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Aquaporin-11 Control of Testicular Fertility Markers in Syrian Hamsters

John L. Shannonhouse[1], **Henryk F. Urbanski**[2], **Shih-Lung Woo**[3], **Li An Fong**[3], **Scott D. Goddard**[4], **William F. Lucas**[4], **Edward R. Jones**[4], **Chaodong Wu**[3], and **Caurnel Morgan**[1,3,*]

[1]Institute for Neuroscience, Texas A&M University, College Station, Texas 77843, USA

[2]Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, Oregon 97006, USA

[3]Department of Nutrition & Food Science, Texas A&M University, College Station, Texas 77843, USA

[4]Department of Statistics, Texas A&M University, College Station, Texas 77843, USA

Abstract

The present study sought novel changes to the hamster testicular transcriptome during modulation of fertility by well-characterized photoperiodic stimuli. Transition from long days (LD, 14 h light/day) to short days (SD, 10 h light/day) triggered testicular regression (61% reduction of testis weight, relative to LD) in SD-sensitive (SD-S) hamsters within 16 weeks. After 22 weeks of SD exposure, a third cohort of hamsters became SD-refractory (SD-R), and exhibited testicular recrudescence (137% testis weight gain, relative to SD-S). Partial interrogation of the testicular transcriptome by annealing-control-primer-modified differential display PCR provided several candidates for regulation of testicular functions. Multiple linear regression modeling indicated the best correlation for aquaporin 11 (*Aqp11*) with changes in testis weight. Correlations were also strongest for *Aqp11* with expression levels of reference cDNAs that control spermatogenesis (*Hspa2* and *Tnp2*), steroidogenesis (*Cox2*, *3βHsd*, and *Srebp2*), sperm motility (*Catsper1*, *Pgk2*, and *Tnp2*), inflammation (*Cox2*), and apoptosis (*Bax* and *Bcl2*). Moreover, siRNA-mediated knockdown of testicular *Aqp11* mRNA and protein reduced *Hspa2* and *Tnp2* mRNA levels, and it increased *3βHsd* mRNA levels. It also reduced mRNA levels for Sept12, which is a testis-specific inducer of spermatogenesis. These results suggest a central role for testicular *Aqp11* signaling in the coordinate regulation of crucial components of fertility.

[*]**Corresponding Author**: Caurnel Morgan, 218C Kleberg Center, Department of Nutrition & Food Science, Texas A&M University, College Station, TX 77843-2253; Tel, 979-458-1849; Fax, 979-458-3704; camorgan@tamu.edu.

Keywords

photoperiod; RNA interference; short interfering RNA; differential display PCR; mathematical modeling

1. INTRODUCTION

Men are attempting to father children at increasingly older ages, but male fertility gradually declines with older age (Crosnoe and Kim, 2013). Although distal regulators of male reproductive physiology, such as gonadotropins, have been studied extensively, proximal regulators have been incompletely characterized. The understanding of gradual changes in male fertility at the testicular level can be facilitated through the study of testicular physiology in animal models. In particular, male Syrian hamsters provide a model for finding conserved mechanisms that underlie normal reversible reduction of testicular function by photoperiodism (Morales et al., 2007; Pastor et al., 2011; Crosnoe and Kim, 2013) and irreversible reduction of testicular function by aging (Pastor et al., 2011; Morales et al., 2007). By contrast, laboratory rats and mice, show relatively minor changes in testicular function during modulation of the photoperiod (Francisco et al., 2004; Yellon and Tran, 2002).

