Abstract

LOCALIZATION AND CHANGES OF NUCLEAR PROGESTERONE RECEPTORS IN ZEBRAFISH OOCYTES AND ADJACENT FOLLICULAR CELLS

by

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The nuclear progesterone receptor (Pgr) is one of the major mediators for progestin signaling during oocyte ovulation in vertebrates. However, any roles Pgr may play in oocyte growth, and especially in the final oocyte maturation, are controversial. Due to the small size of the oocytes and difficulty in separation of encircled follicular cells from oocytes, little information is available on the location and changes of Pgr in vertebrates. We study the roles of the Pgr in the ovaries of zebrafish, which release eggs daily, by localizing and comparing the changes of Pgr in the oocytes and enclosed follicular layers at all developmental stages and from different times of the day or after treatment with various hormones. The *pgr* transcript and Pgr protein were expressed in oocytes and follicular cells of early developmental stages (stage I and II). In contrast, Pgr was expressed abundantly in the follicular cells surrounding late developmental stage oocytes (stage IV) but completely absent from stage IV oocytes. Furthermore, the most significant daily changes of *pgr* transcript were observed in the stage IV follicular cells with the highest level observed at 6 am prior to ovulation and the lowest level at 9 pm.

The levels of *pgr* transcript were upregulated by 17 alpha, 20 beta-dihydroxy-4-pregnen-3-one (DHP) in both stages of I-II and IV oocytes. Intriguingly, estrogen (E2) and human chorionic gonadotropins (hCG) significantly suppressed *pgr e*xpression in stage I-II oocytes, while enhancing *pgr* expression in stage IV oocytes. Presence of Pgr in both oocytes and follicular cells of early stages (stages I-II) suggest roles of Pgr in oocyte growth. Abundant expression and dramatic changes of Pgr in the follicular cells of late stage (stage IV) indicate roles of Pgr during oocyte ovulation. Absence and lack of changes of Pgr in the stage IV ooctyes do not support the role of Pgr in final oocyte maturation.

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INTRODUCTION

All steroids generate slow genomic signaling via altering transcripts in the nucleus, or rapid nongenomic signaling by fast modification of phosphorylation at the cell membrane or in the cytosol (Tokumoto *et al.*, 2005; Bhattacharya *et al.*, 2007; Zhu *et al.*, 2008). The nuclear steroid receptors, including progesterone receptors (PGRs), are major receptors for achieving genomic signaling with their ligands, and may also play roles in nongenomic signaling of the steroids (Mulac-Jericevic *et al.*, 2000; Conneely *et al.*, 2003). Interestingly, a membrane progestin receptor (mPR) belonging to a novel G-protein coupled receptor-like receptor family and with no structural similarity to the classical steroid receptors mediates rapid nongenomic actions of progestin signaling during oocyte maturation in zebrafish and *Xenopus* (Zhu *et al.*, 2003; Hanna *et al.*, 2006; Josefsberg Ben-Yehoshua *et al.*, 2007; Zhu *et al.*, 2008). Similarly, mPR also mediates rapid nongenomic actions of progestin in sheep, rats, mice, and human (Ashley *et al.*, 2006; Liu and Arbogast, 2009; Nutu *et al.*, 2009; Labombarda *et al.*, 2010). In contrast, any roles of PGR in mediating progestin-dependent nongenomic signaling, particularly during oocyte maturation, have been questioned (Bayaa *et al.*, 2000; Zhu *et al.*, 2003; Peluso, 2006).

Nuclear Progestin Receptors

The nuclear progestin receptor (PGR or nPR) belongs to the superfamily of nuclear receptors. Ligand-induced activation causes translocation of the PGR from the cytoplasm to the nucleus (and/or intranuclear change in localization) where they affect transcription by association with various target gene promoters (Edwards, 2005).

Progesterone responsiveness itself is primarily controlled by PGR expression (Merlino *et al.*, 2007).

Similar to other steroid receptors, the PGRs consist of a variable amino terminal or A/B region, a highly conserved DNA-binding or C region, a variable hinge region (D), a conserved ligand-binding or E region, a variable carboxy terminal region and several transcriptional activation functions (AF) (Mangelsdorf *et al.*, 1995). The hinge region contains nuclear localization and export sequences that allow the receptor to constantly shuttle between the nucleus and cytoplasm. Addition of progesterone causes a shift in equilibrium towards the nucleus (Edwards, 2005). The zebrafish progestin receptor of molecular weight 69KDa identified in our laboratory (Hanna *et al.*, 2010), shares a high degree of sequence homology in its ligand- and DNA-binding regions with PGRs from other species (Figure 1).

Multiple isoforms of PGR have been reported in several species of birds, mammals, fish, amphibians, and reptiles (Conneely *et al.*, 1989; Kastner *et al.*, 1990; Custodia-Lora and Callard, 2002; Liu *et al.*, 2005). The isoforms in birds and mammals arise from a single gene as a result of alternative splicing or alternative translation at two alternative AUG signals (Kraus *et al.*, 1993). In contrast, the two subtypes of PGR found in *Xenopus* and *A. japonica*, derive from distinct gene products (Ikeuchi *et al.*, 2001; Liu *et al.*, 2005). While the physiological functions of the multiple forms of the PGR are not yet fully understood, some evidence suggests that PGR-B is a strong activator of gene transcription while PGR-A is a ligand-dependent trans-repressor of PGR-B and other nuclear receptors (Edwards, 2005). The existence of two PGR isoforms that create functionally different receptors leads to speculation that the transcription of a single gene

from multiple promoters provides enhanced control over gene expression (Kastner *et al.*, 1990). However, since only one PGR has been identified in zebrafish (Chen *et al.*, 2010; Hanna *et al.*, 2010), control of gene expression in zebrafish likely reflects ancestral forms of regulation. Thus, knowledge of localization, daily changes in expression and hormonal regulation of PGR in zebrafish may provide unique knowledge.

The PGRs are major receptors for mediating genomic effects of progestins at the nucleus. Upon activation of a PGR by a progestin ligand, such as 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-DHP), a number of events transpire. First, the receptor dissociates from its protein chaperones. In the case of PGRs, these associated proteins include Hsp40, Hsp70, Hop, Hsp90 and p23 which maintain the PR in a conformational state conducive to ligand binding (Cintron and Toft, 2006). After dissociation, conformational changes and phosphorylation occur (Camacho-Arroyo *et al.*, 2007). As well, homo- and/or hetero-dimerization of the receptor complexes are formed with cofactors that modify chromatin organization, the nuclear receptor binds to the hormone response element (HRE) of its target gene where further phosphorylation occurs, and the basal machinery of transcription is recruited (Edwards, 2005; Camacho-Arroyo *et al.*, 2007). When no longer required, the phosphorylated PR is degraded by the ubiquitin-proteasome pathway (Camacho-Arroyo *et al.*, 2002).

Nongenomic actions of PGRs. The PGR may also mediate nongenomic actions of progestins. Both PGR and mPR are able to activate MAPK signaling in target cells (Boonyaratanakornkit *et al.*, 2001; Zhu *et al.*, 2003), one requirement for mediating final oocyte maturation. In *Xenopus*, over-expression of xPGR results in the acceleration of progesterone-induced maturation (Bayaa *et al.*, 2000) while the addition of anti-sense

xPGR oligonucleotides inhibits progesterone-induced maturation (Tian *et al.*, 2000). Similar results were also obtained in reptiles (Custodia-Lora and Callard, 2002).

However, evidence continues to build to discount a possible role for PGR in nongenomic actions. First, nongenomic actions require PGR to localize at the surface of the oocyte membrane (Thomas, 2008; Zhu *et al.*, 2008). Small quantities of xPGR-1 (~5%) have been shown to be present in oocyte membrane fractions by Western blot (Bagowski *et al.*, 2001). However, membrane localization of the *Xenopus* PGR has not been detected despite efforts by other groups (Bayaa *et al.*, 2000; Tian *et al.*, 2000). Also, nongenomic actions are characterized by rapid signaling. Measurements of dissociation/association rates for the seatrout mPR (<10 minutes) and PGR (1-1.5 hours) undermine PGR as key mediator of these actions (Zhu *et al.*, 2008). Finally, the PGR antagonist RU486 weakly induces oocyte maturation and fails to block progesterone in *Xenopus* (Edwards, 2005).

Genomic actions of PGRs during ovulation. The transcription of PGR and several other ovarian genes associated with ovulation are enhanced by GnRH and LH/hCG (Kang *et al.*, 2003; Motola *et al.*, 2006). This increase in PGR is important as steroid-dependent ovulation involves genomic mechanisms regulated by a nuclear receptor (Pinter and Thomas, 1995). Progesterone action occurring through genomic mediation is critical for ovulation in non-mammalian species, such as frogs and at least several teleost fishes (Goetz and Theofan, 1979; Schuetz and Lessman, 1982; Pinter and Thomas, 1995) Progesterone also plays important functions in ovulation in mammalian species. PGR-A is necessary to mediate the ovulatory response to progesterone in mice (Conneely *et al.*, 2002). In rats, progesterone is able to control the timing of ovulation by modulating the

expression of adenylate cyclase activity in granulosa cells (Graham and Clarke, 1997; Ko and Park-Sarge, 2000). The presence of PGRs in most follicular cell types provides support that the process of ovulation is regulated by progesterone (Graham and Clarke, 1997). Finally, PGR knockout (PGRKO) and PGR- A knockout (PGRAKO) mice are unable to ovulate despite forming oocytes that develop normally and are fertilizable (Lydon *et al.*, 1995; Conneely *et al.*, 2002; Kim *et al.*, 2009).

PGR isoforms. As mentioned, the PGR isoforms appear to have distinct physiological roles. The ratio of PGR-A to PGR-B can vary significantly according to cell type and physiological conditions, even though the two PGR isoforms are co-expressed in tissues (Edwards, 2005) and this differential expression contributes to cell-specific responses (Turgeon and Waring, 2006). It is interesting that differential expression of PGR isoforms in the follicular layer of cells versus the oocyte has been noted in *Xenopus* (Liu *et al.*, 2005), but the relative expression of PGR-A to PGR-B in oocytes has not been described in any fish species.

Since zebrafish has only one identified *pgr*, regulation may occur differently than in species containing multiple isoforms. For instance, in mammary tissue of ovariectomized mice PGR-A is extensively co-localized with estrogen receptor (ERα) and its' expression is increased in the presence of estrogen suggesting direct regulation of PGR-A by ERα (Aupperlee and Haslam, 2007). On the contrary, PGR-B is not co-localized with ERα and induction of PGR-B expression coincided with decreased PGR-A levels and increased progesterone levels (Aupperlee and Haslam, 2007). Determining the effect of estrogen and progesterone in a species containing only one form of PGR, such

as the zebrafish, may provide insight into the evolutionary costs or benefits of a multiple isoform system.

Furthermore, PGR-A and PGR-B exhibit distinct trans-activation properties when expressed individually with PGR-A able to act as a strong trans-dominant suppressor of PGR-B (Giangrande *et al.*, 2000). Perhaps, regulation of zebrafish PGR will require mediation through slightly different biochemical pathways than other species. However, differential interactions with co-regulators, despite identical ligand-binding domains, and the ability of different ligands to induce distinct conformational changes in each PGR isoform affect transcriptional regulation (Conneely *et al.*, 2002) so this provides one path of differential regulation by the same isoform.

Cellular expression and localization of nuclear progesterone receptor (PGR) in ovaries. It is known that progestin plays an invaluable role in reproductive function and PGRs are found in many reproductive tissues, including the ovaries and the testes. In *Xenopus*, the PGR subtypes are known as xPGR-1(~80 kDa) and xPGR-2 (~70 kDa) (Liu *et al.*, 2005). In stage VI oocytes, xPGR-1 was identified in the follicular cells while xPGR-2 was expressed mainly in the nucleus of oocytes (~74% of total), but also in the cytoplasm of oocytes (~21%) (Liu *et al.*, 2005). In turtles, PGR-A has been detected in the ovary and kidney, PGR-B in the gastrointestinal tract and both isoforms in the heart and spleen (Custodia-Lora and Callard, 2002). The tissue distributions of two PGR subtypes in *A. japonica* were clearly different from one another. *ePGR2* mRNA was detected in gill, spleen, testis, brain, and ovary while *ePGR1* mRNA was observed in kidney, spleen, liver, and testis (Ikeuchi *et al.*, 2002). In other non-mammalian species, PGRs have been detected mainly in oviduct of chicken (Conneely *et al.*, 1989) and ovary

of seatrout (Pinter and Thomas, 1995). In mammals studied to date, PGRs have been identified mainly in uterus and ovary with expression in the ovary restricted primarily to granulose cells of follicles (Graham and Clarke, 1997; Robker *et al.*, 2009). However, no study has been done on the localization and comparison of PGR in follicular cells and oocytes in vertebrates with the exception of *Xenopus* (Bagowski *et al.*, 2001).

