

### Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis.

Antoine D. Rolland, Aurélie Lardenois, Anne-Sophie Goupil, Jean-Jacques Lareyre, Rémi Houlgatte, Frédéric Chalmel, Florence Le Gac

### ▶ To cite this version:

Antoine D. Rolland, Aurélie Lardenois, Anne-Sophie Goupil, Jean-Jacques Lareyre, Rémi Houlgatte, et al.. Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis.. PLoS ONE, 2013, 8 (1), pp.e53302. 10.1371/journal.pone.0053302 . inserm-00825763

### HAL Id: inserm-00825763 https://inserm.hal.science/inserm-00825763

Submitted on 24 May 2013  $\,$ 

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

### Profiling of Androgen Response in Rainbow Trout Pubertal Testis: Relevance to Male Gonad Development and Spermatogenesis

# Antoine D. Rolland<sup>1</sup>, Aurélie Lardenois<sup>2</sup>, Anne-Sophie Goupil<sup>1</sup>, Jean-Jacques Lareyre<sup>1</sup>, Rémi Houlgatte<sup>3,4</sup>, Frédéric Chalmel<sup>2</sup>, Florence Le Gac<sup>1</sup>\*

1 INRA, UR1037 LPGP, SFR Biosit, Biogenouest, Campus de Beaulieu, Rennes, France, 2 Inserm, U1085, IRSET, Université de Rennes I, Campus de Beaulieu, Rennes, France, 3 Inserm, UMR1087, l'institut du thorax IRT - UN, Nantes, France, 4 Université de Nantes, Nantes, France

#### Abstract

The capacity of testicular somatic cells to promote and sustain germ cell differentiation is largely regulated by sexual steroids and notably androgens. In fish species the importance of androgens is emphasized by their ability to induce sex reversal of the developing fries and to trigger spermatogenesis. Here we studied the influence of androgens on testicular gene expression in trout testis using microarrays. Following treatment of immature males with physiological doses of testosterone or 11-ketotestosterone, 418 genes that exhibit changes in expression were identified. Interestingly, the activity of testosterone appeared stronger than that of 11-ketotestosterone. Expression profiles of responsive genes throughout testis development and in isolated germ cells confirmed androgens to mainly affect gene expression in somatic cells. Furthermore, specific clusters of genes that exhibit regulation coincidently with changes in the natural circulating levels of androgens during the reproductive cycle were highlighted, reinforcing the physiological significance of these data. Among somatic genes, a phylogenetic footprinting study identified putative androgen response elements within the proximal promoter regions of 42 potential direct androgen target genes. Finally, androgens were also found to alter the germ line towards meiotic expression profiles, supporting the hypothesis of a role for the somatic responsive genes in driving germ cell fate. This study significantly increases our understanding of molecular pathways regulated by androgens in vertebrates. The highly cyclic testicular development in trout together with functions associated with regulated genes reveal potential mechanisms for androgen actions in tubule formation, steroid production, germ cell development and sperm secretion.

Citation: Rolland AD, Lardenois A, Goupil A-S, Lareyre J-J, Houlgatte R, et al. (2013) Profiling of Androgen Response in Rainbow Trout Pubertal Testis: Relevance to Male Gonad Development and Spermatogenesis. PLoS ONE 8(1): e53302. doi:10.1371/journal.pone.0053302

Editor: Bin He, Baylor College of Medicine, United States of America

Received July 25, 2012; Accepted November 27, 2012; Published January 3, 2013

**Copyright:** © 2013 Rolland et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the AGENAE program (www.agenae.fr), INRA-CIPA-OFIMER European community IFOP grant. This research received funding from the French National Research Agency (ANR-06-GANI-014 "Spermgen") and from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under grant agreement n°222719, project LIFECYCLE. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

\* E-mail: legac@rennes.inra.fr

#### Introduction

The hypothalamic-pituitary-gonadal axis regulates sexual maturation and spermatogenesis through the release of two gonadotropins from the anterior pituitary upon GnRH stimulation, the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) [1]. The control of spermatogenesis by gonadotropins mainly relies on LH-induced testosterone (T) production by Leydig cells, as observed in mammals: T is indeed sufficient to restore complete spermatogenesis in hypogonadal mice [2,3] or hypophysectomized rats [4,5] whereas FSH promotes Sertoli cell proliferation and differentiation and is thus responsible for the final spermatogenetic capacity of the adult testis [6,7].T binds to the nuclear androgen receptor (AR) that, upon homodimirization, regulates expression of genes containing cis regulatory elements termed Androgen Response Elements (AREs). In mammals, AR is expressed in Leydig, peritubular and Sertoli cells in the adult testis [8]. Consequently, the control of spermatogenesis by T is more likely to occur by modulating somatic signaling rather

than by a direct effect on the germ cells. This was further evidenced by the ability of  $AR^{-/-}$  spermatogonial stem cells to colonize recipient testes [9] and by the spermatogenetic defects observed in selective AR knockout in testicular somatic cells [10,11,12]. A number of studies have addressed the specific question of testicular gene modulation by androgens and, despite the use of various models (for review, see [13]), the way T regulates spermatogenesis are still not fully understood. Notably, only a few direct androgen-target genes have been identified so far and the specific role of T at specific steps in male gonad development is still unclear. In this regard, seasonally-breeding fish species such as the rainbow trout, Onchorynchus mykiss, represent an interesting model to investigate those molecular pathways regulated by androgens in the testis. The seasonal spermatogenetic cycle, together with the cystic organization of the trout testis, tends to synchronize the developmental state of somatic cells at any time. On the other hand, the endocrine landscape in teleost fish species appears somehow more complex than in mammals. In addition to T and androstenedione ( $\Delta 4$ ) several 11-oxygenated androgens such as 11β-hydroxyandrostenedione (11βOHA), 11-ketoandrostenedione (11KA), 11β-hydroxytestosterone (11βOHT) or 11-ketotestosterone (11KT) are found in males and exhibit distinct plasma profiles during fish development and throughout the reproductive cycle [14]. Much evidence points towards 11KT being a potent androgen for promoting spermatogenesis in a few fish species [15,16,17,18,19]. Contradictory results were obtained for T in this regard: A direct stimulatory effect was reported on spermatogenesis in the Mummichog and goldfish [20,21] whereas it was found to inhibit the 11KT-induced spermatogenesis in the African catfish in vivo [22]. In this study we investigated the influence of androgens on gene expression in the trout testis at the onset of puberty. Immature males were submitted to short-term supplementation of either T or 11KT and testicular androgen-responsive genes were identified using microarrays. The use of expression data from testes at various developmental stages [23] further allowed identifying relevant genes that exhibit differential expression coincidently with changes of androgen circulating levels along the reproductive cycle. In addition, it enabled us to distinguish between the somatic or germ cell origin of responsive genes. Functions associated with regulated genes reveal potential mechanisms for androgen actions. Finally, we performed a computational phylogenetic footprinting analysis and pointed out potential direct target genes of androgens whose promoters harbour evolutionary conserved AREs.

#### **Materials and Methods**

#### **Ethics Statement**

Experimental research on animal reported here was performed in conformity with the principles for the use and care of laboratory animals in compliance with French and European regulations on animal welfare. Furthermore, experimenters were delivered an authorization given by the French "Direction des Services Vétérinaires" to conduct or supervise experimentations on live animals.

#### Animals and Hormonal Treatment

Rainbow trout (*Oncorhynchus mykiss*) were maintained under natural temperature and photoperiod at the INRA experimental fish farm (Drennec, France).

Prepubertal male trout weighting 150+/-34 g were submitted to androgen supplementation using subcutaneous implants (Innovative Research of America). Pellets used as implants were a readyto-implant form, using Matrix-Driven Delivery (MDD) Pellet System (>21 day release; Innovative Research of America, USA). Trout were anesthetized using phenoxyethanol and pellets were implanted in the dorsal muscle, just behind the dorsal fin, using a 10-gauge trochar. The control group (n = 14) received an empty pellet.

