al.,* 1993).

Studies have also focused on early expression of these *PRs*. In zebrafish it was found that *nPR* was initially detected in the ovulated oocyte, but then was not detected until 24 and 48 hours post fertilization (hpf) (Pikulkaew *et al.,* 2010). *mPRα* and *mPRβ* were also detected in the ovulated oocyte and continued to be expressed for the first 4 hours. However, in another study done in zebrafish, *nPR* was found to be present beginning at 8 hpf (Chen *et al.,* 2010). This same study also reported that at 4 weeks post hatch, *nPR* levels were higher in the female ovarian tissue than male testicular tissue. Then by 8 weeks there was a 20-fold increase in both males and females. The males then maintained this expression until 12 weeks, whereas, the *nPR* expression in females began to decrease (Chen *et al.,* 2010).

Connecting Everything Together

Previous studies show that the OE plays a role in the regulation of reproduction in some fish by allowing specific ligands to induce action potentials in olfactory sensory neurons. It appears that the receptors present in the OE that enable this to occur are mPRs that can bind progestogenic pheromones released by conspecifics. While there has been one study that shows *mPRs* mRNA transcripts are present in the OE of goldfish and zebrafish, specifically *mPRγ-1*, *mPRγ-2,* and *mPRβ* (Kolmakov *et al.,* 2008), there have not been any studies that specifically connect how PRs in the OE can cause reproductive changes in fish after exposure to pheromones released from a member of the opposite

sex. More research is needed to support these findings and influence further research into the role of mPRγ-1, mPRγ-2, and mPRβ in the transduction of pheromonal signaling.

I hypothesize that all four *mPRs* will be expressed in the OE, but not nPR, and that the expression of mPR will be greatest in reproductive adults and lower in juveniles and photothermally shifted, non-reproductive adult FHMs. Finally, I expect that there is a sexual dimorphism in the expression of *mPRs* and that males will have higher expression of *mPRs* than females.

Materials and Methods

Fish Husbandry

This research was conducted at the University of Maryland, College Park, Maryland. The fish in this study were housed in an environmental chamber in sixteen, ten-gallon tanks (Figure 5A). Eight tanks were designated for the adult groups and eight tanks were designated for the juvenile group. The adult tanks were divided into two, five-gallon sections. One side housed reproductively mature males and females (150-220 dph). The other side housed male and female subadults (120-150 dph) (Figure 5B). Both sides contained two males and four females. Two breeding tiles were placed on each side. The juvenile tanks contained 25 young fish (80-100 dph). Fish were obtained from Aquatic BioSystems Inc. (Fort Collins, CO).

Once the fish were placed into the system they were held at 24 ± 0.5°C and a light:dark cycle of 15:9 hours for two weeks. Ammonia levels (< 0.05mg/L) and pH levels (7.4-7.6) were tested weekly. A 10-15% water change was also performed weekly.

Anesthetization and Dissections

After the two-week acclimation period, the reproductively active adults and the juveniles were sacrificed. They were placed into an anesthetic overdose solution of buffered MS-222 (500 ppm, pH ~7.4). The wet mass and fork length of each fish was measured. Next, the OEs were collected and snap-frozen in liquid nitrogen. Each side was collected separately. The right side was used for quantitative PCR and the left side was saved at -80°C for future use. The gonad was collected, weighed, and one lobe was fixed to determine sex and reproductive status, at the gross morphological and histological levels. The other lobe was snap-frozen for future research by others. As the tank is the statistical unit, the two male OEs in each tank were pooled to represent the male unit of that tank and four female OEs in each tank were pooled to represent the female unit of that tank.

Photothermal Manipulation

The remaining subadults went through a faux regression towards quiescence (non-reproductive) season. This was achieved through lowering the room temperature by 0.5°C daily and decreasing the photoperiod by ten minutes daily. Once 12°C was reached, one male and female were sacrificed and checked for reproductive quiescence by gonadosomatic index and histology of the gonads. They were then examined for early stages follicular development in the females and early stages of spermatogenesis in males (as described by Dietrich and Krieger, 2009) At this time, based on histology, the fish did not appear to be fully non-reproductive. I continued to drop the air temperature and photoperiod and sampled at 10°C and 8°C water temperature. Once reaching 8°C water

temperature the environmental chamber was at 4°C air temperature and could not be lowered past this point. However, I did continue to lower the photoperiod for two weeks and allowed the fish to acclimate to 8°C. After two weeks, the same procedure used in the previous groups for dissecting samples was employed for this group of adult fish.

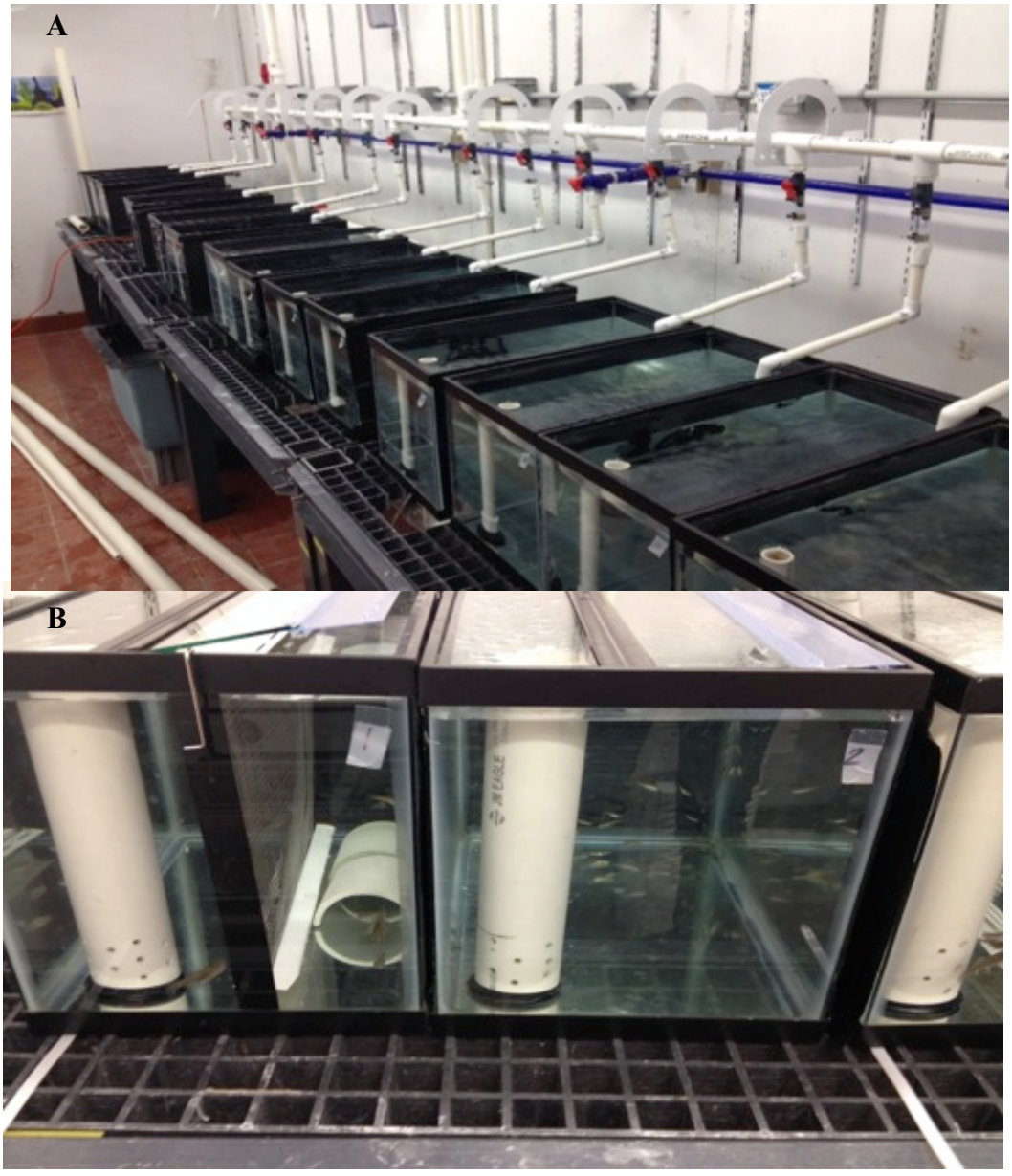


Figure 5: A. Layout of all sixteen tanks. B. Tank on left has a divider with the non-reproductive adults on the left and the reproductive adults on the right. Right tank contains juveniles.

Histology

All chemicals were purchased from Thermo Fisher Scientific (Waltham, MA) or VWR International (Radnor, PA). Fixatives were made following protocols taken from Humason's Animal Tissue Techniques (Humason, 1997). Gonadal samples were collected from both males and females, then immediately placed in Davidson's fixative for 24 hours. The samples were stored in 10% neutral buffered formalin until they are ready for sectioning. Samples were dehydrated by placing them in graded ethanol series from 75 to 100% for one-hour each, cleared in xylene for two one-hour baths, and cleared through four, one-hour wax baths. Wax baths were held within a vacuum oven set at 57°C. Every hour, pressure increased 5mmHG starting at -10mmHg and reaching -25mmHg for the last hour. Samples were then embedded in paraffin wax. Samples were sectioned frontally at 5 μm using a rotary microtome. Sections were floated on a water bath and mounted onto a glass slide. These slides were stained using Harris' hematoxylin and counterstained with eosin-Y, mounted with permount, and coverslipped following standard procedures depicted in (Humason, 1997). Finally, these tissues were observed using a Carl Zeiss Axioplan microscope (model number 451888) (Oberkochen, Germany) and for follicular development and oocyte maturation in the females and development of spermatozoa in the males (Smith, 1978). Micrographs were taken using a Carl Zeiss AxioCam MRC by Zeiss imaging software (Oberkochen, Germany).

Quantitative Real Time PCR

The right side of the OE was used for quantitative real time PCR (qPCR). Procedure for qPCR followed recommendation by Bustin *et al., 2009*. Tissue was

homogenized in a lysis buffer using a tissue homogenizer (Fisher Scientific® PowerGen™ Model 125 Homogenizer) and purified using a Genesee Scientific Quick-RNA™ MicroPrep kit (San Diego, CA). This kit provided a DNase step to ensure our final RNA product is DNA free. Since the sample size is based on the number of tanks used, all samples were homogenized separately, but all the females from the same tank, and all the males from the same tank were pooled prior to continuing with the RNA purification process. This created a sample size of eight for each sex and each life stage. Quantification and quality assurance of RNA was determined using Experion™ RNA StdSens chips and Experion™ Automated Electrophoresis station (Bio-Rad). The Experion™ software performs an automatic RNA integrity assessment with RNA quality indicator (RQI). The RQI was used to determine if a sample was acceptable for use. An RQI between 7-10 was acceptable. Total RNA (500 ng) was used for reverse transcription reactions using Invitrogen™ SuperScript® III First-Strand Synthesis System (Grand Island, NY). SYBR® Green qPCR was used to detect gene expression of each progesterone receptor using Bio-Rad's CFX96™ Real-Time System. A standard curve of known cDNA concentrations for each PR and RPL8 was used to determine the absolute quantity of transcripts present in the tissue samples. The standards were made by amplifying the open reading frame for each gene via PCR, running each sample on an agarose gel, and then extracting the PCR product. The samples were then quantified using a GE Healthcare Life Sciences GeneQuant spectrophotometer (Pittsburgh, PA). The copy number of each gene was determined by the following formula: (X g/μL DNA/[plasmid length product in base pairs * 660]) * 6.022 * 10^{23}. For each standard this was diluted so that each standard began with =1.5x10^^{10} amplicons. From this starting

concentration, a 4-fold serial dilution was created for each known standard and each unknown was compared to the qPCR results of that curve. Reproductive adults and juveniles were performed on the same plate (Figures C.1-C.6 A) while non-reproductive adults were performed on a separate plate (Figures C.1-C.6 B). All samples were duplicated on the qPCR plate and an average of the results were taken. Results collected were normalized by ribosomal protein L8 (*Rpl8*) gene expression.

