Triacylglyceride physiology in the shortfinned eel, *Anguilla australis* – the effects of androgen.

Abbreviated title: Eel triacylglyceride physiology – 11-KT

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Abbreviations: 11-ketotestosterone (11-KT), androgen receptor alpha (Ar-α/ar-α), androgen receptor beta (Ar-β/ar-β), apolipoprotein B (Apob/apob), apolipoprotein E (ApoE/apoe), fatty acid transport protein (Fatp/fatp), lipoprotein lipase (Lpl/lpl), low density lipoprotein receptor (Ldlr/ldlr), microsomal triacylglyceride transfer protein (Mttp/mttp), triacylglyceride (TAG), very-low density lipoprotein (VLDL), vitellogenin receptor (Vtgr/vtgr).
ABSTRACT

The importance of androgens (especially 11-ketotestosterone) during previtellogenesis in eels is well established. In wild pubertal migrants, circulating 11-ketotestosterone levels correlate with a number of morphological and molecular changes. Here, we test the prediction that this correlation represents a causal relationship by artificially raising the levels of circulating 11-ketotestosterone in pre-pubertal non-migratory female and pubertal, migratory male shortfinned eels (Anguilla australis) using sustained-release hormone implants. In females, increases in hepatosomatic index and transcript copy numbers of hepatic apolipoprotein B and microsomal triacylglyceride transfer protein indicated increased re-packaging of endogenously sourced triacylglycerides. These changes in liver measures were reflected in increased concentrations of serum triacylglycerides. However, despite a small increase in gonadosomatic index, ovarian lipoprotein receptor transcript abundances were not affected by 11-ketotestosterone. Interestingly, no such changes in hepatic gene expression were detected in a dose-response experiment using males. We propose that the androgens are inducing the observed changes in previtellogenic females, although it remains unclear to what extent these effects are direct or indirect.

Supplementary key words: 11-ketotestosterone, androgen receptor, apolipoprotein B, apolipoprotein E, fatty acid transport protein, lipoprotein lipase, low density lipoprotein receptor, microsomal triacylglyceride transfer protein, time-course experiment, triacylglyceride, vitellogenin receptor, very-low density lipoprotein
1. INTRODUCTION

The confirmed existence of an ovarian androgen receptor in various vertebrate clades (human (4), rat (43), rhesus monkey (51), pig (3), fish (e.g., Atlantic croaker (1) and shortfinned eel (24))) has fueled support for the notion that low concentrations of androgens are important for the normal progression of oogenesis (34, 49). Indeed, primordial and preantral follicles, the mammalian equivalents of fish previtellogenic oocytes (oocytes in meiotic arrest as found in pre-pubertal fish), have been shown to respond to androgen treatments. Treatment with testosterone was shown to double the number of growing follicles of all classes in rhesus monkeys (15) and to cause as much as a six-fold increase in the number of preantral follicles in humans (50). Furthermore, testosterone administration induced multiple ovarian cysts and increased the number of small follicles in primates (48). Despite the convincing evidence for a role of androgens in increasing ovarian follicle numbers, high levels of androgens in mammalian females are generally associated with lowered reproductive success (i.e., polycystic ovary syndrome (10, 35), premature ovarian failure (18, 41), uterine hyperplasia (42, 45)) and in extreme cases, total infertility (16, 37).

In wild early-pubertal migrant eels (Anguilla australis), serum concentrations of 11-ketotestosterone (11-KT) are naturally high, reaching between 20-60 ng/ml in females (26) and 50-100 ng/ml in males (28). The increase in androgen concentration occurs simultaneously with an array of morphological and molecular changes such as increased gonad and oocyte size. There is compelling experimental evidence to suggest that these correlations are, in fact, causal, because several of these morphological changes can be readily induced in eels by androgen treatment, both in vivo (increased ovary size: 29, 36) and in vitro (increased oocyte size: 11, 24). Studies in other species of fish have provided similar morphological results; androgen treatment increased oocyte size in salmon (Oncorhynchus kisutch: 12) and cod (Gadus morhua: 20) in vitro and in cod in vivo (21), although it failed to induce the same changes in hapuku in vivo (Polyprion oxygeneios: 19).