Four androgen supplementations were realized : 2 doses of testosterone (T) were compared to detect potential dose response effects. For the lowest T dose (T1 = 0.1 mg or 0.666  $\mu$ g/g body weight; n = 5), a single time point (day 7) was investigated. For the highest T dose (T2 = 0.2 mg or 1.333  $\mu$ g/g body weight), 2 time points were investigated to eventually discriminate between early and late responses (day 7, n = 4; day 14, n = 5). The response to 11KT, a major androgen in fish physiology with low metabolisation potential, was also studied, although only at one dose and one time point (0.25 mg or 1.667  $\mu$ g/g body weight, day 7; n = 7) for which the highest response to T was obtained.

Following supplementations, the testes were recovered, weighed to determine the gonadosomatic index (GSI) and rapidly

immersed in Bouin's solution for histological study or frozen until use for microarray analysis. Blood samples were collected and circulating androgen levels were measured by specific radioimmunoassay (RIA) as previously described [24]. These experiments were conducted in January and February, when most animals were immature (containing spermatogonia only in terms of germ cells). The testicular histology of both treated and control animals was also carefully analysed.

#### cDNA Microarray Experiment

Microarray hybridization and raw data production. cDNA microarrays (platform: http://www.ncbi. nlm.nih.gov/geo/query/acc.cgi?acc = GPL3650) were generated by CRB GADIE (http://crb-gadie.inra.fr/) as previously described [25]. Procedures for RNA extraction, cDNA target synthesis using [alpha-33P] dCTP, microarray hybridization and raw data production have been previously described in details [23].

**Normalization procedure.** Expression data were normalized as previously described [23]. Briefly, raw data were corrected for the amount of spotted cDNA (Si/Vi). To avoid the bias affecting relative gene expression levels, the corrected signal of each spot was further multiplied by the median vector signal of all arrays for this same spot ((Si/Vi) x medVi). Expression values were then log2-transformed and submitted to a quantile-quantile normalization [26] using the AMEN software (http:// sourceforge.net/projects/amen/; [27]. Raw data as well as a normalized expression file are available at the GeneOmnibus public data repository (http://www.ncbi.nlm.nih.gov/geo/query/ acc.cgi?acc = GSE16030).

Statistical and cluster analyses. Non-informative clones for which too little cDNA was spotted (oligonucleotide signal <3times the background level in more than 20% of samples) were removed from the analysis. Androgen-responsive genes were then identified by comparing the control group to each of the treated groups (T1, "T2, day7", "T2, day14", 11KT) using the multi-class Limma statistical test (FDR 1%) [28]. All differentially-expressed transcripts were submitted to the PAM algorithm (Euclidian distance measure) and grouped into 4 expression clusters. Three pairwise comparisons were also performed to identify genes differentially-regulated by testosterone and 11KT at a comparable time point (*i.e.* at day 7) using the Limma statistical test: control vs "T2, day7", controls vs 11KT, 11KT vs "T2, day7". Here, a Bonferroni adjustment was used to obtain a global risk of type I error equals to 1%. Thus, for any one comparison to be considered significant, the p-value had to be less than 0.0033, that is 1% divided by the number of pairwise comparisons. For heatmap representations, unlogged data were loaded into the MeV software (www.tm4.org/mev/) and normalized according to the following formula: Value = [(Value) - Mean(Row)] / [Standard]deviation(Row)].

**Metaanalysis.** Expression data from testes at different developmental stages and isolated germ cells populations [23] were used to investigate the developmental pattern and the cell type in which androgen-responsive genes are expressed. Briefly, samples in this dataset included - Testes in early stages containing slowly-dividing type A spermatogonia (Stage I) or growing numbers of actively-dividing type B spermatogonia (Stages IIa and IIb) - Maturing testes also containing meiotic spermatocytes (Stage IIIb) and post-meiotic spermatids (Stage V) - Spawning testes containing essentially mature spermatozoa (stage VIII) (see [29,30] for a more precise description of all stages) - Fractions of isolated germ cells enriched in spermatogonia, spermatocytes or spermatids obtained as previously

described [31]. Genes differentially-expressed during testis development (F's statistic permutation test, FDR 5‰) were compared to androgen-responsive genes.

#### Trout Microarray Annotation and Functional Data Mining

Trout cDNAs spotted onto the microarray were annotated as previously described [23]. GeneOntology (GO) terms associated to fish genes and their rat, mouse and human orthologs were extracted from the Ensembl database (version 52). Biological mining of expression clusters was then performed by searching for over- and under-represented functional GO terms (Gaussian hypergeometric test) as compared to all well-measured genes, using the AMEN software.

#### Prediction of Androgen Response Elements

As there is yet no genome sequence available for the Oncorhynchus mykiss or any other salmonid we used that of Gasterosteus aculeatus (Stickleback) as a reference. Stickleback Ensembl genes were identified as previously described [23] and their Transcriptional Start Sites (TSS) were localized using the ensGene.txt file from UCSC (http://genome.ucsc.edu/) [32]. AREs were predicted within genomic regions from -10000 base pairs (bp) up to +2000 bp with respect to each TSS. Validated ARE motif matrices (M00447, M00481, M00953, M00956 and M01201) from the Transfac Professional database (release 2009.1; http://www.biobase-international.com) [33] were predicted using the MATCH program [34]. Core and matrix similarity cutoff values were set at 0.8. To reduce the number of false positive and focus on motifs that are more likely to be functional, hits sequences were required to be conserved between species [35]. For each predicted motif, a cross-species conservation score was processed by averaging the base-by-base phasCons scores calculated between 8 vertebrates (chicken, fugu, human, medaka, mouse, stickleback, tetraodon, and zebrafish) as provided by the UCSC genome browser [36] and only motifs with such a crossspecies conservation score  $\geq 0.8$  were considered. Similarly, we also screened for the presence of putative Estrogen Response Elements (EREs) in the same gene promoter regions using the validated M00191 Transfac matrice.

#### qPCR Experiments

Total RNAs were first treated with the turbo  $\text{DNA-free}^{\text{TM}}$  kit (Ambion) and 2 µg were submitted to reverse-transcription (RT) using random hexamer primers and 200 units of MMLV reverse transcriptase (Promega). Real-time PCR assays were performed on the StepOne<sup>TM</sup> Real-Time PCR System (Applied Biosystems) using 1:30 diluted RT products, primers (300 nM) and Fast SYBR<sup>®</sup> Green Master Mix (Applied Biosystems). Relative expression levels were normalized using a reference gene, rps15 (clone 1RT58B15\_B\_A08), designed on the basis of its invariant expression (low standard deviation) both in androgen treatment and in spermatogenesis development microarray experiments (Figure S1). Real-time PCR oligonucleotide primers were designed using the perlprimer software (http://sourceforge.net/projects/ perlprimer/), verified with the oligoanalyser 3.1 web interface (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) to avoid self- and hetero-dimer formation as well as hairpin structures, and matched (BLAST algorithm) against the SI-GENAE trout contig collection (som.8 version) to avoid nonspecific annealing to other transcripts (Table S1). Aberrant values, as determined by Dixon test (p<5%), were removed and statistical analyses were performed using the Statistica software using the non-parametric test of Mann & Whitney (n individuals = 5 to 7).

#### Results

#### Short-term Supplementations of Immature Male Trout Induce Physiological Androgen Circulating Levels

We treated prepubertal males in early stages of maturation (stages I-II), for which low basal androgen levels (1 to 2 ng/ml) should make the detection of responsive genes more convenient and sensitive, and we used implants with moderate releasing rates so that possible toxicological effects would be very limited.

Supplementation of immature males with either T or 11KT resulted in a significant increase in corresponding blood plasma androgen concentrations (Figure 1A). T circulating levels were increased 8 and 28 times in animals treated for 7 days with low (T1) and high (T2) T doses, respectively (Figure 1A). Similarly, 11KT circulating levels were increased 32 times in animals treated for 7 days with this hormone as compared to controls (Figure 1A).



