11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, *in vitro*

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Abstract

Previtellogenic ovarian fragments from eel, *Anguilla australis*, were cultured *in vitro* in a chemically defined medium containing steroids and/or peptide hormones for 18 days in order to investigate their involvement in control of early oocyte growth. 11-Ketotestosterone (11-KT), but not estradiol-17 β , induced a significant 10–20% increase in diameters of previtellogenic oocytes and oocyte nuclei in a dose-dependent manner. Effects were greatest for 100 nM 11-KT, a dose that is within the physiological range seen in very early vitellogenic eels in the wild. The effect was not accompanied by obvious ultrastructural changes in the oocytes other than an apparent increase in nuclear size. Similarly, treatment with recombinant human IGF-I resulted in increased oocyte diameters, whereas no such effect was seen after treatment with heterologous insulin, GH, leptin, or human chorionic gonadotropin. Interestingly, lipid supplementation also resulted in an increase in oocyte diameter, and greater radioactivity in ovarian explants following incubation with ¹⁴C-triglycerides and 11-KT, but not FSH, suggesting that the androgen may play a role in lipid accumulation into the oocyte. Our results implicate hormones from both the reproductive and the metabolic axes in control of previtellogenic oocyte growth in a teleost fish.

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Introduction

The development of a fertilizable egg in oviparous vertebrates requires a substantial accumulation of nutritive resources for the development of the future embryo. Most of these resources are yolk proteins, which are laid down during vitellogenesis, the major growth phase known to be under gonadotropin and estrogen control (reviewed in Nagahama *et al.* 1995). Prior to the entry into vitellogenesis, oocytes from a typical teleost fish will have attained a 10- to 100-fold increase in diameter when compared with the oogonial stage. The oogonial stage is characterized by active proliferation of germ cells followed by their entry into the first meiotic division. In Japanese huchen (*Hucho perryi*), the steroid hormones estradiol-17 β (E2) and 17,20 β -dihydroxy-4-pregnen-3-one have been implicated in controlling oogonial proliferation and entry into meiosis respectively (Higashino *et al.* 2003). In contrast, very little information is available on the factors that regulate the previtellogenic or primary growth phase (Tyler *et al.* 2000, Patiño & Sullivan 2002, Campbell *et al.* 2006), despite their potential importance in controlling oocyte

recruitment, and hence, in governing egg batch size (fecundity) at spawning.

The role of pituitary hormones in previtellogenic oocyte growth is not clear; a recent study identified a peak of follicle-stimulating hormone (FSH) around the time of ovulation in rainbow trout, *Oncorhynchus mykiss*, and it was suggested that this peak may be related to the stimulation of development of a new batch of oocytes for spawning in the following year (Santos *et al.* 2001). Studies on coho salmon, *Oncorhynchus kisutch*, also deduced an important role for FSH in very early stages of oogenesis, well before estrogen-mediated vitellogenesis is initiated (Campbell *et al.* 2006). In contrast, many early studies on hypophysectomized teleost fish have failed to identify a clear role for pituitary hormones on previtellogenic oocytes in a number of fish species, such as catfish, *Heteropneustes fossilis* (Sundararaj & Goswami 1968). A study by Khoo (1979) likewise indicated that previtellogenic growth may be pituitary independent in hypophysectomized goldfish, *Carassius auratus*. In very early vitellogenic

silver eel, *Anguilla anguilla*, diameters of oocytes in hypophysectomized fish 2–3 weeks after surgery were slightly smaller than those of controls, but no other histological differences were apparent (Fontaine *et al.* 1976). It is possible that the effects of hypophysectomy on early stages of oogenesis were missed in these studies due to the seasonal absence of FSH or the focus on vitellogenic oocytes. Regardless, Khoo identified the apparent stimulatory effects of steroids, especially estrogens, on formation of cortical alveoli (Khoo 1979). More recently, prolonged treatment with 11-ketotestosterone (11-KT) *in vivo* was found to increase the size of previtellogenic oocytes in shortfinned eel, *Anguilla australis* (Rohr *et al.* 2001). However, the authors did not determine whether these androgen effects were direct or indirect (through feedback on the brain and/or pituitary). Organ culture may prove useful to address this issue, but few researchers have taken this approach for the study of oocyte growth. Among those who have a positive effect of testosterone on incubated vitellogenic ovarian tissue from goldfish was seen: the proportion of previtellogenic oocytes was higher after testosterone treatment than that in controls, while survival of vitellogenic oocytes was prolonged (Remacle *et al.* 1976). In mammals, spontaneous progression of primordial follicles to primary follicles maintained *in vitro* has been reported, and the presence of an inhibitory substance affecting this progression has been postulated (Fortune *et al.* 2000). Using *in vivo* models, androgens have recently been implicated in stimulating growth of primordial ovarian follicles (Vendola *et al.* 1999a). That these androgen effects are direct was supported by the presence of androgen receptors (ARs) in the ovary and clinical observations. Androgen effects appear to be mediated, at least in part, through insulin-like growth-factor-I (IGF-I), given the increase in both IGF-I and IGF-I receptor mRNA levels that occurred after androgen treatment (Vendola *et al.* 1999b). Other growth factors, such as growth and differentiation factor-9 (Nilsson & Skinner 2002), kit-ligand/stem-cell factor (Parrott & Skinner 1999) and fibroblast growth factor (Nilsson *et al.* 2001), and hormones from the growth axis, such as insulin (Kezele *et al.* 2002) may also play a role throughout the development of mammalian ovarian follicles. Crosstalk between the metabolic/nutritional and reproductive systems is likely to operate also in fishes, as there is ample evidence that nutritional status affects oocyte recruitment and fecundity for a range of species (Tyler & Sumpter 1996). A role for IGF-I in such crosstalk is probable (Campbell *et al.* 2006).

