

# Effects of reproductive stage and 11-ketotestosterone on LPL mRNA levels in the ovary of the shortfinned eel

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**Abstract** To understand the dynamics of lipid uptake into the ovary and the potential role that lipoprotein lipase plays in this event, changes in LPL transcript abundance during oogenesis were measured in both wild-caught and pituitary homogenate-induced artificially maturing eels. Also, the effects of 11-ketotestosterone (11-KT) on LPL mRNA levels were investigated in vivo and in vitro. Normalized ovarian LPL transcript abundance increased as oogenesis advanced, and it rose particularly rapidly during midvitellogenesis, corresponding to pronounced increases in ovarian lipid deposits and LPL activity. Furthermore, LPL mRNA levels were dramatically increased following 11-KT treatment in vivo, findings that were reinforced as trends in ovarian tissue incubated in vitro. **■** Ovarian LPL appears to be directly involved in the uptake of lipids into the eel ovary, an involvement that appears to be controlled, at least in part, by the androgen 11-KT.—Divers, S. L., H. J. McQuillan, H. Matsubara, T. Todo, and P. M. Lokman. **Effects of reproductive stage and 11-ketotestosterone on LPL mRNA levels in the ovary of the shortfinned eel.** *J. Lipid Res.* 2010. 51: 3250–3258.

**Supplementary key words** androgens • hormones/growth factors • lipoprotein lipase • lipid transfer proteins

It is well established in teleosts that lipids form the major component of the body nutrient pool in terms of energy storage (1, 2). The main lipid storage sites in fish include liver, muscle, and mesenteric fat (2), but the relative importance of each tissue depot varies among fish species (3). The major lipid reserve in freshwater eels (*Anguilla* spp.) is composed primarily of triacylglycerides (TAG), which are stored predominantly in the carcass, and secondarily in the liver (4, 5). Storage is preceded by TAG uptake from the circulation in which it is transported within transient intestine-derived chylomicrons or in liver-derived lipoproteins (6).

Lipoproteins, particles that transport lipids through the blood stream, are distinguished by density and size: from large, low density macromolecules, such as chylomicrons and very low density lipoproteins (VLDL) (high lipid and low protein content), to smaller, high density macromolecules, such as high density lipoproteins and vitellogenin (low lipid and high protein content) (7). The main lipoprotein component of eel plasma is VLDL (~44% of total plasma lipoproteins), and TAG is its major lipid class (57–66% of VLDL composition (8)). Associated with the lipoproteins is apolipoprotein CII (apoCII), a peptide that has the ability to specifically activate the enzyme lipoprotein lipase (LPL) (9). LPL is attached to the luminal endothelium and located in close proximity to the adipocytes, where it hydrolyses bound TAGs to free fatty acids (FFA). These FFAs, in turn, are sequestered by the surrounding tissue and re-esterified for storage as TAG (e.g., adipose tissue) or oxidized for energy (e.g., muscle tissue (10)). As the catabolism of FFA provides the main source of energy for many species (11), LPL is considered a key enzyme in whole body lipid metabolism and balance, and the extrahepatic rate-limiting enzyme in the hydrolysis of circulating TAG (12–14).

Aside from their importance in generating energy, the lipid composition of eggs is a key determinant of egg quality in marine fish (reviewed in Ref. 15); however, there is almost no information about when and how neutral lipids are incorporated into the oocyte or the role that LPL plays in this event. Likewise, the fate and, in particular, the movement of neutral lipid from storage depots to the ovary throughout oogenesis is largely unknown, despite the obvious relevance of these issues for aquaculture operations. Freshwater eels are highly suitable experimental models for the study of lipid movement and uptake as they

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Abbreviations: 11-KT, 11-ketotestosterone; apo, apolipoprotein; BL, body length; E2, estradiol-17 $\beta$ ; ELF-I, elongation factor-1 $\alpha$ ; GSI, gonadosomatic index; SPH, salmon pituitary homogenate; TAG, triacylglyceride.

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cease feeding at the onset of puberty. Thus, eels rely totally on endogenous TAG stores to both fuel a long-distance spawning migration and complete gametogenesis.

We aimed (i) to determine the potential involvement of LPL in lipid uptake into the eel ovary throughout (induced) oogenesis, and (ii) to investigate the effects of treatment with 11-ketotestosterone (11-KT). The latter aim was proposed in view of the documented effects of 11-KT on lipid uptake in vivo (16) and in vitro (17, 18) and on the demonstrated ability of 11-KT to increase eel oocyte diameters in vitro (17).

## MATERIALS AND METHODS

### Animals and experimental design

Experiments were conducted within the guidelines of the University of Otago Animal Ethics Committee (protocols 32/05 and 51/05).

### Experiment 1: Lipid content, LPL mRNA levels, and LPL enzyme activity in the ovary and liver of wild shortfinned eels

Eight previtellogenic (PV; "yellow") and eight early vitellogenic (EV; "silver") shortfinned eels (*A. australis*) were captured during early summer and early autumn, respectively, to obtain baseline data on lipid physiology. Eels were caught by commercial fyke nets set overnight in Lake Ellesmere, Christchurch, New Zealand and euthanized in 0.3 g/l benzocaine prior to sampling. Body length and body, liver, and gonad weights were determined for purposes of normalization and calculation of the gonadosomatic index (GSI; gonad weight as percentage of total body weight). After removal of the ovary, a small amount of tissue was fixed in phosphate-buffered 4% paraformaldehyde for oocyte staging. Ovarian tissue was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until determination of total lipid content, LPL activity, and quantitative PCR (qPCR).