**Figure 1. Androgen plasma levels in male trout.** A : Increased androgen plasma concentrations following testosterone and 11-ketotestosterone supplementation. Androgen circulating levels were measured in stage I to II males supplemented for 7 or 14 days with testosterone (0.1 mg, T1; 0.2 mg, T2) or 11-ketotestosterone (0.25 mg, 11KT) as well as in corresponding controls (Cont). B : Natural testosterone (grey) and 11-ketotestosterone (black) circulating levels throughout the male trout reproductive cycle. Roman numerals (I-VIII) indicate testicular developmental stages. Mean values  $\pm$  standard deviation are presented.

doi:10.1371/journal.pone.0053302.g001

The induced androgen circulating levels correspond to physiological concentrations since natural T and 11KT circulating levels reach up to 90 and 170 ng/ml respectively during the trout reproductive cycle (Figure 1B). Additionally, by restricting the hormone supplementation to a few days, the gonadosomatic index of treated animals was unchanged and no striking change in cellular composition could be observed by qualitative histology (data not shown). These treatments were therefore found optimal to further investigating the influence of androgens on trout testicular gene expression.

### Identification of Androgen-responsive Genes in the Trout Testis

We first identified androgen-regulated genes by comparing testicular gene expression in controls and in all 4 treated groups. Among 7916 accurately measured cDNAs, 485 were differentially expressed (FDR <1%) in testes of supplemented animals (Figure S2, panel A). Overall the majority of these changes occurred through up-regulation. Expression signals of only 100 cDNAs were decreased following androgen treatment (cluster 1) whereas 327 were increased: 203 cDNAs showed the highest up-regulation at day 7 following T implantation (T2 showing generally more effects than the lower dose T1) (cluster 2) and 124 showed the highest induction at day 14 (cluster 3). In addition, a smaller cluster of 58 cDNAs exhibited down-regulation at day 7 and up-regulation at day 14 (cluster 4). Extensive information for all the androgen-responsive genes, including expression data, cluster information and corresponding annotations, is provided in File S1.

We next compared the effects of different androgen treatments at day 7 and found that the influence of T was stronger than that of 11KT: Among the 485 androgen-regulated genes, 84 genes were indeed significantly regulated by T only (T2, day 7) while 6 genes were regulated by 11KT only (p<0.0033) (Figure S3; these genes are marked "T only" and "11KT only" in File S1). In addition, all the 26 genes regulated by both androgens were found to be more influenced by T treatment (p<0.0033; Figure S3; marked "T >11KT" in File S1).

### Androgens Mainly Affect Gene Expression in Testicular Somatic Cells

The expression profiles of responsive genes were then further investigated during pubertal development of the gonad, using expression data obtained from trout testes in different maturation states (early stages I to V of spermatogenesis and late stage VIII of sperm excretion in the efferent ducts) and from isolated germ cell fractions enriched in spermatogonia, spermatocytes or spermatids [23].

Consistent with a physiological response to androgen supplementation we found that most of the androgen-responsive genes were also differentially expressed during the course of the reproductive cycle (Figure S2, panel B). More importantly, we showed that androgen-responsive genes were preferentially expressed in somatic cells: Among the 304 androgen-regulated genes that were identified as differentially-expressed during spermatogenesis, 242 were indeed found in somatic expression clusters (A–D in table 1) while only 62 belonged to germ cell expression clusters (E–I in table 1) (Figure 2).

Strikingly, most up-regulated genes were found in the "somatic" clusters A or C of the spermatogenesis study (Table 1 and Figure 2), which exhibit a high or a very high expression in stage VIII testes, coinciding with the greatest circulating androgen levels during the reproductive cycle (Figure 1B). Conversely, out of the 29 somatic genes that were down-regulated following experimental androgen

supplementation (cluster 1), 22 were found in cluster B of the spermatogenesis study, which exhibits low or very low expression in stage VIII testes (Table 1 and Figure 2).

The dynamics of these somatic genes are therefore highly consistent with a regulation by endogenous androgens during the natural course of the reproductive cycle, and with a role in testis maturation or in the spermatogenetic processes. Important to note is that, while T and 11KT exhibit highest circulating levels during late phases of the reproductive cycle, they first significantly increase at the recrudescence phase (Stages I-II-III, panel in Figure 1B). Therefore part of the responsive genes may be tightly regulated by androgens at the onset of spermatogenesis.

Importantly, a few genes from the germ cell expression clusters also exhibited remarkable features following androgen supplementations : Out of the 35 androgen-responsive genes from "spermatogonial" expression clusters (E and F), 24 were downregulated in treated animals (cluster 1). On the other hand, 15 of the 16 genes from the meiotic/post-meiotic expression cluster (H) were up-regulated (clusters 2 and 3) following androgen treatment (Table 1 and Figure 2). This indicates that, in addition to regulating gene expression in supporting somatic cells, androgen treatment would result in a shift in germ cell expression profiles, from mitotic spermatogonia to meiotic spermatocytes.

## Potential Direct Targets of Androgens are Revealed by Promoter Analysis

Like in most studies that aimed at identifying androgen-target genes in the testis, the picture obtained in our experimental scheme is likely to result from a complex cascade of gene, protein or endocrine regulations, with only a small number of genes being under the direct control of the AR.

To identify genes potentially directly regulated by androgens we searched for AREs within the promoter of androgenregulated genes. Because the Oncorhynchus mykiss genome was not available we used that of Gasterosteus aculeatus as a reference and considered evolutionary conserved AREs only (i.e. sequences with a high evolutionary conservation score across up to 8 vertebrates). A total of 775 matching sequences were assigned to the promoter regions (-10000/+2000 bp relative to the TSSs) of 283 out of the 395 trout androgen-regulated genes that also displayed a clear ortholog in Gasterosteus aculeatus (Figure 3). We next focused on those androgen responsive genes that also exhibit differential expression during spermatogenesis and, when restricting the exploration to a more proximal promoter -2000/+400 bp relative to the TSS), we identified 53 genes (18.7%) that presented at least one putative conserved ARE. We found that 42 of these genes belonged to somatic cell expression clusters, which represent the most promising direct targets of the AR in this study (column labeled "predicted as "Somatic" with a proximal ARE" in File S2).

Of note is that searching for conserved AREs was compulsory for us in order to reveal AREs that might also be present in the trout genome. While this method fails to identify a subset of species-specific regulatory elements, it allows focusing on sequences that are more likely to be functional given that important DNA regulatory regions tend to be conserved between species [35]. All information regarding androgenresponsive genes that contain at least one conserved ARE in their promoter regions (-10000/+2000 bp relative to the TSSs) is reported in the File S2.



**Figure 2. Expression of androgen-responsive and developmentally-regulated genes.** Heatmap representation of specific expression clusters of androgen-regulated genes that also display differential expression during trout spermatogenesis. Subgroups of genes were established by crossing 4 clusters (1 to 4) of androgen-responsive genes and 9 clusters (A to I) of spermatogenesis as indicated in table 1. For these genes, we present expression signals obtained: - in testes from controls and androgen-supplemented animals, and - in testes at various developmental stages and in enriched fractions of isolated germ cells. Normalized expression values are shown according to the scale bar while histograms represent averaged fold changes to the control (androgens data) or to the stage I (spermatogenesis data)  $\pm$  SEM for each cluster. T1 and T2 correspond to animals supplemented with testosterone implants of 0.1 and 0.2 mg, respectively; 11KT indicates animals treated with 11-ketotestosterone implants of 0.25 mg; d7 and d14 correspond to animals treated for 7 and 14 days, respectively. Roman numerals (I–V and VIII) indicate testicular developmental stages. Sg, Sc and St correspond to isolated germ cell populations enriched in spermatogonia, spermatocytes and spermatids, respectively. The annotation of each gene is accessible in the searchable File S1. doi:10.1371/journal.pone.0053302.g002

## Functional Implication of Androgens are Revealed by GeneOntology Terms Analysis

We performed a GeneOntology (GO) term analysis to evaluate the functional impact of testicular changes in expression mediated by androgens (Figure 4 and Figure S4). Importantly a statistical enrichment was found for genes involved in "sex determination" within genes that were upregulated mainly at day 7 (cluster 2). The same cluster was enriched in genes involved in "regulation of cyclic nucleotide metabolic process". Genes that were up-regulated later on, at day 14 (clusters 3 and 4), were found to bear common and overlapping functions related to "collagen metabolism" and "collagen organiTable 1. Summary of androgen-responsive clones that also exhibit differential expression during spermatogenesis.

