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1 **Sertoli Cell Proliferation in the Adult Testis Is Induced by Unilateral Gonadectomy in**
2 **African catfish**

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20 Financial support: ECP was supported by Consejería de Educación y Cultura, CARM (Erasmus Program
21 grant) and AGL by the Ramón Areces Foundation (Spain) during their stay in Utrecht
22
23 Abbreviations: 11-KT, 11-ketotestosterone; OHA, 11β-hydroxyandrostenedione; 5-bromo-2’-
24 deoxyuridine, BrdU; amh, anti-müllerian hormone; cga, glycoprotein hormones, alpha polypeptide;
25 fshb, follicle stimulating hormone, beta polypeptide; fshr, follicle stimulating hormone receptor;
26 gnrhr, gonadotropin releasing hormone receptor; lhb, luteinizing hormone, betapolypeptide; lhcgr,
27 luteinizing hormone/choriogonadotropin receptor; PE, pituitary extract; rtqPCR, real-time
28 quantitative PCR; A_{und}, single undifferentiated spermatogonium type A; ULG, unilateral gonadectomy
29

Abstract

Survival and development of male germ cells depends on their close contact with Sertoli cells. In the cystic spermatogenesis found in fish, one germ cell clone, initially a single undifferentiated spermatogonium type A, is enclosed by and accompanied through spermatogenesis by a group of Sertoli cells. Previous work showed that after forming such spermatogenic cysts, Sertoli cells proliferated mainly during the mitotic expansion of the spermatogonial clone in the cyst. Here, we used unilateral gonadectomy (ULG) as experimental model to study Sertoli cell proliferation at the start of cyst development in adult African catfish testis. Four days after surgery, we observed a particularly strong increase in the number of mitotic Sertoli cells along with a significant increase in the number of mitotic single type A spermatogonia. Proliferation of pairs of spermatogonia or of larger germ cell clones, however, did not change. At the same time, pituitary transcript levels of the three gonadotropin-subunits (*cga,* glycoprotein hormones, alpha polypeptide*; fshb,* follicle stimulating hormone, beta polypeptide*; lhb,* luteinizing hormone, beta polypeptide) were not different between sham-operated and ULG males. However, expression of the gonadotropin-releasing hormone receptor gene *gnrhr1* was significantly reduced after ULG, and Lh plasma levels were slightly elevated. In the testis remaining after ULG, Fsh receptor (*fshr)* mRNA levels increased significantly but luteinizing hormone/choriogonadotropin receptor (*lhcgr)* mRNA levels did not change. Circulating androgen levels did not differ between groups, but testicular androgen release increased significantly 2- to 3-fold after ULG. Considering the strong steroidogenic potency of Fsh and the expression of the *fshr* gene by Leydig cells in catfish, we explain the absence of an effect of ULG on circulating androgen levels by an Fshr-mediated, compensatory increase in the steroid production of the remaining testis, perhaps supported in addition by the increased Lh plasma levels. Since Fsh is a major stimulator of mammalian Sertoli cell proliferation, we propose that ULG-induced activation of the Fsh signalling system also promoted Sertoli cell proliferation and – possibly as a consequence of that – proliferation of single type A spermatogonia, providing the basis for an increased spermatogenic capacity.

Keywords: unilateral gonadectomy, compensatory response, testicular androgen secretion, Sertoli cell proliferation, Fsh signalling

1. Introduction

Sertoli cells play a pivotal role in the development and functioning of the vertebrate testis [40,46,51]. In mammals, Sertoli cells proliferate until puberty [15,21,51], and their numbers stabilize during the first wave of spermatogenesis when the number of primary spermatocytes increases [16,32,39]. These events coincide with the formation of the Sertoli cell barrier, tubular lumen that forms as a result of increased fluid production by Sertoli cells, mainly under the influence of androgens in adult animals, and an elaborated cytoskeleton, jointly constituting morphological and functional markers of Sertoli cell differentiation [16,39]. The constant contact and support that Sertoli cells provide is critical for germ cell survival and development during spermatogenesis. Since each Sertoli cell supports a certain, species-specific number of germ cells during spermatogenesis in mammals and fish [15,29,33,41, 25], the Sertoli cell number per testis ultimately dictates the magnitude of testis size and sperm production [15,22,33]. In mammals, the pituitary gonadotropin follicle-stimulating hormone (FSH) is a major stimulator of the proliferation of Sertoli cells [32,50], until they attain a non-mitotic state during puberty [9,23].

In fish, spermatogenesis takes place in cysts within the seminiferous tubules. A spermatogenic cyst forms when one or two Sertoli cells enclose a single undifferentiated spermatogonium type A (A_{und}) [46]. These initial spermatogenic cysts can either be in a quiescent state, for example during the resting period between seasonal waves of spermatogenesis, or self-renew to produce more single type A_{und} spermatogonial cysts, a state frequently observed during the initial phase of the seasonal testis growth period. Alternatively, instead of self-renewing, a single type A_{und} spermatogonium in a cyst can differentiate and expand by successive mitotic divisions to form an isogenic, synchronously developing germ cell clone interconnected by cytoplasmic bridges. After a species-specific number of mitotic divisions, germ cell differentiation continues via meiosis and spermiogenesis, i.e. the 'metamorphosis' of the haploid spermatids emerging from meiosis into flagellated spermatozoa. During development and until spermiation, when the cysts open to release the spermatozoa into the lumen of the spermatogenic tubules, the germ cell clone is bordered by the cytoplasmic extensions of a single layer of Sertoli cells. Hence, in cystic spermatogenesis (observed in fish and amphibians), a given Sertoli cell is usually in contact with only a single germ cell clone that is accompanied throughout the spermatogenic process. In adult amniote vertebrates (reptiles, birds

and mammals), on the other hand, any given Sertoli cell is in contact with different germ cell clones (hence being in different stages of spermatogenesis) along its basal, lateral, and apical surface area.