Time frames and treatment regimens for photoperiodic modulation of reproductive capacity are established in Syrian hamsters (Gaston and Menaker, 1967). Following transfer from long days (LD) consisting of at least 12.5 h of light per day, to short days (SD) consisting of less than 12.5 h of light per day, male hamsters undergo testicular regression, which is characterized by marked reductions in spermatogenesis, steroidogenesis, sperm motility, and testis weight (Butler et al., 2008; Morgan et al., 2003b; Morgan and Shannonhouse, unpublished data; Jin et al., 2002). Moreover, nocturnal secretion of melatonin from the pineal gland is crucial for mammalian photoperiodism (Goldman, 2001). Decreased testosterone secretion begins within three weeks of SD exposure, and atrophy begins within 6 weeks (Urbanski, 1990; Berndtson and Desjardins, 1974; Gaston and Menaker, 1967). After 20 weeks of SD exposure, refractoriness to SD results in spontaneous recrudescence of testicular morphology and function (Butler et al., 2008; Nelson and Zucker, 1987). During SD exposure, chronic elongation of the period of melatonin secretion from the pineal gland (Tamarkin et al., 1976) mediates SD-induced inhibition of hypothalamic gonadotropin-releasing hormone signaling, as well as pituitary gonadotropin and prolactin secretion (Tamarkin et al., 1976; Richardson et al., 1982), which precede testicular atrophy by one to three weeks. Although SD-induced changes at the hypothalamic or pituitary levels of the reproductive axis are documented, mechanisms underlying changes at the testicular level are poorly understood.

Differential display PCR (DD-PCR) is useful to assess changes in gene expression without prior knowledge of gene sequences (Liang and Pardee, 1992). Thus, it is useful for the hamster model whose genomes have not been sequenced completely. Although it is a potent gene discovery tool, the original DD-PCR approach is plagued by false-positive results (Liang and Pardee, 1992; Dilks et al., 2003). Annealing control primer (ACP) technology,

however, has been found to markedly enhance PCR specificity, and it has been used to reduce false-positive results during the use of ACP-DD-PCR (Kim et al., 2004). The objective of the present study was to find novel changes in the testicular transcriptome that correlate with the photoperiodic modulation of reproductive capacity in Syrian hamsters, using ACP-DD-PCR.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

Syrian golden hamsters (*Mesocricetus auratus*), HsdHan:AURA (Harlan, Indianapolis, IN) or Lak:LVG(SYR)BR (Charles River, Kingston, NY), were purchased at age 8 weeks. Hamsters were group-housed in long days (LD) (14 h light, 10 h dark, lights on at 0600h) at 22 ± 1 °C for 2 weeks. LabDiet 5001 (Purina, Richmond, IN) and water were provided *ad libitum*. At age 10 weeks, some hamsters were exposed to short days (SD) (10 h light: 14 h dark, lights on at 0800 h) for 16, 16, or 22 weeks, respectively. After euthanasia by decapitation, testes were excised and weighed. Procedures used in this study were approved by Institutional Animal Care and Use Committees.

2.2. RNA extraction

Testis total RNA was extracted using TriZol (Invitrogen, Carlsbad, CA) or a guanidinium isothiocyanate method, as we have described (Morgan, 2012; Morgan et al., 2003a). Samples were homogenized in buffered 4M guanidinium isothiocyanate and sodium lauroyl sarcosine. RNA was precipitated in 3M LiCl and centrifuged. Protein was removed by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. DNA was digested with DNase I or extracted with acidic phenol.

2.3. Annealing Control Primer - Differential display PCR

The GeneFishing DEG Kit and instructions (SeeGene, Rockville, MD) were used. Reverse transcription was performed with MMLV reverse transcriptase and dT-ACP1 primer. PCR was performed in duplicate with SeeAmp ACP Master Mix and arbitrary primers. cDNA bands were visualized on 2% agarose gels.

2.4. Reverse transcription PCR

RT reactions were performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and either oligo $dT_{12\text{-}18}$ or random hexamers, as we have described (Morgan, 2012; Morgan et al., 2003a). PCR was performed with ReadyMix Taq PCR Mix (Sigma, St. Louis, MO). Absence of genomic DNA contamination was determined by PCR performed on RT reactions with or without RT. PCR co-amplifications were divided into two stages. First, 12-μl pre-amplification reactions were performed with primers for lower abundance cDNA. Second, 10 μl of pre-amplification reaction were combined with 10 μl of reaction mix containing the other primer. Conditions and primer sequences are provided in Table 1.