### Experiment 2: Lipid content, LPL mRNA levels, and LPL enzyme activity in the ovary and liver of artificially maturing shortfinned eels

As eels do not undergo vitellogenesis in captivity and mature eels cannot be sourced from the wild (19), an induced-maturation trial was carried out, which allowed for the sampling of eels in different stages of reproductive development. Twenty-three wild-caught EV eels were purchased from a commercial eel processor (Gould Aquafarms, Christchurch, New Zealand) and housed in 1,000 l recirculating tanks. Water temperature was maintained at  $20^{\circ}\text{C}$  and salinity at 30 ppt. Fish were injected intramuscularly once every two weeks with  $10\text{ mg}\cdot\text{kg}^{-1}$  salmon pituitary homogenate (SPH) (20), consisting of acetone-dried Chinook salmon pituitary homogenized in eel Ringer (for Ringer composition, see Ref. 21). Control fish were injected with an equivalent volume of eel Ringer solvent only. Three fish were induced to undergo final oocyte maturation and ovulation using the method outlined in Ref. 20. SPH-treated eels were sampled at various stages of oocyte development after euthanasia in  $0.3\text{ g}\cdot\text{l}^{-1}$  benzocaine; Ringer-injected controls were sampled at the same time. Attempts were made to sample ovarian tissue from three fish at each developmental stage [e.g., EV; mid-vitellogenesis (MV); late vitellogenesis (LV); migratory nucleus (MN); and postovulation (P-OV)], but retrospective oocyte staging indicated that this was not achievable in all instances. Likewise, insufficient animals were avail-

able for sampling at week 10, and only two control fish were available for sampling when SPH-treated fish were ovulating. After removal of the ovary, tissue was sampled as described above (Experiment 1).

### Experiment 3: Effects of 11-KT on ovarian and liver LPL mRNA levels and lipid content in vivo

Fourteen PV shortfinned eels were divided between two treatment groups and implanted intraperitoneally with a pellet (95% cholesterol, 5% cellulose) containing placebo or 2.5 mg 11-KT, as described previously (22). Fish were maintained for two weeks in fresh water at  $16\text{--}17^{\circ}\text{C}$  before terminal anesthesia in  $0.3\text{ g}\cdot\text{l}^{-1}$  benzocaine. Liver and ovarian tissues were collected and frozen for quantification of LPL mRNA levels by qPCR.

### Experiment 4: Effects of 11-KT on ovarian LPL mRNA levels in vitro

In vitro trials were run on ovarian tissue from PV shortfinned female eels, in the presence or absence of 11-KT. Five females were used for both experiments 4.1 and 4.2, and ovarian cultures were maintained for 18 days, whereas experiment 4.3 utilized ovaries from six fish maintained for 3 days, all at  $16^{\circ}\text{C}$ . Ovarian fragments were retrieved and incubated in a supplemented L-15 medium in vitro in a dose response design ( $0\text{--}1,000\text{ nM}$  11-KT) (experiment 4.1) or in the presence or absence of  $100\text{ nM}$  11-KT as described previously (17) (experiments 4.2 and 4.3). At the end of incubation, tissues were recovered and frozen for measurement of LPL mRNA levels by qPCR.

### Histological analysis

Fixed tissue was dehydrated, embedded in paraffin, and sectioned at  $5\text{--}6\text{ }\mu\text{m}$  according to standard procedures. Sections for oocyte staging were stained with either hematoxylin and eosin or Mallory trichrome. Oocytes whose nuclei were visible in the section were staged as previously described (20, 23, 24). The most developmentally advanced oocytes within the ovary determined the oocyte stage assigned to individual fish.

### Determination of total lipid content

Lipid content was quantified using an adaptation of the method used by Folch et al (25). Ovarian or liver tissue ( $100\text{--}150\text{ mg}$ ) was macerated using razor blades and transferred to a test tube containing 4 ml  $\text{CHCl}_3\text{:MeOH}$  (2:1). After mixing by vortex for 60 s, 1 ml 0.8% NaCl was added, and mixing was repeated for 60 s. Following centrifugation at  $4,000\text{ g}$  for 4 min, the lower  $\text{CHCl}_3$  phase was transferred to a preweighed 4 ml glass tube. The  $\text{CHCl}_3$  was evaporated, and after desiccation for 24 h, the tubes were weighed to determine the total amount of lipid present.

### LPL activity assay

LPL activity was measured in ovarian tissue collected from eels in experiments 1 and 2. Ovarian homogenates (for details, see Ref. 26) were centrifuged at  $12,000\text{ g}$  for 2 min at  $4^{\circ}\text{C}$ , and then the supernatant was removed and placed on ice for immediate use. Two aliquots of homogenate from each eel were assayed: one aliquot contained apoCII (from VLDL of human plasma; Merck) in apoCII buffer ( $10\text{ mM}$  Tris-HCl, pH 8.5) to stimulate LPL activity (27); the other aliquot contained the equivalent volume of only apoCII buffer to estimate LPL-independent lipase activity. The assay mixture consisted of  $0.016\text{ mM}$  [carboxyl- $^{14}\text{C}$ ] triolein ( $102\text{ mCi}/\text{mmol}$ ; Perkin-Elmer, Wellesley, MA);  $50\text{ mM}$   $\text{MgCl}_2$ ;  $25\text{ mM}$  PIPES (pH 7.5);  $0.05\%$  BSA (FA-free);  $3\text{ }\mu\text{g}$  apoCII or apoCII buffer only; and  $7\text{ }\mu\text{l}$  of ovarian homogenate in a total volume of  $67\text{ }\mu\text{l}$ . After incubation at  $25^{\circ}\text{C}$

on a shaking platform for 2 h, the reaction was stopped, and the oleate was extracted. After centrifugation (1,000 rpm for 15 min), the supernatant was removed and measured for radioactivity by scintillation counting (Trilux Scintillation and Luminescence Counter, Wallac). LPL activity was calculated as the difference between total and LPL-independent oleate release, and expressed in mU. As some tissue from wild PV and EV eels was used up to develop and validate the assay, quantitative data from these groups could be gathered only from four of eight fish in each group.