In vitro approaches have previously proven extremely valuable model systems for assessing hormonal control of spermatogenesis in eels, *Anguilla japonica*

(Miura *et al.* 1991). Because of the paucity of data on factors controlling previtellogenic growth in teleost fishes and their potential involvement in controlling fecundity, we used a long-term ovarian explant culture system to determine the direct effects of steroids (11-KT, E2) and peptide hormones (gonadotropin, growth hormone, leptin, IGF-I, and insulin) on growth of shortfinned eel previtellogenic ovarian follicles. Trials were repeated several times to ensure that responses were robust and assess whether they were affected by histological protocol. The robust and significant effects of 11-KT prompted follow-up studies aimed at assessing the effects of 11-KT on lipid uptake and AR abundance in ovarian explants *in vitro*.

Materials and Methods

Animals

All eels used for experimentation were previtellogenic female shortfins (400–800 g) in the ‘yellow stage’ mostly obtained from commercial eel-processing companies. Animals were caught by fyke nets and held captive by commercial fishermen for a variable length of time (up to about 3 weeks) until their delivery to these processing companies. Female eels used in Trial IVB, reared to experimental size (500–1000 g) from the glass eels stage and kindly donated by Dr Peter Lee (NIWA, Auckland, New Zealand), were used after 1 week of arrival in the University of Otago facilities. Experiments were conducted in accordance with the guidelines of the University of Otago Animal Ethics Committee (protocols AEC 65-00 and ET 23-02).

Biochemicals

Steroids (11-KT, E2) were obtained from Steraloids (Newport, RI, USA) and Leibovitz L-15 medium from Sigma Chemical Co. Human chorionic gonadotropin (hCG; Lot CR127) and ovine growth hormone (oGH; NIH-oGH-15, Lot AFP9220A) were a gift from the National Institute of Health (NIH/NIDDK, Bethesda, MD, USA). Porcine and bovine insulin and recombinant human leptin (rhLEP; Lot 062K1603) were purchased from Sigma Chemical Co., while recombinant human IGF-I (rhIGF-I) was obtained from GroPep (Adelaide, Australia). Medium additives were purchased from Life Technologies (Gibco BRL: proline, aspartate), BDH (Poole, UK: glutamate), or Sigma (lipids, see Trial IV). Labeled triglyceride, [carboxyl-¹⁴C]triolein (102 mCi/mmol), was obtained from Perkin–Elmer (Wellesley, MA, USA).

Experiment I: in vitro effects of 11-KT or E2 on eel ovarian explants

Trial IA

Three eels were purchased in August 2001 from Gould Aquafarms (Leeston, New Zealand) and euthanized in 0.3 g/l benzocaine. Eels were bled and briefly submersed in 70% ethanol prior to the removal of ovaries under sterile conditions (Miura *et al.* 1991). Ovaries were placed in supplemented Leibovitz L-15 medium (suppL15) containing 10 mM HEPES, 1.7 mM L-proline, 0.1 mM L-aspartic acid, and 0.1 mM L-glutamic acid and, with scissors and forceps, cut into small fragments of 1–2 mm in each dimension (Miura *et al.* 1991).

Using 24-well Costar culture plates, fragments were incubated in 1 ml suppL15 containing 0, 1, 10, 100, or 1000 ng/ml 11-KT (~3, 30, 300, or 3000 nM 11-KT; Steraloids). Porcine insulin (pINS, 1 mg/l) was added to maintain tissue health as recommended for the eel testis explant culture system of Miura *et al.* (1991). Streptomycin sulfate (0.1 g/l; Sigma Chemical Co.) and penicillin (100 000 IU/l; Sigma Chemical Co.) were added to inhibit bacterial growth. Attempts to float the tissue on elder pith, as described for eel testicular fragments (Miura *et al.* 1991) were unsuccessful and tissues were therefore submersed instead. Two replicate incubations were used for each treatment. Incubations were maintained for 18 days at 15 °C, the medium being changed every third day. At the end of experimentation, explants were processed using standard histological methods, embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin. Images were analyzed for oocyte diameters as described in 'Image analysis' towards the end of this section.

Trial IB

Eels were obtained in November 2001 from Gould Aquafarms. For this trial, five eels that had served as placebo controls in the preceding week were used. These controls had been injected intraperitoneally (1 ml/kg) with a 50:50 mix of vegetable oil and vegetable shortening only and held in recirculating tanks at 16±0.5 °C for 1 week. They were killed and processed for tissue culture as outlined under Trial IA.

Ovarian explants were exposed to 0, 1, 10, 100, or 1000 nM of 11-KT or E2 in suppL15 medium (340 mOsm) with 1 mg/l pINS and antibiotics added and maintained as described for Trial IA. Two replicate incubations were used for each treatment. Explants were preserved in Baker's calcium formol and processed for routine methacrylate sectioning at 2 µm (Lokman *et al.* 1998).

Trial IC

In December 2002, three eels were acquired from a commercial fish exporting company (Ngai Tahu Fisheries, Dunedin, New Zealand). Fish were transferred to the laboratory and maintained in tanks supplied with recirculating water of 15±0.5 °C for 1 week.

Eels were killed and dissected as before (Trials IA & IB), and ovarian tissue incubated in single replicates at 15 °C for 18 days in suppL15 medium containing 1 mg/l pINS, 100 IU/l penicillin, and 1 mg/l streptomycin. 11-KT was tested at doses of 0, 1, 10, 100, or 1000 nM. The zero dose was done in duplicate. Tissue handling was minimized by conducting the experiments in 1.5 ml of suppL15 in 12-well culture plates and changing the medium every sixth, rather than every third day.

At the end of the trial, ovarian fragments were fixed in phosphate-buffered (0.1 M, pH 7.4) 2.5% glutaraldehyde and postfixed in 1% phosphate-buffered OsO₄ to prevent shrinkage of oocytes during processing. Tissues were then embedded in epoxy resin, sectioned at 2 µm, and stained with toluidine blue. In addition, ultrathin 80 nm sections were prepared from explants incubated with 0 or 100 nM 11-KT from each of the three eels, and viewed with a Philips CM100 transmission electron microscope to examine ultra-structural changes.