2.5. cDNA cloning and sequencing

Differentially represented bands from the ACP-DDPCR screening were excised from agarose gel and they, along with reference cDNAs generated by RT-PCR, were cloned using the TOPO-TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Plasmid containing the screened and reference cDNAs were prepared using the Wizard SV System (Promega, Madison, WI), and sequenced at the Gene Technology Laboratory facility (Texas A&M University). Information on rat orthologs of candidate hamster cDNA clones are provided in Table 2.

2.6. RNA interference

Aqp11 template cDNA (204 bp) was prepared by ligating T7 RNA Polymerase promoters onto amplicon termini with T4 DNA Ligase. The polymerase promoter sequence is: 5'-TAATACGACTCACTATAGGGAGAY-3'. Complementary sense and antisense RNAs (cRNAs) were transcribed *in vitro* using T7 RNA Polymerase, as we have described (Morgan, 2012; Morgan et al., 2003a). These cRNAs were incubated in hybridization buffer (20 mM HEPES at pH 7.9 and 0.1 M NaCl) in a thermal cycler at 50 °C, 94 °C, and stepping down 2 °C per cycle to reach 60 °C. *Aqp11* dsRNA was purified from agarose gel and digested with ShortCut RNase III into a heterogeneous mix of short interfering RNAs of 18–25 bp (siRNA), according to manufacturer's instructions. Hamsters at ages 16-18 weeks, under light ether anesthesia (Jana et al., 2002), received intra-testicular injections: unilateral, *Aqp11* siRNA (1 μg); and contralateral, vehicle (0.9% saline). After 72 h, subjects were sacrificed by CO_2 asphyxiation, decapitated, and testes were excised and stored at −80 °C. Testes were cut with RNase-free razor blades into 3-mm sections in an acrylic matrix (Stoelting, Wood Dale, IL) on dry ice. Samples were punched from frozen sections with a 1-mm micropunch for RNA extraction. The siRNA reagents were purchased from New England Biolabs (Ipswich, MA).

2.7. Western blotting

Lysates were prepared by homogenizing frozen testis samples in lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 50 mM sodium pyrophosphate, 0.1 M sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM benzamidine, and 2 mM PMSF). AQP11 (1:500 dilution) was analyzed by Western blot, as we have described (Wu et al., 2005), using AQP11 antiserum Alpha Diagnostics (San Antonio, TX).

2.8. Statistical analyses

Either repeated measures one-way ANOVA and Student-Newman-Keuls *posthoc* test or Student's *t*-test was used to determine statistical differences between means (± SEM). Paired Student's t-test was used to determine statistical differences by Western analysis. If the normality test failed, Kruskal-Wallis One Way ANOVA on Ranks was performed. Linear and multiple linear regressions were used to determine the relationship between testis weight and gene expression. Statistical analyses were performed using InStat 3.0 (GraphPad, San Diego, CA). Multiple linear regression with a forward variable selection algorithm was

performed using SAS-Enterprise Guide 4.3 with 0.05 significance level for entry (SAS Institute Inc., Cary, NC).

3. RESULTS

3.1. Putative Photoperiodic Regulation of Testicular cDNA Expression

Hamsters were exposed to 16 weeks of long days (LD), 16 weeks of short days during the photosensitive period (SD-S), or 22 weeks of short days into the photorefractory period (SD-R). After screening duplicate cDNA samples prepared from the testis of hamsters sequentially subjected to LD, SD-S, or SD-R with two annealing control primers (ACPs) that were chosen randomly, nine candidate bands were selected for cloning and sequencing because they were differentially represented on agarose gels (Fig. 1A). Each ACP consists of a: (a) 3′-end with an annealing nucleotide sequence complementary to a site on template cDNA; (b) 5′-end with an arbitrary sequence; and (c) central regulator that is comprised of at least one universal base that regulates the annealing portion in association with annealing temperature. Information on the hamster candidate and reference cDNA clones are provided in Tables 3 and 4, respectively. Seven of the nine cDNA fragments were cloned successfully. Upon sequencing and BLAST searching the NCBI GenBank database, six candidates were determined to contain aquaporin-11 (*Aqp11*), ataxin-7-like protein 1 (*At7l1*), coronin actin-binding protein-1c (*Coro1c*), dual specificity phosphatase-3 (*Dusp3*), nuclear interactor of Arf and Mdm2 (*Niam*), and zinc finger protein-639 (*Zfp639*). A seventh band containing a sequence with an undetermined identity (*Jls1*) was AT-rich, and therefore provided only poor targets for PCR primers. The two remaining cDNA candidates were ~400 bp and less than 100 bp, respectively, and repeated attempts to clone them were unsuccessful.