### Cloning of partial *A. australis* LPL cDNA

Total RNA was extracted from an ovary of an EV eel according to the Trizol® protocol (Invitrogen). Complementary DNA was generated from 1 µg total RNA using oligo-dT<sub>18</sub> and Superscript III (Invitrogen) reagents. A 474 bp fragment of eel LPL cDNA was amplified by PCR using degenerate primers designed around a conserved region of published teleost LPL sequences and an *A. japonica* LPL sequence (T. Todo, unpublished observations). The sequence for the forward primer was 5'-TNATYCATGGRTGGAC-3'. The sequence for the reverse primer was 5'-CCRTTRGGGTAGATRTC-3'. The PCR protocol consisted of 2 min at 94°C, followed by 30 cycles of 94°C for 15 s, 47°C for 20 s, 72°C for 40 s, and a final extension of 3 min at 72°C, utilizing Bioline reagents. The amplified PCR product was extracted from an agarose gel with the QIAEX II Agarose Gel Extraction Kit (QIAGEN, Hilden, Germany) and ligated into the pGEM®-T Easy Vector (Promega, Madison, WI). After transfection and amplification in competent *E. coli* (strain XL-1 Blue), plasmid was isolated using the Plasmid Mini Kit (QIAGEN, Hilden, Germany) and sequenced (Allan Wilson Centre, Massey University, Palmerston North, New Zealand). The identity of a 441 bp cDNA (474–33 bp from degenerate primer sequences) was confirmed (BLAST alignment, NCBI), and nested primers (forward: 5'-CACCTCCGCTGCTACACCAAGCTTGT-3'; reverse: 5'-GTTGGGGTAGATGTCCACGTAGCCCA-3') were subsequently designed for phylogenetic and sequence analysis. An amplicon (339 bp) was obtained by PCR using the cDNA, reaction volume, and reagents described previously. The PCR protocol consisted of 2 min at 94°C, followed by 30 cycles of 94°C for 15 s, 68°C for 20 s, and 72°C for 35 s, followed by a final extension of 3 min at 72°C. The nested PCR product was sequenced as described above and aligned with other lipases to ascertain homology using the neighbor joining method (DNASIS v3.5).

### Real-time PCR for quantification of LPL mRNA levels

Transcript levels were measured by real-time quantitative PCR (Stratagene Mx3000P, Agilent Technologies, CA). The reaction mixture contained 50 ng reverse-transcribed RNA, 50 nM forward primer (5'-AGCTTACCTTCTGGGATACAG-3'), 150 nM reverse primer (5'-TGTTTGGTGTCTAGTTGTCTCCT-3'), 10 µl Platinum SYBR Green Supermix (Invitrogen, Auckland, New Zealand), and distilled water to a final volume of 20 µl. All reactions were carried out in duplicate (LPL) or triplicate [elongation factor-1α (ELF-I)]. The PCR protocol consisted of 2 min at 50°C, 2 min at 95°C, followed by 30 cycles of 95°C for 15 s, 61°C for 30 s, and 72°C for 30 s, followed by 1 min at 95°C, 30 s at 55°C, and 95°C for 30 s. After estimation of the copy number using a standard curve with known amounts of linearized pGEM T-Easy-LPL construct, LPL transcript levels were expressed as a whole-organ copy number or normalized over mg wet tissue weight, over µg total RNA, or over the house-keeping gene ELF-I (17, 22) as appropriate (see detailed outline below).

As the full-length cDNA sequences for LPL and ELF-I are not available for the shortfinned eel, primers could not be optimally

designed to cover intron/exon boundaries to control for genomic contamination. Therefore, possible contamination with genomic DNA in each sample was estimated by qPCR assay of RNA extracts (nonreverse-transcribed). Genomic contamination was less than 0.1% for LPL and less than 0.003% for ELF-I.

### Data transformation and statistical analysis

*Experiments 1 and 2.* The morphometric changes in female eels undergoing gonadal maturation are not isometric. Eel total length is known not to change during maturation, but body weight, width, and depth do change, prompting the use of an allometric eel model (AEM) to correct the various lipid and gene transcript abundance analyses for body size. First, the relationship between body weight and total body length [condition index (CI)] was established using an ordinary least squares regression (28, 29) based on measurements of female shortfinned eels collected from Lake Ellesmere by members of our laboratory over the last decade or so. The regression equation, fitted only to fish ( $n = 99$ ) whose total length fell within the range of 565–982 mm of the fish used in the current study, was determined on the basis of Le Cren (30) and Cone (29) to model weight ( $W$ ) in mm as a function of length ( $L$ ) in mm, yielding condition index (CI):

$$CI = (W/L^b) * 10^n$$

where  $b$  equals the slope of the least squares regression, and  $n$  is chosen so that CI is a mixed number instead of a very low decimal figure (31). For shortfinned eel, these parameters were estimated as  $b = 2.91$  and  $n = 5$ .

The slope from the regression equation was used to generate a standardized, allometric eel length:

$$AEM = L^{2.91}$$

where total length  $L$  is measured in meters. This model provides the best possible standardization for body size, given that it is sex-, size-, and location-specific to the fish used in this study. Standardization was achieved by dividing whole-animal or organ lipid or transcript data by the AEM. Use of ELF-I as a normalizer was not possible for ovarian data from experiments 1 and 2 (ANOVA indicated that mRNA levels differed significantly between developmental stages); therefore, total RNA was used as a normalizer instead. Given our interest in the total capability to accrue lipids, most of our data are not normalized over ELF-I, but rather, presented as a whole-organ mRNA copy number.

Data were evaluated for normality using P-P plots and Shapiro-Wilk analysis and for homogeneity of variance using Levene's test. Wild eel data were analyzed using Student's 2-tailed  $t$ -test. Data from experiment 2 were analyzed using one-way ANOVA (for this purpose, all controls were grouped together), followed by Hochberg's posthoc tests to account for the largely different sample sizes among groups and by Games-Howell test to account for lack of homogeneity of variance.