Further adding support to the involvement of androgens in reproduction of female fish is recent
research that clearly shows that *in vitro* co-treatment of 11-KT with triacylglyceride (TAG) rich very-low density lipoprotein (VLDL) resulted in significant oil droplet accumulation in oocytes from *Anguilla japonica* and *Anguilla australis* (11 and 7, respectively) – this suggests that 11-KT plays an important role in lipid (namely TAG) transfer and/or accumulation into oocytes. An involvement in other aspects of lipid physiology might be anticipated on the basis of these findings. Indeed, increases in mRNA levels of hepatic lipid packaging genes as well as genes involved in ovarian lipid uptake correlate closely with the natural increase in 11-KT in wild-caught *A. australis* (6). However, with the exception of ovarian lipoprotein lipase (*lpl*) expression, which increases both with androgen treatment and as oogenesis progresses (9), the underlying genetic mechanisms through which these androgens exert their effects are yet to be determined. In keeping with a role for androgens in increasing ovarian *lpl* mRNA levels, these steroids are likely to affect TAG physiology in liver and ovary to co-ordinate the movement of these resources from one depot to another. To test this prediction, 11-KT concentrations in previtellogenic female and pubertal male were artificially elevated using sustained-release hormone implants and the relative expression of the hepatic genes involved in lipid uptake (*lpl*; apolipoprotein e, *apo*e) and packaging (apolipoprotein b, *apo*b; microsomal triacylglyceride transfer protein, *mttp*) as well as ovarian lipid uptake (*apo*e; *lpl*; fatty acid transport protein-1, *fatp1*; vitellogenin receptor, *vtgr*; low density lipoprotein receptor, *ldlr* – females only) was analyzed. The concentrations of 11-KT, cholesterol and TAGs in the serum were also examined. Additionally, *in situ* hybridization was used to visualize the localization of *apo*b and *apo*e at the mRNA level and immunohistochemistry to visualize Apob at the protein level. The mRNA levels of both androgen receptors (alpha (ar-α) and beta (ar-β)) were also examined in both tissues with the aim to elucidate the mechanism of 11-KT action.
2. METHODS

2.1 Animal collection and husbandry

All fish were captured in fyke nets in Lake Ellesmere (see: 26 for details on location and capture) and transported to our fish keeping facilities. They were held in recirculating freshwater tanks (5-6 eels / 200 L) on ambient autumn photoperiod (12L:12D) and temperature (10-14°C). Fish were given one week to acclimate before the experiment commenced. Fish were not fed for the duration of the experiments, eliminating exogenous TAG interference. All manipulations were approved by the University of Otago Animal Ethics Committee, in line with national guidelines defined in the Australian and New Zealand Council for the Care of Animals in Research and Teaching.

2.2 Experiment I: Effects of 11-KT on TAG physiology in females

Seventy-two non-migrant (oil droplet stage) female eels (body weight = 636 ± 19 g) were anesthetized (0.15 g/L benzocaine) and implanted intraperitoneally with sustained-release pellets containing cholesterol and cellulose (placebo controls) or with 1 mg of 11-KT added to the matrix (see 27 for details). Implantation was followed by six terminal samplings, the time between each sampling being tripled, i.e. 3 hr after implantation, 9 hr, 27 hr, 81 hr, 243 hr (10 days) and 729 hr (30 days) after implantation. At each sampling, six controls and six 11-KT treated fish were euthanized (0.30 g/L benzocaine). Prior to implantation, a further six fish were euthanized to gain baseline data. Total length and weight were recorded before the tail was removed and the blood was collected from the caudal vein for radioimmunoassay and cholesterol and TAG assays (see section 2.4). Total liver and ovary weight were recorded in order to calculate somatic indices (organ weight/body weight: hepatosomatic index; gonadosomatic index). A portion of the liver and ovary were snap-frozen in liquid nitrogen and stored at -70 °C until required for qPCR analysis (section 2.5). Hepatic tissue was also fixed in 4 % paraformaldehyde for in situ hybridization and immunohistochemistry (section 2.6).
2.3 Experiment II: Effects of 11-KT on TAG physiology in males