Previous work on Sertoli cell proliferation in adult fish showed that the main increase in Sertoli cell number per cyst occurred during the period of mitotic proliferation of spermatogonia when germ cell number and hence cyst size rapidly increased [5,25,29,47]. In tilapia, Sertoli cell proliferation stopped with the establishment of tight junctions between Sertoli cells, coinciding with the completion of meiosis [4]. However, little information is available with regard to Sertoli cell proliferation at the beginning of the spermatogenic process when cysts are formed. The formation of new cysts usually takes place at the start of seasonal testis growth, e.g. after the winter solstice in Atlantic salmon [54], or after the summer solstice in Atlantic cod [2], or is a continuous process in species with continuous spermatogenesis after puberty, such as in African catfish [47] and zebrafish in captivity [25]. A single spermatogonium A_{und} and one or two Sertoli cells constitute the initial cyst. Considering the strict dependency of germ cell survival on Sertoli cell support, we speculate that during the formation of new cysts, the initial step is to generate additional germ cell support capacity via Sertoli cell proliferation; newly formed spermatogonia A_{und} would then find the support guaranteeing their survival.

Searching for a possibility to study Sertoli cell proliferation at the beginning of cyst development, we have used unilateral gonadectomy (ULG) or hemicastration in adult African catfish, a species showing continuous spermatogenesis after puberty in captivity. ULG was developed as an experimental model to study prepubertal Sertoli cell proliferation in mammals and testis growth in fish, and it was shown that ULG resulted in a compensatory growth response of the remaining testis in rainbow trout [38], and in several studies in mammals, such as in rat [12] and in rhesus monkey [34]; in mammals, ULG usually is most effective when applied before puberty. Here, we focused on the examination of the short-term response of the remaining testis to ULG by analysing proliferative activity, gene expression levels, and *in vitro* androgen release in samples collected 4 days after surgery together with analysing pituitary mRNA levels and circulating androgen and luteinising hormone (Lh) levels.

2. Materials and Methods

2.1. Experimental animals, sampling, and surgery

African catfish were bred and raised in the laboratory as described before [48]. Fish culture and experimentation was consistent with the Dutch national regulations; the Life Science Faculties Committee for Animal Care and Use has approved all experimental protocols.

For the present study, 12 months old adult males were used. An initial blood sample was taken from all fish. Anaesthesia and surgery were carried out as described previously [13], except that only one testis was removed. In sham-operated animals, the body wall was opened and closed but both testes were left in place. Fish were euthanized by decapitation for sampling 4 days after the surgical procedure. Three different experiments were carried out, each one involving 6 sham-operated and 6 ULG animals. In the first experiment, proliferation in testis tissue was evaluated using 5-bromo-2'-deoxyuridine (BrdU; Sigma, St. Louis, MO) as marker. In the two other ULG experiments, blood samples were collected before decapitation to quantify Lh and androgen (11-ketotestosterone, 11-KT and testosterone, T) plasma levels. In one of these two experiments, testis tissue was collected and used for a short-term testis tissue culture experiment to quantify the release of the androgen 11β-hydroxyandrostenedione (OHA) under basal and pituitary extract-stimulated conditions. In the other experiment, pituitary, testis, and seminal vesicle tissue samples were collected for the quantification of expression levels of selected genes by quantitative PCR. In African catfish the seminal vesicle, which is not homologous to seminal vesicles in mammals, develops from the caudal part of the testis as several finger-like protrusions with lumina that communicate with those of the seminiferous tubules and the efferent duct [27]. The tunica propria of these protrusions contains cells similar to Leydig cells [56] that produce steroids [42]. The single-layered, columnar epithelium lining the lumen is derived from Sertoli cells [27].

1 *2.2. Sertoli and germ cell proliferation analysis*

2 On the morning of the fourth day after surgery, sham-operated and ULG fish received an
3 injection of 50 mg BrdU per kg body weight. Six hours later, a tissue slice from the cranial third of the
4 testis was immersed in Bouin's fixative where the picric acid concentration was lowered by using
5 warm (40°C) saturated, instead of boiling saturated, picric acid solution. After overnight fixation at
6 room temperature, the tissue was dehydrated in graded alcohol and embedded in paraffin wax,
7 according to conventional techniques.

8 For the immunocytochemical detection of BrdU on 5 µm paraffin tissue sections of African
9 catfish testis, the cell proliferation kit RPN20 was used according to the manufacturer's guidelines
10 (Amersham Biosciences; Piscataway, NJ). After BrdU detection, the sections were counterstained
11 with haematoxylin.

12 We then quantified the number of BrdU-positive cells at 300-fold magnification in testes of
13 sham-operated animals and in the remaining testis after ULG. To this end, 3 sections separated by at
14 least 100 µm were used from each male (n = 6 males per treatment group). Three view fields were
15 analysed per section, and the number of BrdU-positive Sertoli cells representing one animal was
16 calculated by dividing the sum of BrdU-positive Sertoli cells in the sections of a given male by 9. The
17 number of BrdU-positive, single type A spermatogonia, pairs, or larger groups of spermatogonia was
18 counted in a similar manner. The results are expressed as the number of BrdU-positive cells (Sertoli
19 cells or single type A_{und} spermatogonia) or the number of spermatogonial groups per field at 300-fold
20 magnification.

21
22 *2.3. Short-term catfish testis tissue culture*

23 Testis tissue was collected 4 days after surgery and mixed into two independent pools (sham-
24 operated and ULG, respectively) that served to study basal and pituitary extract-stimulated androgen
25 release as described previously [48]. In brief, tissue was chopped into fragments of ~2 mm^3 in Petri
26 dishes containing Earle's balanced salt solution pH 7.2 (M199 EBSS), supplemented with HEPES (0.02

M), penicillin G, and streptomycin (100 units/ml each; all compounds from GIBCO-Life Technologies, Grand Island, NY, USA). To remove tissue debris and suspended sperm, the fragments were filtered over two layers of medical gauze, re-suspended in fresh medium, incubated for 15 minutes in a metabolic shaker (30 cycles per minute at 25°C), and filtered again over medical gauze. Randomly selected fragments (4-6 fragments per well with a total wet weight of 25-40 mg) were placed in the wells of a 24-well culture plate (Costar, Cambridge, MA, USA) in 1 ml of medium. Six replicates were incubated with a pituitary extract (PE) from adult African catfish that was calibrated for its Lh content [48]; the PE also contained Fsh but an assay for catfish Fsh is not available. The Lh concentrations used were 0, 10, 100, and 1000 ng/ml medium. After 18 hours of incubation at 25°C, the medium was collected, heated for 1 hour at 80°C, and centrifuged at 15,000 xg for 15 minutes at 4°C. The supernatants were stored frozen until quantification of androgen levels.