Relative to LD, SD-S apparently reduced *Aqp11* mRNA levels 77%, and SD-R increased them 293%. SD-S and SD-R apparently decreased and increased *At7l1* mRNA levels 63% and 113%, respectively (Fig. 1A). SD-S and SD-R apparently decreased and increased *Dusp3* mRNA levels 23% and 18%, respectively (Fig. 1A). SD-S and SD-R apparently decreased and increased and *Niam* mRNA levels 37% and 2%, respectively (Fig. 1A). SD-S and SD-R apparently decreased *Jls1* mRNA levels by 68% and 202%, respectively (Fig. 1A). By contrast, *Coro1c* and *Zfp639* mRNA levels appeared to go in the opposite directions of those of the other cDNAs (Fig. 1A). SD-S and SD-R apparently increased and decreased *Coro1c* mRNA levels by 277% and 72%, respectively (Fig. 1A). *Zfp639* mRNA was barely detectable in LD and SD-R and poorly quantifiable, but apparently changes in SD-S and SD-R samples were well in excess of 100% (Fig. 1A).

As expected, relative to LD, SD-S reduced testis weight 61%, and relative to SD-S, SD-R increased testis weight 137% (Fig. 1B). Linear regression analysis of the differential representation of mRNAs, determined after agarose gel electrophoresis, revealed the highest correlation for *Aqp11* with testis weight (r^2=0.68, $p < 0.01$) across all three photoperiodic treatment groups (Fig. 1C). The correlations for cDNAs, ranking from highest to lowest, were *Dusp3* (r^2=0.52, $p < 0.01$), *Niam* (r^2=0.45, $p < 0.01$), *Zfp639* (r^2=0.34, $p < 0.05$) and *At7l1* (r^2=0.14, p=0.170), and *Coro1c* (r^2=0.06, p=0.910).

3.2. Verification of Photoperiodic Regulation of Testicular cDNA Expression

Candidate mRNA levels were assessed by relative semi-quantitative RT-PCR using beta-actin (*Actb*) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an internal standard. Linear regression analyses for *Aqp11* and *Dusp3*, normalized to *Actb* or *Gapdh*, yielded similar results (*Aqp11*, r^2=0.75 and *Dusp3*, r^2=0.97). The internal standard was, therefore, selected to provide the best agarose gel resolution of amplicons for targets that were co-amplified with internal standards. Robust differences were found in expression of cDNAs isolated by ACP-DDPCR using 5 LD, 5 SD-S, and 4 SD-R samples, but the differences were smaller when reference mRNAs levels were assessed. Sample sizes were, therefore, increased to 7 LD, 7 SD-S and 6 SD-R in subsequent analyses. Tables 5A and 5B contain one-way ANOVA of candidate and reference mRNA levels, respectively, from the three treatment groups. Results of linear regression analyses of testis weight and candidate mRNA levels from these treatment groups are described in Table 6.

3.3. RT-PCR Analysis of Reference cDNA Expression

In addition to candidate cDNA clones isolated by ACP-DD-PCR screening, mRNA levels were assessed for reference cDNA clones that influence various cell functions (Table 4) that were deemed to be important for fertility. Table 5B contains results from one-way ANOVA analyses of means and SEM for reference mRNA levels. Results of linear regression analyses for testis mass and reference mRNA levels from separate treatment groups are described on Table 6.