In order to investigate differences in TAG physiology between the sexes and eliminate any role the ovary itself may play in driving any changes in TAG packaging; a second experiment was carried out using males. The experimental design is explained in detail in Lokman et al. (23). Briefly, 25 male migrant eels (body weight = 122 ± 18 g; testes with early Type B spermatogonia) were anesthetized and implanted intraperitoneally with sustained-release pellets (see Section 2.2) that did, or did nor, contain 11-KT (0, 0.01, 0.03, 0.10 or 0.30 mg). Fish were euthanized four weeks after implantation and total length and total weight were recorded. Hepatosomatic index was calculated as above and a portion of liver tissue was snap-frozen for qPCR analysis (section 2.5).

2.4 Blood analysis

Blood samples were allowed to clot and then centrifuged at 4 °C and 1000 g for 10 min before the serum was aspirated and stored at -70 °C.

2.4.1 Radioimmunoassay

Serum samples from 11-KT treated female fish were diluted 100 fold and volumes of 30 µl from both 11-KT treated fish and placebo control fish triple-extracted in 1 ml diethyl ether exactly as described by Damsteegt et al. (6) and following the protocol documented in Lokman et al. (26). Samples were spread across two assays, the intra-assay co-efficient of variation was 0.044 (n = 10) and 0.054 (n = 10) respectively, whilst the inter-assay co-efficient of variation was 0.026 (n = 2). The minimum detectable level was 0.30 ng/ml and recovery was between 87% and 89%. No corrections were made for recovery as percentages were deemed to be adequately high.

2.4.2 Cholesterol and TAG assays

Total serum cholesterol and triacylglycerol concentrations were measured by enzymatic assay using CHOD-PAP and GPO-PAP reagents (Roche) as described by Damsteegt et al. (6).

2.5 Quantitative polymerase chain reaction
Primer sequences are listed in either Damsteegt et al. (6) or Setiawan et al. (39). All qPCRs were carried out exactly as explained in Damsteegt et al. (8) and in keeping with the guidelines for the minimum information for publication of quantitative real-time PCR experiments (MIQE (2)). If all samples for one target gene could not fit on a single plate, the six initial controls were re-run on subsequent plates as quality controls. The inter-assay co-efficient of variation was less than 0.13 (n = 2) for each target gene. Efficiencies of amplification ranged from 94 % to 105 % for the different target genes. Two housekeeping genes (eel elongation factor one alpha, **eef1α**; ribosomal protein L36, **l36**) were used to normalize target gene copy numbers after statistical analyses showed that neither were significantly effected by androgens (c.f. (38)).

2.6 In situ hybridization and immunohistochemistry

Localization of Apob/*apob* at the mRNA (**in situ** hybridization) and protein (immunohistochemistry) levels as well as *apoe* at the mRNA level was carried out exactly as described by Damsteegt et al. ((8): Apob/*apob*) and Damsteegt et al. ((6): *apoe*). Analysis was performed on the six control and six 11-KT treated fish from the first and final sampling points (3 hr and 729 hr after implantation). Results from a typical (average) individual were captured using an Olympus BX51 microscope coupled to an Olympus SC100 camera.