Nuclear PGRs are generally associated with genomic actions that occur inside of the nucleus in cells. However, it has been proposed that rapid nongenomic actions, such as those seen in oocyte maturation, may be mediated through association of these classical receptors with the cell membrane (Bagowski *et al.*, 2001). For instance, post-translational modification of the receptor could impart the ability to associate with the plasma membrane which would make sense if nuclear PGRs have a role in nongenomic signaling (Edwards, 2005). Also, interaction of PGRs with trafficking proteins could result in translocation to the oocyte membrane (Edwards, 2005).

Studies of PGRs in ovaries have generally been limited to immunohistochemical and *in situ* localization because the use of biochemical and molecular analyses of PGR changes are challenging and unreliable due to difficultly in separation of oocytes from the follicular layer of cells. Previously, chemical removal of the follicles has not proven reliable and the complete removal of the follicular layer has been difficult to verify. Additionally, the accepted means of removing the follicular layer, through mechanical removal by forceps, has proven time-consuming and has limited study to oocytes in later stages of development. Also, damage to the oocyte membrane during this process could result in yolk proteins leaking out of the oocyte and being collected along with the follicular layer which could interfere with accurate quantification and localization of the

PGR transcripts. Furthermore, damage caused to the oocytes during this process could lead to degradation of the transcripts and protein, thus inhibiting detection of the PGRs. The protocols for chemical and mechanical removal of the follicular cells were developed in the current study to ensure this novel procedure produced results consistent with the proven, though arduous, procedure.

Determining the cellular localization and changes of PGRs in two cellular compartments, i.e. oocytes and follicular cells, is important so that the signaling pathways and molecules mediated by the PGRs can be correctly identified. Specifically, cellular localization and changes provide evidence for roles of PGRs in genomic (i.e. oocyte growth, ovulation) and/or nongenomic actions (i.e. oocyte maturation).

Changes of the PGR during oocyte growth, maturation and ovulation. The expression of PGR transcript and protein has been shown to change during different phases of the reproductive cycle in humans and chickens (Graham and Clarke, 1997). This demonstrates the importance of regulation of PGRs for proper reproductive function. In species with two PGR isoforms, such as humans, chickens, and mice, the levels of both isoforms may change independently of one another altering their relative abundance and providing support for the hypothesis that PGR expression levels are physiologically relevant (Punyadeera *et al.*, 2003; Gava *et al.*, 2004; Camacho-Arroyo *et al.*, 2007). Additionally, chicken PGRs' expression levels differ among progestin target tissues and according to different hormonal and environmental conditions such as those experienced during avian sexual maturity and the seasons of the year (Camacho-Arroyo *et al.*, 2007). During the turtle reproductive cycle, the level of PGR-A protein remains constant in the ovary, but PGR-B protein levels go through several cycles of increase and decrease

(Custodia-Lora and Callard, 2002). In humans, the A:B ratio in lower segment myometrium changes from approximately 0.5:1 in preterm specimens to around 1:1 at term and more than doubles to 2.5:1 during labor, mostly due to increases in levels of PGR-A (Merlino *et al.*, 2007). Variances in the relative expression of the isoforms at different points in the reproductive cycle has also been demonstrated in the Rhesus Macaque and in mice (Bethea and Widmann, 1998; Gava *et al.*, 2004).

Several studies have postulated that variance in the expression of the PGR isoforms, particularly with regards to their relative abundance to one another, plays an important role in the processes of oocyte growth, maturation and ovulation. In humans, variance in the abundance of PGR isoforms is easily explained as different promoters in the *PGR* gene exhibit differential regulation (Bethea and Widmann, 1998). According to Merlino et al. (2007), the extent of PGR-A repression of PGR-B in most cells is directly related to the relative abundance of each. Thus far we have identified only one *pgr* in zebrafish, but it is likely that this single *pgr* gene plays a similar physiological role to PGRs from other species. Therefore it is important to determine whether the zebrafish PGR undergoes similar environmental- and hormone- dependent changes in its expression levels and the mechanism of its regulation during the zebrafish daily reproductive cycle.

Hormonal regulation of the PGRs. As PGR is an important mediator of progestin function, it is crucial to understand its regulation by hormones. Hormones involved in the direct and indirect regulation of PGRs include follicle-stimulating hormone (FSH), leutinizing hormone (LH), estrogens and progestins (Beato *et al.*, 1995; Jalabert, 2005; Drummond, 2006).

The gonadotropins FSH and LH are key factors in ovarian and follicle development. The expression of *FSHR* mRNA occurred during the recruitment and growth of follicles while *LHR* mRNA expression increased during final oocyte maturation and ovulation (Kwok et al., 2005). Additionally, *in vivo* experiments with several teleost species have linked estradiol to increased expression of LH mRNA in the pituitary gland (Aroua *et al.*, 2007). Teleost maturation-inducing hormone (MIH), which is necessary for oocyte maturation, is synthesized in the follicles under the control of LH (Jalabert, 2005). In zebrafish and other teleost species, the MIH is 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-DHP) and it is a potent inducer of Pgr-mediated transcriptional activity (Todo *et al.*, 2000; Hanna *et al.*, 2010). LH-dependent release of MIH results in activation of PGRs . The induction of PGR-A and PGR-B in preovulatory follicles in response to LH has also been demonstrated in mice (Natraj and Richards, 1993) and it is understood that the ability of LH to stimulate transient expression of PGR mRNA and proteins is necessary as follicle rupture is LH-dependent (Conneely *et al.*, 2002). Thus, LH plays an important role in regulating oocyte maturation and ovulation. As such, its effects on PGR expression need to be studied.

In several mammalian and bird tissues, expression of PGR is positively controlled by estrogen and negatively impacted by progesterone in most target tissues (Graham and Clarke, 1997). Estrogen is a known inducer of PGR-A and PGR-B transcriptional activity (Punyadeera *et al.*, 2003). Boney-Montoya *et al.* (2010) have identified a number of estrogen response elements (EREs) in *PGR* gene that allow for dynamic regulation of the *PGR* expression. In a study on ovariectomized mice, the addition of estrogen up-regulated PGR-A and enhanced progesterone-induced increase of PGR-B while

progesterone down-regulated PGR-A (Aupperlee and Haslam, 2007). *In vitro* studies have demonstrated the ability of progestin treatment to effect levels of PGRs. In *Xenopus,* treatment of isolated follicle cells with progesterone results in the degradation of xPGR-1 (Liu *et al.*, 2005).

Elucidation of the possible roles of FSH, LH, estradiol, progesterone and its derivatives (i.e. 17,20β-DHP), a maturation-inducing steroid in zebrafish, in the hormonal regulation of PGR during the processes of oocyte growth, maturation and ovulation in the zebrafish will be accomplished by the addition of these hormones under *in vitro* conditions.

Difficulties in study of PGRs. The inability to collect completely denuded stage I-III oocytes for detection of PGR transcripts and proteins by PCR and Western blotting, respectively, has limited study in the field to mature oocytes. To my knowledge, this study would be the first to specifically localize PGR in early and late developmental stages of oocyte at various times throughout the zebrafish daily reproductive cycle. The elucidation of the molecular mechanisms that promote oocyte growth, maturation and ovulation has potential applications for improving fertility and for *in vitro* culture systems for oocytes from domestic animals and humans (Thomas and Vanderhyden, 2006).

MATERIALS AND METHODS

Defolliculation of Oocytes

Zebrafish, obtained from a local pet store, were sacrificed by decapitation and ovaries were extracted and immediately placed in 5 mL 50% L15 media in 6cm x 1.5cm Petri dishes. Samples were gently pipetted up-down several times to separate individual oocytes for collection according to the stage of development. Oocytes were separated as early stage (stage I-II Ø=100-200 μm) or late vitellogenic stage oocytes (stage IV Ø=500-625 μm) determined by a micrometer under a stereomicroscope (Nikon) and transferred into separate petri dishes for defolliculation treatment. Prior to defolliculation treatment, total oocytes including follicular cells and enclosed oocytes from each stage of development were collected, placed in TRIzol reagent (Life technologies), homogenized, and frozen immediately for total RNA extraction at a later time.

50% L15 media was removed from the samples and replaced with 5mL 0.001% collagenase (Sigma-Aldrich) in Ca^{2+}-free Ringer's solution (116mM NaCl, 2.9mM KCl, 5mM HEPES, pH 7.2). All samples were shaken at 60 RPM for one hour and fifteen minutes at room temperature. Additionally, the samples were gently pipetted for three minutes during each ten minute period. The late stage vitellogenic oocytes were pipetted with a 9" disposable Pasteur pipette (Fisher Scientific) (Ø=1000 μm tip opening), while the stage I-II sample was pipetted using a pipette with a smaller (Ø=300 μm) opening. The smaller pipette was made by heating a pipette tip over a Bunsen burner and stretching the glass to create a narrower tip. During the one hour and fifteen minute time period, free stage IV follicular layer was verified under a stereomicroscope and collected by pipette after each pipetting period.

After the one hour and fifteen minute time period, the follicular cells were carefully removed from Ringer's solution using a pipette while viewing underneath a stereomicroscope to ensure that no oocytes were collected. The follicular cells were placed in separate 1.5 mL microcentrifuge tubes and centrifuged for 1 minute at 13000 RPM (Eppendorf Centrifuge 5415D) to collect the follicle cells. The supernatant was then discarded.

The stage I-II and stage IV oocytes collected above were washed twice in Ringer's solution to remove any free follicle cells. Next, they were stained by the addition of 1 µL of propidium iodide (20 µg/mL, Sigma) in 3 mL of Ringer's solution into the petri dishes for a period of 15 minutes. The samples were washed twice in Ringer's solution and the oocytes were examined under the fluorescent inverted microscope (Axiovert 200M, Zeiss) to verify the removal of the follicular layer of cells from the oocytes. Stage I-II and stage IV completely denuded oocytes (100% removal of follicular layer) were selected off and placed into new 1.5 mL microcentrifuge tubes.

Total RNA Isolation

The sonicator tip was cleaned in 10 M NaOH for 15 minutes and rinsing with distilled water prior to tissue homogenization. Additionally, the sonicator tip was rinsed with distilled water and sequentially dipped in ten sterilized deionized water bathes between homogenization of each sample. The Ringer's solution was removed from the collected samples and 500 µL of TRIzol Reagent was added. Samples were immediately homogenized using a sonicator (Sonic Dismembrator Model 100, Fisher Scientific) and purified following the manufacturer's instructions. The concentration of total RNA extracted from each sample was quantified using a biophotometer (Eppendorf).

RT-PCR

First-strand cDNA was synthesized utilizing the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. The same quantity of RNA was run on a gel to check for degradation and to ensure that the quantity of RNA used to synthesize cDNA was equal. The 10 µL reaction consisted of 4 µL total RNA (0.5 µg), 0.5 µL 10 mM dNTP, 0.5 µL oligo dT primer (0.5g/L), 1 µL10X RT Buffer, 2 µL 25 mM $MgCl_2$, 1 µL 0.1 M DTT, 0.5 µL RNase Out Recombinant Phase RNase Inhibitor and 0.5 µL (25 units) Superscript II Reverse Transcriptase. After the reaction was terminated and inactivation of RNase Out, 0.5 µL (1 unit) RNase H was added to each sample.

Gene specific primers for PGR were utilized to amplify cDNA from oocytes and follicular cells. Each 10 µL PCR reaction mixture consisted of 4.15 µL deionized water, 2 µL Green Taq Polymerase 5X Buffer (Promega), 0.6 µL 25 mM $MgCl_2$, 0.2 µL 10 mM dNTP, 1 µL each of 100 nM gene-specific forward and reverse primer, 0.05 µL GoTaq Flexi DNA Polymerase (Promega) and 1 µL cDNA. The reactions were carried out in a Mastercycler gradient machine (Eppendorf) under the following PCR conditions: 95 ^{o}C for 2 minutes, 30 cycles at 95 ^{o}C for 30 seconds, 56 ^{o}C for 30 seconds, 72 ^{o}C for 1 minute followed by a 10 minute elongation time at 72 ^{o}C.