Statistical Analysis

Data analysis was performed using SAS 9.3 (SAS Institute, Cary, NC). An LSMEANS statement using the DIFF adjustment for Tukey's was used for comparison of least square means. Two different models were used. One to compare the gene expressed in each group, and one to compared the groups to one another. In the first model sex, life stage, and gene were still fixed effects. For the second model, only sex and life stage were included as fixed effects. For both models, the design was blocked by tank to ensure no significant differences were observed among all the tanks. For our second model, comparisons were only observed for reproductive adults versus juveniles, and reproductive adults versus non-reproductive adults. Results were deemed significant if the $p < 0.05$.

Results

Fish

At reproductive photoperiods and temperatures adult fish were actively spawning and laying eggs on breeding tunnels provided. Juveniles were not breeding and none appeared to show any sexual dimorphisms in coloration and size. At non-reproductive photoperiods and temperatures, reproduction was not observed below 20 °C and a light:dark cycle below 14 h:10 h.

Histology

Histology confirmed immature gonads for both juvenile male and female FHMs (Figure 6A and 6B). Juvenile female ovaries contained only primary follicles with perinucleolar oocytes. Juvenile males had small sized seminiferous tubules, which were surrounded by small cysts containing early stage spermatogonia, with little to no spermatozoa in the lumen. Histology also confirmed reproductively active adult male and females (Figure 6C and 6D). Adult females had all stages of oocytes present; perinucleolar oocytes, cortical alveolar oocytes, and early/late vitellogenic oocytes. During dissections, it was also noted that 2 females were ovulating. Reproductive males had much large cysts, and large diameter seminiferous tubules with lumens full of spermatozoa. Cysts containing the various stages of spermatogenesis are present, but are much smaller in comparison to the large lumen. Compared to juveniles, by direct observation, both reproductive females and males had large gonads. Gonadosomatic index (GSI), mass of gonad/mass of weight *100%, was not calculated for juveniles because gonads were too small for dissection; therefore, no comparison can be made

between GSI of juveniles and reproductive adults. As for the non-reproductive adult group, we cannot confidentially say that they are indeed non-reproductive. While during this time period no eggs were being deposited and fertilized, from the histology we do not see full regression of late stage oocytes in females and regression of spermatozoa production in the males (Figure 6E and 6F). A comparison of GSI can be found in Table 1. Surprisingly, non-reproductive females had a significantly higher GSI than reproductive females ($p<0.001$). There was no significant difference between GSI of non-reproductive males and reproductive males ($p=0.113$).

Table 1: The gonadosomatic index (GSI) calculated for all four adult treatment groups. GSI = (gonad mass/ fish mass) x 100.

Group	GSI
Reproductive Female	11.56%
Non-Reproductive Female	17.78%
Reproductive Male	1.28%
Non-Reproductive Male	1.09%

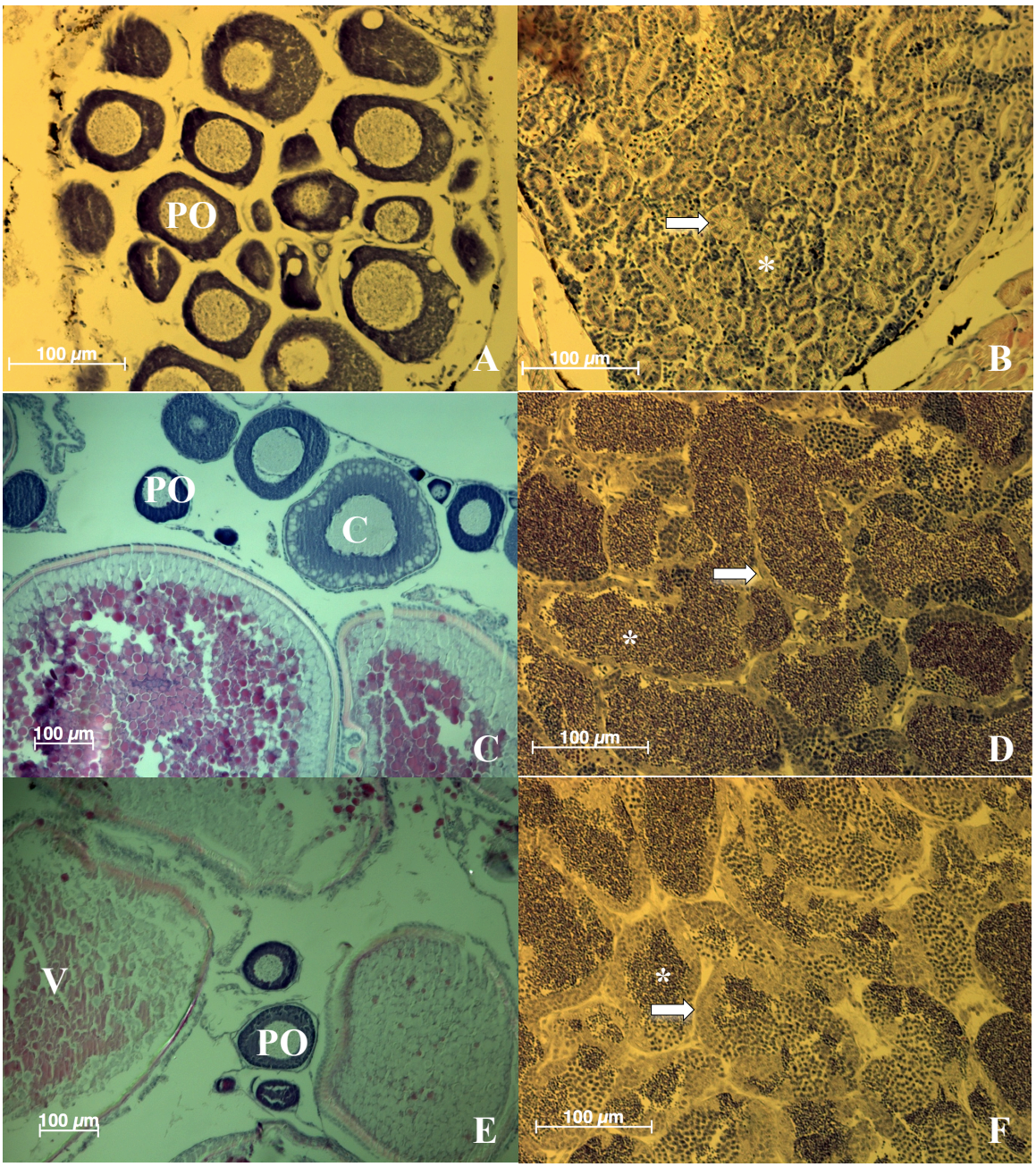


Figure 6: Micrographs of H and E stained gonad sections. A. Juvenile female. B. Juvenile male. C. Reproductive female. D. Reproductive male. E. Non-reproductive female. F. Non-reproductive male. PO refers to the early stage perinucleolar oocytes, CO refers to the slightly later stage cortical alveolar oocytes, V refers to the later developed vitellogenic oocytes. An asterisk (*) points out the lumen of the seminiferous tubules. In juveniles (B) this space is emptied, while in the adults (D and F) it is filled with spermatozoa. The arrow points out cysts, which make up the seminiferous tubules, and are filled with the varying stages of gametes proceeding through spermatogenesis.

Quantitative Real Time PCR

All four *mPRs* were expressed, whereas *nPR* was not (Figure 7). When examining the effect of sex between each group and the interaction across stages, we found that sex had no effect on *PR* gene expression (See Appendix D for figures). From this point on comparisons were made based on life stages as a whole, where sex was combined (Figure 8).

In the juvenile group, we found that *mPRα* and *mPRβ* both have significantly higher expression than *mPRγ-1* and *mPRγ-2* ($p_{\alpha,\gamma\text{-}1}<0.0001$, $p_{\alpha,\gamma\text{-}2}<0.0001$, $p_{\beta,\gamma\text{-}1}<0.0001$, $p_{\beta,\gamma\text{-}2}<0.0001$) (Table 4 for p-values). However, *mPRγ-1* and *mPRγ-2* had no significant difference in expression and the same is found for *mPRα* and *mPRβ* (p=1.00, p=0.999 respectively). As for the reproductive adult group, similar results were seen as with the juvenile group; however, only *mPRα* is expressed significantly higher than *mPRγ-1* and *mPRγ-2* ($p_{\alpha,\gamma\text{-}1}=0.002$, $p_{\alpha,\gamma\text{-}2}=0.005$). In the non-reproductive adult group we found no significant differences in the expression of all mPR genes (p=1.00 for all groups).

When comparing juveniles to the reproductive adults it was observed that all genes except *mPRγ-2* significantly differed (Figure 8). From the juvenile life stage to the sexual mature adult stage *mPRγ-1* increased, whereas *mPRα* and *mPRβ* decreased ($p_{\alpha}=0.024$, $p_{\beta}=0.006$, $p_{\gamma\text{-}1}=0.005$). Although not significant, there was a decreasing trend for *mPRγ-2* ($p_{\gamma\text{-}2}=0.24$)(Figure 8, Table 4).

Finally when examining changes from reproductively active adults to adults that have been raised in photoperiod and temperature not conducive to reproduction, it was found that there was only a significant change in the expression of *mPRα* ($p_{\alpha}=0.024$) and *mPRγ-2* ($p_{\gamma\text{-}2}=0.044$). As fish were photothermally shifted to a non-reproductive state

mPRγ-2 increased, whereas mPRα expression decreased. It is important to remember that although the non-reproductive group of fish were not actively spawning, histology of the gonads did not confirm a non-reproductive state, therefore these results may be distorted and not fully representing non-reproductive fish. Summary of these results are in Table 2.