2.7 Statistics

All statistics were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software). Firstly, data were tested for normality and equal variances using the Shapiro-Wilk and Bartlett’s tests, respectively. Data were log-transformed when these assumptions were not met (i.e. levels of 11-KT in the serum, GSI, transcript copy number of hepatic and ovarian *lpl* and ovarian *fatp1*), then re-checked to confirm normality and equal variances. Two-way ANOVAs (time after implantation*treatment) were carried out to identify any differences between group means in Experiment I. Where interaction and/or time effects were significant, one-way ANOVAs followed
by post-hoc Tukey comparisons were carried out. Where treatment effects were significant,
unpaired two-tailed T-tests with Bonferroni adjustments (i.e. $p: 0.05/6 = 0.0083$) were performed
for each control and the corresponding treatment group at each individual time point to avoid Type I
error associated with multiple testing. One-way ANOVAs were carried out to determine if 11-KT
dose had an effect on males in Experiment II, significant contrasts being identified using Tukey
post-hoc comparisons.

3. RESULTS

3.1 Experiment I: Effects of 11-KT on TAG physiology in females
All fish remained healthy throughout the experiment and implant wounds were no longer visible by
the cessation of the experiment.

3.1.1 Radioimmunoassay
The intraperitoneal insertion of sustained-release 11-KT pellets successfully raised the levels of 11-
KT in the serum (Figure 1). A significant difference was detected after as little as 3 hr ($t = 7.291$, $df$
= 10, $p < 0.0001$). Levels remained high and significantly different from the controls for the
duration of the experiment (for all time points: $t > 5.368$, $p < 0.0001$).

3.1.2 Cholesterol and triacylglycerides
No changes in the concentrations of cholesterol in the serum were detected between any of the
control and 11-KT treated groups at any of the different sampling time points (Figure 2A). The
concentrations of TAGs in the serum significantly increased with 11-KT treatment after 81 hr ($t =
2.245$, $df = 10$, $p < 0.05$) and stayed elevated for the remainder of the experiment (243 hr (10 days):
t = 2.775, $df = 10$, $p < 0.05$; 729 hr (30 days): $t = 2.388$, $df = 10$, $p < 0.05$) (Figure 2B).

3.1.3 Somatic indices
Significant increases were found in both the HSI ($F_{5,30} = 6.214$, $P < 0.0005$) and GSI ($F_{5,30} = 7.504$,
P < 0.0001) in the latter stages of 11-KT treatment (Figure 3A and B). After 243 hr (10 days),
significant increases in relative liver weight were evident in the 11-KT treated eels (t = 2.117, df = 10, p < 0.05); with continued treatment, this enlargement became more prominent such that after 729 hr (30 days), livers in the 11-KT treated fish were more than 30 % heavier than their control counterparts (t = 5.113, df = 10, p < 0.001). A significant difference in gonad size was found after 729 hr (30 days), the GSI of the 11-KT treated eels being more than three times that in the control group (t = 5.113, df = 10, p < 0.001).

3.1.4 Target gene transcript abundance in the liver

In females, significant increases in the transcript copy numbers of both lipid packaging genes were evident in the liver of 11-KT treated fish (Figure 4A and B). The transcript copy number of mttp in the liver of 11-KT treated fish increased steadily from 9 hr after implantation until it peaked at 81 hr (F5,29 = 8.522, P < 0.0001), at which time mRNA levels were significantly higher in 11-KT treated fish than in control fish (t = 2.933, df = 10, p < 0.05). Relative mttp transcript copy numbers then decreased until reaching levels similar to those in control fish after 729 hr (30 days). Transcript copy number of apob increased after 243 hr (10 days) (t = 3.638, df = 10, p < 0.001) and remained significantly higher (t = 1.985, df = 10, p < 0.05) than those in control fish until the completion of the experiment. Transcript copy numbers of apoe in the livers of 11-KT treated fish remained similar to levels in control fish for the majority of the experiment until significantly increasing after 243 hr (10 days) (t = 6.363, df = 10, p < 0.0001) and remaining high until 729 hr (30 days) (t = 2.954, df = 10, p < 0.05) (Figure 4C). No differences were found between lpl mRNA levels in the livers of 11-KT treated and control eels, nor were any trends present (data not shown). Transcript copy numbers of ar-α were significantly elevated in the liver of 11-KT treated fish after just 3 hr (t = 2.350, df = 10, p < 0.05) and remained significantly higher until 81 hr post implantation (t = 3.364, df = 10, p < 0.001; Figure 4D). Thereafter, ar-α expression remained elevated in the androgen-treated fish, but hepatic transcript abundance was no longer different than that in the controls. The mRNA levels of ar-β were also significantly higher in the livers of 11-KT treated fish, although not until after 243 hr treatment (Figure 4E).
3.1.5 Target gene transcript abundance in the ovary