2.4. Hormone quantification

The main androgen produced by African catfish testis tissue is OHA [57]. A RIA developed for OHA [44] served to quantify its levels directly from heat-treated incubation media [48]. The standard curve ranged from 2,000 to 10 pg per tube. The results were expressed as ng of OHA released per milligram of testis tissue incubated.

In African catfish, the main androgen produced by testis tissue, OHA, is converted to 11- KT, the main circulating androgen in fish [8], in liver and seminal vesicle tissue [11]. Catfish testis tissue also releases T [57], a steroid important for the feedback regulation of pituitary gonadotroph cells [36] and hypothalamic Gnrh neurones [14]. Therefore, established RIA systems [44], adjusted to African catfish blood samples, were used to quantify T and 11-KT in blood plasma samples, as described previously [48].

Circulating Lh levels were quantified with a RIA system using intact African catfish Lh as iodinated tracer and for the standard curve (ranging from 1,250 to 25 pg per tube), and an antiserum against the Lh, beta polypeptide [49].

All hormone samples that were statistically compared with each other were analysed in a single assay, thus excluding inter-assay variation. The intra-assay coefficient of variation ranged between 2 and 9% for the different hormones and standard curve concentrations.

2.5. Quantification of mRNA levels

Pituitaries and testes were dissected and immediately flash frozen in liquid nitrogen. RNA was isolated using the Fast RNA Pro Green kit (Bio 101 Systems; MP Biomedicals, The Netherlands), according to the manufacturer's recommendations, and cDNA was synthesized as described previously [37]. Real-time quantitative PCR (rtqPCR) was used to determine the pituitary levels of the two gonadotropin releasing hormone receptor (Gnrhr) type mRNAs, *gnrhr1* and *gnrhr2*, respectively [7] and the three gonadotropin subunits (*lhb*, *fshb*, *cga*) [37,59], and the testicular and seminal vesicle mRNA levels of the Lh receptor (*lhcgr*) [58] and the Fsh receptor (*fshr*) [6], using the $\Delta\Delta$Ct method. A detailed description of the rtqPCR procedure has been given previously [6,17]. Primers and fluorogenic probes (Table 1), specific for the mRNAs mentioned above, or for the endogenous control (catfish *28s* rRNA), were designed with Primer Express software (Applied Biosystems, Foster City, CA), according to the manufacturer's guidelines, as described previously [26], and were purchased from Applied Biosystems. The PCR efficiency and whether the relationship between Ct and log of the starting copy number was linear were tested for all primer/probe sets using 10-fold dilution series of cDNA. For all primer/probe sets, the slope of the standard curves was close to -3.32, and the correlation coefficients were close to unity over four orders of magnitude, indicating maximal PCR amplification.

2.6. Data analysis

Data were analysed using GraphPad PRISM (version 4.0, GraphPad Software, San Diego, CA). Normally distributed data (Dallal-Wilkinson test) were analysed using one-way ANOVA with Tukey's *post hoc* tests for multiple groups, and with the Student t test for two groups. Non-Gaussian data were analysed using a non-parametric test (Mann Whitney test). All data in figures and tables are presented as mean $\pm$ SEM. The significance level considered was $P < 0.05$.

1 **3. Results**

2 After surgery, the animals recovered within ~3 min from anaesthesia. All animals were
3 feeding the next morning and there was no surgery-related loss of animals. Four days after surgery,
4 the single testis remaining after ULG was more hydrated than the testes of sham-operated animals.
5 When tissue slices were prepared with a razor blade to generate samples for morphological or gene
6 expression analysis, more liquid was released from the remaining testis after ULG.
7

8 *3.1. Proliferation*

9 Sertoli cells in the S-phase of the cell cycle (i.e. BrdU-positive) were present in all testis
10 samples analysed. Similar to our previous observations [47], we found that many of the BrdU-
11 positive Sertoli cells were observed in association with BrdU-negative spermatogonia (Fig. 1).
12 Analysing samples 4 days after ULG showed that the number of proliferating Sertoli cells had
13 increased strongly (14-fold) in the remaining testis, compared with testis from sham-operated males
14 (Fig. 2). Also for BrdU-positive single type A spermatogonia (Fig. 1, arrowheads), we recorded a
15 significant 2.5-fold increase in their number when compared with sham-operated males (Fig. 2). The
16 numbers of more advanced stages of the spermatogonial population (pairs or larger groups of
17 spermatogonia) showed no statistically significant changes (Fig. 2).
18

19 *3.2. Endocrine Data*

20 Just prior to surgery, plasma hormone levels were not significantly different between sham-
21 operated control and ULG fish (data not shown). Although one testis was removed surgically,
22 circulating androgen levels showed only minor reductions ($P > 0.05$) four days after surgery while the
23 Lh plasma levels had increased slightly but significantly in the ULG group (Fig. 3). Testicular androgen
24 production in primary tissue culture, on the other hand, was significantly different between the
25 treatment groups. Tissue from the remaining testis after ULG released 2- to 3-fold more androgen
26 than testis tissue from sham-operated controls (Fig. 4), both in the absence and in the presence of
27 pituitary extract. Œstradiol-17β remained below the limit of detection (10 pg/100 µl of medium) in
28 all cases (data not shown).

3.3. Gene expression levels

In the pituitary, the mRNA levels of all three gonadotropin subunits were slightly but not significantly reduced in the ULG group (Fig. 5). The mRNA levels of the two *gnrhr* behaved differently: *gnrhr1* mRNA levels were significantly reduced in the pituitary after ULG, while there was no change in the *gnrhr2* mRNA levels. Since the PCR efficiencies of the two *gnrhr* mRNAs are similar and the analysis of both *gnrhr* mRNA expression levels was performed in the same experiment, comparison of the Ct-values (data not shown) indicated that *gnrhr1* mRNA was ~30-fold more abundant in the pituitary than *gnrhr2* mRNA.

Gonadotropin receptor expression was quantified in testicular and seminal vesicle tissue. Expression of the *lhcgr* gene was not different between the sham-operated and ULG groups in both testis and seminal vesicle tissues (Fig. 6). The levels of *fshr* mRNA, on the other hand, had increased significantly after ULG in testicular and, even more clearly, in seminal vesicle tissue.