*Experiments 3 and 4.* Eels used in experiment 3 had comparable body weights, obviating the need to apply AEM corrections. Data were tested for normality and homogeneity of variance (as above), and following normalization over the ELF-I copy number, analyzed by ANOVA to compare treatment means. Likewise, in vitro data from experiment 4 were analyzed following normalization over ELF-I by ANOVA, using treatment (fixed variable) and fish (random variable) in the model.

## RESULTS

### LPL cDNA sequence analysis

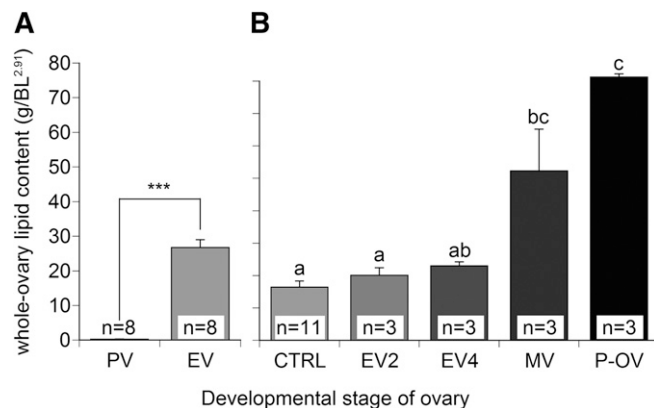
A partial cDNA, obtained by nested PCR, of 339 bp (accession no. HM775157) could be translated into a deduced amino acid sequence of 113 residues that corresponded to residues 143–255 of zebrafish LPL (*Danio rerio*; NP\_571202). The deduced amino acid sequence included two of the three sites that make up the catalytic triad, and the respective amino acids were 100% conserved. The deduced partial protein further contained a predicted lipid-binding site with extremely high conservation (15/18 residues) compared with the human ortholog, and it retained the proposed apoCII binding site (Lys-196 in zebrafish). Using neighbor-joining phylogenetic analysis (data not shown), homology with LPL orthologs proved higher than that with other lipases. Sequence identity ranged from 82% in teleost fish, such as rainbow trout (*Oncorhynchus mykiss*; accession no. AAK69707) and zebrafish (NP\_571202) to around 70% in mammals (e.g., human AAH11353 and rat NP\_036730).

### Experiment 1: Lipid content, LPL mRNA levels, and LPL enzyme activity in the ovary and liver of wild shortfinned eels

**Ovary.** With the initiation of puberty, the ovary increased in relative size (GSI) from  $0.2 \pm 0.03\%$  in PV eels in the chromatin nucleolus or early oil droplet stage to  $3.1 \pm 0.11\%$  ( $P < 0.01$ ) in EV eels. Over the same period, the lipid content of the whole ovary increased approximately 100-fold ( $t = -14.94$ ,  $df = 7.9$ ,  $P < 0.05$ ) in the EV ovary (Fig. 1A) of eels that were of standardized, equal size (i.e., AEM-corrected). This dramatic increase in ovarian lipid content coincided with a significant ( $t = -10.33$ ,  $df = 12.8$ ,  $P < 0.05$ ) 30-fold increase in the whole-organ ovarian LPL mRNA copy number by the time vitellogenesis had started (Fig. 2A). When the LPL mRNA copy number was expressed per mg ovary, this increase remained significant, although the magnitude of the change (2-fold) was much lower (data not shown).

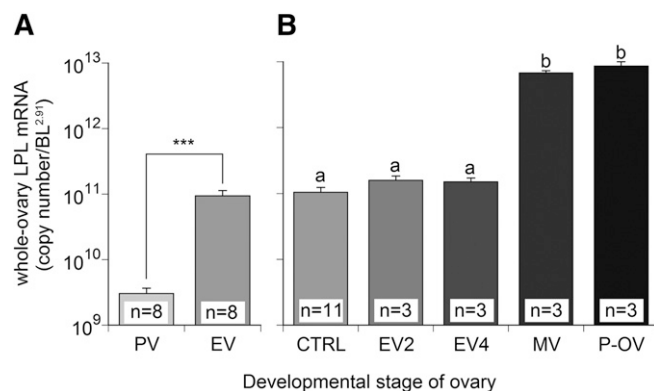
Total LPL activity in the ovary displayed a profile with developmental stages very similar to lipid content and transcript levels, LPL activity being about 30-fold greater ( $t = -4.67$ ,  $df = 6$ ,  $P = 0.003$ ) in wild EV eels ( $98.29 \pm 42.22$  mU LPL/ $BL^{2.91}$ ) than in PV eels ( $3.1 \pm 1.43$  mU LPL/ $BL^{2.91}$ ) (Fig. 3A). This large increase reflects both increases in organ size and absolute activity, as seen by the 3-fold increase in LPL activity expressed per mg ovary in EV compared with PV fish (data not shown).