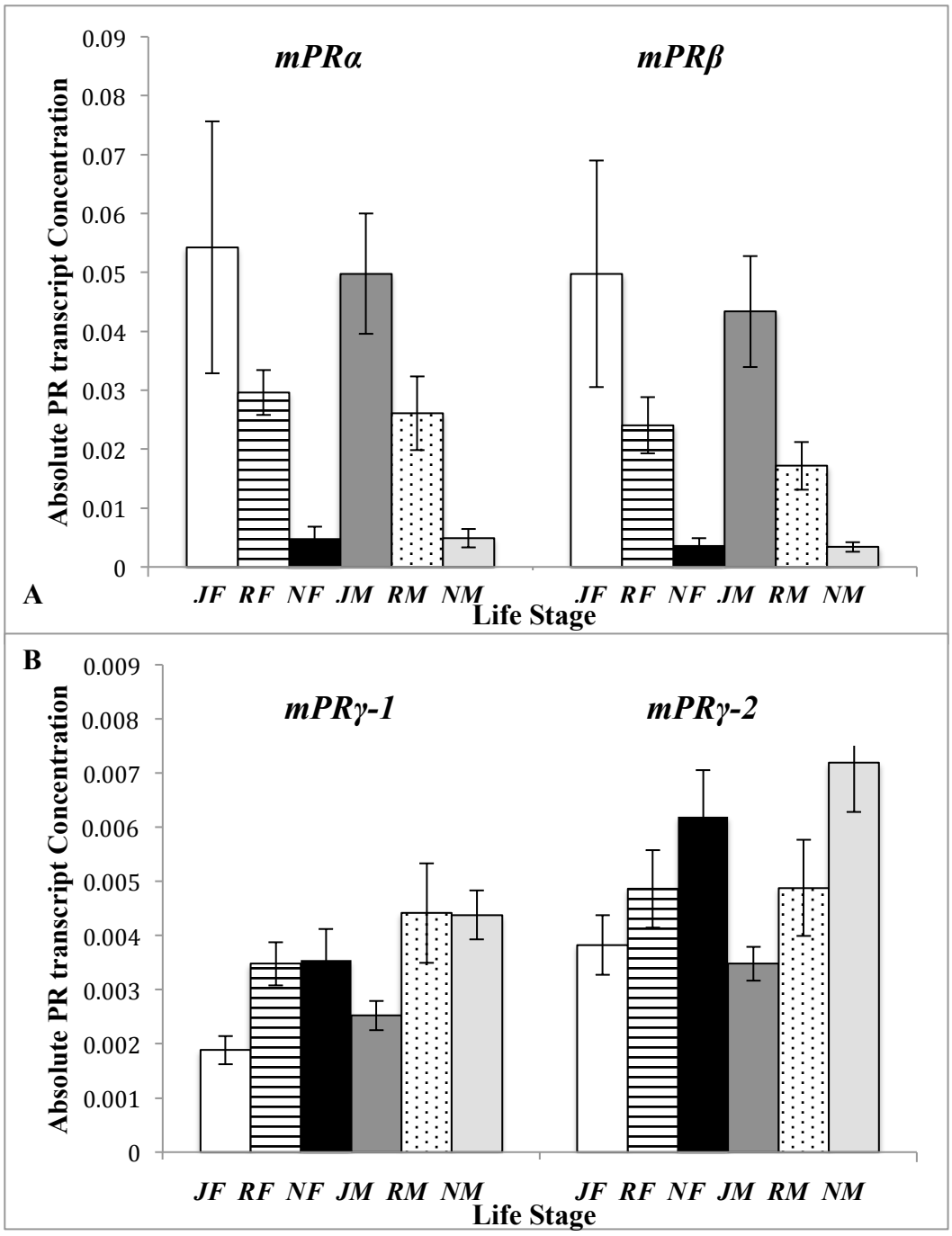


Figure 7: Progesterone receptor (PR) expression levels in the olfactory epithelium of all six experimental groups. (A) *mPRα* and *mPRβ* (B) *mPRγ-1* and *mPRγ-2*. Absolute quantification of PR expression was determined by the use of a standard curve comprised of a serial dilution of PR cDNA and SYBR Green QPCR. Values were then normalized by *ribosomal protein L8 (RPL8)*. n=8 (except reproductive male=7 and juvenile female=6).JF=juvenile females, RF= reproductive adult females, NF=non-reproductive adult females, JM=juvenile males, RM=reproductive adult males, NM=non-reproductive adult males.

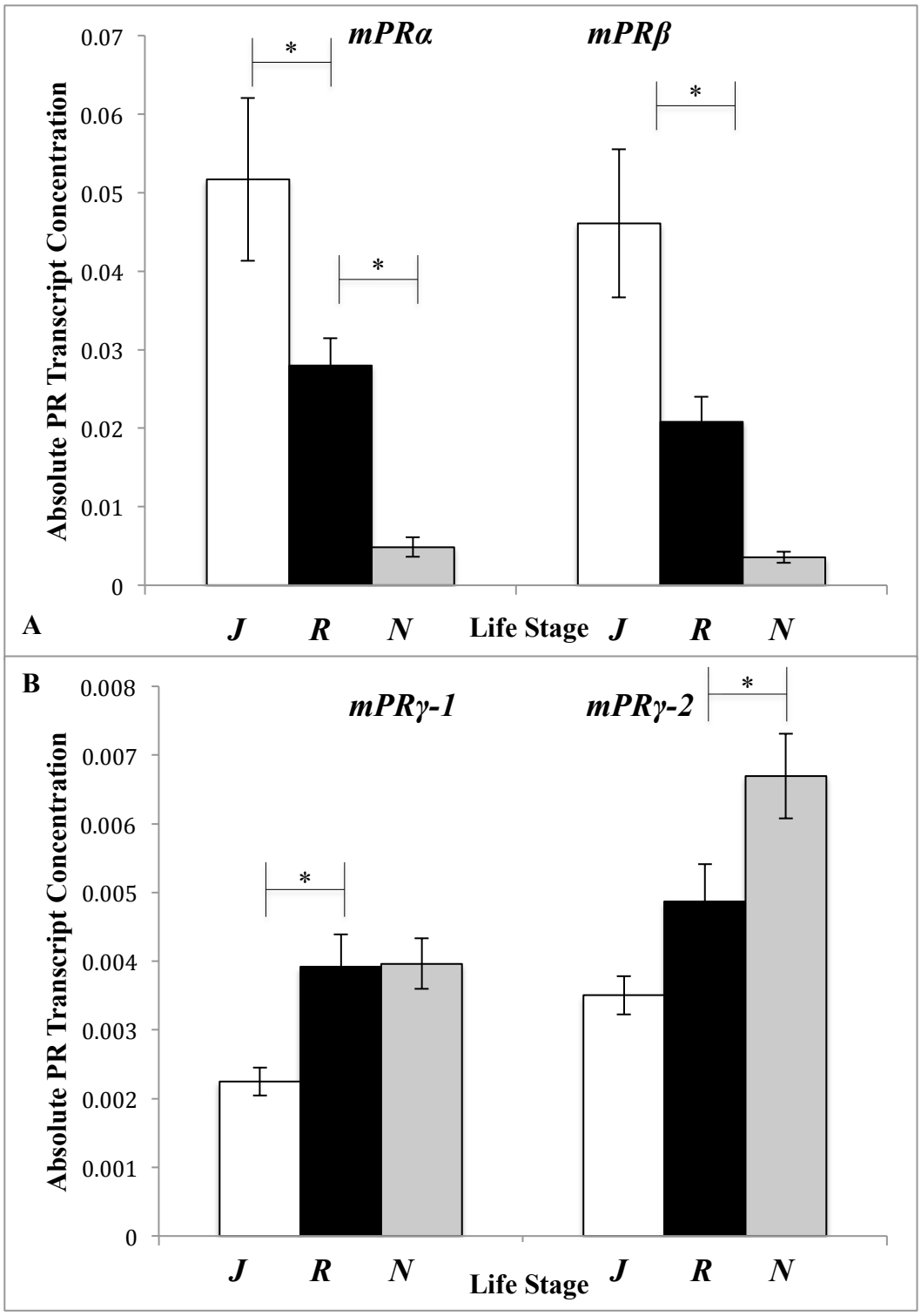


Figure 8: Progesterone receptor (PR) expression levels in the olfactory epithelium from Figure 7 but with sexes combined. (A) *mPRα* and *mPRβ* (B) *mPRγ-1* and *mPRγ-2*. SAS results showed there was no statistical significant between sexes in each life stage. Both male and female data was averaged and SEM is shown. * Represents a significant difference in gene expression either between juveniles and reproductive adults or reproductive adults and non-reproductive adults. No comparison was performed between juveniles and non-reproductive adults. Sample size of juveniles=14, reproductive adults=15 and non-reproductive adults=16. J=juveniles, R=reproductive adults, N=non-reproductive adults

Table 2: Summary of qPCR results. (A) Comparing *mPR* gene expression in each group individually. Arrow refers to first gene mentioned expression compared to second gene mentioned expression. N.S.=no difference

	mPRα **vs.** ***mPRβ***	***mPRα*** **vs.** ***mPRγ-1***	***mPRα*** **vs.** ***mPRγ-2***	***mPRβ*** **vs.** ***mPRγ-1***	***mPRβ*** **vs.** ***mPRγ-2***	***mPRγ-1*** **vs.** ***mPRγ-2***
Juveniles	**N.S.**	↑	↑	↑	↑	**N.S.**
Reproductive Adults	**N.S.**	↑	↑	**N.S.**	**N.S.**	**N.S.**
Non-reproductive Adults	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**	**N.S.**

Table 3: Summary of qPCR results comparing reproductive adults to both non-reproductive groups for each mPR. Arrows represents the change of the reproductive adult compared to the other group. N.S.=no difference.

	mPRγ-1	***mPRγ-2***	***mPRα***	***mPRβ***
Reproductive Adults vs. Juveniles	↑	**N.S.**	↓	↓
Reproductive Adults vs. Non-reproductive Adults	**N.S.**	↓	↑	**N.S.**

Table 4: Comparisons were made between each gene for each treatment group, between juvenile and reproductive adults for each gene, and between reproductive adults and non-reproductive adults for each gene based off of qPCR data. p values for all comparison were performed in SAS and are based off of data normalized by the housekeeping gene RPL8. *Represents significant value.

Comparison	P value
Juvenile: *mPRα* vs. *mPRγ-1*	<0.0001*
Juvenile: *mPRα* vs. *mPRγ-2*	<0.0001*
Juvenile: *mPRα* vs. *mPRβ*	0.998
Juvenile: *mPRβ* vs. *mPRγ-1*	<0.0001*
Juvenile: *mPRβ* vs. *mPRγ-2*	<0.0001*
Juvenile: *mPRγ-1* vs. *mPRγ-2*	1.000
Reproductive: *mPRα* vs. *mPRγ-1*	0.002*
Reproductive: *mPRα* vs. *mPRγ-2*	0.004*
Reproductive: *mPRα* vs. *mPRβ*	0.984
Reproductive: *mPRβ* vs. *mPRγ-1*	0.130
Reproductive: *mPRβ* vs. *mPRγ-2*	0.192
Reproductive: *mPRγ-1* vs. *mPRγ-2*	1.000
Non-Reproductive: *mPRα* vs. *mPRγ-1*	1.000
Non-Reproductive: *mPRα* vs. *mPRγ-2*	1.000
Non-Reproductive: *mPRα* vs. *mPRβ*	1.000
Non-Reproductive: *mPRβ* vs. *mPRγ-1*	1.000
Non-Reproductive: *mPRβ* vs. *mPRγ-2*	1.000
Non-Reproductive: *mPRγ-1*vs. *mPRγ-2*	1.000
mPRα: Juvenile vs. Reproductive	0.024*
mPRα: Reproductive vs. Non-Reproductive	0.024*
mPRβ: Juvenile vs. Reproductive	0.006*
mPRβ: Reproductive vs. Non-Reproductive	0.073
mPRγ-1: Juvenile vs. Reproductive	0.008*
mPRγ-1: Reproductive vs. Non-Reproductive	0.999
mPRγ-2: Juvenile vs. Reproductive	0.226
mPRγ-2: Reproductive vs. Non-Reproductive	0.044*

Discussion

Many anatomical and physiological changes occur during life stage transitions. In the present study, gonadal histology and *PR* mRNA gene expression in in the OE of three distinct fathead minnow (FHM) life stages were examined. Histological analysis clearly identifying the differences between juvenile gonads and reproductive adults gonads. However, it was found that the non-reproductive adults were not fully quiescent. Gene expression analysis revealed that all four *mPRs*, and not *nPR*, were expressed in the OE across all life stages. Gene expression levels of *mPRs* varied from group to group in a manner that was not expected. Compared to the reproductive adults, juveniles expressed higher levels of *mPRα* and *mPRβ*, and non-reproductive adults expressed higher levels of *mPRγ-1* and *mPRγ-2*.

Gonadal Histology

Upon examining the histological differences between the gonads from juveniles and the gonads from adults, substantial changes consistent with what was expected were observed. In juveniles, germ cells in early stages of development were apparent in both sexes. As the fish mature, the gonad is able to increase in size due to the maturation process of these germ cells. As the germ cells of females undergo oogenesis, the follicle fills with yolk that will eventually nourish the growing embryo and the post-hatch larva (Lubzens *et al.,* 2010). Spermatogenesis is the process of maturing the primary spermatogonia to fully functioning spermatozoa in reproductive male adults. The lumen of the seminiferous tubules fills with spermatozoa, increasing the size of the testes. Spermatogenesis in fish occurs synchronously through cysts that are formed from Sertoli cells (Schultz *et al.,* 2010). This corresponds to our results as cysts with the various

stages of spermatogenesis were observed. Our results also concur with a histological study performed on FHMs by others. Vitellogenic oocytes begin to form in females as early as 60 days post-fertilization (dpf, and continued to grow until the end of sampling at 150 dps (Uguz, 2008). While most of the juvenile female samples had only primary follicles, some show signs of larger follicles beginning to form. In males, spermatogenesis was not observed until 120 dpf past the age of our juveniles, where we observed mostly early spermatogonia. This also confirms that sex could be determined via histology at the age we collected the juveniles at (84-104 dpf).