We used multiple linear regression modeling of testis weight and mRNA levels for *Aqp11*, *Zfp639*, *At7l1*, *Dusp3*, and *Niam* with forward stepwise selection. We set significance levels for admission into, retention in, and exclusion from the model at 0.05, 0.35, and 0.15, respectively. Values for *Bax*, *Bcl2*, *3βHsd*, *Cox2* and *Srebp2* were forced into the model because their expression levels are known markers of testicular function. In other words, they were included in the model whether or not they predicted testis weight. The model admitted only Aqp11 and Zfp639 ($F_{7,4}$=48.65, p=0.001, adjusted r^2 =0.97). Only *Aqp11* had a statistically significant relationship with testis mass in the model ($F_{6,11}$= 9.57, p = 0.013, adjusted r^2 = 0.8237).

3.4. *Aqp11* mRNA Knockdown

Hamsters received unilateral intra-testicular injections of *Aqp11* small interfering RNA (siRNA), and contralateral injections of vehicle control. The results are described, relative to vehicle control. *Aqp11* siRNA reduced *Aqp11*/*Actb* mRNA levels 34% (Fig. 2A-2B), and it reduced AQP11 protein levels 33% (Fig. 2C-2D). Candidate and reference mRNA levels were also assessed. *Aqp7* mRNA levels, an index of off-target effects of *Aqp11* siRNA, did not change (Fig. 2E-2F). *Aqp7* has the highest homology to *Aqp11* of aquaporin family members expressed in testis, and it is thought to govern spermatogenesis (Calamita et al., 2001a).

Given *Aqp11* expression and potential importance in spermatids (Yeung, 2010; Yeung and Cooper, 2010), four spermatid-specific reference cDNAs were also included to evaluate *Aqp11* siRNA. Human cation channel sperm associated protein (*CATSPER1*), in sperm

flagellum, has been shown to be activated by elevated intracellular pH, and extracellular progesterone and prostaglandins (Lishko et al., 2011). Expression of human *CATSPER1* and murine *Catsper1* are required for sperm hyperactivation and fertility (Lishko et al., 2011; Carlson et al., 2003). Low expression levels of heat shock protein A2 (*HSPA2*) in human semen have been associated with infertility (Motiei et al., 2012) and previous studies suggest murine *Hspa2* is required for meiosis and spermatogenesis (Zhu et al., 1997). Phosphoglycerate kinase 2 (*Pgk2*), which catalyzes an ATP-generating step in glycolysis and is expressed only during spermatogenesis, has been found to be essential for normal sperm motility and male fertility in mice (Danshina et al., 2010). Transition protein (*Tnp2*) is required for chromatin condensation and histone removal, and it helps maintain fertility in mice (Zhao et al., 2001; Shirley et al., 2004). Septin 12 (SEPT12) is testis-specific and crucial for differentiation of male germ cells, and SEPT12 mutations have been found in infertile men (Kuo et al., 2012).

Aqp11 siRNA reduced *Tnp2*/*Actb* mRNA levels 54% (Fig. 2G-2H), and there was a linear relationship for *Tnp2* and *Aqp11* mRNA levels across both treatment groups (r^2=0.39, $F_{1,22}$=15.67, $p < 0.001$) and in siRNA samples alone (r^2=0.34, $F_{1,14}$=8.77, p=0.010). *Aqp11* siRNA reduced *Sept12*/*Actb* mRNA levels 49% (Fig. 2I-2J), and there was a linear relationship with *Aqp11* mRNA levels across both treatment groups (r^2=0.71, $F_{1,22}$=57.85, $p < 0.001$), and with siRNA alone (r^2=0.34, $F_{1,14}$=114.57, $p < 0.001$). *Aqp11* siRNA reduced *Hspa2*/*Actb* mRNA levels 18% (Fig. 2K-2L), and there was a linear relationship across treatment groups (r^2=0.23, $F_{1,22}$=7.83, p=0.010), but not in siRNA samples alone. *Aqp11* siRNA increased *3*β*Hsd*/*Actb* mRNA levels 36% (Fig. 2M-2N), but there was no linear relationship for *3*β*Hsd* and *Aqp11* mRNA levels across treatment groups or in siRNA samples alone. There were no effects of *Aqp11* siRNA on *Bax* (Fig. 2O-2P), *Bcl2* (data not shown), *Pgk2* (Fig. 2Q-2R), *Srebp2* (Fig. 2S-2T), *Catsper1* (Fig. 2U-2V), or *Cox2* (Fig. 2W-2X) mRNA levels, normalized to *Actb*.