Experiment II: in vitro effects of growth factors and pituitary hormones on eel ovarian fragments

Trial IIA

This trial was conducted using ovarian tissue from the same eels as described under Trial IB above. Duplicate explants were incubated in suppL15 containing 0, 1, 30, or 1000 ng/ml oGH, hCG, pINS, or rhIGF-I. The cultures were maintained in the presence of 100 nM 11-KT (see effects of 11-KT *in vitro* in Trial IA, Results section) and antibiotics for 18 days at 16 °C, and the medium was changed every third day. Tissues were harvested, preserved, and processed as described for Trial IB.

Trial IIB

The effects of IGF-I were studied in more detail using a dose-response design with explants prepared from the eels described under Trial IC and using identical culture additives. Recombinant hIGF-I (1, 10, 100, and 1000 ng/ml) dose-response treatments were done without replication, except for the controls (0 ng/ml rhIGF-I), which were done in duplicate. All cultures were supplemented with 100 nM 11-KT. After incubation, explants were fixed as described under Trial IC and prepared for light microscopical observations.

Trial IIC

A third trial with rh-IGF-I dose responses (0–1000 ng/ml) was conducted in September 2005. To assess whether IGF-I effects required the presence of androgen, cultures were set up for ovarian tissue from seven previtellogenic eels in suppl15, with or without addition of 100 nM 11-KT. Replication was only between, not within, individual fish. Media, medium changes, and duration of the experiment were as described in Trial IC. Tissues were processed for routine embedding in methacrylate resin and oocyte diameters were determined by microscopy and image analysis (cf. Trial IB).

Experiment III: in vitro effects of leptin on eel ovarian fragments*Trial IIIA*

Effects of rhLEP were investigated in February 2004 using the *in vitro* system validated above. A total of five previtellogenic eels were used, and ovarian tissue was incubated without replication according to Trial IA with or without leptin (0–1000 ng/ml) in the presence or absence of 100 nM 11-KT and 1 mg/l pINS.

Trial IIIB

A replicate experiment with leptin was conducted a year later, using the same fish, incubation conditions, and evaluation protocols as described under Trial IIC. 11-KT was again used at 100 nM, whereas leptin was added as 0, 1.25, 12.5, 125, or 1250 ng/ml. All single-run cultures were further supplemented with 1 mg/l bovine insulin.

Experiment IV: effects of lipid supplementation on 11-KT-stimulated oocyte growth in vitro*Trial IVA in vitro accumulation of lipids*

Phospholipid (PL; 1,2-diacyl-sn-glycero-3-phosphocholine, Sigma Chemical Co.) or a fatty acid supplement (FAS) containing linoleic acid and oleic acid in BSA (Sigma Chemical Co.) were added to cultures of ovarian explants (same fish as used for Trial IIC) containing suppl15 and 1 mg/l bovine insulin. PL was added at 100 µg/ml, and FAS at 60 ng/ml linoleic acid + 30 ng/ml oleic acid. Cultures were run without replication in the presence or absence of 11-KT at 100 nM for a total of 18 days and processed as described under Trial IIC.

Trial IVB in vitro accumulation of ¹⁴C-triolein

Ovarian tissue was recovered from five female eels in June 2006 as described for Trial I. For each female, tissue was placed on a piece of soft plastic and finely chopped with razor blades. Chopped tissue was divided into two approximately equal-sized portions. Portions were again

equally divided, and so on, until a total of 16 approximately equal portions had been obtained. These were placed in wells of a 24-well plate and incubated in bovine insulin-supplemented L15 with or without 100 ng/ml native coho salmon FSH (kindly donated by Dr Penny Swanson, Northwest Fisheries Science Center, Seattle, WA, USA), and in the presence or absence of 100 nM 11-KT. To all wells, 80 nCi of [carboxyl-¹⁴C]triolein was added, and duplicate incubations were accordingly set up for either 1 or 4 days. After incubation, the medium was aspirated off, and the tissue was washed with 1 ml eel Ringer for 30 min at room temperature (ca 18 °C) at 200 rpm. Washing was repeated once thereafter and as much as possible of the tissue was transferred to a plastic centrifuge tube. After any remaining medium was removed, the tissue was frozen at –20 °C and subjected to three freeze–thaw cycles to burst cell membranes. Further cell lysis was achieved using 100 µl proteinase K (10 mg/ml in Tris-EDTA, pH 7.5) for 3 h at 37 °C. Sample volume was increased by addition of 100 µl TE buffer, and lysis completed by adding 100 µl of 3 M NaOH and incubation at 65 °C for 2 h. [Carboxyl-¹⁴C]triolein accumulation was then estimated by scintillation counting (Wallac, Turku, Finland).

Experiment V: in vitro effects of 11-KT on ovarian AR gene expression*Experimental design*

Ovarian tissue from five female eels was incubated with 0, 1, 10, 100, or 1000 ng/ml 11-KT as described in Trial IC. Tissue was harvested after 18 days, transferred to a plastic centrifuge tube, and flash-frozen in liquid nitrogen. Total RNA was extracted using Trizol Reagent (Invitrogen) and 1 µg was reverse transcribed using random hexamer primers and Superscript III (Invitrogen). cDNAs were stored at –80 °C until analysis for mRNA levels by quantitative real-time PCR (QPCR).