**Liver.** Albeit not as dramatic as the increase seen in the ovary, there was about 40% more lipid ( $t = -2.56$ ,  $df = 14$ ,  $P = 0.02$ ) in the liver of EV eels ( $1.88 \pm 0.17$  g/ $BL^{2.91}$ ) than in that of PV eels ( $1.33 \pm 0.12$  g/ $BL^{2.91}$ ) (Fig. 4A). Although an increase in total liver lipid was observed, there was no significant difference in liver LPL mRNA transcript abundance, regardless of whether this was expressed as whole-organ message ( $t = 0.511$ ,  $df = 14$ ,  $P = 0.617$ ) (Fig. 5A),

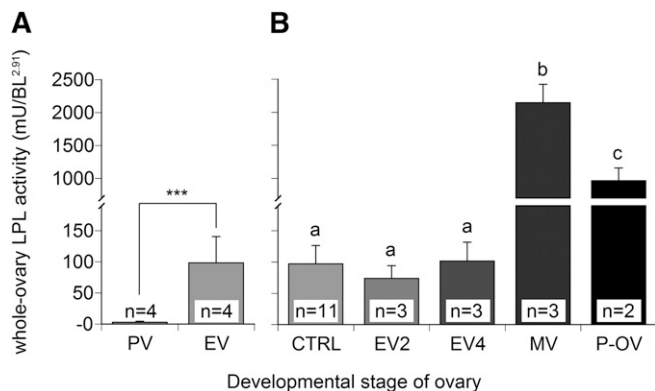


**Fig. 1.** Total ovarian lipid content in eel, *Anguilla australis*, at different stages of reproductive development. Eels were either collected from the wild (A) or repeatedly treated with salmon pituitary homogenate or Ringer solvent (CTRL, Ringer solvent controls; sampled at different time points, but analyzed collectively as controls did not differ over time) to induce gonadal development (B). Data are standardized for body length using an allometric correction ( $BL^{2.91}$ , body length; see text for details). All data are presented as means  $\pm$  standard error and sample sizes are given at the base of each bar. Different letters above bars indicate significantly different treatment means ( $P < 0.05$ ). Asterisks indicate that differences are significant at  $P < 0.001$ . Abbreviations: EV, early vitellogenic after 2 (EV2) or 4 (EV4) weeks of pituitary homogenate treatment; MV, midvitellogenic; P-OV, postovulatory ovary; PV, previtellogenic.

analyzed per mg tissue, or normalized over ELF-I (data not shown). Furthermore, there was no difference in hepatosomatic indices between the developmental stages (data not shown).



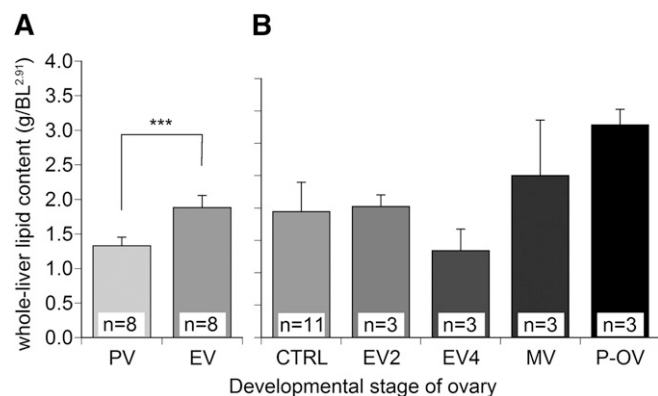
**Fig. 2.** Total ovarian mRNA copy number of lipoprotein lipase (LPL) in eel, *Anguilla australis*, at different stages of reproductive development. Eels were either collected from the wild (A) or repeatedly treated with salmon pituitary homogenate or Ringer solvent (CTRL, Ringer solvent controls; sampled at different time points, but analyzed collectively as controls did not differ over time) to induce gonadal development (B). Data are standardized for body length using an allometric correction ( $BL^{2.91}$ , body length; see text for details). All data are presented as means  $\pm$  standard error and sample sizes are given at the base of each bar. Different letters above bars indicate significantly different treatment means ( $P < 0.05$ ). Asterisks indicate that differences are significant at  $P < 0.001$ . Abbreviations: EV, early vitellogenic after 2 (EV2) or 4 (EV4) weeks of pituitary homogenate treatment; MV, midvitellogenic; P-OV, postovulatory ovary; PV, previtellogenic.



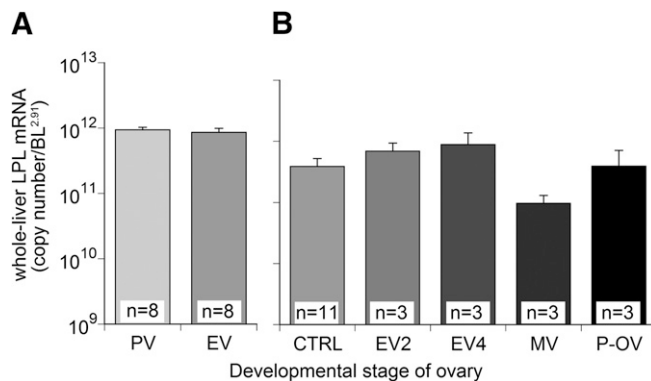
**Fig. 3.** Total ovarian activity of apolipoprotein-CII-dependent lipoprotein lipase (LPL) in eel, *Anguilla australis*, at different stages of reproductive development. Eels were either collected from the wild (A) or repeatedly treated with salmon pituitary homogenate or Ringer solvent (CTRL, Ringer solvent controls; sampled at different time points, but analyzed collectively as controls did not differ over time) to induce gonadal development (B). Data are standardized for body length using an allometric correction ( $BL^{2.91}$ , body length; see text for details). All data are presented as means  $\pm$  standard error and sample sizes are given at the base of each bar. Different letters above bars indicate significantly different treatment means ( $P < 0.05$ ). Asterisks indicate that differences are significant at  $P < 0.001$ . Abbreviations: EV, early vitellogenic after 2 (EV2) or 4 (EV4) weeks of pituitary homogenate treatment; MV, midvitellogenic; P-OV, postovulatory ovary; PV, previtellogenic.