	1 - Downregulated	2 - Upregulated at day 7	3 - Upregulated at day 14	4 - Down at day 7, Up at day 14
A - Somatic	4	65	40	б
B - Somatic, low in stage VIII	22	5	22	9
C - Somatic, high in stage VIII	1	40	6	3
D - Somatic+isolated spermatogonia	2	7	7	4
E - Spermatogonia - type A	19	1	1	4
F - Spermatogonia - type B	5	2	2	1
G - Germline	2	3	2	0
H - Meiotic/Post-meiotic	0	10	5	1
I - Isolated germ cells only	1	3	0	0

Subgroups of clones were established by crossing 4 clusters of androgen-responsive genes (1 to 4, present study) and 9 clusters (A to I) of genes differentially expressed during trout spermatogenetic development [23]. Bolded numbers indicate subgroups of genes that are represented in Figure 2. doi:10.1371/iournal.pone.0053302.t001

zation", "ion transport" or several developmental processes such as "ossification", "skin development" and "visual perception" (Figure 4). When focusing on androgen-regulated genes with "somatic" expression (clusterA-D), additional processes were also



Figure 3. Identification of conserved Androgen Response Elements within the promoter of androgen-regulated genes. A : The relative number of genes displaying 0 to 14 conserved AREs is presented. Conserved AREs (cAREs) were searched in a region of -10000/+2000 base pairs relative to the TSS in the promoter of 395 androgen-responsive gene orthologs in Gasterosteus aculeatus. B : Frequency of conserved AREs in the promoter regions of androgenresponsive genes with respect to the TSS, expressed by number of sites per kilobase of DNA.

doi:10.1371/journal.pone.0053302.g003

found enriched such as "positive regulation of hormone secretion" "regulation of transcription factor import into nucleus" or "water transport".

Overall these particular expression changes suggest an important role for androgens in remodelling extracellular matrix and the seminiferous tubule structure, which was also evidenced at the "Molecular function" (e.g. "extracellular matrix structural constituent", "structural molecule activity") and "Cellular component" levels (e.g. "extracellular region" and "extracellular matrix part") (Figure S4).

#### Real-time PCR Validation of Candidate Genes

We choose several somatic transcripts possibly involved in paracrine regulations and representative of the 3 major androgenresponsive clusters to perform qPCR measurments (Figure 5). We confirmed the strong inhibition of 4 genes that are also downregulated during the spermatogenetic maturation process: inhba and *clu* are inhibited by both T and 11KT whereas *amh* and *angpt17* are mainly influenced by T. In addition, the moderate downregulation of inha following 7 days of T supplementation and the strong and wide inhibition of star by T were also confirmed. Noteworthy is that these 2 genes exhibited high expression in stage VIII testes. Among the up-regulated genes we investigated, *il13ra2*, cxcl14, cldn11 and zpacp were confirmed to be increased by T and 11KT (Figure 5), while the up-regulation of fgf12 was confirmed statistically for 11KT only (data not shown).

We also intended to validate the regulatory effect of androgen treatments on several somatic genes encoding for transcription factors of interest for testis pubertal development (Figure 5). tbx1, whose expression strongly decreases throughout the reproductive cycle, was confirmed to be down-regulated, while dmrt1, a testisspecific factor, was up-regulated by the two androgens. Surprisingly, when investigating the expression of sox9, only sox9a was confirmed to be highly up-regulated after both T and 11KT treatment whereas sox9b was down-regulated by T. It is likely that sox9a mRNA actually cross-hybridizes onto the sox9b cDNA probe, resulting in its apparent up-regulation of both factors when using cDNA microarrays.

The expression of 8 germ cells transcripts was also further investigated. In those cases the statistical validation was rendered difficult post probably because of the low amplitude of androgen effects, notably for genes preferentially expressed in spermatogonia and down-regulated by androgens (noc21, bop1, eprs, prpf8 and



#### Androgen-responsive genes

#### Androgen-responsive and developmentallyregulated genes

Cluster   Cluster <t< th=""></t<>
Biological Process 4993 82 153 90 45 182 31 2'   sex determination 10 0/0 3/0 0/0 0/0 2/0 0/0 0/   cGMP biosynthetic process 5 0/0 3/0 0/0 0/0 2/0 0/0 0/0   regulation of cyclic nucleotide metabolic process 8 0/0 3/0 0/0 0/0 3/0 0/0
sex determination 10 0/0 3/0 0/0 0/0 2/0 0/0 0/0   cGMP biosynthetic process 5 0/0 3/0 0/0 0/0 2/0 0/0
cGMP biosynthetic process 5 0/0 3/0 0/0 0/0 2/0 0/0 0/0   regulation of cyclic nucleotide metabolic process 8 0/0 3/0 0/0 0/0 3/0 0/0
regulation of cyclic nucleotide metabolic process 8 0/0 3/0 0/0
ossification 39 0/1 0/1 5/1 1/0 3/1 0/0 0/   inorganic anion transport 69 3/1 2/2 8/1 3/1 8/3 0/0 0/   skin development 10 0/0 0/0 3/0 1/0 3/0 0/0 0/   collagen fibril organization 12 0/0 0/0 7/0 1/0 5/0 2/0 0/   collagen biosynthetic process 5 0/0 0/0 5/0 1/0 3/0 2/0 0/   phosphate transport 32 0/1 1/1 5/1 3/0 4/1 0/0 0/   collagen catabolic process 9 0/0 1/0 2/0 3/0 2/0 0/   visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 0/0 0/   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 0/0 0/0
inorganic anion transport 69 3/1 2/2 8/1 3/1 8/3 0/0 0/   skin development 10 0/0 0/0 3/0 1/0 3/0 0/0 0/   collagen fibril organization 12 0/0 0/0 7/0 1/0 5/0 2/0 0/   collagen biosynthetic process 5 0/0 0/0 5/0 1/0 3/0 2/0 0/   phosphate transport 32 0/1 1/1 5/1 3/0 4/1 0/0 0/   collagen catabolic process 9 0/0 1/0 2/0 3/0 2/0 0/   visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 0/0 0/   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0 0/0
skin development 10 0/0 0/0 3/0 1/0 3/0 0/0 0/   collagen fibril organization 12 0/0 0/0 7/0 1/0 5/0 2/0 0/   collagen biosynthetic process 5 0/0 0/0 5/0 1/0 3/0 2/0 0/   phosphate transport 32 0/1 1/1 5/1 3/0 4/1 0/0 0/   collagen catabolic process 9 0/0 1/0 2/0 3/0 2/0 0/   visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 0/0 0/   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 1/0 0/0 0/0 0/0
collagen fibril organization 12 0/0 0/0 7/0 1/0 5/0 2/0 0/   collagen biosynthetic process 5 0/0 0/0 5/0 1/0 3/0 2/0 0/   phosphate transport 32 0/1 1/1 5/1 3/0 4/1 0/0 0/   collagen catabolic process 9 0/0 1/0 2/0 3/0 2/0 0/   visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 0/0 0/   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 1/0 0/0 0/0 0/0
collagen biosynthetic process 5 0/0 0/0 5/0 1/0 3/0 2/0 0/   phosphate transport 32 0/1 1/1 5/1 3/0 4/1 0/0 0/   collagen catabolic process 9 0/0 1/0 2/0 3/0 2/0 0/0 0/   visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 0/0 0/   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 1/0 0/0 0/0 0/0
phosphate transport 32 0/1 1/1 5/1 3/0 4/1 0/0 0/   collagen catabolic process 9 0/0 1/0 2/0 3/0 2/0 0/0 0/0 0/   visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/0   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 0/0 0/0 0/0   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 0/0 0/0 0/0
collagen catabolic process 9 0/0 1/0 2/0 3/0 2/0 0/0 0/0   visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/0   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 4/0 0/0 0/0   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 1/0 0/0 0/0 0/0
visual perception 74 2/1 1/2 5/1 6/1 8/3 1/1 0/   positive regulation of hormone secretion 12 1/0 1/0 0/0 2/0 4/0 0/0 0/   regulation of transcription factor import into nucleus 7 0/0 2/0 0/0 1/0 0/0 0/0 0/0 0/0 0/0
positive regulation of hormone secretion 12 $1/0$ $1/0$ $0/0$ $2/0$ $4/0$ $0/0$ $0/$
regulation of transcription factor import into nucleus 7 $0/0$ $2/0$ $0/0$ $1/0$ $3/0$ $0/0$ $0/$
water transport 7 0/0 2/0 1/0 0/0 3/0 0/0 0/
mRNA transport 35 3/1 0/1 0/1 2/0 0/1 3/0 0/
nuclear mRNA splicing, via spliceosome 99 5/2 2/3 0/2 2/1 1/4 5/1 0/
RNA splicing 159 7/3 2/5 0/3 3/1 2/6 6/1 0/
proton transport 50 0/1 3/2 2/1 0/1 0/2 0/0 3/