QPCR

Four micrograms of total RNA, extracted from the testis of a silver-stage male shortfinned eel, was reverse transcribed using oligo-dT_{15–18} and Superscript III. Shortfinned eel AR α and AR β cDNA fragments were obtained by PCR, with primer design guided by Genbank Accession Nos AB023960 and AB025361 respectively for the Japanese eel AR cDNAs. Primers used for AR α were forward 5'-TCCATGTTCTCAACATCCTGGAG-3' and reverse 5'-GAAGTTCTTCATCCGGATGCAGTG-3', whereas AR β cDNA was amplified using forward 5'-TCCACAAATCTTCCTTGCTGAA-3' and reverse 5'-GGGTTTCAACTGTCCAATTTTCT-3'. PCR products of 360 and 374 bp in length were obtained after 34 (AR α) or 31 (AR β) cycles (20 s at 94 °C, 20 s at 58 °C and 30 s at

72 °C) using Roche PCR reagents. PCR products were electrophoresed on agarose gels, excised using the QIAEX Gel Extraction Kit (Qiagen) and ligated into the pGEM T-Easy vector (Promega) according to the manufacturer's instructions. Following amplification in *Escherichia coli* XL-1 Blue and recovery of plasmid by Plasmid Midi Kit (Qiagen), cDNAs were sequenced and aligned with the Japanese eel homologs. Accordingly, 99.7 and 98.6% sequence identity was obtained at the nucleotide level for the α - and β -subtype respectively. At the amino acid level, both subtypes showed 100% sequence identity, confirming that the amplified fragments encoded the AR. cDNAs were recovered from the plasmid by restriction enzyme digestion, and standards (10^0 – 10^5 copies/ μ l) generated for use in QPCR.

Primers for QPCR were designed with primer express as follows: AR α forward, 5'-GTACGTGGATGACCAGATGACAGT-3'; AR α reverse, 5'-CCAGCCCAGAGCGAACAC-3'; AR β forward, 5'-AACGATTGCACCATTGATAAGCT-3'; AR β reverse, 5'-CATTCCGGCCTCAAGCA-3'. Samples were heated for 2 min at 50 °C and 2 min at 95 °C and then cycled 40 times between 95 °C (15 s) and 58 °C (30 s) and 72 °C (30 s), before a final extension step of 1 min at 72 °C using a Stratagene MX-3000P Real Time thermal cycler. PCR products were subsequently subcloned into pGEM T-Easy vector and confirmation sequenced.

QPCR was carried out on duplicate samples of 50 ng cDNA or on 1 μ l of standard using Invitrogen's SYBR Green qPCR Supermix in a reaction volume of 20 μ l on a single plate for each target. Primers were used at final concentrations of 400 (AR α reverse) or 800 nM (other primers), determined on the basis of a primer dilution matrix. Amplicons were obtained as described in the previous paragraph. A melting curve was generated at the end of the run to ensure that only a single amplicon was amplified. MX-3000P (Stratagene, La Jolla, CA, USA) software generated standard curves and AR copy number estimates in each sample.

AR copy number was expressed per microgram of total RNA or normalized over copy number of elongation factor-1 α (ELF). For the latter approach, we amplified an ELF fragment by PCR using forward primer 5'-ATGGGAAAGGAAAAGATCCACATCA-3' and reverse primer 5'-TCAAGCTTCTTGCCAGAACGACGGT-3' at 25 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s), and extension (72 °C, 30 s). Primer design was based on the ELF sequence of zebrafish, *Danio rerio* (Accession No BC064291). An 1163 bp cDNA, located within the open reading frame, was cloned into the pGEM T-Easy plasmid vector and sequenced, as described for AR above. The sequence had 88.4 and 94.8% sequence identity with the zebrafish cDNA and deduced amino acid sequences respectively.

Plasmids, linearized with Spe I, were agarose gel purified and used as standards in QPCR. QPCR for ELF was conducted using a primer pair (150 nM forward

5'-AATCCTGAAGCCTGGTATGGTGGT-3' and 100 nM reverse 5'-ACGTTGAAGCCAACATTGTCACCG-3') nested within the 1163 bp ELF fragment to generate amplicons of 126 bp after 40 cycles of 95 °C (15 s), 62 °C (30 s), and 72 °C (30 s) and melting curve analysis on the Stratagene MX-3000P, as described above. MX-3000P software was again used to generate a standard curve and estimate target gene copy number in each sample.

Image analysis

Microscope images were captured using a Nikon video camera and analyzed through NIH Image software (version 1.62) at low power. Starting with a calibration slide, a scale bar length of 100 μ m was selected. During sample image capture, a field of vision was chosen that had maximum occupancy by oocytes; the scale bar was then pasted into the image and the image stored for analysis at a later time. The calibration slide was repeatedly re-scanned during a photographic session to ensure that the magnification did not change.

For analysis, all intact oocytes in an image were first counted. A proportion of oocytes was then used for data collection; thus, only the largest oocytes (20% of total number) were selected to eliminate those oocytes that may have been sectioned off-center. To find these oocytes, the largest 25% of oocytes (by eye) were numbered and oocyte areas were measured by NIH image software. Oocyte diameters were subsequently calculated by taking the square root of oocyte surface area and multiplying this value by $4/\pi$. Data were then sorted by oocyte diameter and the largest 80% (i.e. 20% total oocytes) retained and means calculated to yield a single value for each image, thereby avoiding pseudoreplication. As much as possible, all images from any one experiment were analyzed by the same operator.

Validation of image analysis

The procedures outlined above were applied independently (1 month apart; no communication on result, etc.) by two different authors (PML, KANG) to the 0 and 30 ng/ml rhIGF-I samples of Trial IV to assess reliability and consistency of oocyte diameter measurements. We extended the validations by comparing the same two treatments after counting not only the largest 20%, but also the largest 10% and largest 40% during image analysis.

Statistical analysis

Figures were primarily drawn on the basis of the percentage change (mean $\pm$ S.E.M.) of response variables to circumvent the large error bars that otherwise result due to fish-to-fish variation, obscuring treatment effects. Accordingly, controls were given arbitrary values of '1'.

Replicates from the same animal were averaged for graphical presentation, but not for statistical analyses.