The next life stage was from a reproductively active adult to a reproductively quiescent adult, initiated due to seasonal changes. It was expected that in decreased photoperiod and water temperatures, the gonads would regress as the response to reproduce declined. During regression of the ovaries, it was expected that the follicle would become atretic. The atretic follicle would degenerate and be reabsorbed by the body (Saidapur, 1978). Histologically, one should observe breakdown of the nuclear membrane, a yolk mass that is liquified, an increased invasion of in-folding follicular layers, and phagocytosis of the granulosa cells (Coward & Bromage, 1998). The vitellogenic oocytes would disappear, and there should be a predominance of primary follicles. This is not what was observed in the fish collected for this study. Instead, we continued to see later stage oocytes with no appearance of atresia.

During testicular regression in males, it was expected to see the formation of collagenous capsules containing degenerating germ cells and blood cells, the cysts would appear as a colloidal mass, and the lumen would be emptied of spermatozoa (Singh *et al.,* 2010). Once again, our results were not fully consistent with the work done in other fish

species. We continued to see spermatozoa in the lumen, although, based solely on observation, there appeared to be fewer spermatozoa in this group of fish than the reproductive group. Both results, from male and female, suggest that the fish are just beginning the regression process and have not fully reached a quiescent state, as was anticipated.

The lack of fully quiescent fish was most likely due to limitations on time. The nonspawning environment induced for this experiment occurred in 45 days. This timetable did not allow for photoperiod or temperature to reach what may have been necessary for full quiescence, and/or fish should have been at those conditions longer to fully regress. To get results based off of fully non-reproductive adults, is crucial that this portion of the experiment is repeated to allow for enough time to induce a full change in the reproductive status of these fish. Also, we began the induced non-reproductive season with subadult fish as opposed to already sexually mature adults. This may mean that subadult fish are still able to mature under the decreasing photoperiods and temperatures. If this experiment were to be repeated, it would be necessary to begin the nonspawning induction with reproductive adults.

Specified spawning seasons are crucial for maintaining adequate energy budget during times when nutrients are not as readily available. For most seasonal breeders, this time is during the winter when day length is short and temperatures are low (Mommsen & Walsh, 1988). While resources are limited, it would be beneficial for fish to save energy for maintenance and growth, therefore, reducing the energy put towards reproductive growth. At the same time, it would not be beneficial for offspring to be born during the time of year when nutrients are low. By ensuring that hatching occurs during

times of prime nutrient availability, offspring have a greater chance of growing and surviving to adulthood.

Another interesting observation was that the GSI of the non-reproductive females was larger than the GSI for reproductive females. Most studies examining GSI post reproductive seasons show that the GSI of non-reproductive females should be lowered due to a lack of late stage oocytes, as seen in walleye pollock (*Theragra chalcogramma*) (Stahl & Kruse, 2008). However, since the fish of this study were still in a regressing state and not fully quiescent the oocytes may be beginning the apoptosis process. Histological analysis of oocytes undergoing apoptosis in the zebrafish shows evidence of hypertrophy and hyperplasia occurring in the granulosa cells (Üçüncü & Çakici, 2009). This would cause an enlargement of the oocyte, adding to the weight of the ovary, and is consistent with why the GSI of non-reproductive females would be larger than the reproductive females. If full regression was to take place, the granulosa cells would eventually be engulfed by phagocytes and leave behind a large cavity called the atrium (Üçüncü & Çakici, 2009). At this point, it would be expected that the space would fill with primary follicles and become smaller in size. These results could also be affected by the phase of the spawning cycle of the reproductive females. When studying FHMs throughout a reproductive cycle it was found that their GSI was lowest the day of spawning compared to the 45% GSI increase two days later just before the peak spawning period (Jensen et al., 2001). If a large portion of the female fish from my study had recently spawned, which was possible since eggs were observed to be deposit on the breeding tile, then this could also explain why there is a discrepancy with my results compared to other studies that showed lowered GSI post spawning season. If the large

mature oocytes are causing the increased mass of the ovaries, then once they are deposited after ovulation this would cause the ovary mass to decrease.

Progesterone Receptor Gene Expression in the Olfactory Epithelium

It was hypothesized that due to the specific reproductive function of these receptors, a greater abundance of *PR* transcripts would be found in the reproductive adults than in the juveniles. While changes in gene expression occurred, these results were not completely as predicted. It was observed that mPRα and mPRβ were expressed higher in juveniles than in the reproductively active adults. The other two *mPRs*, *mPRγ-1* and *mPRγ-2,* while still expressed in juvenile OEs, increased over the pubertal transition as expected. It is interesting to find that receptors crucial for coordinating reproduction are found during this non-reproductive life stage in FHMs.

This may imply that these receptors have other regulatory functions that could be important for the transition from juvenile to adults. mPRs are hypothesized to be in crypt cells, and in a study examining the odorant tuning of crypt cells in rainbow trout, isolated crypt cells in juveniles responded to a wide variety of odorants, whereas adults only had a response to sex steroids and extracts from conspecifics (Bazáes & Schmachtenberg, 2012). This odorant tuning change overtime could possibly be due to the change in the expression pattern of *mPRs*. These receptors may not be initially tuned to detect pheromones only, but require some adjustment during the transition from juvenile to adult. It could even be possible that the initial expression patterns help activate the onset of puberty. Once reaching the age conducive to start reproducing, pheromones from conspecifics could trigger a response from the hypothalamic-pituitary-gonadal axis to stimulate the onset of puberty. This has been previously demonstrated in mice, where

females exposed to the odor of male urine had an earlier onset of puberty compared to the age of puberty in mice not exposed to male urine (Flanagan *et al.,* 2011). Similar results have long been known for swine, where puberty onset occurs in a gilt when she comes in direct contact with a boar (Pearce & Paterson, 1992). mPRs present in juvenile fish prior to being reproductive may be functioning as the trigger for puberty in FHMs. Once initial growth of the gonad has finished for both sexes, pheromones secreted by conspecifics may activate the onset of puberty. This process would be important, especially in FHMs, due to seasonal breeding and the importance of needing reproductively active fish during the mating season.

It was hypothesized that reproductive quiescence would decrease the expression of all *mPRs*. However, qPCR results showed that while *mPRα* and *mPRβ* declined, *mPRγ-1* and *mPRγ-2* increased. These results could be due to the fact that the fish were not fully quiescent; therefore if the fish were reproductive quiescent the results may differ. However, expression of *mPRγ-1* and *mPRγ-2* is consistent with the *mPRγ* mRNA expression in the ovaries of the channel catfish (*Ictalurus punctatus*) (Kazeto *et al.,* 2005). The spawning season of channel catfish is early to mid-July and ovarian regression is seen starting in August with reproductive quiescence occurring during the winter months. When assessing the mRNA abundance of *mPRγ* in catfish ovarian follicles, it was found that the expression of *mPRγ* levels peaked at the time when the reproductive cycle was in a quiescent state (Kazeto *et al.,* 2005). The initially high level of *mPRα* and *mPRβ* mRNA expression in reproductive adults and the decrease in mRNA expression in non-reproductive adults suggests that mPRα and mPRβ, and not mPRγ-1

and mPRγ-2, are the receptors responsible for coordinating reproduction in the OE of FHMs.

While it is suspected that mPRγ-1, mPRγ-2, and mPRβ play a role in pheromone communication in goldfish and zebrafish, *mPRα* was not detected in goldfish or zebrafish OE transcriptome (Kolmakov *et al.,* 2008). The difference in receptor expression already demonstrates a species difference among goldfish, zebrafish, and FHMs. This suggests that there may also be a difference in which mPR(s) are responsible for pheromonal communication among teleost fish.

The question still remains: what is the role of mPRγ-1 and mPRγ-2 and why would their expression levels increase in the non-reproductive state? While it is clear that mPRs likely play a role in reproductive behaviors, they are potentially important for other social behaviors. The presence of mPRs outside of the spawning season corresponds with work done in waigieu seaperch (*Psammoperca waigiensis*). Peaked progesterone levels were measured in November and measurements of significantly high levels of progesterone continued into December even though their spawning season is March through September (Pham *et al.,* 2012).

Detecting progestogens from others in conspecifics may also help with social hierarchy. Salmonids, rainbow trout, and cichlids are known to form social hierarchies when limited resources are available (Chapman, 1966; McCarth*y et al.,* 1992; O'Connell *et al.,* 2013). A dominant fish is able to gain access to more food, space, and mates (McCarth*y et al.,* 1992). In *Astatotilapia burtoni*, dominant males and females have higher circulating progestogen levels than the levels in subordinates (O'Connell *et al.,* 2013). Male FHMs are also known to show dominance after exposure to pheromones

excreted by females. The dominant male will defend the breeding nest and the subordinate male is less likely to defend a nest in the presence of a dominant male (Danylchuk & Tonn, 2001). Although all these studies presented were performed with reproductively active adults, it may be possible that this hierarchy continues past the spawning season. This may lead to better reproductive success during the next spawning season because it can help ensure that the dominant males and females are already hormonally primed to reproduce right away and it may allow for them to already have a mate lined up.

These non-reproductive functions could also account for why there were no sexually dimorphic observations in the expression pattern of *mPRs* in any of the groups examined in this study. This is surprising because it was expected that males would have a higher abundance of *PRs* than females. This was based on results seen in our pilot study (Appendix A). Males had all around higher *PR* expression than females. However, this was based off of a sample size of one male and one female; therefore, no statistics were performed on the data collected. The tissue collected for this pilot study was also from fathead minnows of different strains, ages, and environmental conditions than the one used in this thesis study. Also the higher expression in males than females was expected due to the fact that females are the progestogen secreting sex and, therefore, males would be the ones required to perceive progestogens as pheromones. The possible social function of mPRs could explain why there is no difference between both sexes. This could be because both males and females are responding to progestogens as another form of social stimuli then both sexes would have to express mPRs in a similar manner. It is also possible that females are able to respond to their own progestogen secretions. This

may allow for feedback that continues the female's reproductive activity and allows for a continuation of ovulation until her egg supply is depleted.

Finally, it is important to think about the redundancy of having all four mPRs present in the olfactory epithelium at each life stage no matter the expressional pattern. Is it possible that all four are able to respond to the same pheromones? Do some respond to free progestogens, while others respond to the conjugated forms? Does the expression of one affect the expression of another? Or is each PR responsible for a specific reproductive function? At this time cross-talk has been shown to occur between mPRs and nPRs in human myometrium cells that help with maintaining a quiescent state of the myometrium during pregnancy, and helps with progesterone withdrawal at the time of labor (Karteris *et al*., 2006). It may be possible that mPRs within the OE work together to coordinate the crucial reproductive behaviors that help males and females find each other to spawn.

Future Direction

This study showed that in different life stages, *mPRs* in the OE vary in their mRNA expression. While some studies suggest that pheromonal communication occurs by progestogens binding to mPRs in the OE, how this mechanism occurs has not been explored in great detail. This information is critical for understanding how fish are able to coordinate finding a mate and spawning at the right time. Furthermore, no studies have looked at why *mPRs* are expressed in non-reproductive life stages. As seen in this study, specific *mPRs* have higher mRNA expression in non-reproductive fish than in reproductive fish. While I have made suggestions as to why this may occur, further studies need to be performed to determine the non-reproductive role of mPRs in the OE.

There is a potential ecotoxicology aspect to this research. It is important to determine the potential effect pollutants in the water can have on normal reproductive behaviors. Environmental gestagens have been measured in paper mill plant effluents, wastewater treatment plant effluents, and runoff from animal agriculture (Koplin et al., 2002; Jenkins et al., 2003; Change et al., 2009; Bartelt-Hunt et al., 2012). These studies have measured gestagen compounds ranging from few to hundreds of ng/L, and these concentrations have been used in exposure studies to determine their biological significance.