For RT-PCR, each sample was run with primers for PGR and β-actin (Figure 5). β-actin was used to confirm that cDNA synthesis was successful and that equal amounts of cDNA were used for the RT-PCR reaction.

in situ Hybridization

Preparation of Probes for *in situ*

PGR cDNAs were linearized with *Spe*I or *Sph*I to generate templates for synthesizing sense or antisense probes, respectively. Sense and antisense digoxigenin-labeled probes were generated using T7 or SP6 RNA polymerases by *in vitro* transcription according to the instructions of the manufacturer (Roche Applied Science, Indianapolis, IN).

Sectioning for *in situ* Hybridization

Whole ovaries were removed and fixed in 4% paraformaldehyde–PBS overnight. The tissues were dehydrated, embedded in paraffin, cut into 5–8 μm thick sections and serially mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA).

in situ Hybridization

Previous *in situ* protocol were followed (Zhu *et al.*, 2007). In brief, sections were processed, re-hydrated, post-fixed with 4% paraformaldehyde for 20 minutes, and then treated with 200mM HCl for 10 minutes followed by 15 minutes digestion of 10 μg/mL protein kinase K. Hybridization was conducted with a digoxigenin-labeled probe (10 ng/probe/slide) in 60 μl buffer (50% deionized formamide/2x SSC/10% Dextran sulfate/0.01% yeast RNA/0.02% SDS) overnight at 55°C. Sections were washed in high-stringency buffer (1x SSC/50% formamide) at 55°C and 1x SSC at room temperature. Sections were then equilibrated with TBS (50mM Tris, pH 7.5, 150mM NaCl) and blocked with 1x Roche blocking solution containing 10% fetal bovine serum and 1% sheep serum in maleic buffer (100mM maleic acid, 150mM NaCl, pH 7.5) for 1 hour at room temperature. To develop the color, sections were

incubated for 1 hour with anti-digoxigenin–AP (Roche Applied Science), diluted 1/2000 with 1x Roche blocking solution containing 10% FCS at room temperature. Sections were washed with TBS, equilibrated in AP buffer (pH 9.5) and incubated with NBT/BCIP substrate (Roche Applied Science) for a few minutes to a few hours at 37 °C in a dark humid chamber. The development of the substrate was examined under a microscope and reactions were stopped by washing with TE buffer (10mM Tris, pH 7.5, 1mM EDTA) twice, then fixed with 4% paraformaldehyde, washed twice with TE buffer, and mounted with 90% glycerol in PBS.

Quantitative Real-Time PCR (qRT-PCR)

Preparation of Standard Curve

Plasmid DNAs containing full length *pgr* cDNA inserts were quantified using a DNA/RNA calculator followed by serial dilution of the plasmids from 10^3 to 10^9 times. The concentrations of undiluted plasmids were 0.100 μM. A growth curve based upon the amount of fluorescence detected at each cycle number was created using SmartCycler software (Cepheid, Sunnyvale, CA). A standard curve was created by using the critical threshold (Ct) value, determined from this growth curve, and the log concentrations of the serially diluted standards. The cDNA standards were measured three separate times to ensure reproducibility.

qRT-PCR Conditions

The *pgr* concentrations in the samples were determined using quantitative real-time PCR (qRT-PCR) with SYBR green dye (Stragtagene, La Jolla, CA) in a Cepheid SmartCycler MX4000 (Cepheid, Sunnyvale, CA). The PCR mixture consisted of 1x Cepheid enhancer additive (1mM Tris, pH 8.0; 0.1 mg/mL bovine serum albumin,

non-acetylated; 0.75M trahalose; 1% tween-20), 10 µL MasterMix (2.5x, Eppendorf), 500 nM forward and reverse primers and 0.25x SYBR green dye.

The PCR conditions were 95 ^{o}C for 2 minutes followed by 30-40 repeats at 95 ^{o}C for 15 seconds, 60 ^{o}C for 30 seconds and 72 ^{o}C for 30 seconds.

Calculation of *pgr* Concentrations in the Samples

Ct values for each sample were determined by the BRAND software. The initial concentrations of *pgr* were interpolated from the standard curve and converted to fmol/µg total RNA.

Immunological Analysis

Production of Polyclonal Antibody for Pgr

Rabbits generated polyclonal antibodies against four synthetic peptides corresponding to the N-terminal (aa 74-87, aa 100-112) or C-terminal (aa 466-479, aa 519-532) part of zebrafish Pgr. The peptides were designed to have low/no homology to other zebrafish proteins and a strong immuno-response was ensured by linking the peptides to keyhole limpet hemocyanin (KLH) to increase the size of the antigen. A N-terminal antibody (aa 100-112) was confirmed to be specific and subsequently used for Western blotting and immunohistochemical (IHC) localization.

Fixation and Embedding

Ovary samples were collected at various time points throughout the zebrafish's reproductive cycle and immediately placed in 10% buffered formalin upon dissection from the zebrafish. Samples were left overnight at room temperature (~21 ^{o}C). After overnight fixation, the samples were dehydrated in a series of ethanol washes (70%, 95%, 100%), twice for 30 minutes each. This was followed

by washes in xylene (15 minutes), xylene:methyl salicytate (1:1- 30 minutes), methyl salicytate (30 minutes) and three paraffin washes at 60 ^{o}C for 1 hour each.

Tissues were embedded in paraffin and placed in the refrigerator (5 ^{o}C). Samples were cut into 8 μm sections using a microtome (Reichert-Jung 2030, Leica, Germany). The sections were positioned on labeled microscope slides with albumen fixative and Milli-Q water. Slides were dried on a hot plate (42 ^{o}C) overnight.

Immunostaining

Slide samples had paraffin removed and were rehydrated by the following series of washes for 5 minutes each: xylene, 100% ethanol, 95% ethanol, 70% ethanol and 1X PBS. Next, sections were incubated in 0.3% hydrogen peroxide (H_2O_2) in 100% methanol for 30 minutes followed by 3 washes in 1X PBS for 5 minutes each. Slides were placed on slide plates and covered with diluted normal serum (Vectastain ABC Kit, Vector Laboratories) for 30 minutes. Excess serum was washed off and slides were placed on slide plate with primary antibody serum (1:250 dilution). The slide plate was placed in a humidity chamber and left overnight at 4 ^{o}C. The next morning the slides were carefully rinsed with distilled water and washed four times in 1X PBS for 3 minutes per wash. Then, the sections were incubated in diluted biotinylated antibody solution (Vectastain ABC Kit, Vector Laboratories) for 30 minutes and washed 3 times in 1X PBS for 5 minutes per wash. Sections were placed back on slide plate with Vectastain ABC reagent (Vectastain ABC Kit, Vector Laboratories) for 30 minutes and then washed 4 times with 1X PBS for 5 minutes per wash. The peroxidase substrate solution (100 mg diaminobenzidine, 64 μL 30% H_2O_2 and 200 mL Tris Buffer [50 mM Tris, pH 7.2])

was prepared immediately prior to slide development. Following development, sections were placed in 1X PBS for at least 5 minutes, then dehydrated through a series of ethanol washes (70%, 95%, 100%) and xylene for 5 minutes each before mounting with a coverslip using Permount.

Western Blotting

Total protein was collected from ovary samples placed in 1X SDS in 1.5 mL microcentrifuge tubes immediately upon removal from zebrafish. The sonicator probe was cleaned with 70% ethanol and rinsed with distilled water prior to homogenization of sample. After homogenization samples were boiled for 10 minutes and placed on ice.

Statistical Analysis

Statistical analysis was performed using SPSS 14.0 (SPSS Inc., Chicago, IL). Descriptive statistics were used to determine means and standard deviations of log concentrations of *pgr* mRNA at various time points and after hormone treatments. Analyses of variance (ANOVAs) were conducted to determine the significance of mean differences between various treatments. Paired t-tests were performed to compare the effects of various hormone treated oocytes to a control. A significant *p* value was set at $\alpha = .05$.

RESULTS

Separation of Follicular Cells and Oocytes from Early (stage I-II) and Late (stage IV) Vitellogenic Oocytes

After collagenase treatment of oocytes, separation of the follicular layer from oocytes was verified with propidium iodide staining (Figure 3). Propidium iodide staining and collection procedures had no negative impact on the integrity of the RNA used for cDNA synthesis and amplification of *pgr* from stage I-II denuded oocytes (Figure 4).

Almost all oocytes had their follicular layers removed via the combination of chemical and mechanical treatment. While the standard 9" disposable glass pipette worked well for removing the follicular layers from late stage vitellogenic oocytes, a smaller pipette size (0.3 mm) was necessary to facilitate the removal of the follicular layer from early stage vitellogenic oocytes.

Expression of *pgr* in Follicular Cells and Denuded Oocytes Analyzed by RT-PCR

The *pgr* transcripts were detected in follicular cells and denuded oocytes of early developmental samples (stage I-II) by RT-PCR (Figure 5). In late (stage IV) vitellogenic oocyte samples, *pgr* transcripts were detected in follicular cells only (Figure 5). These results correspond with our *in situ* localization and immunocytochemical analyses described below.

Expression of *pgr* in Follicular Cells and Denuded Oocytes Analyzed by *in situ* Hybridization

The *pgr* transcripts were detected in the follicular layer of late stage oocytes by *in situ* hybridization using a zebrafish *pgr* antisense probe. The *pgr* transcript was restricted to the follicular layers with no detectable signaling within the late stage oocytes (Figure

6). Interestingly, the *pgr* transcript was also observed in the cytoplasm of early stage oocytes (stages I and II) (Figure 6).

Expression of Pgr in Follicular Cells and Denuded Oocytes Analyzed by Western Blotting and Immunocytochemistry

The Pgr protein (69 kDa) was detected by Western probing in early stage vitellogenic oocyte samples (not shown) and late stage vitellogenic follicular samples using a zebrafish Pgr antibody (Figure 7).

Specific immunocytochemical staining of Pgr was observed only in the nuclei of follicular cells surrounding late stage vitellogenic oocytes (Figure 8). This corresponded to results seen in the Western blotting, *in situ*, and RT-PCR where the Pgr protein and *pgr* transcripts was only detected in the follicular cells of late stage vitellogenic oocytes. Thus, it seems likely that the 69 kDa band representing the zebrafish Pgr is localized specifically to the nucleus of the stage IV follicle cells.

Daily Changes of pgr in Follicular Cells and Denuded Oocytes

Changes in the daily expression of *pgr* mRNA was determined using quantitative real-time PCR (qRT-PCR) (Figure 10). In late stage oocytes, *pgr* transcripts expression decreased slightly, but not significantly, at the 1pm and 9pm time points versus the 6am time point. Expression in early stage oocytes was significantly lower at 6am versus 1pm [$F(2,9)=8.91$, $p=.014$]. In late stage denuded oocytes, expression appeared much lower than in late stage oocytes or late stage follicle cells. Expression of *pgr* transcripts was significantly lower at 6am versus 1pm [$F(2,9)=8.44$, $p=.015$]. Significant differences in *pgr* expression were found between all time points in the follicular cells. Expression

decreased significantly from 6am to 1pm [$F(2,9)=58.58$, $p=.012$], 1pm to 9pm [$F(2,9)=58.58$, $p<.001$], and 9pm to 6am [$F(2,9)=58.58$, $p<.001$].

Daily changes in Pgr expression were assessed using Western blotting. However, the quality of the results did not allow quantification.

Hormonal Effects on pgr Expression

Changes in *pgr* expression in early and late stage oocytes (with intact follicular layers) were determined after 1.5 hours and 3 hours of *in vitro* hormonal treatment (Figure 11). No significant differences in expression were seen in late stage oocytes between the control and treatment with 17,20β-dihydroxy-4-pregnen-3-one (DHP), estrogen (E2), progesterone (P4), or human chorionic gonadotropin (hCG) after 1.5 hours. After 3 hours, *pgr* mRNA expression appeared to increase with DHP, E2, and hCG treatments, but the change was not significant.