Statistically, animal-to-animal variation was controlled for by including animal as a random variable into the model. Thus, effects of hormone treatment on oocyte diameter, nuclear diameter, AR abundance, or lipid accumulation in replicated experiments were tested using a nested univariate model in SPSS 14.0 (SPSS Inc., Chicago, IL, USA), command UNIANOVA, with FISH and REPLICATE as random variables and using the following design subcommand: /DESIGN HORMONE FISH(HORMONE) REPLICATE(FISH). To compare the effects of 11-KT and E2, a two-way univariate ANOVA was run without the zero dose and without interaction in the model.

Experiments that were run in singles were analyzed using hormone treatment as fixed factor and FISH as random factor in a two- or three-way UNIANOVA/DESIGN HORMONE_1 HORMONE_2 HORMONE_1*HORMONE_2 FISH. Interaction between hormone treatments was removed from the model if its effect was not significant. Nesting of fish within hormone treatment could not be done due to insufficient degrees of freedom. Normality of data was confirmed by analyzing the spread of residuals, whereas homogeneity of variances was tested on unnested data using Levene's statistic. Posthoc comparisons of means were done using Tukey. Differences between groups were considered significant for $P < 0.05$.

Results

Culture conditions

Tissues could be maintained *in vitro* without bacterial or fungal contamination. Histologically, atresia was evident in some oocytes in many of the tissue explants, but the incidence of atresia was not quantified. Some shrinkage of oocytes was noted after processing of explants for histology, especially in paraffin-embedded material, for which no corrections were made. Oocytes from all animals were of comparable size and typically in the range of 50–100 μm . Due to the small size of the explants used for incubation, together with the regular changes of the incubation medium, some tissue could either not be recovered, or in rare circumstances, proved to be connective tissue. Variation in sample size during analyses could therefore not be avoided.

Validation

Analyses of images by two independent investigators yielded highly consistent results, as illustrated by regression analysis (Fig. 1). However, a consistent difference between operators in absolute oocyte diameters existed, due to differences in the total number of oocytes counted on a section. These differences were

Figure 1 Reliability of measurements on oocyte diameters in eel explants ($n=20$) by two independent operators.

found to reflect inclusion of the smallest oocytes by one, but not the other operator. As a result, the average oocyte diameter of a sample (based on the 20% largest oocytes in a section) decreased with increasing numbers of total oocytes, thus yielding oocytes that on average were 7–8% smaller in diameter. These differences between operators did not affect the statistical outcome, both treatments yielding similar mean diameters that tended to be higher for explants treated with 30 ng/ml rhIGF-I than for controls (Operator I, $P=0.144$; Operator II, $P=0.065$).

Similarly, basing the average oocyte diameter in a section on counting the largest 10, 20, or 40% in a section did not affect the outcome of the statistical analysis, with rhIGF-I at 30 ng/ml always tending to yield larger oocyte diameters than controls (all $P > 0.05$; data not shown).

Experiment I

Trial IA

Treatment of ovarian fragments with 11-KT resulted in a significant increase in the diameter of eel oocytes ($F_{4,8}=6.803$; $P=0.011$) at all doses, except 10 ng/ml ($P=0.052$ for this group; data not shown). No differences in oocyte diameter were found among any of the 11-KT treatments. Similarly, there were no significant differences between individual fish, but replicates did differ significantly for some fish $\times$ dose combinations ($F_{3,12}=7.151$; $P=0.005$).

Trial IB

Following 18 days of organ culture, oocyte diameters ($F_{1,50}=21.53$; $P < 0.001$) and nuclear diameters

Figure 2 Mean relative oocyte (a) and nuclear diameters (b) after incubation of ovarian fragments from eel (*Anguilla australis*) in the presence of 0–1000 nM 11-ketotestosterone (11-KT) or estradiol-17 β (E2). Steroid effects were significant (*). Letters above bars indicate statistically different oocyte diameters between doses within steroid treatment. Values are mean $\pm$ S.E.M. of 4–5 fish.

($F_{1,50}=20.164$; $P<0.001$) were significantly greater, by nearly 10%, in cultures containing 11-KT compared with those with E2 added (Fig. 2a and b). Treatment with E2 did not significantly alter oocyte or nuclear diameters. By contrast, 11-KT effects were dose dependent (Fig. 2a and b), and differed significantly from control incubations, except for nuclear diameters of oocytes exposed to 10 nM and for diameters of oocytes exposed to 1000 nM.

Trial IC

Application of protocols for use in transmission electron microscopy (TEM) yielded trends for 11-KT that confirmed those obtained by tissues processed for paraffin (Trial I) or methacrylate resin embedding (Trial II), even with a small sample size of three fish. Thus, treatment of ovarian fragments with 11-KT led to a dose-dependent increase in relative oocyte diameters, which was significant in the physiological range (10 and 100 nM) when compared with control incubations

(data not shown). Histologically, ovaries from all females were in the early stages of lipid accumulation and had maximum oocyte diameters ranging between 50 and 70 μ m. Effects of 11-KT on oocyte size were readily visible (cf. Fig. 3a and b). However, the increase in diameter brought about by 11-KT were neither reflected in obvious changes in number of lipid droplets nor in mitochondrial number or Golgi apparatus, at least not when comparing explants exposed to 0 and 100 nM 11-KT (Fig. 3c and d). Atresia (Fig. 3e) and oögonia (Fig. 3f) were occasionally found in ultrathin sections.

Experiment II

Trial IIA

Growth factors, rather than pituitary hormones, tended to affect oocyte diameters *in vitro*. Thus, addition of oGH had no effect ($F_{3,12}=1.374$; $P=0.298$), whereas only hCG at 1 IU/ml resulted in a significantly smaller oocyte diameter compared with other doses after 18 days of culture ($F_{3,12.3}=3.562$; $P=0.047$). In contrast, rhIGF-I induced a highly significant increase in oocyte diameters ($F_{3,12.3}=6.434$; $P=0.007$), essentially in a dose-response fashion (Fig. 4a). Similarly, pINS significantly affected oocyte size ($F_{3,12.6}=7.752$; $P=0.003$), although a dose response was not apparent. Differences between fish were not significant for any of the treatments.