4. Discussion

The response to ULG at the testicular level included a strong stimulation of Sertoli cell proliferation, an elevated proliferation of single type A spermatogonia, an up-regulation of *fshr* mRNA levels and of the androgen release capacity. With respect to the pituitary, we observed moderately elevated plasma Lh levels and a decrease of *gnrhr1* mRNA levels. These multiple compensatory responses of the reproductive axis to ULG indicate that this model constitutes an interesting experimental tool for studying regulatory mechanisms driving spermatogenesis in fish, especially with regard to early stages. This model had been used previously in male fish, but the available information is restricted to long-term morphological aspects of the compensatory growth of the remaining testis [38]. For female fish, on the other hand, several studies have reported in certain detail, morphological, physiological, and molecular aspects of the compensatory responses of reproductive axis to hemi-castration (see [19]).

1 We have found that ULG resulted in an increase of average circulating Lh levels from 1 to 1.8
2 ng/ml four days after surgery. Under standard culture conditions for catfish, spermatogenesis is
3 completed but plasma Lh levels are relatively low (~1 ng/ml) [13]; the same applies to wild animals
4 during most of the year, including the period of full spermatogenesis, and only for males sampled on
5 their spawning grounds a significant ~4-fold increase in circulating Lh concentrations has been
6 recorded [43]. Similarly, plasma Lh levels in two species of salmonid fish were low or undetectable
7 and did not change until after completion of the main testicular growth period [10,20]. These
8 observations suggest a higher relevance of Lh for late spermatogenesis/spawning in male fish, while
9 it is unclear at present if the Lh increase observed in the present study has been of physiological
10 significance for the compensatory effects at the testicular level (see below). It is possible that such an
11 increase in plasma Lh levels reflects a ULG-induced increase in Gnrh-stimulated gonadotropin
12 release. Elevated *gnrh1* mRNA levels were recorded in the brain after ULG in female European sea
13 bass [11], and after exposure of coho salmon pituitary cells to Gnrh, the *gnrhr1* mRNA levels were
14 down regulated [28]. In the present study, we also have observed reduced *gnrhr1* mRNA levels
15 following ULG. Our data would be in line with assuming a stimulation of Gnrh receptors by
16 endogenous ligand that, in turn, triggered Lh but possibly also Fsh release. It is also interesting to
17 note that when administering increasing doses of Gnrh1, lower doses that did not yet induce
18 increases in plasma Lh levels , did increase plasma androgen levels [45]. This can be explained
19 assuming that release of Fsh, the other steroidogenic gonadotropin in fish (see below), responds to
20 lower Gnrh1 doses than Lh release. Unfortunately, an assay to quantify circulating African catfish Fsh
21 levels does not exist. However, ULG in female rainbow trout resulted in significantly elevated plasma
22 Fsh levels for 8 weeks following surgery [55]. Also in a primate model, ULG in males leads to a
23 sustained elevation of FSH levels [35], while plasma LH levels did not show a clear response to ULG in
24 hemicastrated pubescent male goats [1]. Taken together, it seems that with the endocrine activity of
25 the remaining testis, the pituitary response to ULG would be mainly mediated via Fsh and less so via
26 Lh.

27 There are further indications for an activation of Fsh-dependent signalling systems in catfish
28 after ULG. One of them is the strong increase in the number of proliferating Sertoli cells after ULG. It
29 is well established in mammals [15,21,34,51] that FSH is an important stimulator of Sertoli cell
30 proliferation. Also in two species of salmonid fish, plasma Fsh levels increased during the rapid

1 testicular growth phase, i.e. the main period of Sertoli cell proliferation, while plasma Lh levels were
2 low or undetectable [10,20]. In mammals FSH stimulates Sertoli cells to release the TGFβ family
3 member inhibin that exerts a negative feedback on pituitary FSH release, but respective information
4 is not available in fish.

5 While we can present only circumstantial evidence for elevated Fsh plasma levels following
6 ULG in African catfish, we have found significantly increased levels of *fshr* mRNA in testicular tissue.
7 Previous work showed that the *fshr* gene is expressed by Sertoli but also by Leydig cells in African
8 catfish [17]; Leydig cell expression will be discussed further below. Our data does not allow
9 discerning an increase in *fshr* mRNA copy number per cell from an increase in the number of *fshr*
10 mRNA expressing cells. Leydig cells can proliferate in adult male African catfish [47]. Although not
11 quantified properly, there was no obvious difference in the frequency of BrdU-labelled interstitial
12 cells between control and ULG males. On the contrary, in the light of the 14-fold increase in the
13 number of proliferating Sertoli cells, we assume that an increased number of Sertoli cells has in any
14 case contributed to the elevated *fshr* mRNA levels measured after ULG. Changes in *fshr* mRNA levels
15 were also recorded in seminal vesicle tissue, a known Fsh target tissue [6]. The columnar epithelium
16 of the seminal vesicles present in different families of catfishes is derived from Sertoli cells [27] and
17 our present results indicate that the regulation of *fshr* gene expression takes place in a similar way in
18 testis and seminal vesicle tissue. We did not study proliferation in seminal vesicle tissue, so that we
19 cannot relate *fshr* mRNA changes to possible changes in cell numbers.

20 In mammals, postnatal Sertoli cell proliferation is observed in juveniles when only
21 spermatogonia are present in the seminiferous epithelium, and ends as soon as primary
22 spermatocytes start differentiating at puberty, when also tight junctions are established between
23 neighbouring Sertoli cells [16,39]. In previous studies on adult fish, Sertoli cell proliferation also was
24 observed mainly while they were associated with spermatogonia [3,47], and Sertoli cell proliferation
25 stopped – reminiscent of the situation in mammals – when tight junctions formed among Sertoli cells
26 at the beginning of spermiogenesis [4,46]. The quantitative analysis of the changes in Sertoli cell
27 numbers per cysts during tilapia spermatogenesis [47] showed that after a new cyst had formed, the
28 first spermatogonial mitosis took place without Sertoli cell proliferation. Thereafter, the further
29 increasing cyst size was accompanied by increasing Sertoli cell numbers. This indicates that there are
30 two types of Sertoli cell proliferation: one type to generate Sertoli cells that would then associate

with type A_{und} spermatogonia to form new spermatogenic cysts, and another type to accommodate the increasing number/volume of germ cells during the growth of an existing spermatogenic cyst. The present results suggest that the ULG-induced Sertoli cell proliferation, when examined four days after surgery, mainly activated the first type of Sertoli cell proliferation leading to new spermatogenic cysts.