Whilst the ovarian transcript copy numbers of *apob* did not change during the experimental period, those of *apoe* in the ovary of 11-KT treated fish spiked significantly after 729 hr (30 days) (*t* = 5.754, *df* = 10, *p* < 0.001) (Figure 5A and B). Transcript copy numbers of *lpl* in the ovary of 11-KT treated fish increased from 27 hr and continued to increase for the duration of the experiment (Figure 5C). Significantly higher transcript copy numbers of *lpl* were seen in the ovary by 243 hr (10 days) (*t* = 3.078, *df* = 10, *p* < 0.05) with an even greater increase by 729 hr (30 days) (*t* = 5.572, *df* = 10, *p* < 0.001). Levels of *fatp1* mRNA in the ovary were not significantly different between controls and treated fish but an overall downward trend was seen in both the control and 11-KT treated eels throughout the time-course experiment (data not shown). Transcript copy numbers of *ldlr* in the ovary of 11-KT treated fish appeared to decrease when compared to the controls but the only significant difference was observed by 81 hr (*t* = 3.534, *df* = 10, *p* < 0.05) (Figure 5D). The mRNA levels of *vtgr* in the ovary remained constant throughout the treatment with 11-KT (data not shown). Similarly, no differences in transcript copy numbers of *ar-α* were found either between control and 11-KT treated fish or over time (data not shown). In contrast, after 243 hr, levels of *ar-β* were significantly higher (*t* = 2.281, *df* = 10, *p* < 0.05) in 11-KT treated fish than in controls and levels remained higher by 729 hr.

3.1.6 In situ hybridization and immunohistochemistry

*In situ* hybridization localized mRNA of both *apob* and *apoe* in the cytoplasm of the hepatocytes (Figure 6A-D). For both apolipoproteins, staining appeared darker in the tissue collected from the 11-KT treated eels than the control eels by 729 hr (30 days) after implantation. No difference was found 3 hr after implantation with 11-KT or between control time points. A similar trend was detected at the protein level for Apob, the immunohistochemical staining being slightly darker in the liver from 11-KT treated eels (Figure 6E and F). No non-specific staining was visible on any of the sections incubated with either sense cRNA probe. Likewise, control slides incubated without Apob antiserum or anti-rabbit IgG showed no signal.
3.2 Experiment II: Effects of 11-KT on TAG physiology in males

All eels were in good condition throughout the experiment with the exception of two fish from the 0.1 mg 11-KT group which escaped from their tanks and could no longer be used.

3.2.1 Somatic indices

A significant increase in GSI (F_{4,18} = 4.430, P < 0.05) was observed after treatment with both 0.10 and 0.30 mg of 11-KT. No change was detected in relative liver weights of male eels (data not shown).

3.2.2 Target gene transcript abundance in the liver

No changes in transcript abundances (apob, mttp, apoε, lpl, ar-α and ar-β) were identified in the livers of males, nor were any trends present (data not shown).