No significant differences in expression were seen in early stage oocytes between the control and treatment with 17,20β-dihydroxy-4-pregnen-3-one (DHP), progesterone (P4), or human chorionic gonadotropin (hCG) after 1.5 hours. However, E2 significantly decreased *pgr* mRNA expression after 1.5 hours [$t(2)=7.17$, $p=.019$]. After 3 hours, *pgr* mRNA expression appeared to increase with DHP and P4 treatments, but the change was not significant.

Using Western blot analysis, changes in *pgr* mRNA expression in early and late stage oocytes were determined after 1.5 hours and 3 hours of hormonal treatment (Figure 12). In late stage oocytes, P4 significantly up-regulated *pgr* expression after 1.5 hours [$t(2)=-12.529$, $p=.006$]. Treatment with hCG also resulted in a significant increase in *pgr* expression after 1.5 hours [$t(2)=-5.119$, $p=.036$]. Expression levels increased with E2

treatment as well but not significantly. No significant differences were seen after 3 hours of treatment.

Expression of Pgr increased after 1.5 hours of treatment with P4 but not significantly. No significant differences were seen after 3 hours of treatment though Pgr expression appeared to increase with E2 and P4.

DISCUSSION

Presence of pgr in follicular cells and denuded oocytes

Unlike many other animal models, a single locus encoding only one full-length zebrafish *pgr* has been identified (Hanna *et al.*, 2010). In the current study, *pgr* mRNAs and Pgr protein were detected in early developmental and late vitellogenic oocyte samples by RT-PCR, *in situ* hybridization, Western blotting and immunocytochemical analyses. These results are expected as Pgrs are known to be present in the ovaries and total oocyte samples included both the follicular layer and the oocyte itself (Chen *et al.*, 2010; Hanna *et al.*, 2010). In early stage oocyte samples, *pgr* transcripts and Pgr protein were detected in the follicular cells and denuded oocyte. These results correspond to those of Hanna *et. al* (2006) who detected Pgr protein in the follicular layer and oocyte nucleus of stage I-II oocytes using immunohistochemistry. Follicles from late vitellogenic (stage IV) oocytes contained abundant *pgr* mRNA and Pgr protein. Relatively small amounts of *pgr* transcripts, but not protein, were rarely detected in denuded-late vitellogenic oocytes by PCR. However, these amounts were much less than those found in early or late stage vitellogenic follicular cells and not supported by our *in situ* and immunohistochemical results. Detection of any *pgr* transcripts in denuded oocytes is likely due to the contamination of trace amounts of follicle cells in the denuded oocyte samples and excessive cycles of PCR.

It is interesting to note that the amount of *pgr* transcript increased from early stage to late stage follicles. Possibly this involves a mechanism preparing the oocytes for ovulation. In mice, the presence of PGR-A in the granulose cells of preovulatory follicles has been shown to be necessary for successful ovulation (Gava *et al.*, 2004). In fact,

studies of mouse models have demonstrated that PGR-A plays the prominent physiological role in the uterus and ovary with PGR-B being more important in the mammary gland (Edwards, 2005). Thus, the detection of *pgr* transcripts in stage IV follicular cells may be explained by the requirement of Pgr in immature oocytes for ovulation. Additionally, in bovines, PGR-A plays a role in mediating apoptosis of preovulatory follicles (Quirk *et al.*, 2004). Up-regulation of PGR prior to ovulation may facilitate survival of embryos by increasing their resistance to apoptosis. Friberg et al. (2010) have identified novel, early gene targets of PGR that may be involved in the mediation of apoptosis.

Daily changes in pgr expression

Expression of *pgr* mRNA in early stage oocytes was significantly lower at 1pm versus 6am and 9pm. It is probable that *pgr* mRNA expression is high at 6am as previtellogenic and early stage oocytes begin preparing for and beginning vitellogenesis. For instance, a period of intense RNA synthesis characterizes the initial stages of primary oocyte growth (Tyler and Sumpter, 1996; Lubzens *et al.*, 2010). However, while this has received considerable study in amphibians (Wallace and Selman, 1990), there is less information available about RNA synthesis activities in the oocytes of teleosts (Tyler and Sumpter, 1996).

Overall, *pgr* expression appeared much lower in denuded-late stage oocytes compare to folliclular cells of same developmental stage, and detection of *pgr* transcripts did not occur until >30 cycles of PCR. This finding agrees with our *in situ* and immunohistochemical results. Additionally, as pgr is not expected to play a direct role in oocyte maturation (Hanna and Zhu, 2009, Hanna et al., 2010), a process that is mediated

through a G-coupled receptor at the surface of the oocyte, having very little, if any, *pgr* mRNA expression in denuded oocytes makes physiological sense. In late stage oocytes, *pgr* mRNA expression decreased slightly, but not significantly, at the 1pm and 9pm time points versus the 6am time point. In contrast, significant differences in *pgr* expression were found between all time points in the follicle cells collected from late stage oocytes. In particular, the highest levels of transcripts were detected at 6am which is just prior to oocyte ovulation and fertilization. The difference in results between oocyte and follicle cell samples may be explained by equal amounts of total RNA being used for PCR. Since late stage oocytes contain vast amounts of maternal mRNAs (Tyler and Sumpter, 1996; Lubzens *et al.*, 2010), *pgr* mRNA from oocyte samples was greatly diluted relative to follicle cell samples. As published from this study, expression of *pgr* mRNA was localized to the oocyte and follicular layer of early stage oocytes and exclusively to the follicular layer of cells in late stage oocytes by *in situ* hybridization (Hanna *et al.*, 2010). Since Pgr plays a role in oocyte growth and ovulation, processes mediated by the follicular layer of cells (Lubzens *et al.*, 2010), the daily changes in expression observed in follicular cells concur with observations from other studies. Transcription ceases in many vertebrate oocytes prior to fertilization (Moore *et al.*, 1974; LaMarca *et al.*, 1975; Tan *et al.*, 2009). In mice, genes remain silenced during meiotic maturation and even after fertilization (Abe *et al.*, 2010). If transcription is silenced similarly in the zebrafish, it would make sense that levels of *pgr* transcripts, due to its importance in ovulation, are expressed in high levels just prior to this silencing. Nagahama and Yamashita (2008) postulate that maturation inducing steroid (MIS) upregulation of Pgr may be a key step in the ovulatory process of teleosts. Further studies could provide evidence for this

hypothesis by determining whether the amounts of *pgr* transcripts remain stable and Pgr protein expression increases after maturation and fertilization. In summary, since progesterone is an important mediator of female reproductive activity (Conneely *et al.*, 2003) and many of its actions are controlled by PGR (Conneely *et al.*, 2002), better understanding of the daily changes in expression levels of these receptors provides valuable insight into reproductive functioning.

Hormonal regulation of pgr

Expression of Pgr protein significantly increased after 1.5 hours of P4 treatment. This result is somewhat counterintuitive as increased levels of P4 normally serve as a stimulus to decrease Pgr expression (Liu *et al.*, 2005). In isolated *Xenopus* follicles and rat gonadotropes progesterone treatment leads to Pgr degradation, presumably through a proteasome-mediated pathway (Turgeon and Waring, 2000; Liu *et al.*, 2005) while another study demonstrated little effect of P4 on Pgr expression (Xiao and Goff, 1999) However, P4 is able to induce expression of PGR-B in the mouse mammary gland,, but only after prolonged treatment (Aupperlee and Haslam, 2007). It is important to note that these findings occurred in species with multiple isoforms of PGRs while the zebrafish has only one form. Thus, it is possible that the mechanisms regulating zebrafish Pgr expression may be slightly different.

In late stage oocytes after 3 hours of treatment with E2, *pgr* mRNA expression levels were greater than controls. After 1.5 hours, Pgr expression also appeared elevated by E2, though not significantly. E2 is known to increase expression of Pgr in rat gonadotropes and the mouse mammary gland (Turgeon and Waring, 2000; Aupperlee and Haslam, 2007). In rat preovulatory follicles and eel pituitary cells, E2 may also act

indirectly by causing an increase in LH which serves to stimulate PGR expression (Natraj and Richards, 1993; Aroua *et al.*, 2007).

Not surprisingly, human chorionic gonadotropin (hCG), a combination of FSH and LH also elevated expression of *pgr* mRNA after 3 hours. Additionally, hCG significantly up-regulated *pgr* expression after 1.5 hours. LH/hCG is known to play a role in oocyte maturation and ovulation (Motola *et al.*, 2006). These pituitary gonadotropins indirectly mediate these processes by controlling follicular production of steroidal mediators, such as E2 and DHP (Nagahama *et al.*, 1995). In rats, studies have shown that ovulatory action of LH involves mediation by ovarian autocrine/paracrine EGF-like factors (Park *et al.*, 2004; Ashkenazi *et al.*, 2005). Mouse studies have demonstrated that increases in LH levels induce expression of a number of genes in ovarian follicular cells including progesterone receptor (*pgr*) (Lydon *et al.*, 1995). Expression of downstream target genes controlled by PGR then function in an autocrine/paracrine manner to control the ovulatory process (Kim *et al.*, 2009) The ability of these hormones to regulate Pgr expression provides evidence for the importance of their actions during oocyte growth, maturation and ovulation in the zebrafish model.

No significant differences in expression were seen in early stage oocytes between the control and treatment with 17,20β-dihydroxy-4-pregnen-3-one (DHP), progesterone (P4), or human chorionic gonadotropin (hCG) after 1.5 hours. However, E2 significantly decreased pgr mRNA expression after 1.5 hours but no effect was seen at 3 hours. In contrast, E2 increases *pgr* gene expression in MCF-7 breast cancer cells in which eight estrogen response elements (EREs) have been identified within the progesterone receptor (*pgr*) gene (Boney-Montoya *et al.*, 2010). Also, E2 has been shown to upregulate Pgr

levels in various tissues in humans, macaques and mice as well (Bethea and Widmann, 1998; Petz *et al.*, 2004; Aupperlee and Haslam, 2007). Combined with the lack of effect seen after 3 hours of E2 treatment, it seems likely that decreased expression of *pgr* transcripts at 1.5 hours is likely a transient response. Additionally, Pgr protein levels were approximately 3-fold greater than controls after 3 hours of E2 treatment. In MCF-7 cells, maximal levels of PGR mRNA and protein are not reached until after 72 hours of E2 treatment (Nardulli *et al.*, 1988; Read *et al.*, 1988; Wei *et al.*, 1988).

After 3 hours, *pgr* mRNA expression appeared to increase with DHP and P4 treatments. Though the change was not significant, both of these hormones are known activators of Pgr-mediated transcriptional activity (Hanna *et al.*, 2010). Aupperlee and Haslam (2007) demonstrated upregulation of PR-B in mouse mammary glands after prolonged P4 treatment. However, studies in cultured bovine endometrial cells and rat gonadotropes have demonstrated that progesterone has a negative effect on PGR expression (Xiao and Goff, 1999; Turgeon and Waring, 2000).

Conclusion

In conclusion, Pgr was localized in the cytoplasm and follicular cells of early stage oocytes (stages I-II), but only in the follicular cells of stage IV oocytes. Expression levels of *pgr* mRNA varied throughout the daily reproductive cycle of zebrafish. Changes in expression occurred in early (stage I-II) oocytes and follicular cells but only in follicular cells of late (stage IV) oocytes. These findings are consistent with the known genomic role of PGRs in oocyte growth and ovulation, but not maturation. Finally, expression of *pgr* transcripts and Pgr protein were affected by several steroid hormones involved in

reproduction. Together, these results provide support for the roles of PGRs in oocyte growth and ovulation, but not final oocyte maturation.

Interestingly, only one PGR has been identified in zebrafish compared to multiple isoforms identified in other fish, amphibian, bird, and mammalian species. This unique trait makes the study of zebrafish Pgr less complex and further study may provide insight into the evolutionary benefits of multiple isoforms. Additionally, the relationship, if any, between nuclear and membrane progesterone receptors remains to be elucidated to determine whether PGR plays an indirect role in the nongenomic process of oocyte maturation.

Table 1 Primers used for RT-PCR amplification of zebrafish nuclear progestin receptor (*pgr).*

	Primer (5' to 3')
pgr	F1: GGATCACCTTTCTGCGCT R4: GACAACCAGAAGCCTCATC

Table 2 Primers used for quantitative real-time PCR amplification of zebrafish nuclear progestin receptor (*pgr*).