The response to ULG also included an increase in the proliferation of single type A spermatogonia. It is not clear if proliferation of these early spermatogonia is a direct effect of the ULG-triggered changes in the endocrine environment, or secondary to the (presumably Fsh-stimulated) increase in the number of Sertoli cells. In mice, one of the effects of FSH on Sertoli cells is to stimulate the production of glia cell-derived neurotropic growth factor (GDNF), which, in turn, stimulates self-renewal divisions of the spermatogonial stem cells [53]. In zebrafish, Fsh down-regulated anti-müllerian hormone (*amh)* mRNA levels in Sertoli cells, thereby reducing the inhibitory effect of recombinant zebrafish Amh on steroidogenesis and spermatogenesis [52]. In Japanese eel, Fsh stimulated steroid release that in turn modulated Sertoli cell growth factor production, which induced testis growth and spermatogenesis [30]. Irrespective of the mechanism(s) involved, our observations of an increased proliferation of both, single type A spermatogonia and Sertoli cells in response to ULG, would result in an increased number of the initial functional units of fish spermatogenesis, i.e. spermatogenic cysts with a single type A spermatogonium enveloped by one or two Sertoli cells. Hence, the ULG-induced proliferation response in the testis seems to result in new spermatogenic cysts, and hence an increased spermatogenic capacity of the remaining testis.

Different from higher vertebrates, Fsh does not only bind to Fsh receptors on Sertoli cells but is a potent steroidogenic hormone in fish that directly activates *fshr* mRNA-expressing Leydig cells. This has been shown in Japanese eel [31], African catfish [17], and zebrafish [18]. Indeed, Fsh was more potent as steroidogenic hormone than Lh in both catfish [60] and zebrafish [18]. Moreover, Fsh but not Lh was able to up-regulate the expression of steroidogenesis related genes in juvenile eel [24] and in adult zebrafish testis [18]. Therefore, we propose that Fsh, rather than Lh, mediated the up-regulation of the steroidogenic capacity of the testis remaining after ULG. Considering the magnitude of this up-regulation, resulting in a 2- to 3-fold higher androgen release capacity than in control tissue, we moreover propose that this increased capacity can explain the observation that circulating plasma androgen levels were not significantly different between control and ULG males.

1 Collectively, this data set suggests that removal of one testis was compensated by an up-regulation
2 of the androgen production capacity of the remaining testis to the level that allowed attaining
3 normal plasma androgen concentrations. This up-regulation could be mediated mainly by an
4 activation of Fsh/Fshr signalling, although a contribution of Lh to the increased release of androgens
5 cannot be excluded.

6 The increased hydration of the remaining testis might also be related to an activation of
7 steroidogenesis. In mammals, estrogens regulate the fluid balance in the efferent duct system
8 (epididymis) of the testis [23]. Our studies did not provide clear evidence for an involvement of
9 estrogens in the response to ULG. With the up-regulation of testicular androgen release, on the other
10 hand, and hence probably increased intratesticular androgen levels, an up-regulation of expression
11 of ion transporter proteins such as pendrin, a sodium-independent chloride/iodide transporter
12 encoded by the *slc26a4* gene, may have occurred, as has been observed in mature rainbow trout
13 testis [40 schon plus 2].

14 In summary, ULG leads to several compensatory responses of the remaining testis. Strongly
15 increased proliferation of Sertoli cells and an also elevated proliferation of single type A
16 spermatogonia indicate that newly formed Sertoli cells could associate with single type A
17 spermatogonia to form new spermatogenic cysts. Moreover, with a stimulation of the androgen
18 release capacity of the remaining testis that compensates for the loss of one testis, it seems possible
19 that the initially formed new spermatogenic cysts would be stimulated to continue differentiation,
20 thereby also elevating the sperm output of the remaining testis, as observed previously in rainbow
21 trout [38].

1 **Figure Legends**

2

3 Figure 1

4 Proliferating cells in African catfish testis tissue sections. BrdU was detected by
5 immunocytochemistry and sections were counterstained with hematoxilin. BrdU-positive single
6 spermatogonia (arrowheads) or Sertoli cells (arrows) are readily detected after ULG. Bar = 25 µm.
7 Sertoli cells that did or did not incorporate BrdU were found close to each other, suggesting that
8 different spermatogenic cysts behave as independent functional units.

9

10 Figure 2

11 Numbers of BrdU-labelled cells per view field (at 300-fold magnification) of adult male African catfish
12 testis sections prepared from animals 4 days after sham-operation or ULG. Among the
13 spermatogonia, single type A spermatogonia were differentiated from pairs, or larger groups (three+)
14 of proliferating spermatogonia. *, $P < 0.05$ (compared with the respective control value; Mann
15 Whitney test; n = 6 in both sham-operated and ULG groups).

16

17 Figure 3

18 Effect of sham-operation or ULG on plasma androgen (11-KT, 11-ketotestosterone; T, testosterone)
19 and plasma luteinising hormone (Lh) levels. All hormone concentrations are in nanogram per ml of
20 plasma. *, $P < 0.05$ (compared with the respective control value; Student t-test; n = 6 in both groups).

21

22 Figure 4

23 Effect of sham-operation or ULG on androgen (11β-hydroxyandrostenedione, OHA), release from
24 African testis tissue fragments 4 days after surgery. The tissue was exposed to catfish pituitary
25 extract containing luteinising hormone (Lh) concentrations between 10 and 1000 ng/ml. *, $P < 0.05$
26 (compared with the respective control value from sham-operated fish; Tukey test; n = 6 in all
27 groups).
28

Figure 5

Effect of sham-operation or ULG in adult male African catfish on the relative pituitary mRNA levels of the three gonadotropin subunit genes and on the two types of receptors for Gnrh peptides. *, $P < 0.05$ (compared with the respective control value; Student t-test; n = 6 in both groups).

Figure 6

Effect of sham-operation or ULG on the relative mRNA levels of African catfish follicle-stimulating hormone receptor (*fshr)* or luteinising hormone receptor (*lhcgr)* in testicular (*top*) and seminal vesicle (*bottom*) tissue. *, $P < 0.05$ (compared with the respective control value; Tukey test; n = 6 in both groups).