**Figure 4. Enriched biological processes among testicular androgen-responsive genes.** Over-represented "biological process" terms from the GeneOntology (GO) were identified in the 4 expression clusters of androgen-responsive genes (1 to 4) as well as in 3 groups of androgen-regulated genes that exhibit "somatic" (A to D), "spermatogonial" (E and F) and "germline" (G to I) expression profiles during spermatogenesis. Rectangles indicate the observed (left) and expected (right) numbers of genes bearing the corresponding GO term whereas the number of genes exhibiting this GO term on the entire microarray is given on the left. Only GO terms with a p-value of  $\leq 10^{-6}$  and for which at least 3 non-redundant genes were in the cluster were considered as statistically enriched. To avoid redundancy between closely related terms an Ontology Specific Information Rate (OSIR) cutoff of  $\geq 0.95$  was selected [27]. Numbers in bold indicate a statistical enrichment for a given GO term according to the scale bar.

doi:10.1371/journal.pone.0053302.g004

mf17). However, similar profiles for PCR and microarray data were obtained for *eprs* and *prpf8* (data not shown) and a significant down-regulation was confirmed for *noc2l* (Figure 6). Furthermore 3 genes preferentially expressed in meiotic and post-meiotic germ cells, *rsph3*, *mom3* and *bty*, were confirmed to be up-regulated following T supplementation.

#### Discussion

Androgens, in fish species notably, have been shown to masculinize the female gonad, to trigger spermatogenesis onset and to promote testis growth and maturation. In this study we provide new information on molecular pathways regulated by androgens in the testis of a vertebrate.We identified a complement of genes showing transcript regulation in fish testis after androgen supplementation, and a number of evidences support the physiological relevance of the data: Changes in expression were obtained in response to physiological doses of androgens and before any modification of the testis cell composition could be detected. The highly cyclic and partially synchronous development of the trout testis made it a choice model to show that many of these androgen-regulated genes are naturally differentially expressed at particular stages of testicular development, suggesting that they are indeed involved during testis maturation or during spermatogenesis. The model also enabled to predict in which cell type the regulated transcripts are expressed [23]: Regulated transcripts were preferentially found in the somatic compartment of the gonad, which is in agreement with the known expression of the AR in testicular somatic cells ([37,38]; and this work: Figure S5). We notably find many somatic cell genes whose developmental patterns fit well with changes in endogenous androgen circulating levels during natural testis maturation, which further reinforces the physiological significance of the data reported here. With this study we thus provide a substantial body of data and identify genes that likely contribute to pubertal development in the testis. Most of the comprehensive and useful information regarding these androgen-regulated genes - including expression during testis development, promoter analysis as well as annotation - is provided in the File S1 and in the File S2. Taken together these data also allow further speculating on the molecular mechanisms of androgen action in the testis.

The potential impact of androgens could include an active role in the initiation of spermatogenesis. Indeed, besides their effects on somatic cells, androgens altered the germ line towards more differentiated germ cell expression profiles. This would be quite in agreement with experimental data obtained in other fish species that showed androgens to induce spermatogenesis or accelerate this process (for reviews, see [39,40]). In trout, Baron and collaborators found that in early sexual differentiation, a prolonged treatment of female embryos with high doses of androgens promote testicular organization with apparition of meiosis whereas control male gonads contain only spermatogonia [41]. The way androgens act on germ cells and influence their



**Figure 5. Real-time PCR validation of candidate genes.** Relative expression profiles as measured by qPCR in the androgen supplementation experiment (left panel) and by microarray during spermatogenesis (right panel). The mRNA levels measured by qPCR were normalised to the Rps15 gene reference levels whereas microarray data correspond to Log2-transformed signal intensities. Mean values  $\pm$  SEM are are presented. One, 2 or 3 star symbols indicate p<0.05, p<0.01 or p<0.005, respectively, when comparing treated samples and corresponding controls (cont) using the non-parametric test of Mann & Whitney. T1 and T2 correspond to animals supplemented with testosterone implants of 0.1 and 0.2 mg, respectively. 11KT indicates animals treated with 11-ketotestosterone implants of 0.25 mg. Day 7 and Day 14 correspond to animals treated for 7 and 14 days, respectively. The number of biological replicates used for qPCR experiments is indicated in brackets. Roman numbers (I–V and VIII) indicate testicular developmental stages analysed by microarrays. Sg = spermatogonia. Sc = spermatocytes. St = spermatids.



Figure 6. Real-time PCR validation of candidate genes (continued). doi:10.1371/journal.pone.0053302.g006

proliferation and differentiation is likely to be indirect and to involve growth factors and extracellular signaling from the somatic compartment. Importantly, we found that androgens strongly down-regulate inhba and amh transcripts, two members of the Activin family. Activins have effects on many physiological processes, including a stimulatory effect on spermatogonia proliferation [42,43]. Inhibition of the BA subunit would prevent the formation of biologically active activin, particularly activin A (dimer BA-BA), and could therefore have a blocking action on germ cell proliferation and allow their differentiation towards meiosis. This is consistent with our observation of a rapid decrease of this transcript when meiosis develops during the reproductive cycle (stages III-V of spermatogenesis), coincidently with an increase in androgen levels. AMH (or Mullerian inhibiting substance) production in the mouse fetal testis has been linked with male-specific differentiation events associated with the assignment of male gender to PGCs [44]. anh expression and its hormonal regulation were studied in trout during gonad sex differentiation [45] but its role in the adult male gonad is mainly ignored. In vitro, when applied to prepubertal eel and zebrafish testis explants, AMH prevents androgen-stimulated proliferation or differentiation of early spermatogonia [46,47]. For the first time in trout we show that both T and 11KT can down-regulate amh in the early stages of pubertal maturation. Interestingly, we show that amh transcript relative abundance rapidly decreases during the natural reproductive cycle in inverse correlation with the large increase in circulating levels of T and 11KT (this study), and that amh transcripts are preferentially accumulated in Sertoli cells surrounding early spermatogonia [23]. In agreement, accumulation of the ar transcripts in Sertoli cells surrounding early germ cells was demonstrated in zebrafish testis [37]. Our data reinforce the idea that androgen-mediated suppressions of AMH and activin are physiologically required for initiation of active spermatogenesis at puberty. We propose to extend this hypothesis to several downregulated candidates from cluster 1 with known functions in cell fate/growth or differentiation, including inhibin, folistatin like 1, BMP and activin membrane-bound inhibitor (BAMBI), Plateletderived growth factor receptor beta, TGFB-inducible early growth response protein 2, Proliferation-inducing gene 32 protein, secreted retinol binding protein 4 and Angiopoietin-like factor 7. Other genes involved in cell commitment and specification (tbx1, sox8, sox9a and sox9b), were found regulated in androgen-treated prepubertal testes. By regulating the expression of such genes, androgens might influence the differentiation and function of the supporting somatic cells during the reproductive cycle. Furthermore, some of these genes are known as important for sex determination/differentiation (sox9a, dmrt1 and nr0b1) in the trout larvae [41], which emphasizes some similarities of androgen action both in adult testis development and in early gonad differentiation.