	Primer (5' to 3')
pgr	F2: ACAGACAGCATACACCGC R2: TCCACAGGTCAGAACTCC

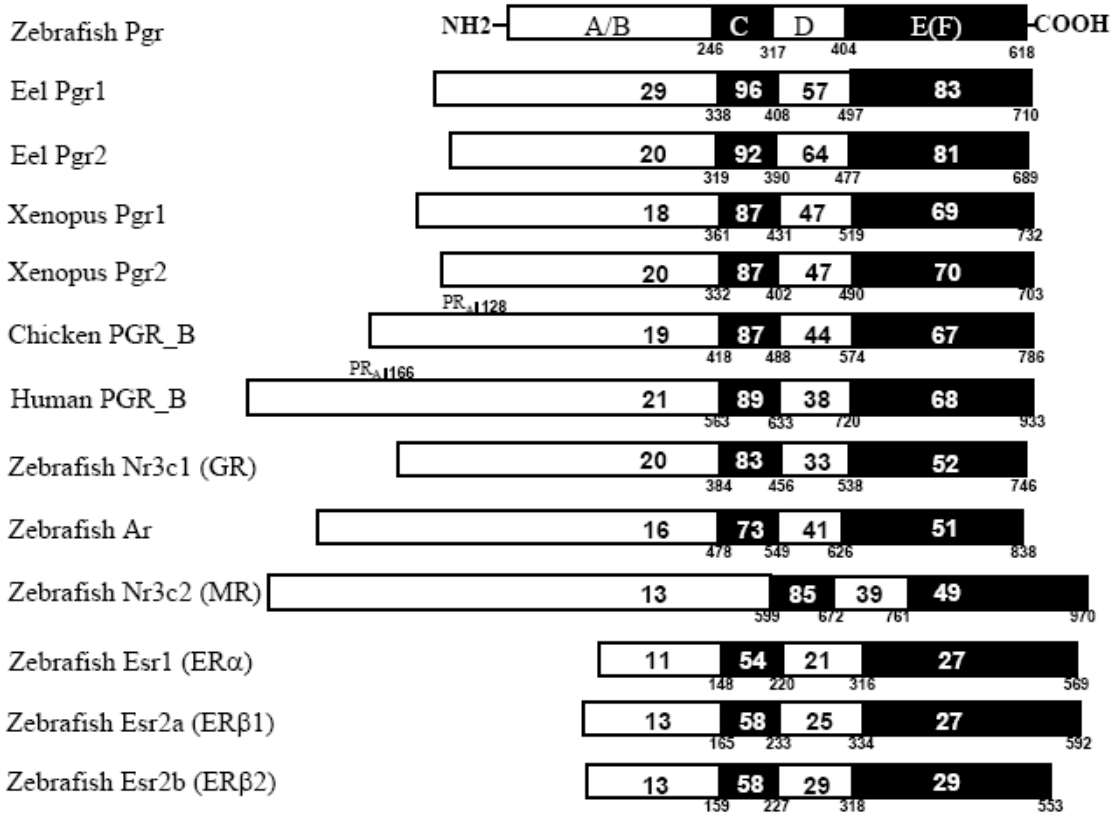

Figure 1. Comparison of amino acid sequence identities of domains of zebrafish PGR and domains of PGRs from other vertebrates; also domains of other zebrafish nuclear receptors. The numbers below the line represent the amino acid position with respect to the translation starting site. (Hanna *et. al*, 2010)

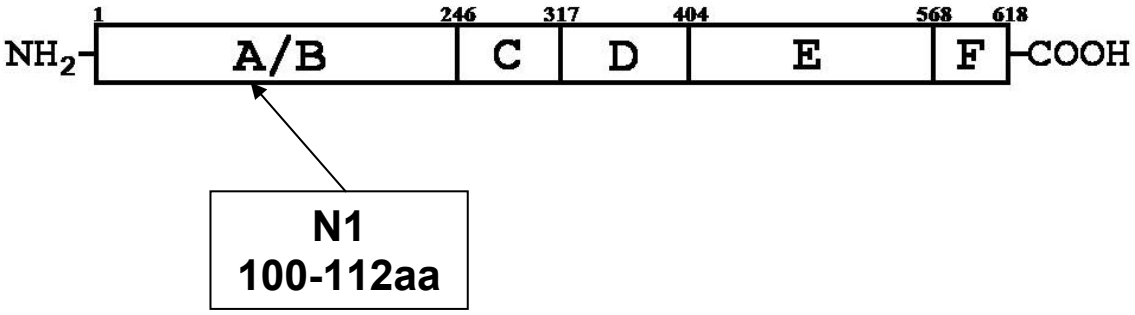

Figure 2. Schematic showing the localization of amino acids that were used for synthesizing a peptide in order to generate an antibody against zebrafish nuclear progestin receptor (Pgr). The numbers in and above the boxes represent the amino acid positions with respect to the translation starting site.

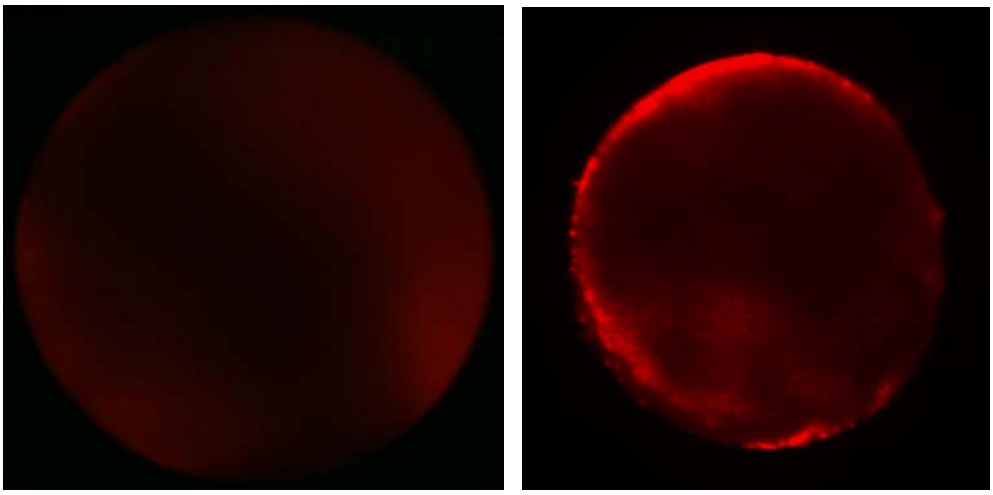

Figure 3. Staining of oocytes with propidium iodide for verification of follicular cell removal; follicular cells present (right) versus denuded occytes (left). (Hanna *et al.*, 2010)

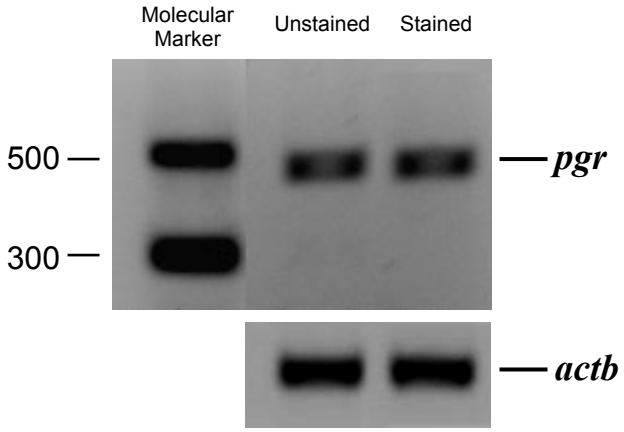

Figure 4. RT-PCR analysis of zebrafish nuclear progestin receptor (*pgr*) and β-actin (*actb*) in stage I-II denuded oocytes either unstained (left) or stained with propidium iodide (right).

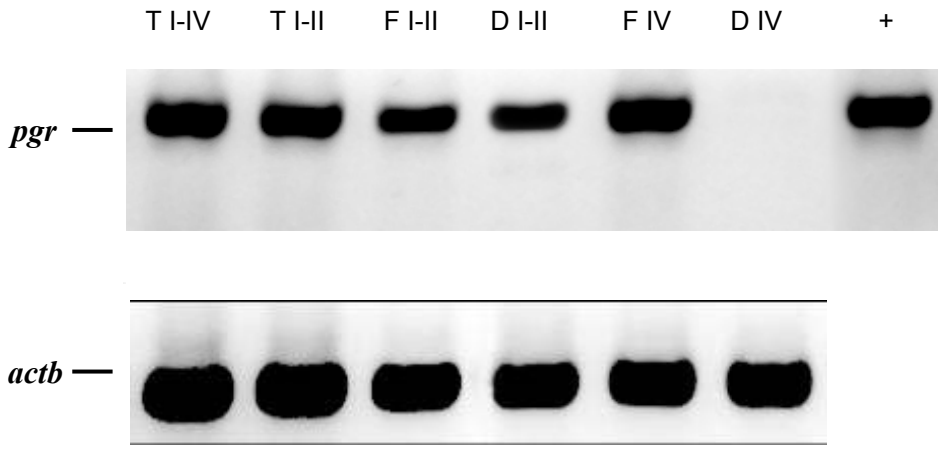

Figure 5. RT-PCR analysis for localization of zebrafish nuclear progestin receptor (*pgr*) in early developmental (stage I-II) and late vitellogenic oocytes (stage IV) and follicular cells. I-II: stage I-II, IV: stage IV, T: total oocyte samples include both follicular cells and enclosed oocytes, F : Follicular cells only, D : denuded oocytes only, + : pgr plasmid used as a positive control in the PCR amplification. β-actin (actb) used as loading control.

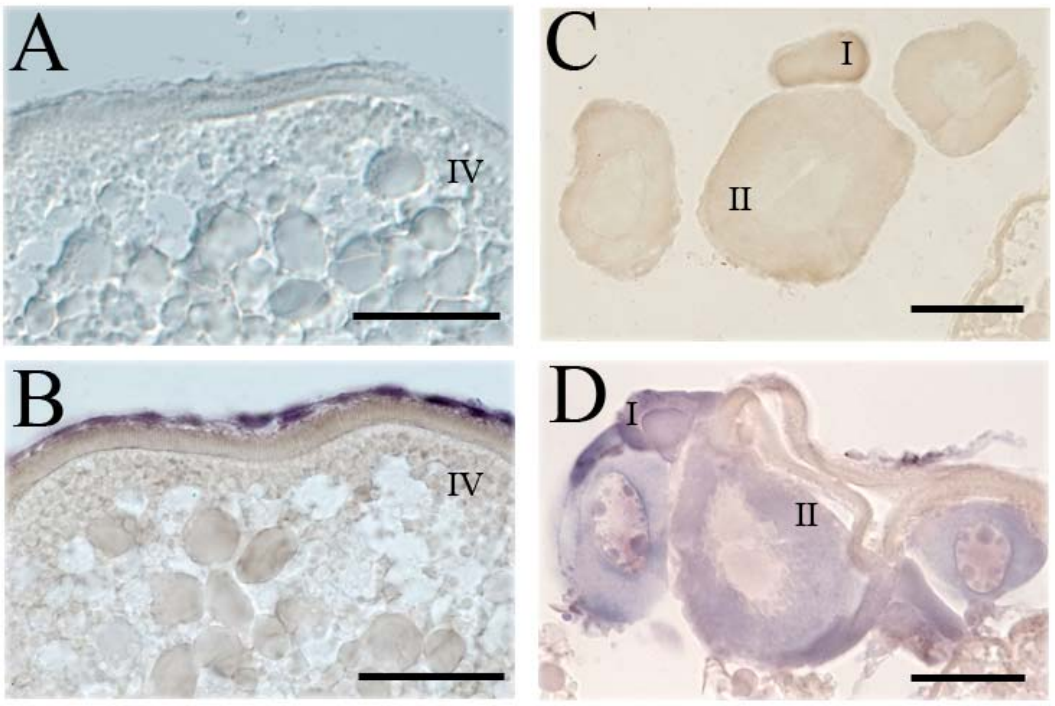

Figure 6. Expression of zebrafish nuclear progestin receptor (*pgr*) transcripts in early developmental (stage I-II) and late vitellogenic oocytes (stage IV) analyzed by *in situ.* Picture A and C: *pgr* sense probe; Picture B and D: *pgr* antisense probe. Scale bar: 50 μm.