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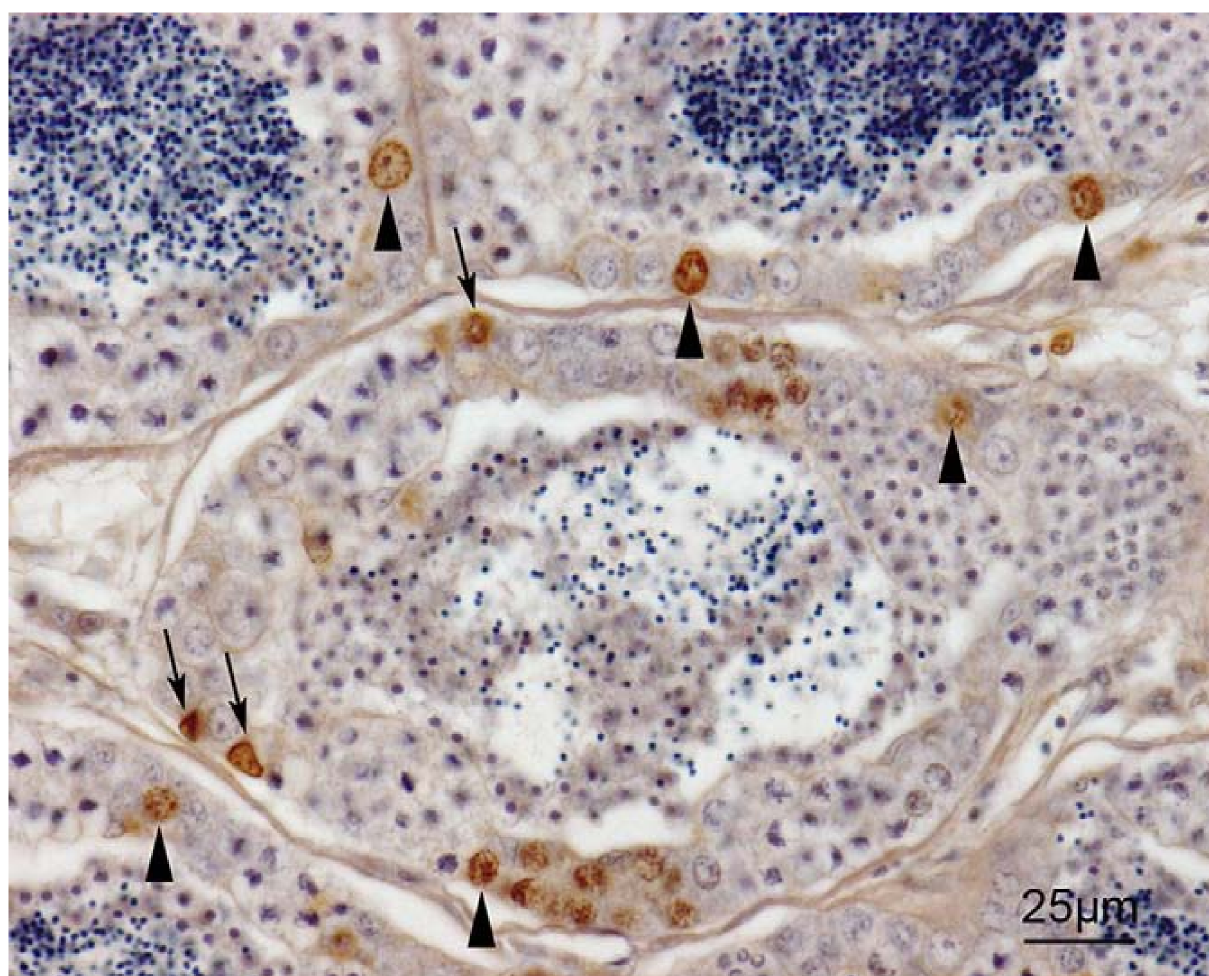