Trial IIB

Responses of ovarian explants to rhIGF-I varied considerably between the three individuals, and a significant treatment effect therefore could not be discerned ($P=0.593$). However, there was a tendency for oocyte diameters to increase with increasing dose of IGF-I (data not shown).

Trial IIIC

Exposure of ovarian explants to IGF-I resulted in a highly significant increase ($F_{4,50}=6.974$; $P=0.011$) in oocyte diameters in an approximately dose-dependent fashion (Fig. 4b). Oocyte diameters of ovarian follicles exposed to control media or those containing 1 ng/ml IGF-I averaged 70 μ m in diameter, increasing to mean values of up to 92 μ m in the highest dose IGF-I groups. Effects of 11-KT were additive and also highly significant ($F_{1,50}=4.157$; $P=0.006$), and the steroid increased mean oocyte diameters from 76 to 87 μ m across treatment groups.

Experiment III: in vitro effects of leptin on eel ovarian fragments

Trial IIIA,B

Leptin did not affect oocyte diameters in either trial (IIIA: $F_{2,29}=0.527$; $P=0.717$; IIIB: $F_{4,55}=0.177$; $P=0.949$;

Figure 3 Micrographs of ovarian tissue from eel (*Anguilla australis*; explants a–d are from the same female) treated *in vitro* with 0 (a, c, e and f) or 100 nM 11-KT (b, d) for 18 days. Note the presence of larger oocytes after exposure with 100 nM 11-KT (b), but the absence of any clear changes in ultrastructure (d). Some atretic oocytes (e) as well as oogonia (f) were present. Abbreviations and symbols: N, nucleus; F, fat; >, lipid droplet; *, atretic oocyte; OG, oogonium; M, mitochondrion. Scale bar light micrographs, 50 μ m; scale bar electron micrographs, 5 μ m (f) or 10 μ m (c–e).

data not shown). Effects of 11-KT were significant only for trial IIIA ($F_{1,29}=16.767$; $P<0.001$; data not shown).

Experiment IV: effects of lipid supplementation on 11-KT-stimulated oocyte growth *in vitro*

Trial IVA *In vitro* accumulation of lipids

Addition of lipids to the incubation medium tended to result in increased oocyte diameters ($F_{2,26}=2.393$; $P=0.111$). Indeed, for six out of seven females, control incubations yielded the smallest oocyte diameters. Oocyte diameters for the control incubation from the seventh fish were unusual, at nearly twice those seen for some other incubations; exclusion of this single data point changed the probability for an effect of lipid addition on oocyte diameters to $P=0.003$ with an average increase of 14 μ m to the oocyte diameter in response to adding PL or FAS (Fig. 5). Regardless of whether the outlying data point was included or excluded, a statistically significant additive effect of 11-KT was observed ($F_{1,26}=9.349$; $P<0.001$; Fig. 5).

Trial IVB *In vitro* accumulation of 14 C-triolein

Accumulation of 14 C-triolein in ovarian fragments was significantly increased (Fig. 6a and b) in the presence of 11-KT ($F_{1,13.1}=6.587$; $P=0.023$), but not FSH ($F_{1,13.2}=1.688$; $P=0.216$), when analyzing data from both days in the same nested ANOVA. Statistical outcome was regardless of whether the non-significant interaction term (11-KT * FSH) was included in the model. Duration of incubation also significantly increased triolein accumulation in ovarian fragments ($F_{1,52}=50.242$; $P<0.001$).

When simplifying the statistical model and analyzing data for both days individually, degrees of freedom were insufficient to detect an effect of 11-KT by 1 day ($F_{1,13}=1.912$; $P=0.19$) and just insufficient to detect an 11-KT effect by 4 days of incubation ($F_{1,14.3}=4.464$; $P=0.053$).

Experiment V: *in vitro* effects of 11-KT on ovarian AR gene expression

AR transcript abundance was low, with maximum levels of around 7000 and 1700 copies/ μ g total RNA for AR α and AR β respectively. QPCR on total RNA without the RT step typically yielded 500–2000 copies of target/ μ g RNA. There was insufficient RNA to assess contamination of cDNA for all samples; an arbitrary threshold of 2000 copies of target/ μ g RNA was therefore set as the minimum detectable level, deeming AR β mRNA levels undetectable in all samples. Samples in which AR α copy number exceeded 2000/ μ g RNA, genomic contamination copy number was subtracted if a value for that sample could be obtained; in the absence of an estimate of copy number due to genomic contamination, the copy number was set at 1000, midway in the contamination range (500–2000 copies). Estimates for AR α copy number could accordingly be obtained for 11 out of 25 samples. Although insufficient for statistical analysis, data on AR α transcript abundance from only these 11 samples hinted at a reduction in copy number with increasing concentrations of 11-KT in the incubation medium, regardless of whether the data were expressed per microgram total RNA or per 10^6 copies of ELF (Fig. 7a and b).

Figure 4 Relative diameter of oocytes after incubation of ovarian fragments from eel (*Anguilla australis*) with 0, 1, 30, or 1000 ng/ml recombinant human insulin-like growth factor-I (rhIGF-I), porcine insulin (pINS), ovine growth hormone (oGH), or human chorionic gonadotropin (hCG) (a), or with 0–1000 ng/ml rhIGF-I in the presence or absence of 100 nM 11-ketotestosterone (b). Explants treated with rhIGF-I had significantly greater mean oocyte diameters (*) than remaining treatments, and its effects were dose dependent ($P < 0.05$). Values are mean $\pm$ s.e.m. of 4–5 fish.