4. DISCUSSION

This study aimed to elucidate whether androgens regulate the changes in hepatic TAG packaging and ovarian TAG uptake potential that are seen as residential feeding eels transition into fasting, migratory silver eels (see: 6). This was done by artificially elevating the levels of 11-KT using sustained-release hormone implants and analyzing the concentrations of cholesterol and TAGs in the serum, together with the transcript abundances of ar-α, ar-β, apob, mttp, apoε, lpl, fatp1, ldlr and vtgr in either liver and/or ovary. In doing so, support was found for our prediction that androgens affect TAG packaging and uptake potential, reflected in changes in mRNA levels of several of the target genes. Accordingly, it appears that treatment with 11-KT can partially mimic the changes in TAG physiology that are associated with the pubertal transition of previtellogenic into early vitellogenic eels seen in the wild.
Levels of 11-KT in the serum of wild female silver eels are typically around 20-60 ng/ml (26) and in males can be as high as 110 ng/ml (28). The hormone implants used in this study successfully raised the levels of 11-KT in females, albeit to a potentially supra-physiological level (100-150 ng/ml), after just three hr and this level was sustained throughout the duration of the experiment (averaging 130 ng/ml after 30 days). Previous studies (25, 35, 39, 40) have successfully elevated the levels of 11-KT in the serum of eels using this same approach, but no time-course of hormone levels was provided. Thorarensen and colleagues (44) carried out a time-course experiment in trout, amongst others analyzing the levels of 11-KT in the serum after implantation of silastic capsules containing 11-KT. However, in their experiment, the first blood sample was taken one week after implanting so no indication was given of how soon levels were elevated. Also, there was notable fluctuation in the recorded levels of 11-KT throughout the experiment, indicating that the implants used in our study are more reliable to quickly elevate and then sustain high and stable levels of 11-KT.

Increases in HSI and transcript copy number of *apob* and *mttp* similar to those identified in wild-caught eels entering vitellogenesis (see: 6) were observed in androgen-treated female eels. These treatment-induced changes were mirrored by increased Apob/apob staining at both the protein and transcript levels after immunohistochemistry and *in situ* hybridization, respectively. Whilst an increase in HSI has been reported in several species of fish following androgen treatment (goldfish (13), catfish (30), trout (5), eels (36) and grouper (53)), this is the first report of an androgen-induced increase, whether direct or indirect, in transcript copy number of hepatic *apob* in fish. Unlike female eels, however, there was no increase in HSI or hepatic *apob* in males; the males used in this study had already undergone silvering (i.e. were ready to migrate) and any changes in liver function may have already been completed. Alternatively, the effects seen in females could be specifically related to mobilization of endogenously stored TAGs that are needed to meet the increased neutral lipid demands of the ovary, but this requires further investigation.
We assumed that the increase in transcript abundances of \textit{apob} and \textit{mttp} in the liver would result in an increase in TAG packaging which, in turn, would be matched by an increase in ovarian TAG uptake – thus leaving TAG concentrations in serum unchanged. However, serum concentrations of circulating TAGs increased significantly. This suggests that the liver is packaging TAGs and releasing them as VLDL at a rate faster than at which the ovary and/or other tissues can sequester them. This suggestion is reinforced by ovarian gene expression profiles; thus, whilst increases in GSI and transcript copy numbers of \textit{lpl} in the ovary were observed (results identical to those previously reported by Divers \textit{et al.} (9)), the transcript abundances of the receptors likely to be involved in TAG uptake (\textit{vtgr} and \textit{ldlr} see: (7)) remained unchanged. Closer examination of the GSI in the 11-KT-treated fish (approximately 1 \%) revealed that it was notably lower than the GSI recorded in early vitellogenic stage wild-caught fish (approximately 3 \%), quite possibly indicating a lower overall-organ lipid uptake potential and thus, a corresponding increase in TAGs in serum of experimentally manipulated animals.