### Experiment 2: Lipid content, LPL mRNA levels, and LPL enzyme activity in the ovary and liver of artificially maturing shortfinned eel

**Ovary.** As oogenesis advanced from the EV stage, the ovary amassed significant amounts of lipid ( $f = 18.175$ ,  $df = 4.18$ ,  $P < 0.05$ ). This accumulation of lipid into the



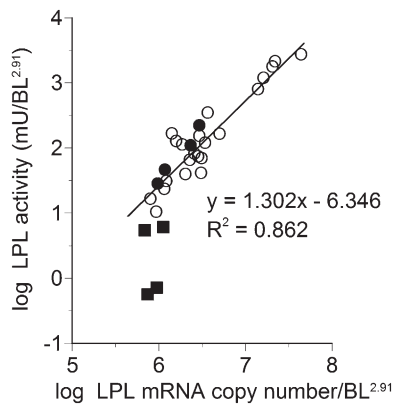
**Fig. 4.** Total hepatic lipid content in eel, *Anguilla australis*, at different stages of reproductive development. Eels were either collected from the wild (A) or repeatedly treated with salmon pituitary homogenate or Ringer solvent (CTRL, Ringer solvent controls; sampled at different time points, but analyzed collectively as controls did not differ over time) to induce gonadal development (B). Data are standardized for body length using an allometric correction ( $BL^{2.91}$ , body length; see text for details). All data are presented as means  $\pm$  standard error and sample sizes are given at the base of each bar. Asterisks indicate that differences are significant at  $P < 0.001$ . Abbreviations: EV, early vitellogenic after 2 (EV2) or 4 (EV4) weeks of pituitary homogenate treatment; MV, midvitellogenic; P-OV, postovulatory ovary; PV, previtellogenic.



**Fig. 5.** Total liver mRNA copy number of lipoprotein lipase (LPL) in eel, *Anguilla australis*, at different stages of reproductive development. Eels were either collected from the wild (A) or repeatedly treated with salmon pituitary homogenate or Ringer solvent (CTRL, Ringer solvent controls; sampled at different time points, but analyzed collectively as controls did not differ over time) to induce gonadal development (B). Data are standardized for body length using an allometric correction ( $BL^{2.91}$ , body length; see text for details). All data are presented as means  $\pm$  standard error and sample sizes are given at the base of each bar. Abbreviations: EV, early vitellogenic after 2 (EV2) or 4 (EV4) weeks of pituitary homogenate treatment; MV, midvitellogenic; P-OV, postovulatory ovary; PV, previtellogenic.

ovary was particularly pronounced around the MV and P-OV stages, when in both instances an approximately 50% increase in the amount of lipid was observed compared with the preceding reproductive stage. The P-OV stage ovary contained on average five times the amount of lipid found in control fish ovaries (Fig. 1B). On attaining the MV stage, total ovarian LPL mRNA transcript abundance increased dramatically ( $f = 56.26$ ,  $df = 4.18$ ,  $P < 0.05$ ) to a level 45-fold greater than that in the advanced EV stage (EV4) and that in control fish (Fig. 2B). This high transcript abundance was maintained or slightly increased ( $\sim 20\%$ ), albeit not significantly, in the P-OV-stage ovary. When expressed per mg ovary, the timing and significance of this increase in LPL mRNA levels remained ( $f = 21.567$ ,  $df = 4.18$ ,  $P < 0.05$ ), but the magnitude of the increase at MV was less pronounced (14-fold increase), and transcript abundance in the P-OV ovary was lower than that in the MV ovary (data not shown). Changes in whole-organ LPL transcript abundance with development were closely mirrored by changes in LPL activity (Fig. 3B); indeed, as much as 86% of the variation in LPL activity could be explained by variation in LPL mRNA levels (Fig. 6).

**Liver.** An increasing trend in total liver lipid content was noted with the reproductive stage (Fig. 4B), but the effects were not significant ( $f = 1.26$ ,  $df = 4.18$ ,  $P = 0.322$ ). Likewise, the hepatic LPL mRNA copy number did not differ between reproductive stages when expressed on a whole-organ basis (Fig. 5B); however, when expressed as copy number per mg wet weight, a significant decrease was observed when fish reached the MV stage (data not shown).



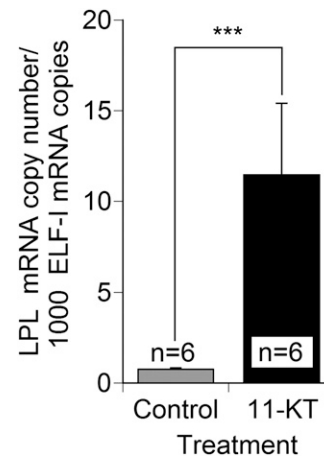
**Fig. 6.** Relationship between whole-ovary lipoprotein lipase (LPL) mRNA levels and whole-ovary LPL activity in eels, *Anguilla australis*, from the wild (■, previtellogenic eel; ●, early vitellogenic eel) and eels repeatedly treated with salmon pituitary homogenate or Ringer solvent (○) and sampled in different stages of gonadal development. Data are standardized for body length using an allometric correction ( $BL^{2.91}$ , body length; see text for details) and presented following  $^{10}\log$  transformation. The regression equation is based on all data points, except the previtellogenic eels.

### Experiment 3: Effects of 11-KT on ovarian and liver LPL mRNA levels and lipid content in vivo

One of the fish in the 11-KT treatment group died of an unknown cause; however, the remaining animals appeared healthy and vigorous. Administration of 11-KT in vivo resulted in a significant increase ( $t = 4.56$ ,  $df = 5.42$ ,  $P = 0.005$ ) in ovarian LPL transcript abundance, 11-KT treated eels ( $0.012 \pm 0.004$  copies LPL per copy ELF-I) displaying a 10-fold increase in the LPL copy number compared with control fish [ $1.1 \times 10^{-3} \pm 0.4 \times 10^{-3}$  copies LPL per copy ELF-I; this increase was even more pronounced when one observation, statistically identified as an outlier, was removed (adjusted mean of control group:  $8 \times 10^{-4} \pm 0.7 \times 10^{-4}$  copies)] (Fig. 7). In the same fish, 11-KT had no significant impact ( $t = -0.90$ ,  $df = 11$ ,  $P = -0.387$ ) on hepatic LPL mRNA transcript abundance (Fig. 8).