Discussion

Since little is known about the factors controlling previtellogenic growth in teleost fish other than two early reports (Remacle *et al.* 1976, Khoo 1979), we exposed eel ovarian fragments to a range of hormones *in vitro*. Exposure to E2 did not affect oocyte diameter after 18 days of culture. Remacle *et al.* (1976) likewise could not detect any effects of estrogens on the goldfish ovary *in vitro*, irrespective of oocyte stage. *In vivo* observations, however, seem contradictory; Yu *et al.* (1979), while feeding estrogen-enriched diets, could not detect any effect on perinucleolar oocytes in ovaries of juvenile coho salmon. In contrast, E2 treatment was correlated with cortical alveolus formation in hypophysectomized goldfish (Khoo 1979). In European silver eels, oocytes increased from an average of around 70 μ m in controls (previtellogenic) to around 180 μ m (very early vitellogenic) after long-term *in vivo* E2-treatment (Olivereau & Olivereau 1979). This increase coincided with an increase in accumulated lipids. However, the

Figure 5 Diameter of oocytes after incubation of ovarian fragments from eel (*Anguilla australis*) under control conditions (C) or after addition of 100 μ g/ml phospholipid (PL; 1,2-diacyl-sn-glycero-3-phosphocholine, Sigma Chemical Co.) or a fatty acid supplement (FAS) containing 60 ng/ml linoleic acid + 30 ng/ml oleic acid. Incubations were done with or without addition of 100 nM 11-ketotestosterone (11-KT). Bars represent the mean $\pm$ s.e.m. of 5–7 fish. Differences between lipid supplements are indicated by differing letters above bars ($P < 0.05$). 11-KT effects were significant ($P < 0.001$).

profound increase in the activity of gonadotropes suggests that the observed gonadal changes are not only attributable to E2 but probably also to gonadotropins.

In contrast to E2, *in vitro* treatment of eel ovarian fragments with 11-KT led to a significant increase in the size of the largest oocytes in all the three trials, irrespective of the method of tissue processing used (paraffin, methacrylate, epoxy resin). The nucleus too, appeared to be larger in oocytes from explants treated with 11-KT, an effect that was confirmed statistically for Trial IB. Ultrastructurally, increased oocyte dimensions were not mirrored by notable changes in cell morphology other than a seemingly larger nucleus (Trial IC); thus, oocytes were recognizably larger when viewed with the electron microscope, but there was no clear evidence for the increased abundance of organelles, such as endoplasmic reticulum or golgi. Similarly, mitochondrial numbers were not clearly altered, although the non-random distribution of mitochondria makes it difficult to ascertain this conclusively. Likewise, there were no histological or ultrastructural indications that the abundance of lipid droplets in the ooplasm had changed. This is not in keeping with the observed lipid accumulation following exposure of eels to 11-KT *in vivo* (Rohr *et al.* 2001) or with the uptake of lipids in the form of radiolabeled triglycerides into the ovary that was stimulated by 11-KT (Expt IVb). These observations, together with the finding that the addition of lipid supplements to the incubation medium tended to increase oocyte diameters in the presence of 11-KT (Expt IVa), suggest that lipids were limited in the medium, thus prohibiting ultrastructural change (Trial IC).

Remacle *et al.* (1976) previously investigated the effect of different hormone preparations on vitellogenic

Figure 6 Accumulation of ^{14}C -triolen in ovarian fragments of eel (*Anguilla australis*) incubated for 1 (a) or 4 days (b) under control conditions (C) or in the presence of 100 ng/ml salmon follicle-stimulating hormone (FSH). Incubations were done with or without addition of 100 nM 11-ketotestosterone (11-KT). Bars represent the mean $\pm$ S.E.M. of five fish. Differences between treatments are indicated by asterisks above bars ($P < 0.05$).

ovarian tissues of goldfish *in vitro*. Interestingly, the integrity of oocytes up to stage IV (cortical alveolus stage) was better in the presence of testosterone and somatic tissues were less hypertrophied when compared with controls. The authors further suspected the development of nests of spermatogonia within the ovarian explants (Remacle *et al.* 1976). In our study, the presence of nests of spermatogonia was not apparent.

Androgens have been implicated in oogenesis in mammals, particularly in the growth of small preantral follicles. These follicles resemble previtellogenic follicles of fishes in that growth progresses slowly, occurs both prior to and after puberty (McGee & Hsueh 2000) and appears to be mostly gonadotropin independent (reviewed by Smits & Cortvrindt 2002). Thus, the number of primordial follicles advancing to the pre- or small antral stage increased in androgen-treated monkeys (Vendola *et al.* 1998, 1999a). Likewise, women treated with testosterone or suffering from androgen excess (polycystic ovarian syndrome, androgen-

Figure 7 Effects of 11-ketotestosterone (0–1000 nM) on androgen receptor- α (AR α) mRNA copy number (a) or after normalizing over elongation factor (ELF; b) in ovarian explants of eel after 18 days of incubation *in vitro*. Sample sizes were 3, 4, 1, 1, and 2 for 0–1000 nM doses respectively. Statistical analyses were not carried out due to small sample sizes.

producing tumors) showed increased numbers of growing follicles (reviewed in Vendola *et al.* 1998). These observations contrast with atretogenic actions of androgens on preantral follicles in immature hypophysectomized rats (Hillier 1987). Although some of the stimulatory effects may be indirect, via surrounding stromal tissue (Vendola *et al.* 1999a), direct androgen effects on ovarian follicles are likely given that AR mRNA abundance in rhesus monkey was the greatest in preantral to small antral follicles (Weil *et al.* 1998). In the mouse, a role for androgens is also apparent in view of the observations that female AR knock-outs had reduced average litter size (Yeh *et al.* 2002, Shiina *et al.* 2006) and an increased incidence of atresia of ovarian follicles (Shiina *et al.* 2006). The latter observations probably result from impaired folliculogenesis, as evidence has recently been mounting for an important role of androgens in folliculogenesis through modulation of expression or effects of local growth factors (e.g., Hickey *et al.* 2005, Shiina *et al.* 2006).