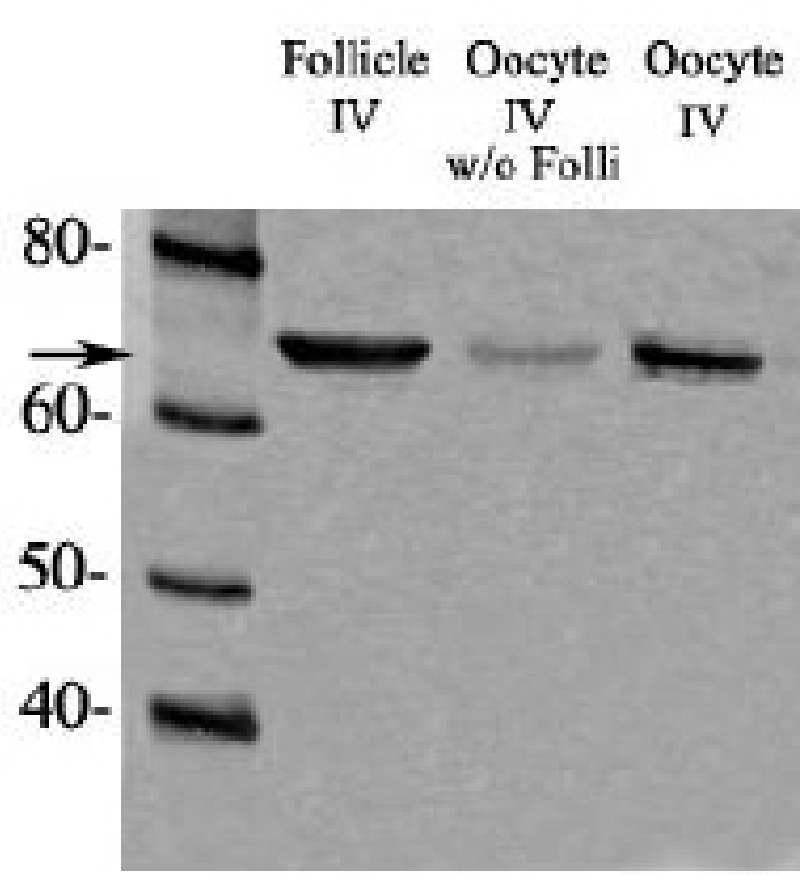

Figure 7: Western analysis of the expression of zebrafish nuclear progestin receptor protein (Pgr) in follicular cell layers without oocytes (Follicle IV), denuded stage IV oocytes (w/o folli: without follicular cells), and stage IV follicular cell enclosed oocytes. Analyses of antibody specificity with (+) or without (-) pre-absorption of antibodies with the synthetic peptides used for antibody generation (right). Arrow indicates expected size of Pgr. (Hanna *et al*., 2010)

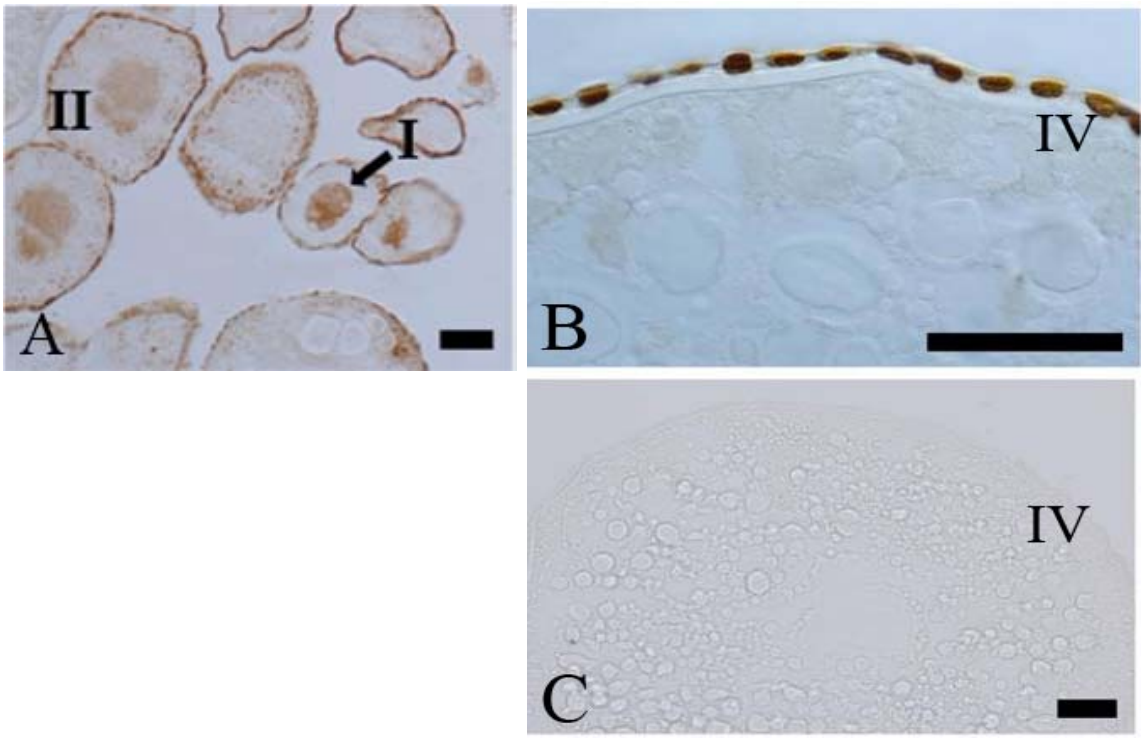

Figure 8. Expression of zebrafish nuclear progestin receptor(Pgr) protein in early developmental (stage I-II) and late vitellogenic oocytes (stage IV) analyzed by immunocytochemistry using a specific zebrafish Pgr antibody developed against A/B region of Pgr. (A) Positive immunostaining in the nuclei and follicular cells of stage I-II oocytes, arrow indicates nucleus of stage I oocyte; (B) Positive immunostaining in the follicular cells of stage IV oocytes; (C) Immunostaining of stage IV oocyte with pre-immune serum. Bar=50 μm

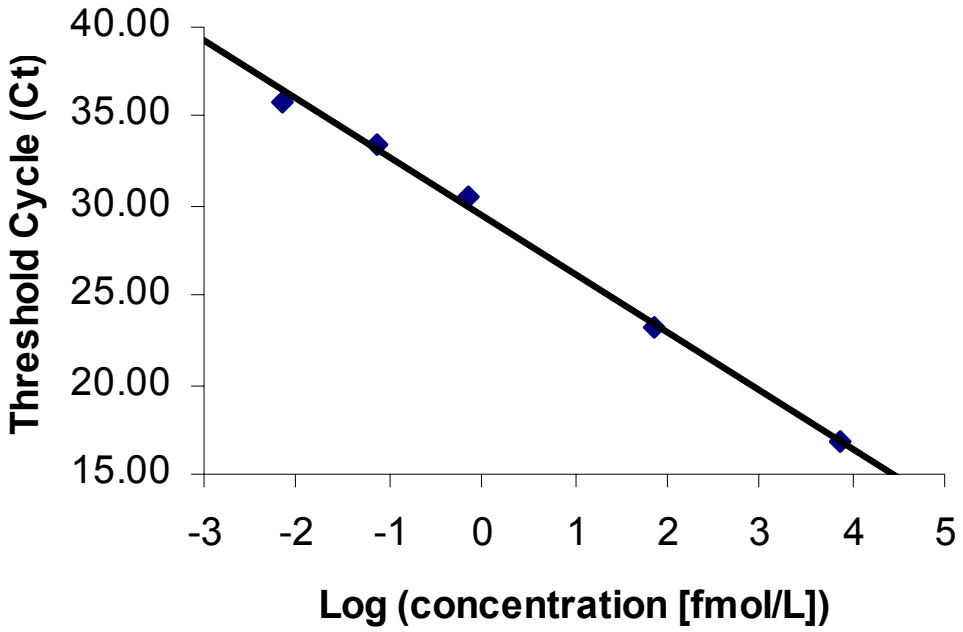

Figure 9. A representative standard curve for qRT-PCR analyses of zebrafish nuclear progestin receptor (*pgr*). The x-axis and y-axis represent Log Concentration (fmol/L) and Threshold Cycle (Ct) of a serially diluted (10^4 to 10^{10} fold) plasmid DNA containing full-length zebrafish *pgr* cDNA inserts.

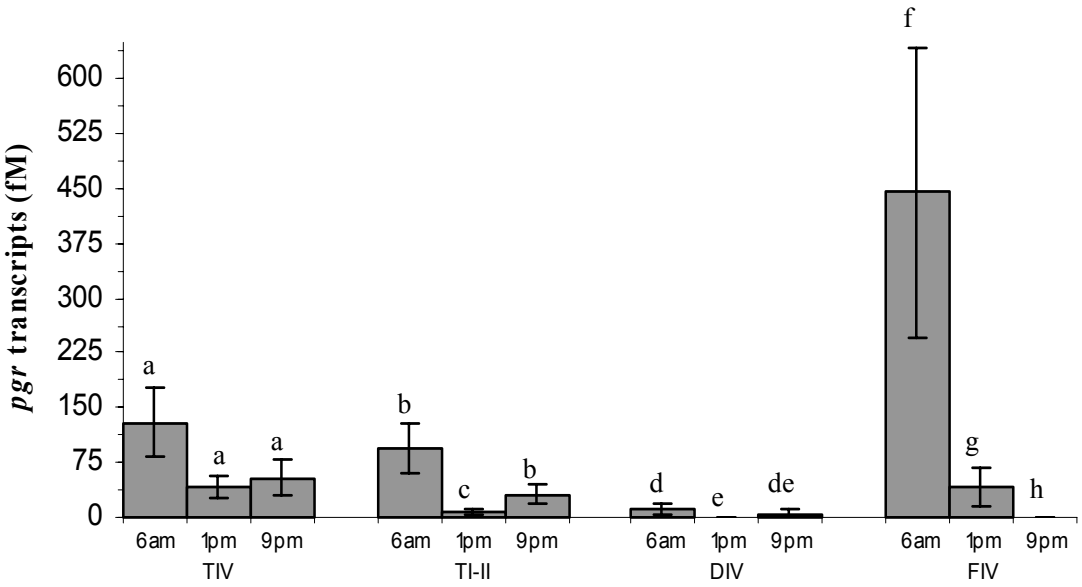

Figure 10. Daily changes of zebrafish nuclear progestin receptor (*pgr)* in denuded-late vitellogenic oocytes (DIV), follicular cells of late vitellogenic oocytes (FIV), and follicular cells enclosed oocytes of late vitellogenic stages (TIV) or early developmental stages (T I-II) analyzed by quantitative real-time PCR (qRT-PCR). Oocytes and follicular cells were collected at 6AM prior to the oocyte maturation and ovulation, and at 1PM and 9PM after the ovulation. Different letters above the bars represent significant differences within each group ($p < 0.05$). Values are means ± SEM from at least three independent experiments.