In addition to their roles during testis recrudescence, other regulated genes also emerge as probably involved in later stages of the reproductive cycle, at the time of sperm excretion. Within up-regulated genes, statistical enrichment was found for genes involved in "water transport" (*aqp1, aqp4* and *itpn/vspn*) and "ion transport", including "chloride transport" and "potassium transport" (*c1qtnf5, c1qtnf6, clic4, col1a1, col5a1, col6a2, col12a1, col18a1, gabra1, gabra3, ppap2a, slc12a4* and *slc26a4*), and "proton transport" (*atp6v0a2, atp6v1f* and *rsph3*). These functions are highly relevant concerning the final maturation of spermatozoa in fish, which relies on seminal plasma pH and Ca2+ concentration, their motility in the male tract, which depends on K+ concentration, and their excretion, which involves H2O transport [48,49]. Strikingly, these genes were also found strongly up-regulated in the ultimate stages of normal testicular development or spermato-

genesis (Figure 2: clusters A, C, G and H). Therefore the related genes reported here represent strong new candidates in sex steroid control of sperm hydration and final maturation in seminiferous tubules and efferent ducts.

An interesting issue regarding androgen function in fish species resides in the respective roles of T and 11KT for promoting male germ cell differentiation: 11KT was shown to be a potent androgen in some species [15,16,17,19], and in other cases T was shown to have no, or only weak positive effects [18,21]. More, T inhibits the positive effects of 11KT on germ cell proliferation and testis maturation in the African catfish [22]. Under our experimental conditions T appeared quantitatively and in several cases qualitatively more active than 11KT (more transcripts significantly modified with stronger regulatory effects following 7 days of treatment) at similar blood plasma concentrations. The mechanisms underlying the different effects of T and 11KT remain unknown. Specific conformation changes of the AR upon ligand binding may account for an efficient interaction with different transcription cofactors. Furthermore, the rainbow trout contains two distinct isoforms of the androgen receptor, ARa1 and AR $\alpha$ 2 [50,51], that may differ in terms of response to 11KT as suggested by the binding affinities of the AR $\alpha$ 2 in the salmon ovary [52]. Importantly, part of the specific effects we observed for T may occur through its metabolisation into active products, either locally or peripherally, including aromatization into estradiol and the subsequent regulation of estrogen-responsive genes. However, no evidence of such estrogenic effects could be noticed: Indeed, according to unpublished microarray data, the genes responding to T did not demonstrate significant changes in estradiol-treated males (Figure S6). Actually, only two out of the 418 androgen-responsive genes were also found to be regulated following estradiol-supplementation. Conversely, at the time points analysed, androgen supplementations did not result in change in expression for the vitellogenin or the choriogenin L genes, two highly responsive genes to estrogenic compounds in many fish tissues, including the testis (Figure S6; [53]). These observations support the idea that T and 11KT have both distinct and common functions in the gonad, even though a more detailed analysis (several time points and several doses) would be required to make final conclusions on the potencies of T compared to 11KT

Although we presume that most changes in expression occurred via the intra-testicular action of androgens, we cannot exclude that the effects we observe after in vivo supplementation partially result from alteration in the circulating levels of gonadotropin or other endocrine factors, or from the regulation of the testicular hormone receptivity. Indeed, positive and inhibitory effects on basal gonadotropin secretion by testosterone during the early-recrudescence phase of the gonadal cycle have been reported [54,55]. In addition, we found that the *fshr* transcript was slightly downregulated and that *lhr* was up-regulated at day 7, suggesting that the responsiveness to gonadotropin might be changed in treated animals. While the expression of  $ar\alpha 1$  and  $ar\alpha 2$  themselves was not modified following androgen treatment (Figure S5), genes encoding factors involved in the androgen or steroid metabolic pathways (cyp17a1, fdxr, hs3db2, star and tdh) or that mediate the response to androgens (nr0b1 and pap2a) were regulated. This is consistent with the modulation of steroidogenesis in an autoregulatory manner [56]. Such feedback loops are important for fine tune local regulation of testicular steroid production, and inhibitory effects may prevent Leydig cell hyperactivity in some stages of puberty.

Finally, when androgens act directly at the testis level, only a small proportion of the regulated genes are expected to be under the direct control of the AR. Using a phylogenetic footprinting approach we identified 42 genes as potential direct targets of the AR in the trout testis. Indeed, those androgen-regulated genes are of particular relevance as they have at least one highly conserved ARE predicted in their proximal promoter region (-2000/+400)with respect to their TSSs) and they are expressed in somatic cells where the AR is also present. This in silico analysis also highlighted genes that harbor two or more putative AR binding sites nearby the transcription initiation start site and are of great interest : Others and we have indeed demonstrated that multiple AREs, cooperate to confer full responsiveness and cell specific expression of some androgen target genes including for example, Igf1 [57], Lcn5 [58], Rhox5 [59] or Slp [60]. Of note is that, in addition to cAREs, we also found potential cEREs within the promoter of androgen-responsive genes (File S2). In silico promoter analyses indeed remain predictions and we we do not expect all predicted response elements to be functional. Instead we believe the somatic genes that respond to androgens and that also display proximal or multiple putative cAREs are preferential candidates for future functional validations.

In conclusion, we have identified a complement of genes expressed in the somatic compartment and predicted to be under the influence of androgens during testicular development in trout. Functions associated with these genes reveal potential mechanisms through which androgen exert their regulatory actions in tubule formation, germ cell differentiation, sperm secretion, and steroid production. Therefore we provide meaningful information on the mechanism by which T and/or 11KT could regulate germ cell production in vertebrates.

#### **Supporting Information**

**Figure S1 Expression profile of the reference gene** *rps15* **in microarray datasets.** Expression of *rsp15* (clone 1RT58B15\_B\_A08) as measured in androgen supplementation and spermatogenesis datasets. Histograms represent means  $\pm$ standard deviation of Log2-transformed signal intensities. Cont correspond to untreated control animals. T1 and T2 correspond to animals supplemented with testosterone implants of 0.1 and 0.2 mg, respectively. 11KT indicates animals treated with 11ketotestosterone implants of 0.25 mg. Day 7 and Day 14 indicate 7 and 14 day post-implantation, respectively. Roman numbers (I–V and VIII) indicate testicular developmental stages. Sg = spermatogonia. Sc = spermatocytes. St = spermatids. (TIF)

Figure S2 Changes in testicular gene expression following androgen supplementation. Heatmap representation of 418 androgen-responsive genes (485 clones) in the trout testis. After statistical filtration, co-expressed transcripts were classified into 4 clusters (1–4) using the PAM algorithm. For these genes, we present expression signals obtained: - in testes from controls and androgen-supplemented animals, and - in testes at various developmental stages and enriched fractions of isolated germ cells. Each line represents the expression signal of a single clone and each column is a sample. Normalized expression values are shown according to the scale bar while histograms represent averaged fold changes to the control (androgens data) or to satge I (spermatogenesis data)  $\pm$  SEM for each cluster. T1 and T2 correspond to animals supplemented with 0.1 and 0.2 mg testosterone implants, respectively; 11KT indicates animals treated with 0.25 mg 11-ketotestosterone implants; d7 and d14 correspond to animals treated for 7 and 14 days, respectively. The gene annotation of each cluster is accessible in the searchable File S1. (TIF)

Figure S3 Expression of genes altered differently by testosterone and 11-ketotestosterone treatments. Heatmap representation of 103 androgen-responsive genes (116 clones) which regulation by testosterone and 11-ketotestosterone differs statistically. Genes are displayed according to their response to testosterone only (T only; Up- or Down-regulated), to 11ketotestosterone only, or to their greater response to testosterone (T > 11KT; Up or Down regulated). Each line represents the expression signal of a single clone and each column is a sample. Normalized expression values are shown according to the scale bar while histograms represent averaged fold changes to the control  $\pm$ SEM for each cluster. T1 and T2 correspond to animals supplemented with testosterone implants of 0.1 and 0.2 mg, respectively; 11KT indicates animals treated with 11-ketotestosterone implants of 0.25 mg; d7 and d14 correspond to animals treated during 7 and 14 days, respectively. The gene annotation of each cluster is accessible in the searchable File S1. (TIF)