In our previous *in vivo* work on shortfinned eels (Rohr *et al.* 2001), 11-KT had a clear effect on the growth of previtellogenic oocytes, but it was not determined

whether this was a direct or an indirect effect, mediated via feedback of this steroid on the brain–pituitary axis. The current findings, based around an *in vitro* bioassay design, comply with the mammalian scenario, and indicate that at least some of the effects seen *in vivo* are likely to have been direct. The presence of AR mRNA, but not AR β mRNA, in the eel ovary, albeit at copy numbers some 2–5 fold lower for the α -subtype than those seen *in vivo* (M Algie and PM Lokman, unpublished observations), or around 20–50 fold lower than those reported in for example, liver or muscle of melengestrol acetate-treated heifers (Pfaffl *et al.* 2002), support this notion. In other species of fish, such as coho salmon (Fitzpatrick *et al.* 1994), Atlantic croaker, *Micropogonias undulatus* (Sperry & Thomas 1999), seabream, *Pagrus major* (Touhata *et al.* 1999), goldfish (Wells & Van Der Kraak 2000), and Japanese eel (Ikeuchi *et al.* 1999), AR transcripts have also been identified in the ovary. However, specific roles of androgens and ARs in the fish ovary have not been described previously, and this is the first study to indicate a possible function, i.e. involvement in control of previtellogenic oocyte growth, at least in eel.

Maximal effects of 11-KT were observed when using doses in the 10–100 nM range (3–30 ng/ml), similar to blood 11-KT levels seen in very early vitellogenic shortfinned eels caught from the wild (20–50 ng/ml; Lokman *et al.* 1998, 2002). Although not a focal point of other investigations, increased levels of androgens (generally testosterone, rather than 11-KT) during previtellogenesis have been reported in a number of fish species, such as striped bass, *Morone saxatilis* (Holland *et al.* 2000) and seahorse, *Hippocampus abdominalis* (Poortenaar *et al.* 2004). In females of other species, such as killifish, *Fundulus grandis* (Greeley *et al.* 1988), channel catfish, *Ictalurus punctatus* (Kumar *et al.* 2000) and sweep, *Scorpius lineolatus* (Dedual & Pankhurst 1992), elevated androgen levels during previtellogenesis were not obvious. Nonetheless, it is likely that androgens do not only serve as precursors for estrogen synthesis, but also that they have a functional role during previtellogenesis in teleost fish in general.

Completion of the previtellogenic growth phase in fish is followed, at some stage, by initiation of puberty, the activation of the brain–pituitary–gonad axis. In eels, puberty coincides, or is just preceded by, ‘silvering’; silvering encompasses dramatic changes in the physiology and behavior of the eel in preparation for a long-distance oceanic migration and is probably mediated by 11-KT (Rohr *et al.* 2001, Lokman *et al.* 2002). Pre-adaptations include increases in eye size, expression of different opsins, jaw remodeling for stream-lining, cardiovascular changes, cessation of feeding, and enhancement of hypo-osmoregulatory ability (reviewed in Lokman *et al.* 2003b). It is believed that silvering only occurs once eels reach a critical condition (fat stores;

Larsson *et al.* 1990). Therefore, it is likely that hormones involved in metabolism and growth modify the activity of the brain–pituitary–gonad axis. However, it is not clear which metabolic hormone is the primary regulator of this axis, and there is no evidence to indicate at which level the axis is most affected. When evaluating the effects of heterologous peptide hormones, namely, gonadotropin, growth hormone, insulin, IGF-I and leptin on the eel ovary *in vitro*, an effect was noted only for IGF-I. It is possible that heterologous hormones were not recognized by the previtellogenic eel ovary, an issue that is impossible to address without extensive research on receptor–ligand interaction for these peptides. Such data is fortunately available for IGF, a structurally highly conserved protein for which the salmon and human recombinant homologs have comparable activities in salmon embryonic cells *in vitro* (Upton *et al.* 1998). Addition of hIGF-I to the incubation medium resulted in a significant increase in eel oocyte diameters, an effect that was additive to that of 11-KT. In the mammalian ovary, IGF-I appears to be important for oogenesis throughout the development of the ovarian follicle. For example, IGF-I can modify ovarian steroidogenesis, and thus appears to play a role in dominant follicle selection (Ginther *et al.* 2004). Similarly, IGF-I can increase the population of gonadotropin-independent small antral follicles (Gong *et al.* 1997) and stimulate growth of preantral follicles, possibly through mediating androgen action (Vendola *et al.* 1999a, 1999b). Wide effects of IGF-I on aspects of oogenesis have been reported in fish also; thus, IGF-I can affect basal or gonadotropin-supported steroidogenesis in vitellogenic or maturing ovarian follicles (Weber & Sullivan 2000, Mendez *et al.* 2005), and IGFs have been implicated in stimulating germinal vesicle breakdown in a number of fish species (e.g., Weber & Sullivan 2000). Very recently, involvement of IGF-I on early stages of oogenesis in salmon was postulated on the basis of its plasma dynamics during previtellogenesis (Campbell *et al.* 2006). Our findings on the eel ovary confirm an involvement in regulating previtellogenic ovarian growth, although the mechanisms that cause actual growth remain to be established.

Noticeable animal-to-animal variation in responses to hormones was observed in our experiments, although responses obtained were consistent among trials, and did not depend on the individual investigator, on the tissue processing technique, or on the number of oocytes measured. Therefore, individual variation cannot be attributed to methodology or data analysis. Instead, there probably is a physiological cause for the differences amongst individuals, for instance in relation to differences in IGF-I receptor and AR abundance, issues that require additional study.

In summary, we have shown using a functional *in vitro* organ culture system that IGF-I and the androgen 11-KT can exert direct effects on the eel ovary, resulting in

additive increases in size of previtellogenic oocytes. Moreover, 11-KT, but not FSH, significantly stimulated the accumulation of radiolabeled triglycerides into the ovary. We conclude that growth factors and androgens are involved in the regulation of previtellogenic oocyte growth in a teleost fish.

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