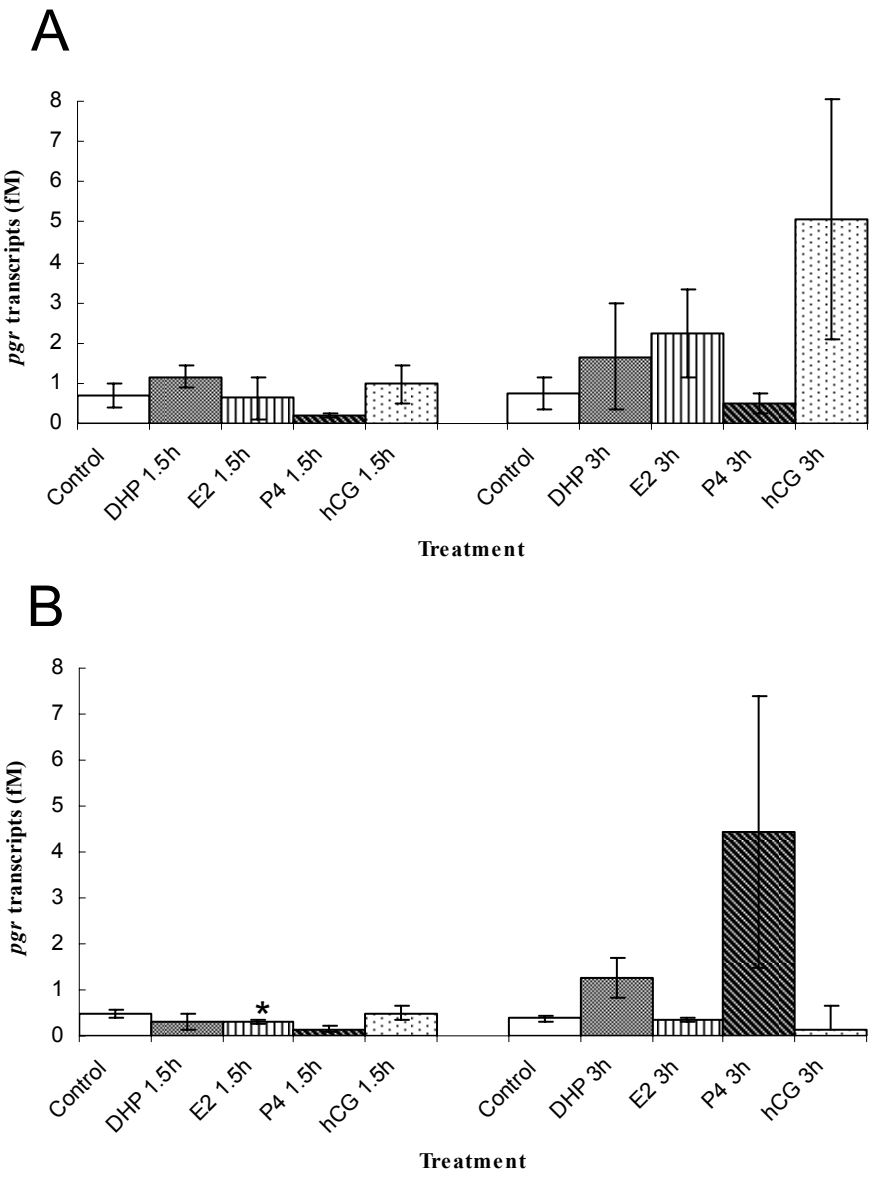

Figure 11. Expression changes of zebrafish nuclear progestin receptor (pgr) transcript in follicular cells enclosed stage IV oocytes (A) and follicular cells enclosed stage I-II oocytes (B) after 1.5 hours and 3 hours of treatment with 17,20β-dihydroxy-4-pregnen-3-one (DHP), estrogen (E2), progesterone (P4), human chorionic gonadotropin (hCG), or with a vehicle (control) analyzed by quantitative real-time PCR (qRT-PCR). * $p<0.05$ compared to controls. Values are means ± SEM from three independent experiments.

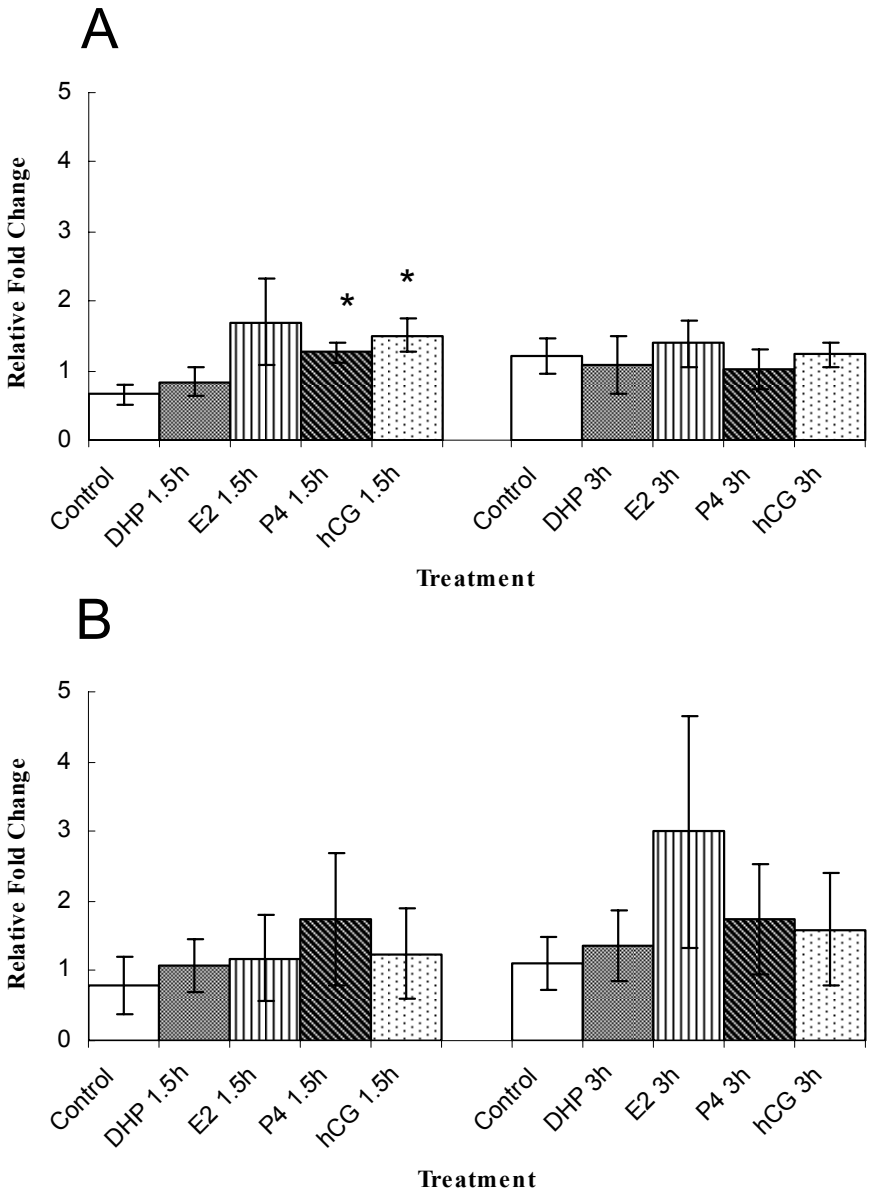

Figure 12. Expression changes of zebrafish nuclear progestin receptor (Pgr) protein in follicular cells enclosed stage IV oocytes (A) and follicular cells enclosed stage I-II oocytes (B) after 1.5 hours and 3 hours of treatment with 17,20β-dihydroxy-4-pregnen-3-one (DHP), estrogen (E2), progesterone (P4), human chorionic gonadotropin (hCG), or with a vehicle (control) analyzed by Western blotting. * $p<0.05$ compared to controls. Values are means ± SEM from three independent experiments.

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Appendix

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

February 6, 2008

Yong Zhu, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. Zhu:

Your Animal Use Protocol entitled, "Studies of Hormones and Receptors in Zebrafish (Danio Rerio)," (AUP #D185b) was reviewed by this institution's Animal Care and Use Committee on 2/6/08. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University
Animal Use Protocol (AUP) Form
Revised, October 5, 2007

Project Title: Studies of Hormones and Receptors in Zebrafish (*Danio Rerio)*

1. Personnel

1.1. Principal investigator and email:

Yong Zhu
zhuy@ecu.edu

1.2. Department, office phone:

Biology, Howell Science N401, 328-6504

1.3. Emergency numbers:

	Principal Investigator:	Other (Co-I, technician, student)
Cell: Pager: Home:	Yong Zhu 252-215-6504 (office) 252-215-9071 (home)	PhD candidate, Nhu Nguyen (Cell 252-412-9439) PhD candidate: Richard Hanna (cell 443-514-6229) MS candidate: Melina Pereira (252-258-8202) MS candidate: Sean Daly (cell 814-3952) MS candidate: Raymond Stewart Lyon Jr (cell 919-219-3288) MS candidate: Brian D. Sufrinko (252-452-4866)

FOR IACUC USE ONLY

AUP # D1856
New/renewal: Renewal
Date received: 2/4/08
Full Review and date: Designated Reviewer and date:
Approval date:
Pain/Distress category: A
Surgery: ✓ Survival: Multiple:
Prolonged restraint:
Food/fluid restriction:
Hazard approval/dates: Rad: IBC: EH&S:
OHP enrollment/mandatory animal training completed :
Amendments approved:

1.4. Co-Investigators if any:

1.5. List all personnel (PI, Co-I, technicians, students) that will be performing procedures on live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name	Relevant Animal Experience
PI: Yong Zhu	22 years
PhD student: Nhu Nguyen	5 years
PhD student: Richard Hanna	4 years
MS student: Melina Pereira	5 years
MS student: Sean Daly	1 year (will be trained by PI)
MS student: Stewart Lyon Jr	1 year (will be trained by PI)
MS student: Brian Sufrinko	1 year (will be trained by PI)

2. Regulatory Compliance

2.1 Non-Technical Summary

Using language a non-scientist would understand, please provide a 6 to 8 sentence summary explaining the overall study objectives and benefits of proposed research or teaching activity, and a brief overview of procedures

involving live animals (more detailed procedures are requested later in the AUP). Do **not** cut and paste the grant abstract.

Using zebrafish as a model, my laboratory focus on: 1) examining physiological functions of pituitary hormones, especially roles of the prolactin/growth hormone superfamily in early development in vertebrates; 2) investigating non-genomic actions of steroids with a focus on physiological functions and signaling mechanisms of the membrane and nuclear progestin receptor family. These studies conducted in my laboratory are novel and using cutting-edge technologies. In past 5 years, we have provided opportunities to a large number of graduates and undergraduates to engage in nationally and internationally recognized research to help them achieve their career objectives and to gain an appreciation for the value of basic science research in our society. We will continue our effort to prepare our students well for the future by providing them with up-to-date knowledge, advanced technical skills and a strong sense that they are participating in exciting science. The basic procedures include rearing and maintaining of health zebrafish colony, embryos, and larvae. Sometime, fish will be subjected to experimental treatments; tissues from various treated fish will require being isolated for further analyses.

2.2. Unnecessary Duplication

Does this study duplicate existing research? Yes ☐ No ■.

If yes, why is it necessary? (note: teaching by definition is duplicative)

2.3 Literature Search to ensure that there are no alternatives to the use of animals

List the following information for each search (please do not submit search results but retain them for your records):

Date Search was performed: 02/04/2008
Database searched: Medline and Premedline
Period of years covered in the search: 1966-present
Keywords used and strategy (must include the word alternatives): growth hormone, prolactin, embryonic development, organogenesis, steroid, progestin, membrane, nuclear, receptor, nonclassical, nongenomic, genomic, animal modes, zebrafish, and alternative methods.

Other sources consulted: publications and communications in related professional journals, and local, national, and international conferences.
No alternatives have been found from search of journal sof Science, Nature, Proceeding of the National Academy of Sciences of the United States of America, Endocrinology, Molecular Endocrinology, Journal of Endocrinology, Biology of Reproduction, General and Comparative Endocrinology. Currently, my lab is the only laboratory in the world conducting the proposed studies using zebrafish as a model.

Narrative indicating the results of the search (2-3 sentences) and why there are no alternatives to your proposed use of the animals in this protocol. If alternatives exist, describe why they are not adequate:
The above keywords were combined in various amalgamations. No publication or communication was found from other laboratories describing the characterizations of membrane progestin receptor in zebrafish.

2.4 Hazardous agents

2.4a. Protocol related hazards

Will any of the following be used in live animals and therefore pose a potential risk for animal care and research personnel:

	Oversight committee/ approval date	Safety procedures attached (Yes/No)
Radioisotopes	Radiation/	No
Ionizing radiation	Radiation/	No
Infectious agents	Biosafety/	No
Toxins (venoms, etc)	Prospective Health/	No
Oncogenic/toxic/mutagenic agents	EH&S/	No
Material of human origin	Biosafety/	No
Cell lines injected or implanted (MAP test)	DCM/	No
Recombinant DNA in animals	Biosafety/	Yes
Nanoparticles	EH&S/	No
Other agents		No

If any hazardous agents are used, please fill out the attached Hazardous Agents Form (Appendix 1).