### Experiment 4: Effects of 11-KT on ovarian LPL mRNA levels in vitro

An 18-day dose response experiment (11-KT at 0, 1, 10, 100, and 1,000 nM) showed an interesting, although not significant ( $f = 1.82$ ,  $df = 4.19$ ,  $P = 0.167$ ) trend, as in vitro ovarian LPL mRNA transcript abundance increased in parallel with the 11-KT dose. Maximum transcript abundance was observed at a physiological dose (100 nM) while at 1,000 nM, transcript levels were equivalent to control (0 nM) incubations (data not shown). Additional in vitro incubations, all on different fish, in the presence or absence of 100 nM 11-KT resulted in somewhat inconsistent observations; addition of 11-KT resulted in increases in LPL mRNA levels by day 18 compared with controls, which was significant in one experiment (experiment 4.2;  $t = -2.45$ ,  $df = 10$ ,  $P = 0.034$ ) (see Fig. 9) but not quite so in another experiment run for three days (experiment 4.3;  $t = -0.197$ ,  $df = 9$ ,  $P = 0.08$ ; data not shown).

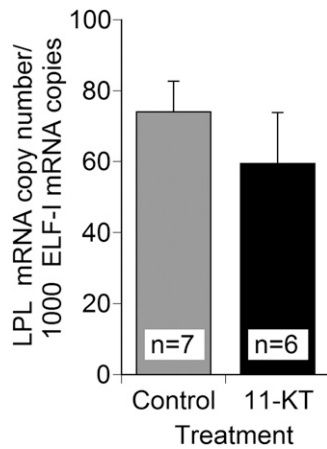


**Fig. 7.** Mean ovarian mRNA copy number of lipoprotein lipase (LPL) in eel, *Anguilla australis*, following a two-week treatment with slow-release implants with or without (CTRL) 2.5 mg 11-ketotestosterone (11-KT). Transcript abundance is normalized over that of elongation factor-1 $\alpha$  (ELF-I). All data are presented as means  $\pm$  standard error; sample sizes are given at the base of each bar. Asterisks indicate that differences are significant at  $P < 0.001$ .

## DISCUSSION

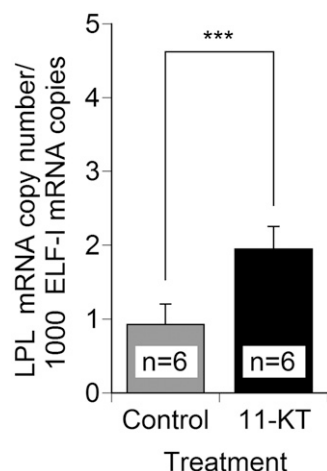
Egg lipids provide the building blocks for membranes and help meet the energy demands of the developing embryo and larva, yet very little is known about the key periods during which these lipids are accumulated and the key mechanisms responsible for their accumulation. We addressed these issues by field sampling and in vivo and in vitro experimentation using the eel, an animal model that accumulates large amounts of egg lipids during prolonged periods of natural fasting. These lipids, whether phospholipids, TAGs, or wax esters, all contain fatty acid moieties, and their assembly in the ooplasm is likely to depend in considerable part on FFA uptake. Thus, our focus in the present study centered on the activity and (mRNA) abundance of LPL, the rate-limiting enzyme in the hydrolysis of TAGs into FFA. Accordingly, we have exposed a very strong correlation between ovarian lipid content and both LPL activity and LPL mRNA levels. Moreover, we have demonstrated a novel role for the androgen 11-ketotestosterone in regulating LPL mRNA levels in the eel ovary.

A partial cDNA was cloned and found to greatly resemble LPL orthologs from a variety of species, the deduced amino acid sequence corresponding more closely with LPL than with LPL2 (red sea bream, BAB20996; data not shown). Moreover, several key amino acid residues involved in enzyme activity (32) and a string of residues associated with lipid binding (33) were highly conserved. Lastly, Lys-196 (equivalent to human Lys-148) was found in the eel-deduced amino acid sequence. Bruin et al. (34) have implicated this residue in binding to apoCII, although no such association was recognized by Goldberg and Merkel (35). Regardless, together these observations strongly indicate that the cloned cDNA does indeed encode part of the eel LPL ortholog.



**Fig. 8.** Mean liver mRNA copy number of lipoprotein lipase (LPL) in eel, *Anguilla australis*, following a two-week treatment with slow-release implants with or without (CTRL) 2.5 mg 11-ketotestosterone (11-KT). Transcript abundance is normalized over that of elongation factor-1 $\alpha$  (ELF-1). All data are presented as means  $\pm$  standard error; sample sizes are given at the base of each bar.

We used the partial LPL cDNA sequence to quantify mRNA levels to allow changes in expression to be tracked. A remarkable increase in whole-organ ovarian LPL transcript abundance was seen in EV eels compared with those in PV when data were standardized over animal size. This increase coincided with a comparable increase in ovarian LPL activity, indicating that LPL mRNA levels are a suitable proxy for LPL activity. This was further reinforced by the great increase in stored lipid by two orders of magnitude, an increase that was reflected by the presence of large numbers of lipid droplets in the cytoplasm of EV oocytes. Hormonal treatment with SPH successfully advanced gonadal development (36, 37) and led to dramatic increases in stored lipid in both the MV and P-OV stages.



**Fig. 9.** Mean ovarian mRNA copy number of lipoprotein lipase (LPL) in eel, *Anguilla australis*, after incubation in vitro with or without 100 nM 11-ketotestosterone (11-KT) for 18 days. Transcript abundance is normalized over that of elongation factor-1 $\alpha$  (ELF-1). All data are presented as means  $\pm$  standard error; sample sizes are given at the base of each bar. Asterisks indicate that differences are significant at  $P < 0.001$ .