In adult FHMs, exposures to the progestins, levonorgestrel (<1 to 30 ng/L) and drospirenone (<1 to 70 μg/L), have deleterious effects on reproduction (Zeilinger et al., 2009). In females, these effects include inhibited egg production, increased numbers of atretic follicles, decreased numbers of other follicular stages, enlarged ovaries, and, at higher exposure concentrations, masculinization, which included development of male coloration and nuptial tubercles. Inhibition of egg production was seen at biologically relevant concentrations of levonorgestrel. For male fish, effects included enlarged testes and advanced spermatogenesis (Zeilinger *et al.,* 2009). In another study, norethindrone caused masculinization of the female FHM and decreased egg production in exposed adult medaka (Paulos*, et al.,* 2010). For a further review on environmental gestagens, refer to Orlando & Ellestad, 2014. Given the presence of *mPRs* in the OE, it is likely that gestagens are able to interfere with normal binding of the endogenous hormones and hinder the normal reproductive function of the OE. This could have dire consequences on normal aquatic life, leading to decreases in the population of many aquatic species and potential decline in aquatic ecosystem health.

Significance

This study is the first to compare *PR* gene expression in the OE of reproductive fish to that of non-reproductive fish. It is also the first to compare progesterone receptor expression between males and females. Progesterone receptors in the OE play a major role in reproduction. Endogenous pheromones secreted by conspecifics have a beneficial role in initiating behavior, oocyte maturation, and sperm maturation.

FHMs are sexually dimorphic and males portray overt reproductive behaviors, such as aggression towards other males, defending the nest, and spawning with females. Studies looking into the expression of *PRs* in the OE and behavioral studies on ways to influence *PR* binding will provide crucial information to make a final linkage between the roles that OEs, PRs, and pheromones play in regulating reproduction in teleost.

Fish are an extremely important commodity, providing a source of high quality protein, amino acids, and omega-3 fatty acids to over 4.3 billion people each year (Tacon & Metian, 2013). As a result of their high nutritional and economic value, the demand for seafood has increased drastically over the past few decades resulting in the decline and collapse of many wild capture fisheries (Villasante *et al.*, 2013; Natale *et al.,* 2013). To help mitigate the gap between supply and demand, and relieve pressures on wild fish stocks, greater emphasis has been placed on aquaculture. A clear understanding of reproductive physiology and morphology is limited for many of the species currently cultured, creating a potential limitation for production rates. One area that is under-investigated is the role that pheromones play in regulating spawning behavior. By increasing our understanding of the role that pheremonal signaling play among individuals and their effects on spawning behavior, we may be able to increase the productivity of fish farms and their resultant contributions to the world's food supply. We

can also better understand the ramifications of environmental gestagens in freshwater systems (Orlando, 2014). If it is found that these gestagens bind to the PRs in the OE and affect reproductive outcomes, then this could lead to decreased fish populations. This would be detrimental to aquatic ecosystem health and information from these studies would be crucial to those who regulate the quantity and quality of the Nation's water resources.

Appendices

Appendix A: Pilot Study of Progesterone Receptor Gene Expression in the Olfactory Epithelium

Prior to my thesis research, a pilot study by our lab was performed to determine the potential presence of *PR* mRNA transcripts and to determine the concentration of RNA extracted from a pooled sample of OEs.

Methods & Materials

Three adult male FHMs and three adult female FHMs were euthanized in a strong dose a MS-222 anesthetic (500 ppm, buffered pH=7.4). The left and right OEs were dissected out, and separately snap frozen in liquid nitrogen. All of the male right side OEs were combined into one pool, and all of the right side female OEs were pooled, giving a sample size of one male and one female. RNA was extracted, quantified, and reverse transcribed into cDNA. Male-pooled sample underwent both PCR and quantitative real time PCR (qPR), while only qPCR was performed on the female pool. Both of these were performed to gain visual and quantitative confirmation of which progesterone receptor mRNA transcripts were present in the OE.

Results

PCR results from male FHMs showed the presence of *mPRα, mPRβ*, and *mPR total γ* (at the time we did not have separate mPRγ-1 and mPRγ-2 primers) and no *nPR* expression (Figure A.1). qPCR confirmed these results. For these results, they were normalized by RPL8 and the no RT results were negligible. At this time we had two primer sets for each splice variant of γ and these results indicated that *mPRγ-2* had the highest expression levels followed by *mPRγ-1*, with lower expression of *mPRα* and *mPRβ* (Figure A.2). These results also demonstrated that male FHMs expressed these receptors

more abundantly than female FHMs. Statistical analysis was not performed on this data because we only had a sample size of one.

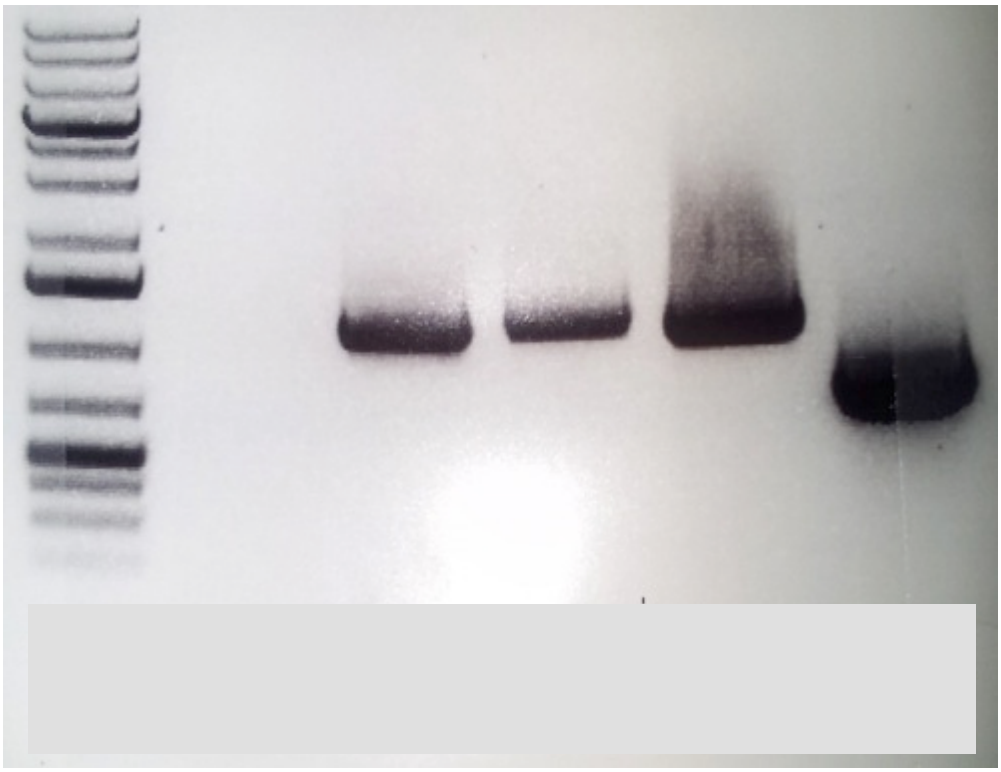


Figure A.1: Progesterone receptor (PR) PCR products run on a 2% agarose gel stained with ethidium bromide, visualized on a Biorad Molecular Imager® ChemiDoc™ XRS. Lanes from left to right: protein marker, nuclear progesterone receptor (*nPR*), *membrane PR (mPR) α, mPR β, mPR total γ* (does not distinguish between *mPRγ-1* and *mPRγ-2*), and ribosomal protein L8 *(RPL8)*.

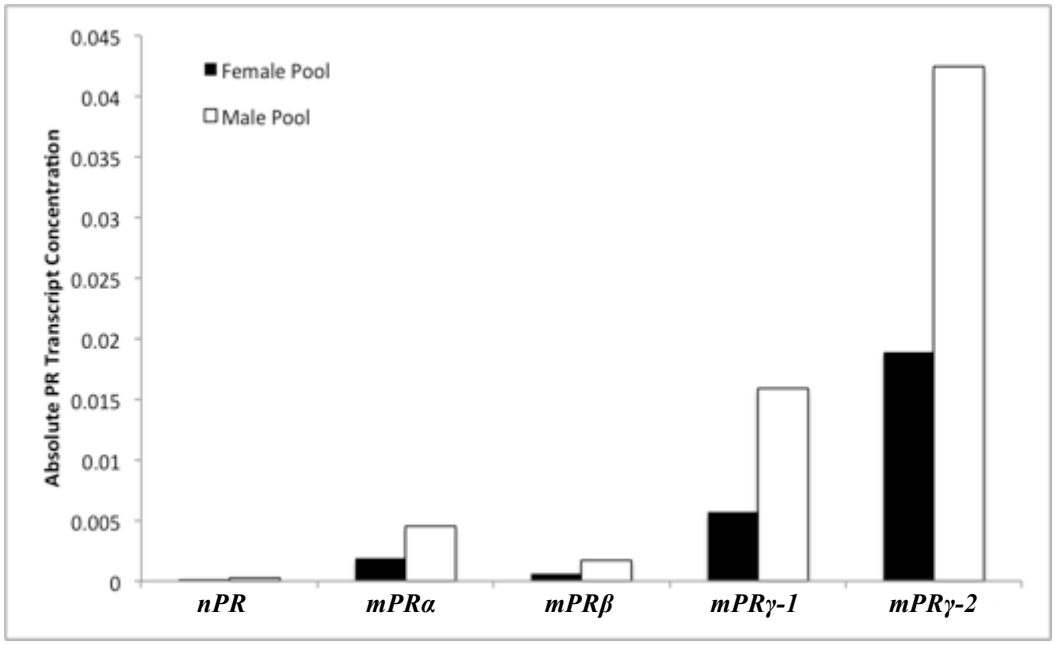
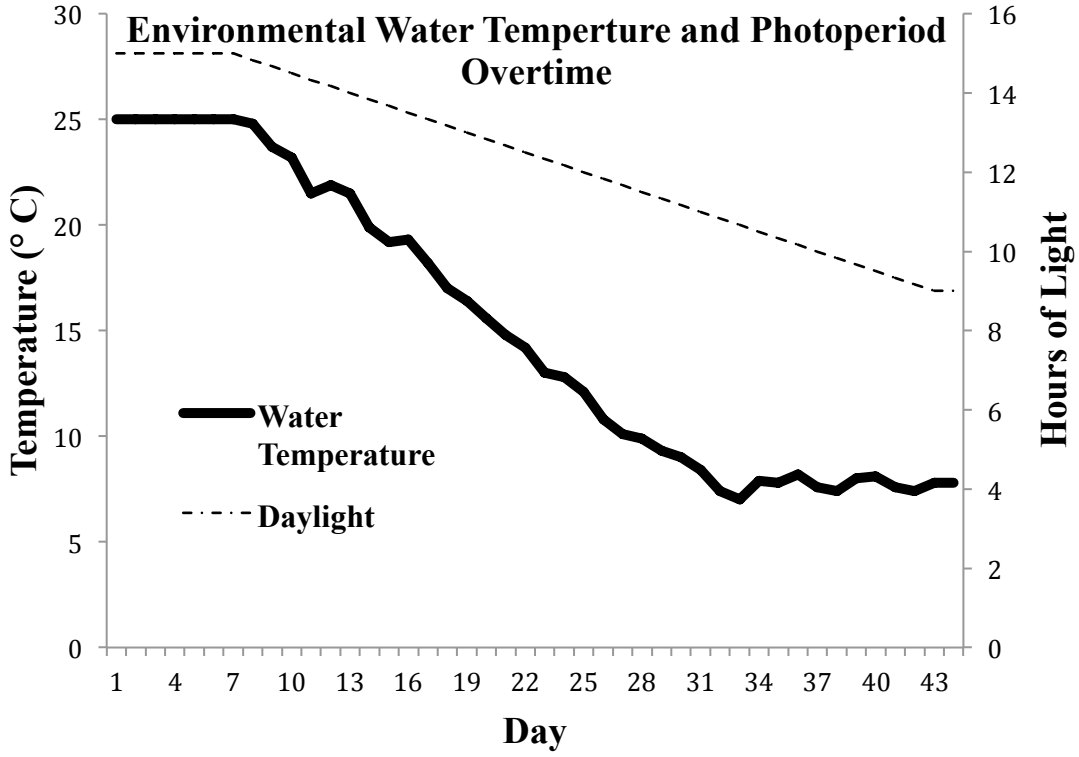


Figure A.2: Progesterone receptor (PR) expression levels in the olfactory epithelium of pooled female and male FHMs, measured by SYBR Green QPCR. Absolute quantification of PR expression was determined by the use of a standard curve comprised of a serial dilution of PR cDNA. Values were then normalized by ribosomal protein L8 *(RPL8*). Expression levels of *RPL8* were not different between sexes. (n=1)

Appendix B: Photothermal Manipulation

Temperature and daylight hours were logged over the month long photothermal manipulation (Figure B.1). The variation in room temperature and water temperature is due to the fact that the water pump produced heat that raised the water temperature by about 4 °C. The room contained a monitor that recorded the temperature at all times which allowed for determination of the maximum and minimum daily room temperature. The water temperature was recorded once a day in the early morning. Temperature and daylight hours were changed every morning. The plateau in temperature and daylight hours was when we began the weeklong acclimation period.