Figure 1

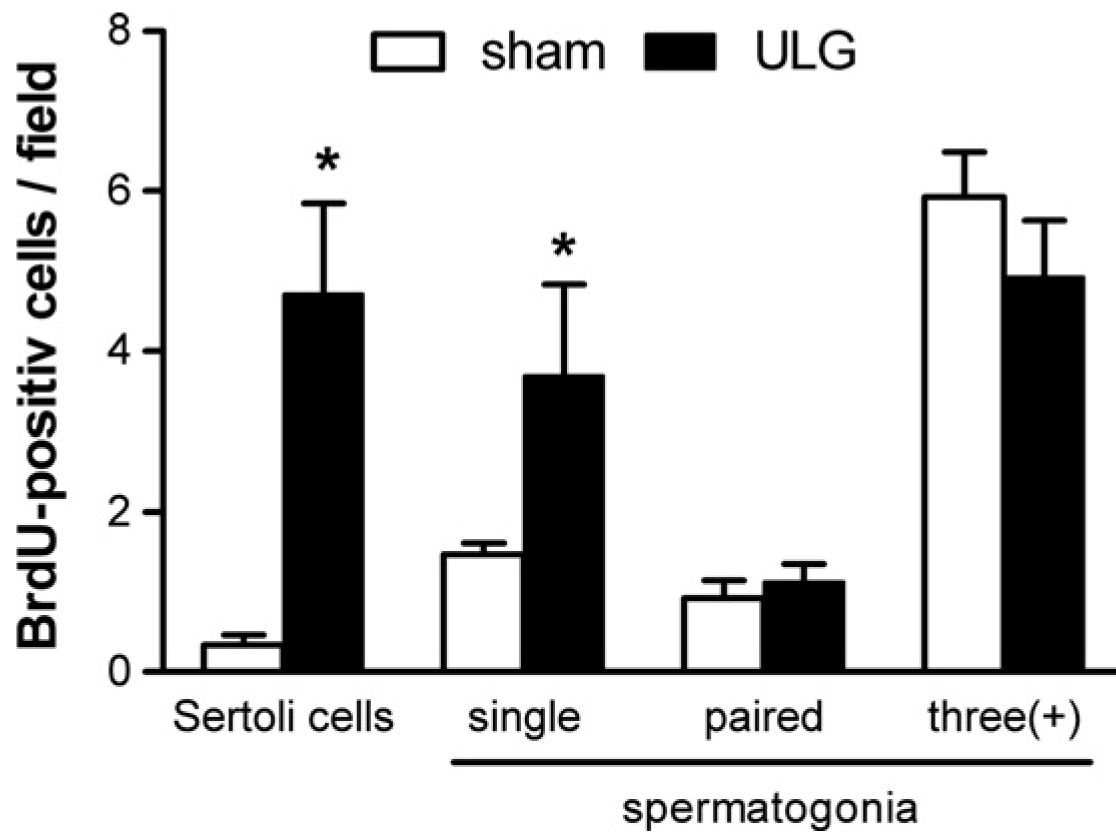


Figure 2

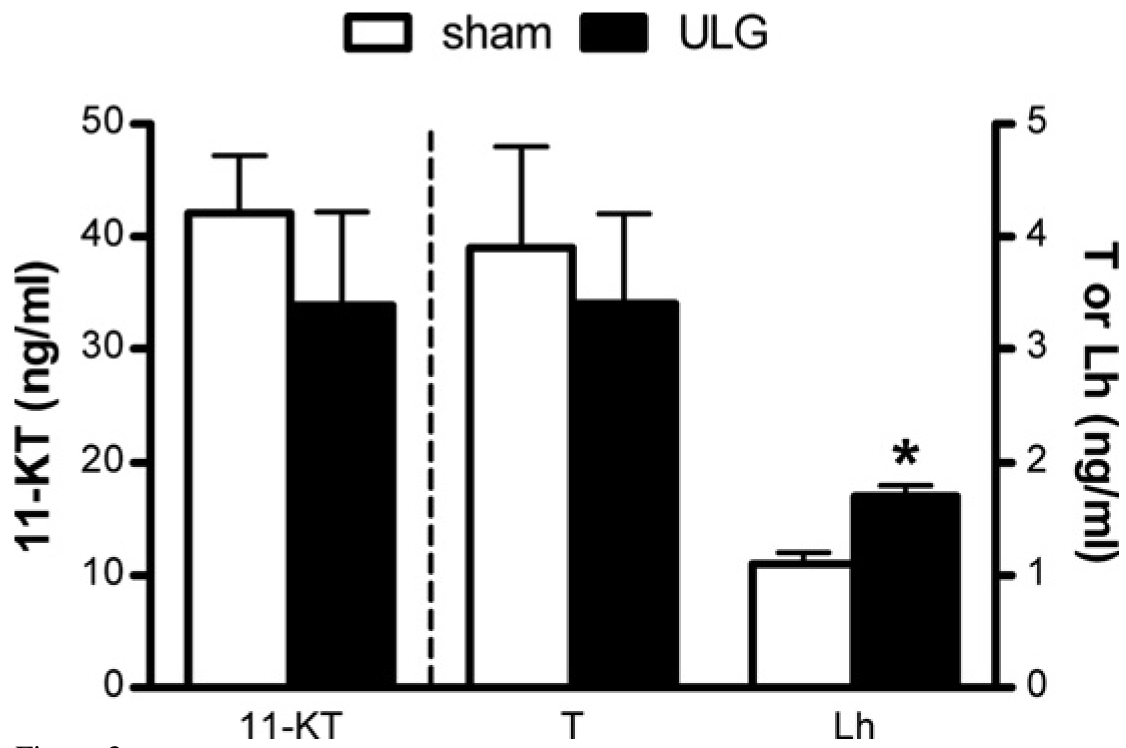
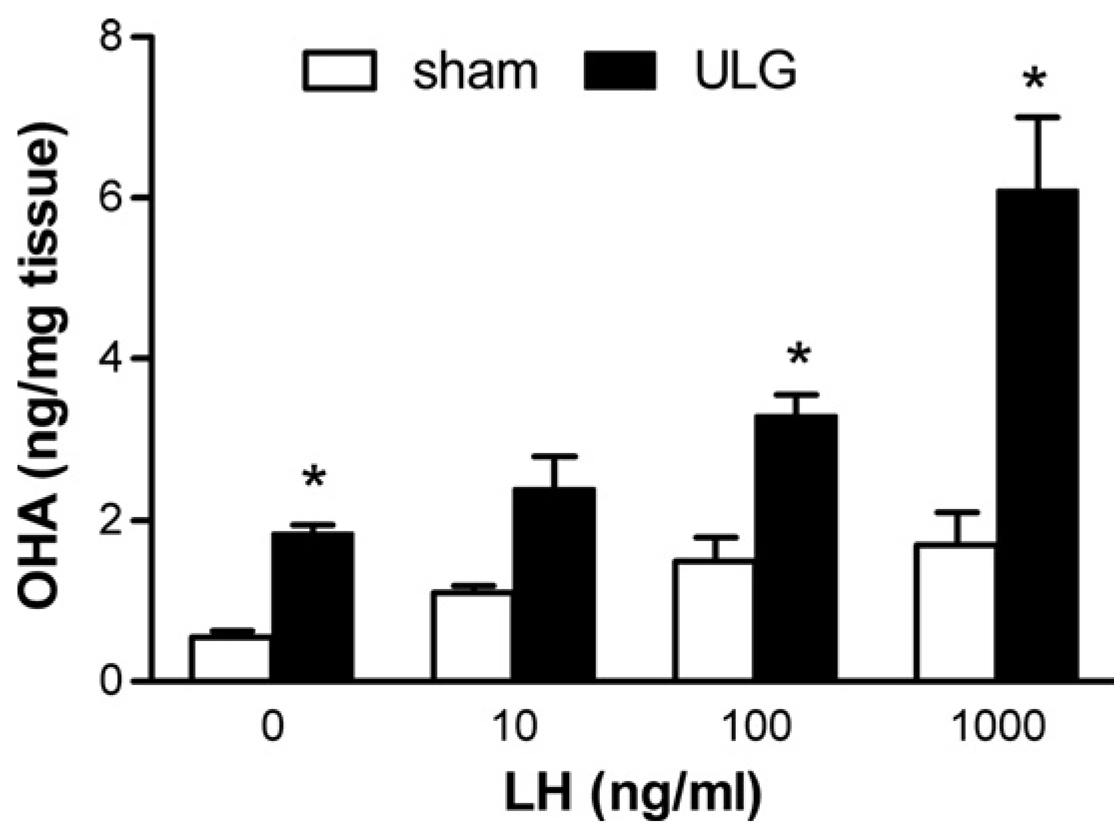


Figure 3


sham
ULG
OHA (ng/mg tissue)
8
6
4
2
0
*
*
*
*
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100
1000
LH (ng/ml)