Finally, because Aqp11 has been shown to be expressed in mouse and rat spermatids (Yeung and Cooper, 2010), expression levels of the four spermatid-specific reference genes were assessed for photoperiodic regulation (Table 5). The mRNA levels of *Catsper1*/*Actb*, *Pgk2*/*Actb*, and *Tnp2*/*Actb*, but not *Hspa2*/*Actb* were influenced by photoperiod. The mRNA levels for each of these four genes were lowest during SD-S exposure, and the levels for *Hspa2*/*Actb* did not differ between SD-S and SD-R.

5. DISCUSSION

In the course of identifying novel changes in the hamster testicular transcriptome that correlate with photoperiodic modulation of fertility, two main findings were produced. First, after a small set of cDNA candidates was identified by ACP-DD-PCR, multiple linear regression modeling indicated a high goodness-of-fit for changes in *Aqp11* mRNA levels during exposure to three photoperiodic conditions. Semi-quantitative RT-PCR allowed for verification of the correlation between *Aqp11* expression and testicular weight. Consequently, for the remainder of the study the focus was on the potential role of *Aqp11* in testicular functions. Second, siRNA-mediated knockdown indicated the important role of *Aqp11* in the tonic regulation of signals required for male fertility.

Photoperiodic manipulation has been established as a strategy for disrupting and restoring fertility in hamsters. Marked reductions in testis weight during SD-S exposure, and its recovery during SD-R exposure, have been shown previously to correlate with steroidogenesis, spermatogenesis, and sperm motility (Kawazu et al., 2003; Gaston and Menaker, 1967; Morgan et al., 2003b; Mayerhofer et al., 1989). Thus, it was expected that photoperiodic manipulation would induce near-global changes in the testicular transcriptome of hamsters. This prediction was supported by results presented herein. Specifically, even partial interrogation of the testicular transcriptome by ACP-DD-PCR allowed rapid identification of *Aqp11* as a candidate to regulate testicular function. Thus, testicular regression and recrudescence in hamsters have continued to provide information to inform the study of reproductive capacity.

Multiple linear regression modeling showed that *Aqp11* fit predetermined criteria for potentially regulating testis function. Although acute changes in *Aqp11* expression are not proposed here to regulate testis weight, proximal factors, such as *Aqp11*, that mediate photoperiodic effects on genes that regulate steroidogenesis and sperm function, are likely to affect structural changes that attend to those effects. The cDNA candidates that were excluded by multiple linear regression modeling were *At7l1*, *Coro1c*, *Dusp3*, *Niam*, and *Zfp639*.

Aqp11 is expressed abundantly in testis and kidney (Yeung and Cooper, 2010; Gorelick et al., 2006). Although aquaporins transport water and solutes across cell membranes, *Aqp11* has not been show to do so (Gorelick et al., 2006). Heterogeneous expression of aquaporins along the male reproductive tract suggests that they modulate local fluid balance (Pastor-Soler et al., 2001; Arrighi et al., 2010; Calamita et al., 2001a; Calamita et al., 2001b). In the testis, *Aqp11* is expressed in elongated spermatids at the time of the shedding of residual bodies before spermiation (Yeung and Cooper, 2010). A reproductive function for *Aqp11* has not been determined in *Aqp11* knockout mice, as 85% have died of renal failure by postnatal day 60, with cysts forming in their renal cortices and large vacuoles of endoplasmic reticulum origin forming in proximal tubule cells (Morishita et al., 2005). Interestingly, *Aqp11* transfected into CHO cells localized to endoplasmic reticulum (Morishita et al., 2004).