Appendix C: Quantitative Real Time PCR Primers, Annealing Temperatures, Standard Curves, and Efficiencies

The table below shows the accession number, qPCR forward and reverse primers, and annealing temperature for each gene (Table C.1). The following figures show the qPCR results for each PR. Reproductive adults and juveniles were performed on the same plate (Figures C.1-C.6 A) while non-reproductive adults were performed on a separate plate (Figures C.1-C.6 B). For each graph, the standard curve is plotted (filled circles) with the trend line, equation, and R^2 value shown. Unknown samples are also plotted on each graph with open squares.

Table C.1: For every gene examined in this experiment this table give the NCBI accession number, both the forward and reverse primers, and the annealing temperature used.

Gene	Accession Number	Forward Primer	Reverse Primer	Annealing Temperature (°C)
RPL8	AY919670	AACTACGCCACAG TCATCTC	AGCAACAACACC AACAACAG	59.0
nPR	JX012230	AGTTTGATGAAAT GAGACAGA	TGACCTTCTTTAC AATCTCG	58.2
mPRα	JX012231	AAGTTCGTTCACA AGCTATT	TTATAGATGCGA TGGAACAC	57.2
mPRβ	JX012232	CTGGAAGCAATAT TTAGAGATTATC	GCACCAATAAGA AGAAGGAT	58.2
mPRγ-1	JX012233	TGGCCTGCTACTCC AGATTC	TCAGTGCAACCC TCACCTAC	57.2
mPRγ-2	JX012234	TCGTGGGTGAACA GCACATT	TAGTGTAGGAAT GGTAGGCCAAGC	57.2

RPL8

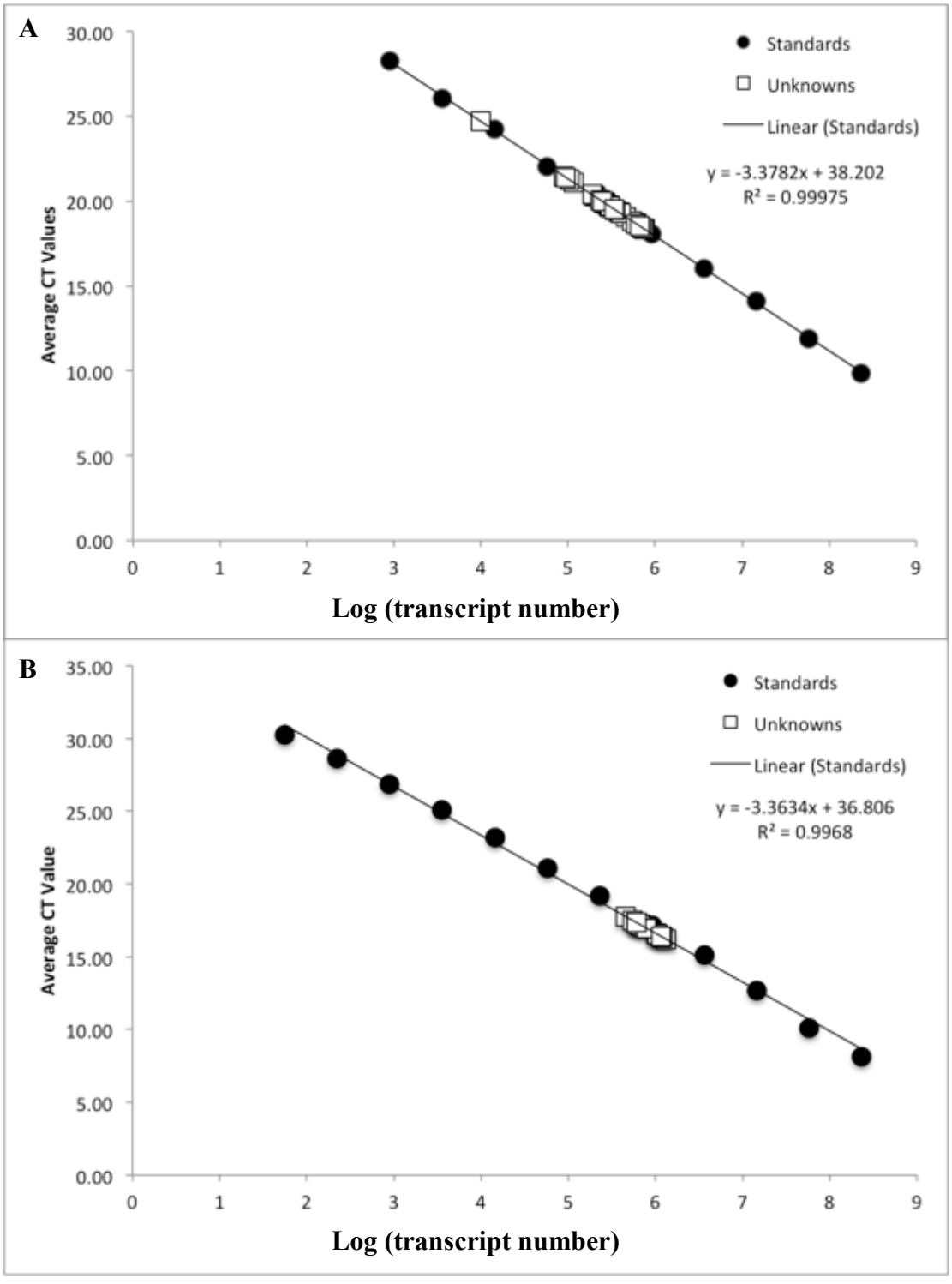


Figure C.1: A. Standard curve and unknowns for reproductive adults and juveniles. (Efficiency=97.7%) B. Standard curve and unknowns for non-reproductive adults. (Efficiency= 98.3%)

mPR α

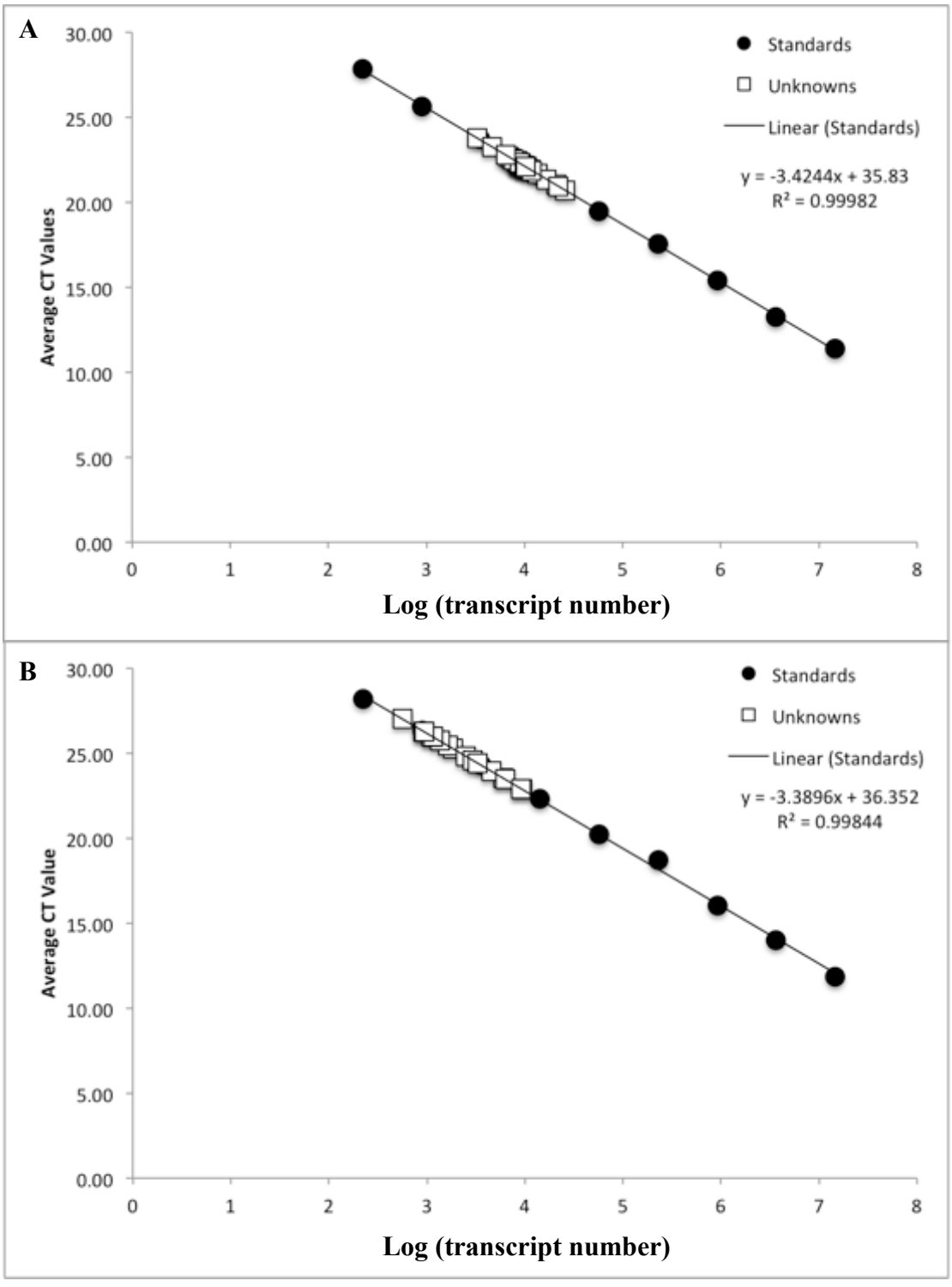


Figure C.2: A. Standard curve and unknowns for reproductive adults and juveniles. (Efficiency=95.9%) B. Standard curve and unknowns for non-reproductive adults. (Efficiency=97.3%)