These increases were again reflected in increased LPL activity and mRNA levels, primarily in the P-OV stage, reinforcing that LPL is likely a key enzyme in lipid uptake in the ovary during oogenesis. This hypothesis is supported by the notion that apoCII-dependent lipase (LPL) activity was, on average, around 2.5-fold higher (i.e., around 70% of total TAG hydrolysis in the ovary) than that of apoCII-independent lipase activity (see the LPL activity assay in “Materials and Methods”; data not shown).

A recent study on sea bass (*Dicentrarchus labrax*) showed a temporal pattern with regard to LPL transcript abundance very similar to that of the eel, except for the rise in LPL activity in prespawning fish (26). In eel, as in rainbow trout (*Oncorhynchus mykiss*), LPL activity was highest in midvitellogenesis (38), closely matched by peak LPL transcript abundance (39). Of particular interest is the notion that ovarian LPL mRNA levels were over 30-fold greater in MV and P-OV stages than in the PV stage in both eel (this study) and sea bass (26), whereas only a 3- to 4-fold increase was seen in rainbow trout (39). It is plausible that this reflects the greater total lipid content (% dry weight) and/or the timing of lipid uptake in the eggs of marine representatives (e.g., Japanese eel, ~40% (40); sea bass, ~31% (41)) compared with that of freshwater-dwelling rainbow trout (23% in trout fed commercial feed (42)).

To compare treatment means, we standardized all animals to a uniform length. Accordingly, whole-organ lipid content, LPL activity, and LPL mRNA abundance could be adequately compared by statistical tests. Indeed, the enormous increase in organ size (~100-fold) during sexual development emphasizes that a several-fold change in lipid content, LPL activity, or LPL transcript abundance per g of ovary is of little consequence if the change in organ size (and thus, whole-organ capacity) is not considered when evaluating the implications (43). We believe that our approach has effectively addressed this important issue.

Total lipid uptake and LPL mRNA levels in the ovary changed dramatically during the (induced) reproductive cycle, peaking during midvitellogenesis, as has also been reported for sea bass (see Ref. 26). A second, less dramatic, increase in LPL activity and transcript abundance was seen when comparing EV with PV eels. However, as only PV fish in the chromatin nucleolus and early oil droplet stages were sampled in this study, it remains unclear whether LPL activity and transcript abundance are consistently higher in EV than in PV fish, or whether a discrete peak occurs during a particular PV stage. Regardless, the changes observed in the ovary bear no resemblance to those in the liver, where no notable changes in LPL mRNA levels associated with the reproductive cycle were observed; thus, changes in LPL mRNA levels appear to be highly tissue-specific.

Captivity is known to have profound impact on reproductive development in the eel; indeed, it is generally accepted that gonadal development is arrested when peri- or postpubertal eels are held in captivity and that, in fact, regression of ovarian follicles can occur (37). Dufour et al. (44) proposed that this arrest is caused by dopaminergic inhibition of gonadotropin-releasing hormone secretion

and action. Our present data suggest that captivity and fasting effects are not restricted to the endocrine system but that they also affect lipid storage, as seen by the reduction in ovarian lipid content in experimental control eels, both over time and compared with eels captured from the wild in the same year and the same location (Fig. 1A versus 1B). While this may be intuitive—fasting will require burning of stored fuels for survival—it is also somewhat surprising. Indeed, the duration of fasting was modest in eel terms (up to four months), and preferential use of carcass lipid over ovarian lipid (lots of which can be stored in adipocytes within the ovary of shortfinned eel (45)) might have been expected, but this did not occur (comparable decreases in ovary and carcass lipid stores were observed) (S. L. Divers, unpublished observations).

The real revelation of this study is the remarkable increase in LPL mRNA levels that were seen after treatment with 11-KT, both in vivo and in vitro. Some caution in interpreting the in vitro data should be exercised, however, as only one out of three experiments yielded a significant effect of androgen on LPL transcript abundance. This is likely to reflect differences in incubation duration (18 days for experiments 4.1 and 4.2 and 3 days for experiment 4.3) and an inherent variability in responses from in vitro studies, possibly related to (i) the use of chemically defined but incomplete media; (ii) the exposure to temperature changes prior to the start of incubation (on/off ice, etc.); and (iii) variation between individual females. Notwithstanding these cautions, as far as we know, this is the first study in which insights into the regulation of the LPL gene in the ovary have been observed in a teleost fish. In eutherian mammals, which evidently do not provision their eggs in the same way as oviparous vertebrates, hormonal effects on LPL transcript abundance have also been demonstrated; in this taxon, both androgens (46) and estrogens (47) decreased triglyceride uptake and/or LPL activity in adipose and hepatic tissue, respectively. In yellow perch (*Perca flavescens*), E2 treatment likewise reduced LPL mRNA levels in the liver (48). This seems different from our findings in the eel, in which estrogens, at least in vitro, were not effective in altering LPL transcript levels in the ovary (one in vitro trial; data not shown), reinforcing the notion that regulation of LPL expression is tissue-dependent.

Increases in LPL transcript abundance in response to androgen complement our earlier observations on androgen actions in the eel ovary. We have previously shown (17, 18) that androgens can induce increases in oocyte diameters and increases in accumulation of radiolabeled triolein or VLDL-associated lipids in the eel ovary in vitro. These observations are in general agreement with earlier findings on the effects of slow-release androgen-implanted eels in vivo (16). The present data suggest that the mechanism responsible for the increase in lipid content in the ovary following exposure to androgen involves, at least in part, an increase in LPL transcript abundance, probably due to increased rates of LPL gene transcription. The link between lipid content or composition and egg quality in teleost fish is well-documented. It is tempting to speculate

that egg quality could be a reflection of the LPL expression pattern in the ovary during oogenesis—a hypothesis that deserves being put to the test in future studies. ■

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