The use of cholesterol in the matrix of sustained-release hormone pellets has been an invalidated area of caution for some years due to the potential for the cholesterol to exert its own effects independent of the incorporated steroid. Of particular importance to this study is the role cholesterol plays in steroid production and its association with the high density lipoproteins. In this experiment, the concentrations of circulating cholesterol in the serum did not change regardless of treatment or the length of time during which the implant remained inside the fish. Indeed, the sustained-release pellets could be easily retrieved from the body cavity of euthanized fish, presumably reflecting the very low solubility of cholesterol in aqueous environments and confirming the compound’s suitability for use in the matrix of sustained-release hormone pellets.
The lack of change in cholesterol concentrations following (non-aromatizable) androgen treatment in this study opposes previous research which has shown that treatment with (aromatizable) testosterone increased levels of circulating cholesterol (32, 52). Similar to the increase seen in wild-caught fish, an increase in cholesterol would most likely be associated with an increase in the high density lipoproteins (HDL and vitellogenin). Indeed, several previous studies have reported the ability of androgens to induce vitellogenin synthesis in the liver of several species of fish (goldfish (13), European eel (33), rainbow trout (31) and fathead minnow (14)). However, the ability of androgens to induce the ovarian uptake of vitellogenin and the downstream formation of yolk granules remains controversial (i.e. the results could be due to the androgens being aromatized to estrogen, a well-documented inducer of vitellogenin synthesis, or to administration of supra-physiological doses inducing non-specific effects). Thus, an early study reported fully matured oocytes in the Japanese eel after 17-MT treatment (22); however, yolk granules were not detected in shortfinned eels after treatment with 11-KT (36) or 17-MT (27). Although it can not be determined without specific histological and/or biochemical investigations, the lack of an increase in cholesterol seen in this study likely reflects a failure to induce hepatic vitellogenin synthesis in these animals.

The lack of change in any of the above-mentioned parameters over time in control fish indicates that the observed effects are likely to be androgen-induced. Indeed, the androgen receptor has been identified in both the liver and the ovary of eels (46) suggesting that direct effects of androgens are possible. Further support for this notion comes from the results of this study which showed that mRNA levels of both androgen receptors were higher in the livers of 11-KT treated female fish than in control fish. However, culture of primary hepatocytes in vitro (47) did not yield any effects of 11-KT on transcript abundance of apob and apoe. This latter finding indicates that the increased hepatic transcript copy numbers of apob and apoe seen in this experiment may not be a direct effect.
Recent in vitro work has shown that androgen treatment can increase the size of shortfinned eel previtellogenic oocytes (7, 24) and induce oil droplet accumulation in the ooplasm of Japanese eel oocytes (11), indicating direct effects. As demonstrated for the liver (above), in the ovary, too, ars are expressed. Moreover, ar-β, but not ar-α, responded to 11-KT by increasing its mRNA abundance, similar results to those previously reported in A. Australis (39). In contrast, no change in transcript levels of either receptor was detected following testosterone treatment in A. japonica (17). Further, few changes in the expression of the genes usually associated with lipid uptake were detected in this study. Whilst the large increase seen in lpl expression indicates 11-KT may be able to regulate Lpl-mediated hydrolysis, it is unlikely that this mechanism is solely responsible for the observed increase in GSI. Previous research has heavily implicated the Ldlr in oocyte lipid accumulation (7), yet, it appears that the expression of this receptor and that of the Vtgr gene is androgen-independent.

We set out to investigate whether the changes in lipid physiology that occur during the silvering transformation in eels are mediated by androgens. Here, we show that 11-KT treatment results in increased expression of its own receptors in both the liver and the ovary. Associated with these increases, whether directly or through another mediator(s), is an increase in expression of hepatic genes involved in lipid packaging (apob, apoe and mttp), probably leading to increased levels of TAG (and presumably, VLDL) in the blood stream. Increased levels of VLDL, in turn, may well contribute to meeting the animal’s energy needs (maintenance, migration). Moreover, increased levels of VLDL, in combination with 11-KT, can drive increased lipid accumulation in the oocyte (7), a likely reflection of increased lpl and apoe gene expression. Androgens, thus, appear to be important drivers of altered lipid physiology that may be essential for both migration and reproduction in anguillid eels.
5. PERSPECTIVES AND SIGNIFICANCE

We have shown that the notable changes in energy budget that are associated with the peri-
migrational period in the eel life cycle and that command a switch from exogenous to endogenous
sourcing of TAGs, can in part be mimicked by androgen treatment of previtellogenic eels in vivo.