mPR β

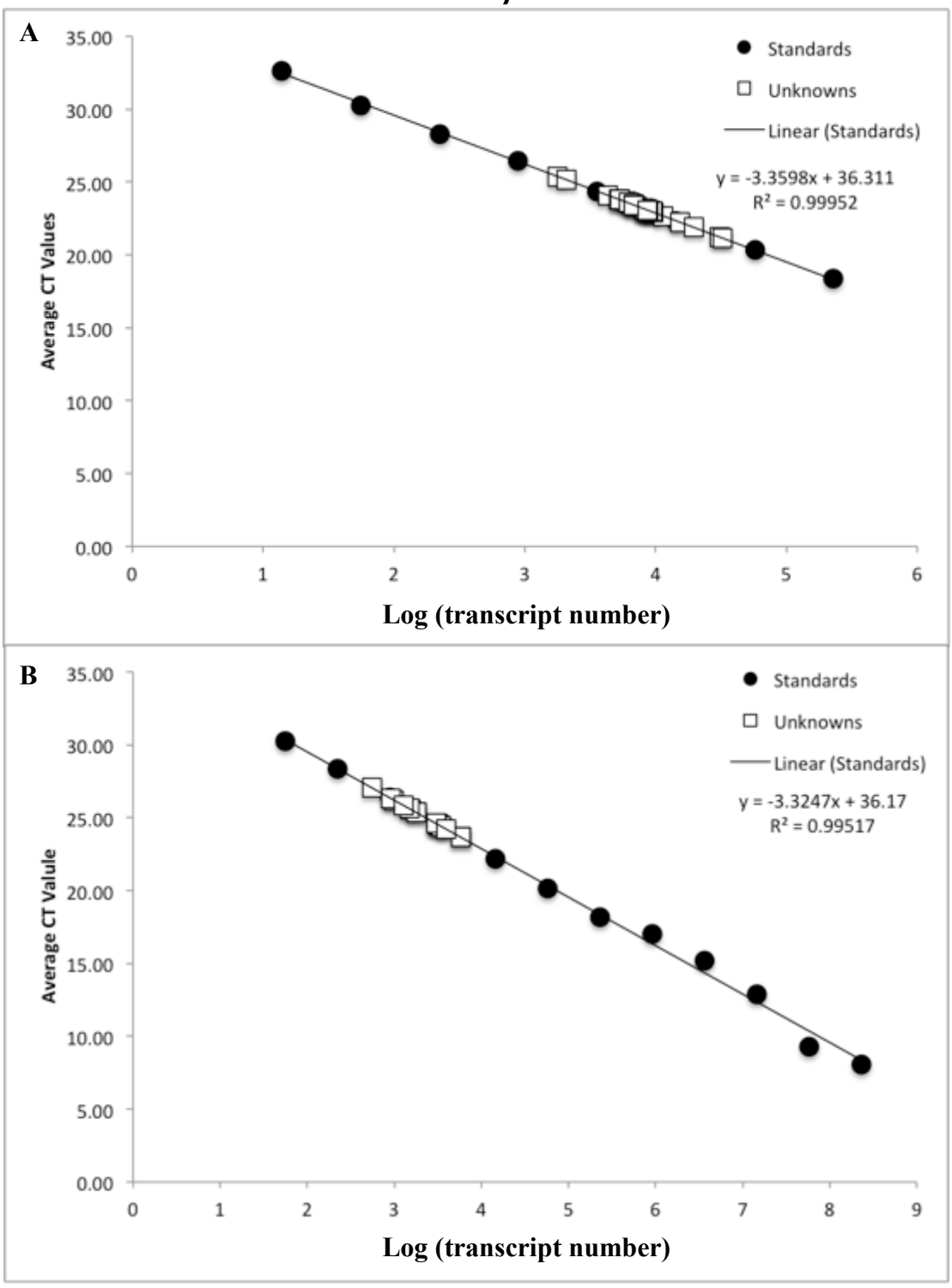


Figure C.3: A. Standard curve and unknowns for reproductive adults and juveniles. (Efficiency=98.4%) B. Standard curve and unknowns for non-reproductive adults. (Efficiency=99.9%)

mPR γ-1

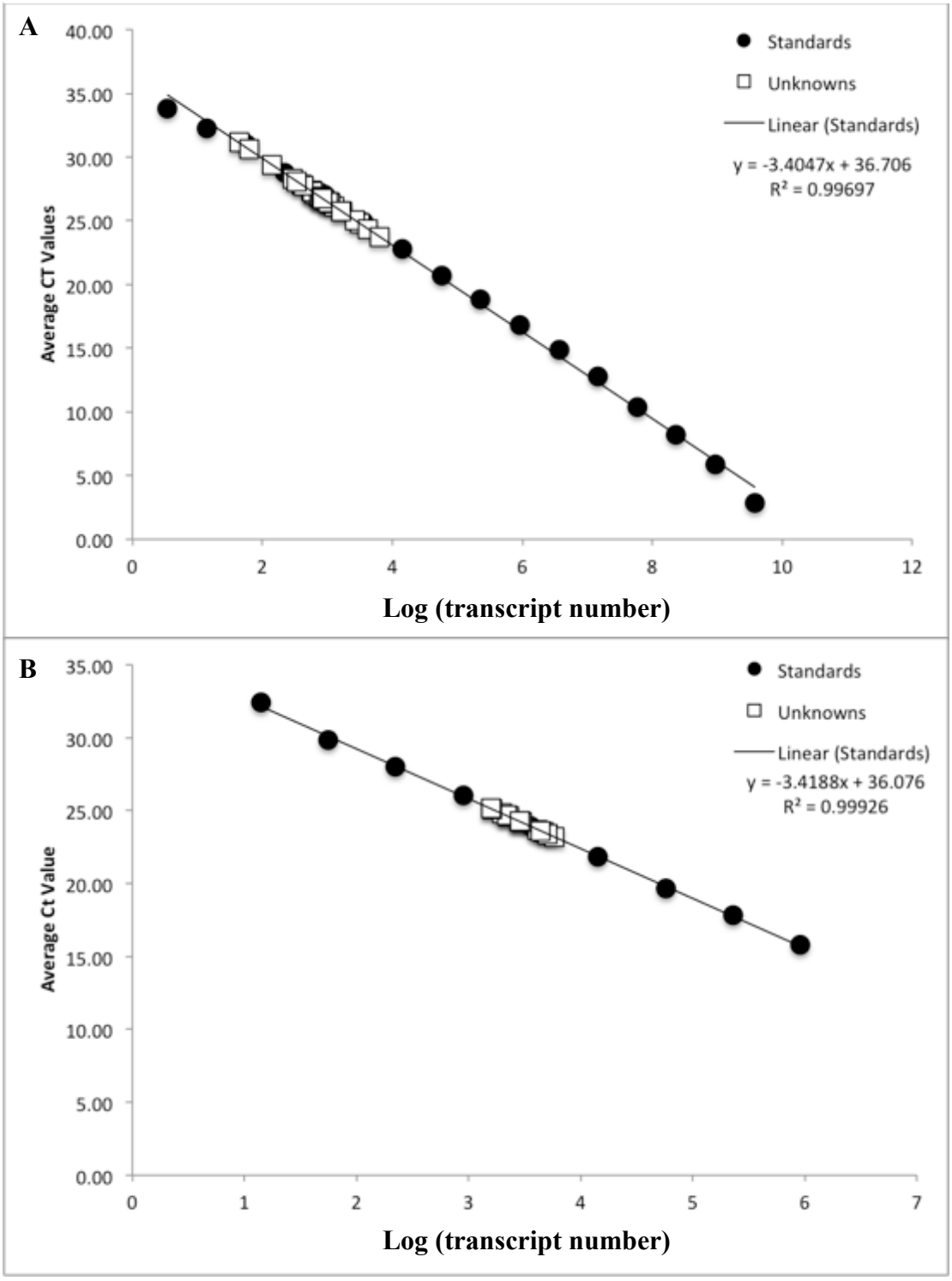


Figure C.4: A. Standard curve and unknowns for reproductive adults and juveniles. (Efficiency=96.7%) B. Standard curve and unknowns for non-reproductive adults. (Efficiency=96.1%)

mPR γ-2

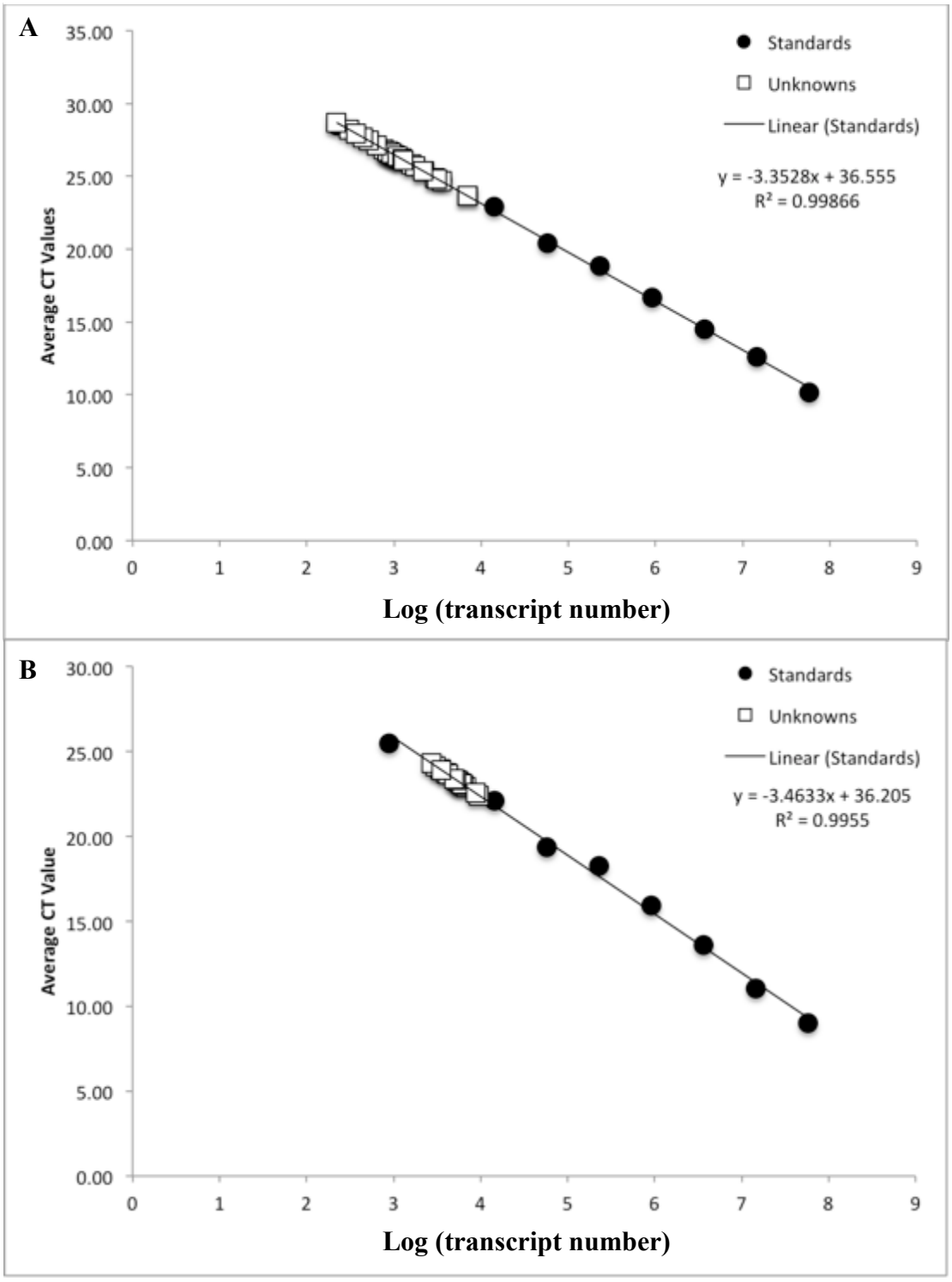


Figure C.5: A. Standard curve and unknowns for reproductive adults and juveniles. (Efficiency=98.7%) B. Standard curve and unknowns for non-reproductive adults. (Efficiency=94.4%)