As might be expected, six of the reference cDNAs, *Catsper1*, *3βHsd*, *Hspa2*, *Pgk2*, *Srebp2*, and *Tnp2*, were expressed at their lowest levels during SD-S. Given the extent to which reduction of spermatogenesis, steroidogenesis, and sperm motility are expected to associate with a major reduction of testis weight during SD-S (Butler et al., 2008; Morgan et al., 2003b; Morgan and Shannonhouse, unpublished data; Jin et al., 2002), the observed changes in the expression levels for these genes reflects a global loss of testicular function. Apoptosis occurs extensively during spermatogenesis in hamsters (Pastor et al., 2011). Reduction of both apoptosis-associated genes, *Bax* and *Bcl2*, during SD-R likely maintains constitutive and induced apoptosis within optimal ranges.

Aqp11 knockdown with intratesticular siRNA selectively altered gene expression. A heterogeneous siRNA mixture of *Aqp11* cDNA fragments was used, as this general strategy has avoided shortcomings of testing a series of single siRNA sequences (Morlighem et al.,

2007). In addition to that of *Aqp11*, expression levels of *Hspa2*, *Sept12*, and *Tnp2* were reduced by *Aqp11* siRNA. Importantly, roles for *Hspa2*, *Sept12*, and *Tnp2* in promoting sperm motility and spermatogenesis is conserved in humans and rodents (Zhao et al., 2001; Adham et al., 2001; Motiei et al., 2012; Kuo et al., 2012; Lin et al., 2009; Dix et al., 1997). Interestingly, this same treatment increased expression of steroidogenic *3βHsd*. Recent studies indicate androgens can reduce spermatogenesis, forming a basis for male contraception (Chen et al., 2005). At 72 h post-injection, treatment with *Aqp11* siRNA did not alter testicular weight. A prolonged knockdown of *Aqp11* expression might have an effect, as SD exposure requires a few weeks to reduce testicular weight (Morgan, 2012; Morgan et al., 2003a). Under the experimental conditions used in the present study, however, longer siRNA treatment increase the likelihood of inducing a confounding immune response with inflammation and cytotoxicity (Beyerle et al., 2011).

By contrast, expression levels of other genes that influence sperm motility (*Catsper1* and *Pgk2*) and steroidogenesis (*Srebp2*) were unaltered by *Aqp11* siRNA. Furthermore, testicular *Aqp11* siRNA did not alter *Aqp7* mRNA levels, providing evidence against off-target effects of *Aqp11* siRNA. Moreover, absence of effects of *Aqp11* siRNA on *Cox2*, *Bax*, and *Bcl2* mRNA levels provides evidence against global pathological effects, as *Cox2* mediates immunogenic responses. Absence of changes in expression of pro-apoptotic *Bax* and anti-apoptotic *Bcl2* genes suggests no effect on basal apoptosis. Although the exploration of mechanisms that underlie *Aqp11* regulation of the expression of multiple genes fell outside the scope of this study, it is likely that such coordinate regulation is brought about by changes in a core testicular function.

The collective results elucidate testicular processes that influence infertility. ACP-DDPCR screening identified candidate genes for control of testicular regression and recrudescence. Although there are previous reports of *Aqp11* expression in testis, to our knowledge the present study is first to provide molecular evidence of a role for Aq11 in specific testicular functions. The close relationship between *Aqp11* expression and testicular weight during photoperiodic modulations suggests that the approach used herein provides a means by which future studies could assess incremental changes in testicular function over finely tuned time courses, in order to elucidate further the proximal control of fertility.