Figure S4 Enriched molecular functions and cellular components GeneOntology terms among testicular androgen-responsive genes. Over-represented "biological process" terms from the GeneOntology (GO) were identified in the 4 expression clusters of androgen-responsive genes (1 to 4) as well as in 3 groups of androgen-regulated genes that exhibit "somatic" (A to D), "spermatogonial" (E and F) and "germline" (G to I) expression profiles during spermatogenesis. Rectangles indicate the observed (left) and expected (right) numbers of genes bearing the corresponding GO term whereas the number of genes exhibiting this GO term on the entire microarray is given on the left. Only GO terms with a p-value of  $\leq 10^{-6}$  and for which at least 3 non-redundant genes belonged to the cluster were considered as statistically-enriched. To avoid redundancy between closely related terms an Ontology Specific Information Rate (OSIR) cutoff of  $\geq 0.95$  was selected [27]. Bolded numbers indicate a statistical enrichment for a given GO term according to the scale bar. (TIF)

**Figure S5 Expression profiles of** *arα1* **and** *arα2*. Expression of *arα1* and *arα2* was measured by qPCR and normalised to *rps15* expression levels. Cont corresponds to untreated control animals. T1 and T2 correspond to animals supplemented with testosterone implants of 0.1 and 0.2 mg, respectively. 11KT indicates animals treated with 11-ketotestosterone implants of 0.25 mg. Day 7 and Day 14 indicate 7 and 14 day post-implantation, respectively. Roman numerals (I-VIII) indicate developmental stages. SgA/B = Type A/B spermatogonia; ScI/II = primary/secondary spermatocytes; St = spermatids (TIF)

Figure S6 Androgen- versus estrogen-responsive genes. A: Heatmap representation of the expression of all the androgenresponsive genes according to the androgen-supplementation experiment (this study) as well as to the estradiol-supplementation experiment (unpublished data). Normalized expression data are displayed according to the scale bar. B: Venn diagram showing the overlap between androgen-responsive genes (this study) and estradiol-responsive genes (unpublished data). C-F: Expression profiles (as determined by microarray analysis) for two estradiolresponsive genes (choriogenin in C, vitellogenin in D) and for two estradiol- and androgene-responsive genes (aldh1l2 in E, mfap2 in E). Histograms represent fold changes to the control  $\pm$  SEM. (TIF) **Table S1 Sequences of primers used in qPCR experiments.** The clone name, the corresponding gene symbol and the sequence of forward and reverse primers (5'-3') used for qPCR measurements are indicated.

#### $(\mathbf{DOC})$

File S1 Androgen-responsive genes in the trout testis. A searchable excel file containing normalized expression data (Log-2 transformed), annotations, and information about "Androgen response" (clusters 1 to 4), "Testicular development" (clusters A to I) and the identification of "conserved ARE" for the 485 androgen-responsive clones. Annotation provided contains the "Clone Name", the "Annotation status" ("None", "Not confident", "Confident"), the "EST Name" and "Organism" retained for annotation, the corresponding "BLAT Score", the "Matching type" ("Exonic", "Intronic" or "Intergenic"), the "Overlapping length" (# base pairs; if <0 corresponds to the distance from the closest gene), the confidence index "n", the "Fish ortholog Ensembl gene IDs" and "Fish ortholog Gene Symbols" and "Descriptions", the "Non redundant ID" (Ensembl Gene ID of fish orthologs according to the following species availability: Gasteosteus aculeatus, Danio rerio, Oryzias latipes or Takifugu rubripes), the "Mammalian ortholog Ensembl gene IDs" (for human, mouse and rat), the "Mammalian ortholog gene symbols" and "Descriptions" and associated GeneOntology terms and IDs ("Biological process", Molecular function" and "Cellular component"). (XLS)

File S2 Identification of genes with putative conserved Androgen Response Elements. An excel file containing all putative conserved Androgen Response Elements (cAREs) and

#### References

- Gharib SD, Wierman ME, Shupnik MA, Chin WW (1990) Molecular biology of the pituitary gonadotropins. Endocr Rev 11: 177–199.
- Haywood M, Spaliviero J, Jimemez M, King NJ, Handelsman DJ, et al. (2003) Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone. Endocrinology 144: 509–517.
- Singh J, O'Neill C, Handelsman DJ (1995) Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. Endocrinology 136: 5311– 5321.
- El Shennawy A, Gates RJ, Russell LD (1998) Hormonal regulation of spermatogenesis in the hypophysectomized rat: cell viability after hormonal replacement in adults after intermediate periods of hypophysectomy. J Androl 19: 320–334; discussion 341–322.
- Elkington JS, Blackshaw AW (1974) Studies in testicular function. I. Quantitative effects of FSH, LH, testosterone and dihydrotestosterone on restoration and maintenance of spermatogenesis in the hypophysectomized rat. Aust J Biol Sci 27: 47–57.
- Meachem SJ, Ruwanpura SM, Ziolkowski J, Ague JM, Skinner MK, et al. (2005) Developmentally distinct in vivo effects of FSH on proliferation and apoptosis during testis maturation. J Endocrinol 186: 429–446.
- Orth JM, Gunsalus GL, Lamperti AA (1988) Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 122: 787–794.
- Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, et al. (2002) Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J Androl 23: 870–881.
- Johnston DS, Russell LD, Friel PJ, Griswold MD (2001) Murine germ cells do not require functional androgen receptors to complete spermatogenesis following spermatogonial stem cell transplantation. Endocrinology 142: 2405–2408.
- Chang C, Chen YT, Yeh SD, Xu Q, Wang RS, et al. (2004) Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proc Natl Acad Sci U S A 101: 6876–6881.
- De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, et al. (2004) A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc Natl Acad Sci U S A 101: 1327–1332.
- Holdcraft RW, Braun RE (2004) Hormonal regulation of spermatogenesis. Int J Androl 27: 335–342.
- Rolland AD, Jegou B, Pineau C (2008) Testicular development and spermatogenesis: harvesting the postgenomics bounty. Adv Exp Med Biol 636: 16–41.

Estrogen Response Elements (cEREs) identified in the promoter of Gasterosteus aculeatus genes. Each line corresponds to one such putative ARE. For genes harbouring several of these putative response elements, as many lines as necessary are presented. Provided is the "Ensembl Gene ID" and associated "Gene Symbol" and "Description", the chromosome location ("Chromosome" and "Strand" of the gene; the "Start" and "End" of the motif), the Transfac "Matrix ID" that matched the motif, the corresponding "Genomic sequence", its position relative to the Transcription Start Site ("-10000/-5000", "-5000/-2000", "-2000/-1000", "-1000/0", "0/+200", "+200/+400", "+400/+1000" and "+1000/+2000"; labelled "0" or "1"), the "Cross-species conservation score" and the "Orientation" of the motif (+ or -). Column D indicates those genes that are "Predicted as "Somatic" with a proximal cARE". If such a gene harbours several putative AREs, then only that (those) identified in the proximal region is (are) bolded. (XLS)

#### Acknowledgments

We thank the SIGENAE informatics team for their support, the PEIMA experimental fish farming facilities for the experimental animals and the INRA-Rennes/BIOSIT functional genomic platform for advice.

#### **Author Contributions**

Conceived and designed the experiments: RH FC FLG. Performed the experiments: ADR AL ASG JJL RH FC FLG. Analyzed the data: ADR AL ASG JJL RH FC FLG. Contributed reagents/materials/analysis tools: AL RH FC. Wrote the paper: ADR FLG.