nPR

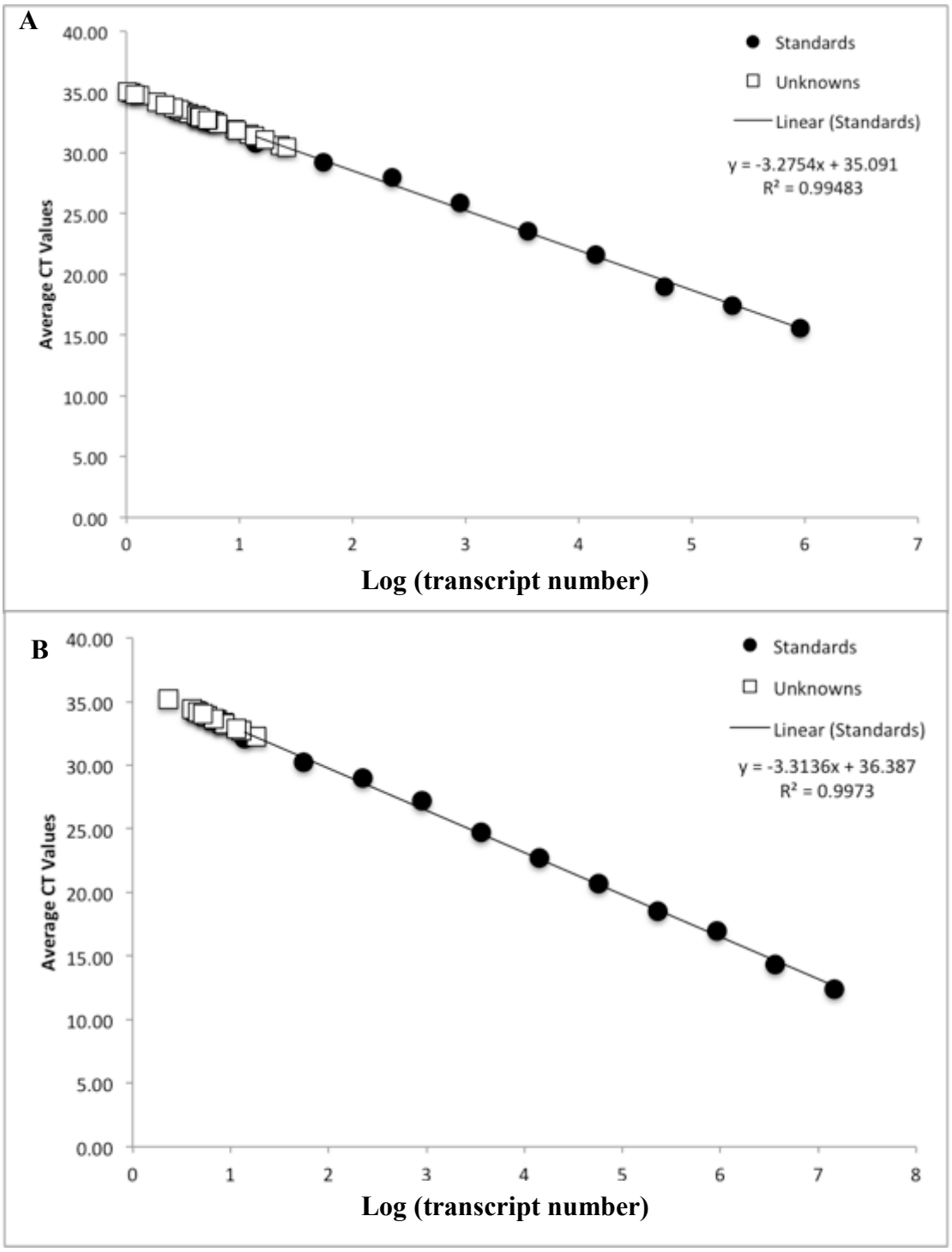


Figure C.6: A. Standard curve and unknowns for reproductive adults and juveniles. (Efficiency=102%) B. Standard curve and unknowns for non-reproductive adults. (Efficiency=100.4%)

Appendix D: Quantitative Real Time PCR Results for each Receptor, and Split by Sex

mPRα* and *mPRβ

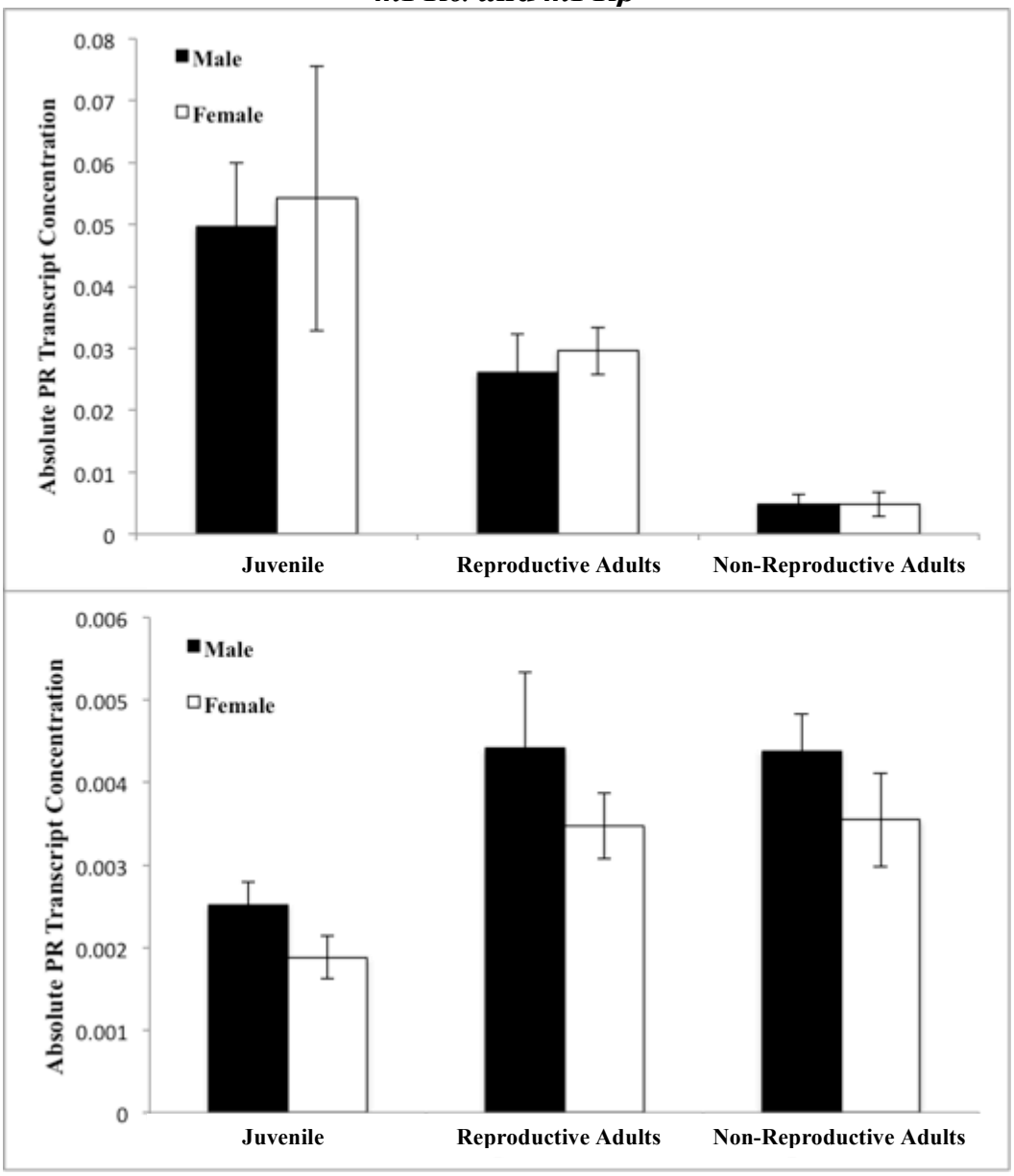


Figure D.1: Comparing *membrane progesterone receptor (mPR) α* (A) *mPRβ* (B) expression levels in the olfactory epithelium between sexes in each life stage group. Absolute quantification of PR expression was determined by the use of a standard curve comprised of a serial dilution of PR cDNA and SYBR Green QPCR. Values were then normalized by ribosomal protein L8 *(Rpl8*). For both genes and all three life stages, there was no significant difference between the sexes. n=8 (except reproductive male=7 and juvenile female=6).

mPRγ-1* and *mPRγ-2

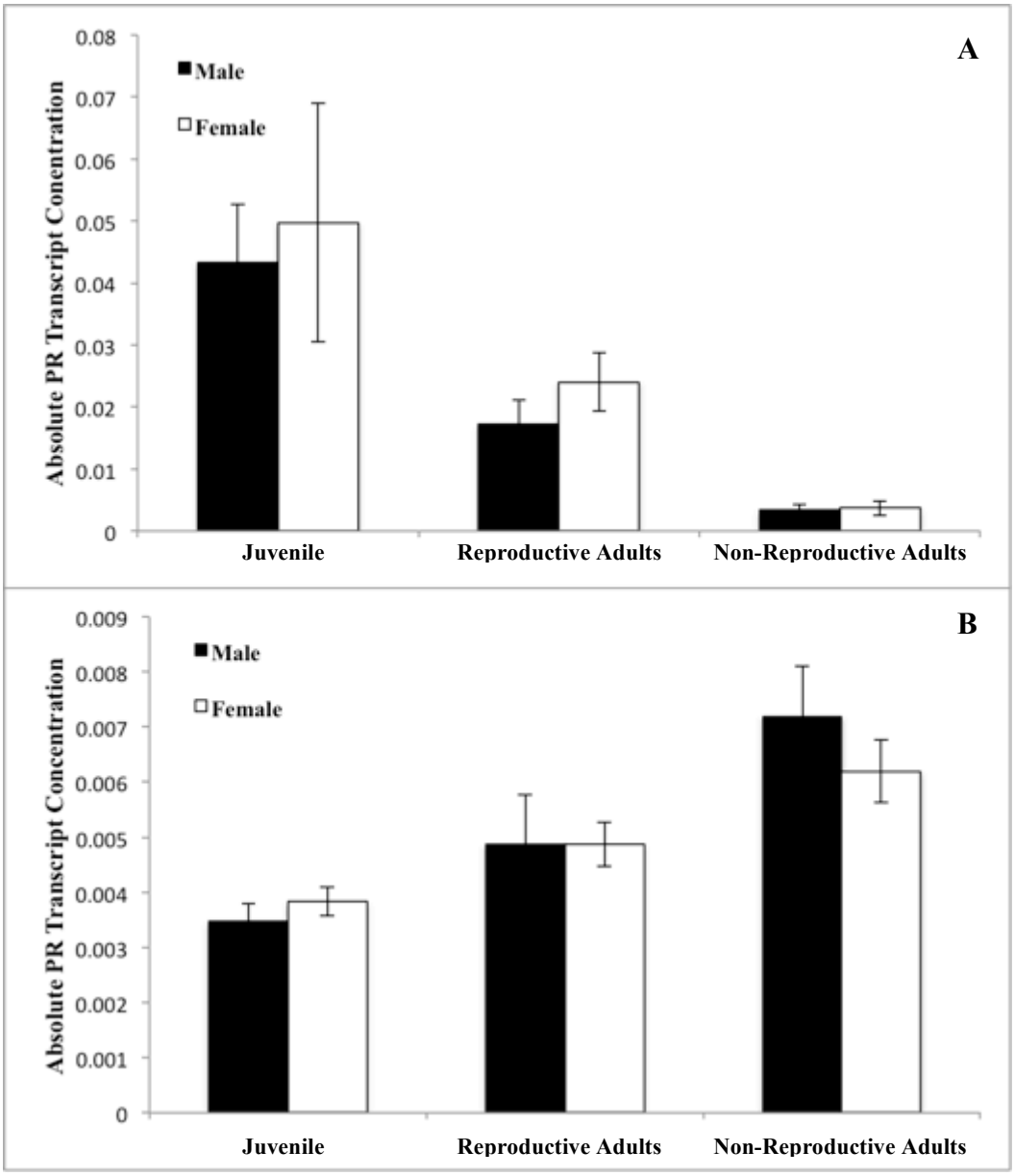


Figure D.2: Comparing *membrane progesterone receptor (mPR) γ-1* (A) *mPR γ-2* (B) expression levels in the olfactory epithelium between sexes in each life stage group. Absolute quantification of PR expression was determined by the use of a standard curve comprised of a serial dilution of PR cDNA and SYBR Green QPCR. Values were then normalized by ribosomal protein L8 *(Rpl8*). For both genes and all three life stages, there was no significant difference between the sexes. n=8 (except reproductive male=7 and juvenile female=6).

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