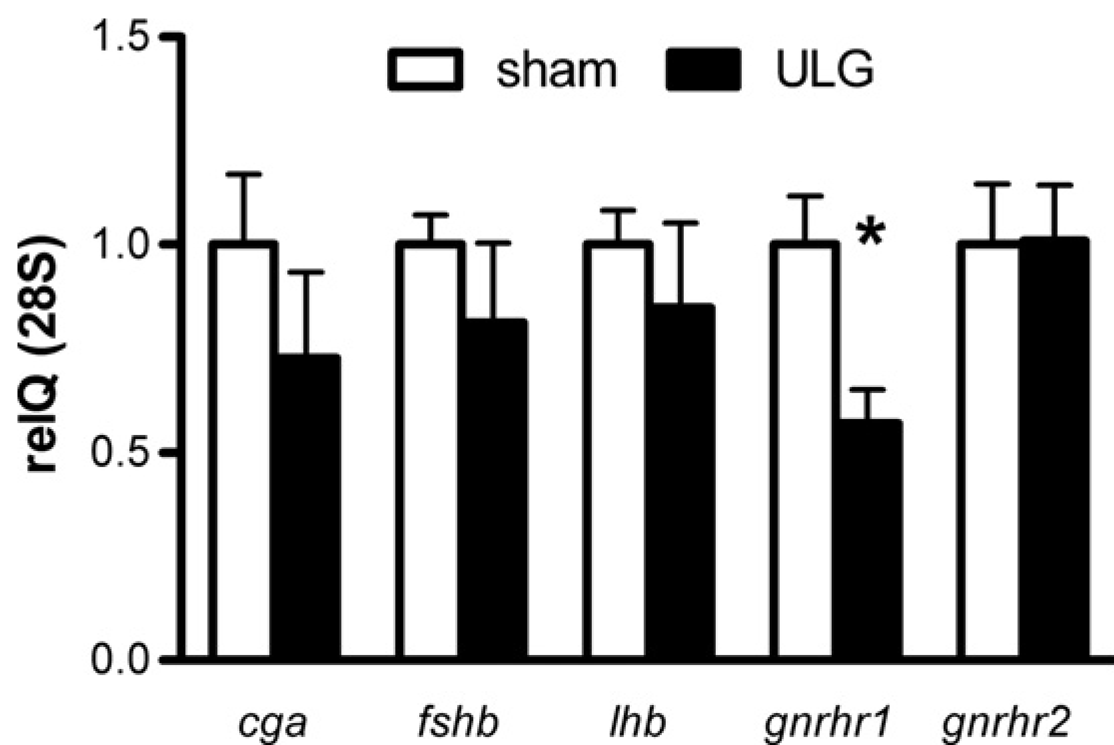

Figure 5

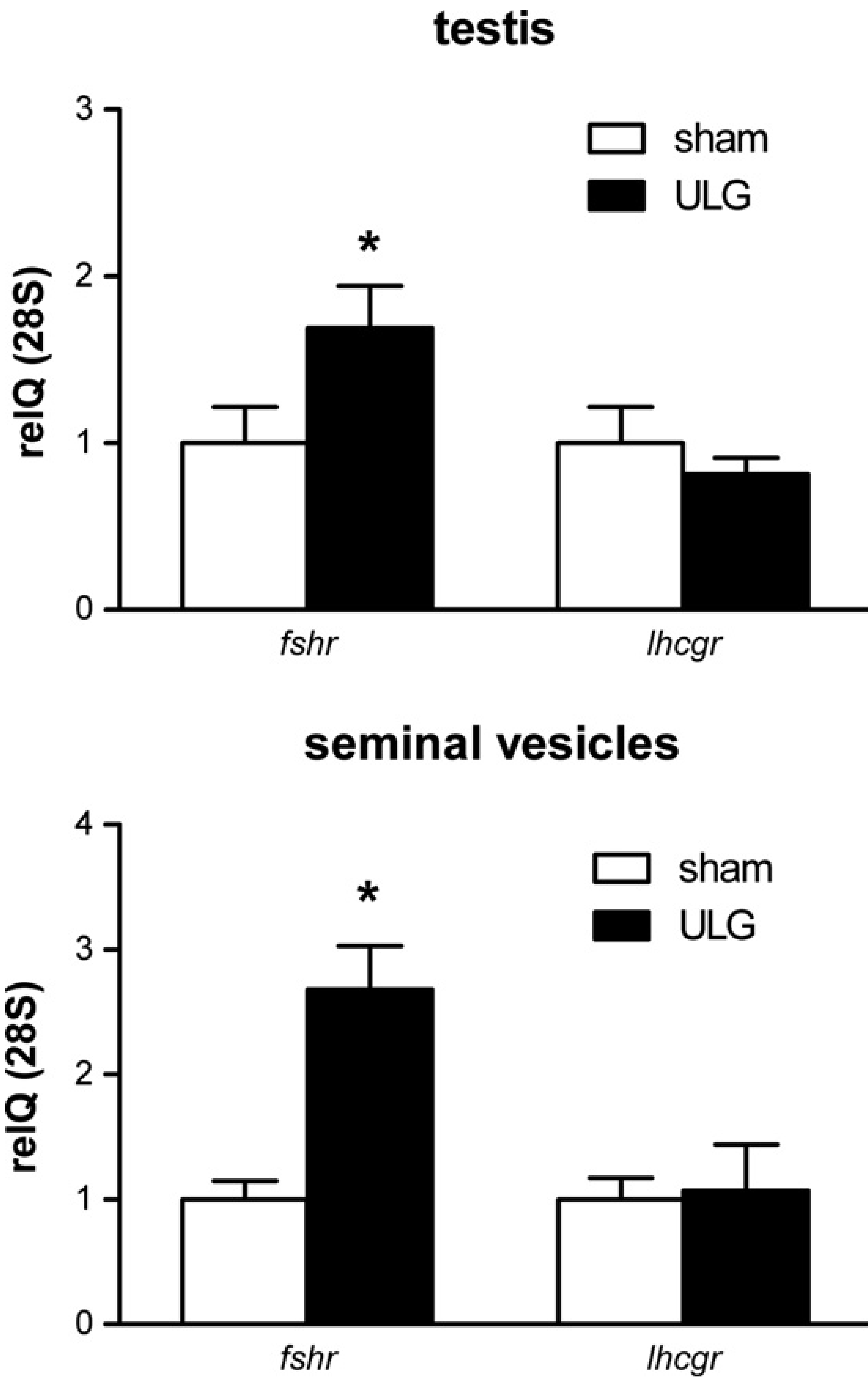

Figure 6