Despite the naturally high levels of 11-KT circulating in the serum of wild-caught early vitellogenic
stage fish, it is not clear at this point whether these effects stem from direct or indirect androgen
action; regardless, this observation, together with the dramatic effects of androgens on lpl mRNA
levels in vivo and on oocyte cytology in vivo and in vitro, implicate androgens as major regulators
of TAG physiology in eels. Whether these findings are particular to members of the Anguillidae or
have application across the Teleostei infraclass remains to be revealed.

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8. FIGURES

**Figure 1**: Mean concentrations of 11-ketotestosterone (11-KT) in the serum of female *Anguilla australis* after treatment with intraperitoneal sustained-release hormone implants containing 0 (○) or 1 mg 11-KT (■). N = 6 for each group. **** indicates a significant difference between control and treatment groups at individual time points at *P* < 0.0001. Error bars show ± one standard error of the mean.

**Figure 2**: Mean concentrations of cholesterol (A) and triacylglycerides (B) in the serum of female *Anguilla australis* after treatment with control (○) or 1 mg 11-ketotestosterone (■) intraperitoneal sustained-release hormone implants. N = 6 for each group. * indicates a significant difference between control and treatment groups at individual time points at the < 0.05 level. Different letters indicate significant differences in 11-KT treated fish over time. Error bars show ± one standard error of the mean.

**Figure 3**: Mean hepatosomatic (A) and gonadosomatic (B) indices of female *Anguilla australis* after treatment with control (○) or 1 mg 11-ketotestosterone (■) intraperitoneal sustained-release hormone implants. N = 6 for each group. * and *** indicate significant differences between control and treatment groups at individual time points at the < 0.05 and < 0.001 levels respectively.
Different letters indicate significant differences in 11-KT treated fish over time. Error bars show ± one standard error of the mean.

Figure 4: Mean transcript copy numbers of microsomal triacylglyceride transfer protein (mttp) (A), apolipoprotein B (apob) (B), apolipoprotein E (apoE) (C), androgen receptor alpha (ar-α) (D) and androgen receptor beta (ar-β) (E) in the liver of female Anguilla australis after treatment with control (□) or 1 mg 11-ketotestosterone (■) intraperitoneal sustained-release hormone implants. N = 6 for each group. *, **, *** and **** indicate a significant differences between control and treatment groups at individual time points at the <0.05, <0.01, <0.001 and < 0.0001 level. Different letters indicate significant differences in 11-KT treated fish over time. Error bars show ± one standard error of the mean.

Figure 5: Mean transcript copy numbers of apolipoprotein B (apob) (A), apolipoprotein E (apoE) (B), lipoprotein lipase (lpl) (C), low density lipoprotein receptor (ldlr) (D) and androgen receptor beta (ar-β) (E) in the ovary of Anguilla australis after treatment with control (□) or 1 mg 11-ketotestosterone (■) intraperitoneal sustained-release hormone implants. N = 6 for each group. * and ** indicate significant differences between control and treatment groups at individual time points at the < 0.05 and < 0.01 levels respectively. Different letters indicate significant differences in 11-KT treated fish over time. Error bars show ± one standard error of the mean.

Figure 6: Micrographs of sections through the liver of female eels, Anguilla australis, after 729 hrs treatment with control (A, C, E and G) or 1 mg 11-ketotestosterone (B, D, F and H) intraperitoneal sustained-release hormone implants. Tissue was subjected to in situ hybridization using antisense
cRNA probes specific for either apolipoprotein B (A and B) or apolipoprotein E (C and D), an immunohistochemistry reaction with apolipoprotein B antiserum (E and F) or stained with hematoxylin and eosin (G and H). Scale bar = 50 μm.
**Graph:**

Title: 11-KT (ng/ml) vs. Time after implantation (hr)

- X-axis: Time after implantation (hr)
- Y-axis: 11-KT (ng/ml)

-_y-axis ticks: 0, 1, 10, 100, 1000

- Data points for time points 0, 3, 9, 27, 81, 243, 729

- Each time point has a bar chart with error bars indicating variability.

- Significant differences are marked with asterisks: ****