- Borg B (1994) Androgens in teleost fishes. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 109: 219–245.
- Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991) Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (Anguilla japonica). Proc Natl Acad Sci U S A 88: 5774–5778.
- Kobayashi M, Aida K, Stacey NE (1991) Induction of testis development by implantation of 11-ketotestosterone in female goldfish. Zool Sci 8: 389–393.
- Amer MA, Miura T, Miura C, Yamauchi K (2001) Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (Hucho perryi ). Biol Reprod 65: 1057–1066.
- Cavaco JE, Vilrokx C, Trudeau VL, Schulz RW, Goos HJ (1998) Sex steroids and the initiation of puberty in male African catfish (Clarias gariepinus). Am J Physiol 275: R1793–1802.
- Leal MC, de Waal PP, Garcia-Lopez A, Chen SX, Bogerd J, et al. (2009) Zebrafish primary testis tissue culture: an approach to study testis function ex vivo. Gen Comp Endocrinol 162: 134–138.
- De Clercq L, Remacle C, Demal J (1977) Gonads of Carassius auratus (teleosts) in organ culture: a new technique and the effects of testosterone. In Vitro 13: 85–90.
- Cochran RC (1992) In vivo and in vitro evidence for the role of hormones in fish spermatogenesis. J Exp Zool 261: 143–150.
- Cavaco JE, Bogerd J, Goos H, Schulz RW (2001) Testosterone inhibits 11ketotestosterone-induced spermatogenesis in African catfish (Clarias gariepinus). Biol Reprod 65: 1807–1812.
- Rolland AD, Lareyre JJ, Goupil AS, Montfort J, Ricordel MJ, et al. (2009) Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. BMC Genomics 10: 546.
- Fostier A, Billard R, Breton B, Legendre M, Marlot S (1982) Plasma 11oxotestosterone and gonadotropin during the beginning of spermiation in rainbow trout (Salmo gairdneri R.). Gen Comp Endocrinol 46: 428–434.
- Rescan PY, Montfort J, Ralliere C, Le Cam A, Esquerre D, et al. (2007) Dynamic gene expression in fish muscle during recovery growth induced by a fasting-refeeding schedule. BMC Genomics 8: 438.
- Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185–193.
- Chalmel F, Primig M (2008) The Annotation, Mapping, Expression and Network (AMEN) suite of tools for molecular systems biology. BMC Bioinformatics 9: 86.

- Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3.
- Gomez JM, Loir M, Le Gac F (1998) Growth hormone receptors in testis and liver during the spermatogenetic cycle in rainbow trout (Oncorhynchus mykiss). Biol Reprod 58: 483–491.
- Gomez JM, Weil C, Ollitrault M, Le Bail PY, Breton B, et al. (1999) Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (Oncorhynchus mykiss). Gen Comp Endocrinol 113: 413–428.
- Loir M (1999) Spermatogonia of rainbow trout: I. Morphological characterization, mitotic activity, and survival in primary cultures of testicular cells. Mol Reprod Dev 53: 422–433.
- Karolchik D, Kuhn RM, Baertsch R, Barber GP, Clawson H, et al. (2008) The UCSC Genome Browser Database: 2008 update. Nucleic Acids Res 36: D773– 779.
- Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, et al. (2006) TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res 34: D108–110.
- Kel ÁÉ, Gossling E, Reuter I, Cheremushkin E, Kel-Margoulis OV, et al. (2003) MATCH: A tool for searching transcription factor binding sites in DNA sequences. Nucleic Acids Res 31: 3576–3579.
- Wasserman WW, Palumbo M, Thompson W, Fickett JW, Lawrence CE (2000) Human-mouse genome comparisons to locate regulatory sites. Nat Genet 26: 225–228.
- Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, Hou M, et al. (2005) Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Res 15: 1034–1050.
- de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J (2008) Functional characterization and expression analysis of the androgen receptor in zebrafish (Danio rerio) testis. Reproduction 136: 225–234.
- Galas JF, Hejmej A, Glogowski J, Bilinska B (2009) Morphological and functional alterations in testes and efferent ducts of homogametic rainbow trout Oncorhynchus mykiss Walbaum. Ann N Y Acad Sci 1163: 398–401.
- Taranger GL, Carrillo M, Schulz RW, Fontaine P, Zanuy S, et al. (2010) Control of puberty in farmed fish. Gen Comp Endocrinol 165: 483–515.
- Schulz RW, de Franca LR, Lareyre JJ, Le Gac F, Chiarini-Garcia H, et al. (2010) Spermatogenesis in fish. Gen Comp Endocrinol 165: 390–411.
- Baron D, Montfort J, Houlgatte R, Fostier A, Guiguen Y (2007) Androgeninduced masculinization in rainbow trout results in a marked dysregulation of early gonadal gene expression profiles. BMC Genomics 8: 357.
- Mather JP, Attie KM, Woodruff TK, Rice GC, Phillips DM (1990) Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. Endocrinology 127: 3206–3214.
- Miura T, Miura C, Yamauchi K, Nagahama Y (1995) Human recombinant activin induces proliferation of spermatogonia in vitro in the Japanese eel Anguilla japonica. Fish Sci 61: 434–437.
- Adams IR, McLaren A (2002) Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. Development 129: 1155–1164.

- Vizziano D, Randuineau G, Baron D, Cauty C, Guiguen Y (2007) Characterization of early molecular sex differentiation in rainbow trout, Oncorhynchus mykiss. Dev Dyn 236: 2198–2206.
- Miura T, Miura C, Konda Y, Yamauchi K (2002) Spermatogenesis-preventing substance in Japanese eel. Development 129: 2689–2697.
- Skaar KS, Nobrega RH, Magaraki A, Olsen LC, Schulz RW, et al. (2011) Proteolytically activated, recombinant anti-mullerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. Endocrinology 152: 3527–3540.
- Alavi SM, Cosson J (2006) Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol Int 30: 1–14.
- Marshall WS, Bryson SE, Idler DR (1993) Gonadotropin action on brook trout sperm duct epithelium: ion transport stimulation mediated by cAMP and Ca2+. Gen Comp Endocrinol 90: 232–242.
- Douard V, Brunet F, Boussau B, Ahrens-Fath I, Vlaeminck-Guillem V, et al. (2008) The fate of the duplicated androgen receptor in fishes: a late neofunctionalization event? BMC Evol Biol 8: 336.
- Takeo J, Yamashita S (1999) Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. J Biol Chem 274: 5674–5680.
- Fitzpatrick MS, Gale WL, Schreck CB (1994) Binding characteristics of an androgen receptor in the ovaries of coho salmon, Oncorhynchus kisutch. Gen Comp Endocrinol 95: 399–408.
- Woods M, Kumar A, Barton M, Woods A, Kookana R (2009) Localisation of estrogen responsive genes in the liver and testis of Murray rainbowfish Melanotaenia fluviatilis exposed to 17beta-estradiol. Mol Cell Endocrinol 303: 57–66.
- Dickey JT, Swanson P (1998) Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (Oncorhynchus kisutch). J Mol Endocrinol 21: 291–306.
- Khan IA, Hawkins MB, Thomas P (1999) Gonadal stage-dependent effects of gonadal steroids on gonadotropin II secretion in the Atlantic croaker (Micropogonias undulatus). Biol Reprod 61: 834–841.
- Schulz RW, Vischer HF, Cavaco JE, Santos EM, Tyler CR, et al. (2001) Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp Biochem Physiol B Biochem Mol Biol 129: 407–417.
- Wu Y, Zhao W, Zhao J, Pan J, Wu Q, et al. (2007) Identification of androgen response elements in the insulin-like growth factor I upstream promoter. Endocrinology 148: 2984–2993.
- Lareyre JJ, Reid K, Nelson C, Kasper S, Rennie PS, et al. (2000) Characterization of an androgen-specific response region within the 5' flanking region of the murine epididymal retinoic acid binding protein gene. Biol Reprod 63: 1881–1892.
- Barbulescu K, Geserick C, Schuttke I, Schleuning WD, Haendler B (2001) New androgen response elements in the murine pem promoter mediate selective transactivation. Mol Endocrinol 15: 1803–1816.
- Adler AJ, Scheller A, Hoffman Y, Robins DM (1991) Multiple components of a complex androgen-dependent enhancer. Mol Endocrinol 5: 1587–1596.