2.4b. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

No

3. Animals and Housing

3.1. Species and strains:	Wild type, mutant or transgenic zebrafish. Wild type zebrafish are normally obtained from local pet stores, and propagated in the laboratory. Disease free mutant or transgenic zebrafish are normally obtained from Zebrafish International Resource Center or other laboratories around the world
3.2. Weight, sex and/or age:	0.001-5 gram, mixed sex, 0-4 years

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project
2000	+ 4000	=6000

3.3. Justify the species and number (statistical justification if applicable) of animals requested:

Zebrafish is easy to maintain and the adult fish spawn daily under laboratory conditions. Zebrafish is a well-accepted model for studying the processes of the development and the reproduction, and has become an alternative model for studying gene functions in vertebrates. My laboratory focuses on physiological functions and molecular mechanisms of the hormones and receptors in vzebrafish using various probes including hormones and receptors that we have developed recently in zebrafish.

Because of small size of the fish, relative large number of fish will be required to provide the minimal amount of tissues for in vitro incubations and manipulations. Number of sampled animals will be varied according to the various experiments. Approximately 5-100 individuals will be sampled each time.

3.4. Justify the number and use of any additional animals needed for this study (i.e. breeder animals, inappropriate genotype/phenotype, extra animals due to problems that may arise, etc.):

Normally, we keep about 100-200 adult breeders for raise zebrafish larvae. Each female capable spawn 110-200 eggs daily. Our experiments need to be repeated at least 3 times in order to accept and reject the results.

3.5. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health problems? Yes ☐ No ■
(if yes, describe)

3.6. If wild animals will be captured or used, provide permissions (collection permit # or other required information:

N/A. Wilde type zebrafish were purchased from local pet store

3.7. Are there any unusual husbandry and environmental conditions required? Yes ☐ No ■ If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, wire-bottom cages, no enrichment, social isolation, etc.):

3.8. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.

4. Animal Procedures

4.1. Will procedures other than euthanasia and tissue collection be performed? Yes ☐ No ■
If animals will be used exclusively for tissue collection following euthanasia (answer "no" above), then skip to Question 5 (Euthanasia).

4.2. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design.

Two typical types of experiments are carried out in my laboratory. The first type of experiment is involvement of studies in zebrafish development. Normally, 8 females and 4 males will be placed in 20-gallon tanks. Two layers of marbles are laid at bottom of the tanks the night prior to collection of embryos. We pay special care on minimal disturbance to parent zebrafish in order to obtain health and a large number of embryos. No tissues collection and no scarifying of adult zebrafish will be conducted in this kind of experiment.
The second type of experiments involved collection of tissues from various stages of zebrafish. For single treatment or one time point analyses, tissues from 2-3 fish will be collected following appropriate anesthesia. Additional 4-15 fish will needed in order to repeat the experiment and conduct statistical analyses. For multiple treatments and multiple time points such as monitoring change of receptors and hormones during daily cycle, normally 5-8 fish are needed for each treatment or each time point (in order to be able to conduct statistical analyses). Typically, 6 treatments or 6 time points (24 hours a day, sampling every 4 hours) are required for each experiment. Total 30-48 fish will be needed for one experiment, and the experiment needs to be repeated at least 3 times in order to publish the results. So, approximately 90-144 individual fish are required for one experiment.

In sections 4.3-4.19 below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

4.3. <u>Anesthesia/Analgesia/Tranquilization/Pain/Distress Management (for procedures other than surgery)</u>
Adequate records describing anesthetic monitoring and recovery must be maintained for all species.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary.

	Agent	Concentration	Dose (mg/kg)	Volume	Route	Frequency	Duration
Pre-emptive analgesic							

APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator:	Campus Phone:		Home Phone:
IACUC Protocol Number:	Department:		E-Mail:
Secondary Contact: Department:	Campus Phone:	Home Phone:	E-Mail:
Chemical Agents Used:		Radioisotopes Used:	
Biohazardous Agents Used:		Animal Biosafety Level:	Infectious to humans?
PERSONAL PROTECTIVE EQUIPMENT REQUIRED:			
Route of Excretion:			
Precautions for Handling Live or Dead Animals:			
Animal Disposal:			
Bedding / Waste Disposal:			
Cage Decontamination:			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment:			
Initial Approval Safety/Subject Matter Expert Signature & Date _____			

Pre-anesthetic							
Anesthetic							
Analgesic Post procedure							
Other							

a. Reason for administering agent(s):

b. For which procedure(s):

c. Method of monitoring anesthetic depth:

d. Methods of physiologic support during anesthesia and recovery:

e. Duration of recovery:

f. Frequency of recovery monitoring:

g. Specifically what will be monitored:

h. When will animals be returned to their home environment:

i. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:

4.4 Use of Paralytics

Will paralyzing drugs be used?

For what purpose:

Please provide scientific justification for paralytic use:

Paralytic drug:

Dose:

Method of ensuring appropriate analgesia during paralysis:

4.5. Blood or Body Fluid Withdrawal/Tissue Collection/Injections/Tail Snip/Gavaging

Please fill out appropriate sections of the chart below:

	Location on animal	Needle/ catheter/ gavage tube size	Route of administration	Biopsy size	Volume collected	Compound and volume administered (include concentration and/or dose)	Frequency of procedure
Body Fluid Withdrawal							
Tissue Collection							
Injection/Infusion							
Tail clip							
Gavaging							
Other							

4.6. Prolonged restraint with mechanical devices

Restraint in this context means beyond routine care and use procedures, and includes rodent and rabbit restrainers, primate chairs, stocks, slings, tethers, metabolic crates, inhalation chambers, and radiation exposure restraint devices).

a. For what procedure(s):

b. Restraint device(s):

c. Duration of restraint:

d. Frequency of observations during restraint/person responsible

e. Frequency and total number of restraints:

f. Conditioning procedures:

g. Steps to assure comfort and well-being:

h. Adverse effects/humane endpoints:

4.7 Tumor and Disease Models/Toxicity Testing

a. Describe methodology:

b. Expected model and/or clinical/pathological manifestations:

c. Signs of pain/discomfort:

d. Frequency of observations:

e. Adverse effects/humane endpoints:

4.8 Treadmills/Swimming/Forced Exercise

a. Describe aversive stimulus (if used):

b. Conditioning:

c. Safeguards to protect animal:

d. Duration:

e. Frequency:

f. Total number of sessions:

g. Adverse signs/humane endpoints:

4.9 Projects Involving Food and Water Deprivation or Dietary Manipulation
(Routine pre-surgical fasting not relevant for this section)

a. Food Restriction
   i. Amount restricted and rationale:

   ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented (weight, etc):

iv. Adverse effects/humane endpoints:

b. Fluid Restriction

i. Amount restricted and rationale:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented:

iv. Adverse effects/humane endpoints:

c. Dietary Manipulations

i. Compound supplemented/deleted and amount:

ii. Duration (hours for short term/weeks or months for long term):

iii. Frequency of observation/parameters documented:

iv. Adverse effects/humane endpoints:

4.10 Endoscopy/Fluroscopy/X-Ray/Ultrasound/MRI/CT/PET/Other Imaging

a. Describe animal methodology:

b. Duration of procedure:

c. Frequency of observations during procedure:

d. Frequency/total number of procedures:

e. Method of transport to/from procedure area:

f. Please provide or attach appropriate permissions/procedures for animal use on human equipment:

4.11 Polyclonal Antibody Production

a. Antigen/adjuvant used:

b. Needle size:

c. Route of injection:

d. Site of injection:

e. Volume of injection:

f. Total number of injection sites:

g. Frequency and total number of boosts:

h. What will be done to minimize pain/distress:

i. Adverse effects/humane endpoints:

4.12 Monoclonal Antibody Production

a. Describe methodology:

b. Is pristane used: [ ] Yes [ ] No
- Volume of pristane:

c. Will ascites be generated: [ ] Yes [ ] No
d. Criteria/signs that will dictate ascites harvest:

e. Size of needle for taps:

f. Total number of taps:

g. How will animals be monitored/cared for following taps:

h. What will be done to minimize pain/distress:

j. Adverse effects/humane endpoints:

4.13 Temperature/Light/Environmental Manipulations

a. Describe manipulation(s):

b. Duration:

c. Intensity:

d. Frequency:

e. Frequency of observations/parameters documented:

f. Adverse signs/humane endpoints:

4.14 Behavioral Studies

a. Describe methodology/test(s) used:

b. If aversive stimulus used, frequency, intensity and duration:

c. Frequency of tests:

d. Length of time in test apparatus/test situation:

e. Frequency of observation/monitoring during test:

g. Adverse effects/endpoints:

4.15 Capture with Mechanical Devices/Traps/Nets

a. Description of capture device/method:

b. Maximum time animal will be in capture device:

c. Frequency of checking capture device:

d. Methods to ensure well-being of animals in capture device:

e. Methods to avoid non-target species capture:

h. Method of transport to laboratory/field station/processing site and duration of transport:

g. Methods to ensure animal well-being during transport:

h. Expected mortality rates:

i. Endpoints for injured/ill animals:

4.16 Field Site/Laboratory Processing

a. Parameters to be measured/collected:

b. Approximate time required for data collection per animal:

c. Method of restraint for data collection:

d. Methods to ensure animal well-being during processing:

e. Disposition of animals post-processing:

f. Endpoints for injured/ill animals:

4.17 Wildlife Telemetry/Other Marking Methods

a. Describe methodology (including description of device):

b. Will telemetry device /tags/etc be removed? If so, describe:

c. Adverse signs/humane endpoints:

4.18 Other Animal Manipulations

a. Describe methodology:

b. Steps to ensure animal comfort and well-being:

c. Adverse effects/humane endpoints for ill/injured animals:

4.19 Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.

A. Location of Surgery:

B. Type of Surgery:

[ ] Nonsurvival surgery (animals euthanized without regaining consciousness)
[ ] Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function
[ ] Minor survival surgery

[ ] Multiple survival surgery
If yes, provide scientific justification for multiple survival surgical procedures:

C. Describe the pre-op preparation of the animals:

1a. Food restricted for _____ hours

1b. Food restriction is not recommended for rodents and rabbits and must be justified:

2a. Water restricted for _____ hours

2b. Water restriction is not recommended in any species for routine pre-op prep and must be justified:

D. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species.

[ ] Sterile instruments

- How will instruments be sterilized:

- If serial surgeries are done, how will instruments be sterilized between surgeries:

[ ] Sterile gloves
[ ] Cap and mask
[ ] Sterile gown
[ ] Sterile operating area
[ ] Clipping or plucking of hair or feathers
[ ] Skin preparation with a sterilant such as betadine
[ ] Practices to maintain sterility of instruments during surgery

E. Describe the following surgical procedures:

1. Skin incision size and site on the animal:

2. Describe surgery in detail (include size of implant if applicable):

3. Method of wound closure:
   a. Number of layers

b. Type of wound closure and suture pattern:

c. Suture type/size / wound clips/tissue glue:

d. Plan for removal of skin sutures/wound clips/etc:

F. Anesthetic Protocol:

	Agent	Concentration	Dose (mg/kg)	Route	Frequency	Duration
Pre-emptive analgesic						
Pre-anesthetic						
Anesthetic						
Analgesic Post Op						
Other						

1. Criteria to monitor anesthetic depth, including paralyzing drugs:

2. Methods of physiologic support during anesthesia and recovery:

3. Duration of recovery from anesthesia:

4. Frequency/parameters monitored during recovery:

5. When will animals be returned to their home environment:

6. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:

G. Recovery from Surgical Manipulations (after animal regains consciousness)

1. Following recovery, what parameters will be monitored:

2. How frequently will animals be monitored:

3. How long post-operatively will animals be monitored:

5. Euthanasia

Please refer to the 2007 AVMA Guidelines on Euthanasia and DCM Guidelines to determine appropriate euthanasia methods.

5.1 Euthanasia Procedure. If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a **scientific justification** must be provided. All investigators, even those doing survival or field studies, must complete this section in case euthanasia is required for humane reasons.

MS-222 (150-200 mg/Liter) will be administrated into buffered water (10% Hank's solution, The Zebrafish Book, Westerfield, 2007) for cardiovascular uptake. The fish will be remained in the solution for approximate 1-2 minutes until stopping of movement and heart beat, which can be easy visualized.

5.2. Method of ensuring death:

Anesthetic overdose (MS-222, see 5.1 for detail) followed by decapitation

5.3. For field studies, describe disposition of carcass following euthanasia:

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: by e-mail Date: 2/4/08

Veterinarian: Karen A. Dypplet Date: 2/6/08

IACUC Chair: Robert S Carroll, DVM Dipl Date: 2/6/08