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HIGHLIGHTS

- Differential display PCR revealed candidate genes for control of fertility in testis during photoperiodic modulation
- Multiple linear regression modeling helped identify aquaporin-11 as the best fit among the candidate genes
- RNA interference of *Aqp11*, using small interfering RNA demonstrated Aqp11 tonically induces markers of fertility
- The collective results provide the first molecular evidence that *Aqp11* regulates fertility

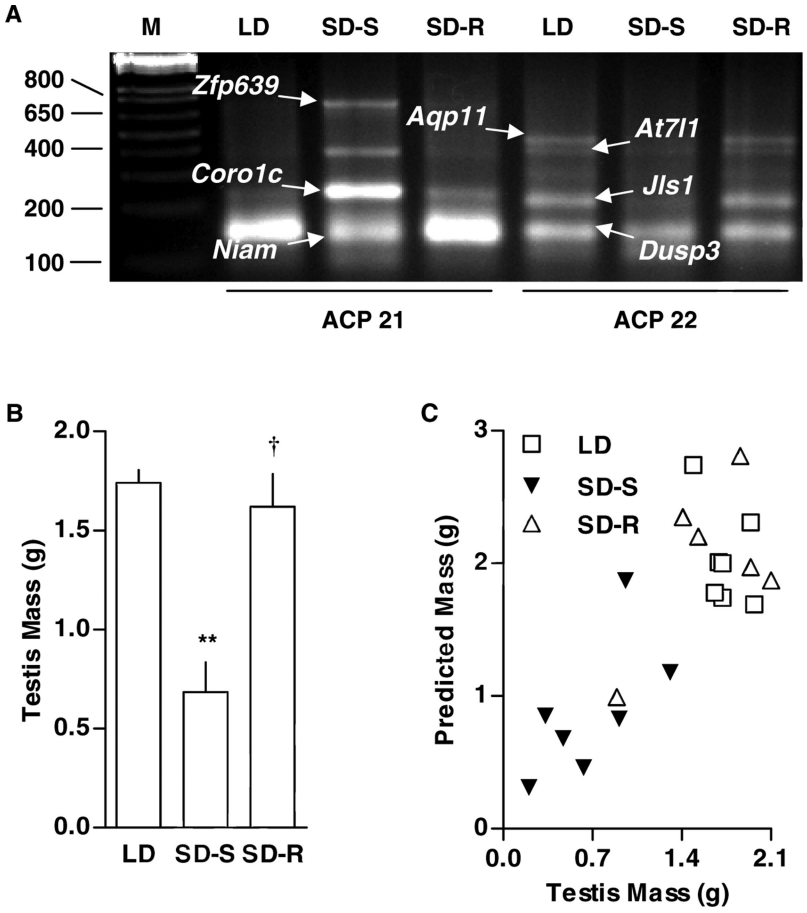

Figure 1. Photoperiodic regulation of testicular expression and functionality

(A) Differential display PCR was used to assess changes in testicular transcript levels of hamsters exposed to long days (LD: 14 h light and 10 h dark for 16 weeks, n = 7), short days during the photosensitive period (SD-S: 10 h light and 14 h dark for 16 weeks, n = 7), or short days during the photorefractory period (SD-R: 10 h light and 14 h dark for 22 weeks, n = 6).Differentially represented cDNAs were extracted from the agarose gel, cloned into the TOPO-TA cloning vector, and sequenced. Among the genes whose roles in testicular function have not been established, we identified aquaporin-11 (*Aqp11*), ataxin-7-like protein-1 (*At7l1*), coronin actin-binding protein-1c, dual specificity phosphatase-3 (*Dusp3*), nuclear interactor of *Arf* and *Mdm2* (*Niam*), a cDNA of undetermined identity (*Jls1*), and zinc finger protein-639 (*Zfp639*). **(B)** Relative to LD exposure, SD-S exposure decreased testis weight by 61%. Relative to SD-S exposure, SD-R exposure increased testis weight by 140%. Means (± SEM) are shown. For LD vs SD-S comparison, **$p < 0.01$. For SD-S vs SD-R comparison, †$p < 0.01$. **(C)** Multivariate regression analysis, across the three photoperiodic treatments, revealed that *Aqp11* gene expression had the highest correlation with testis weight.

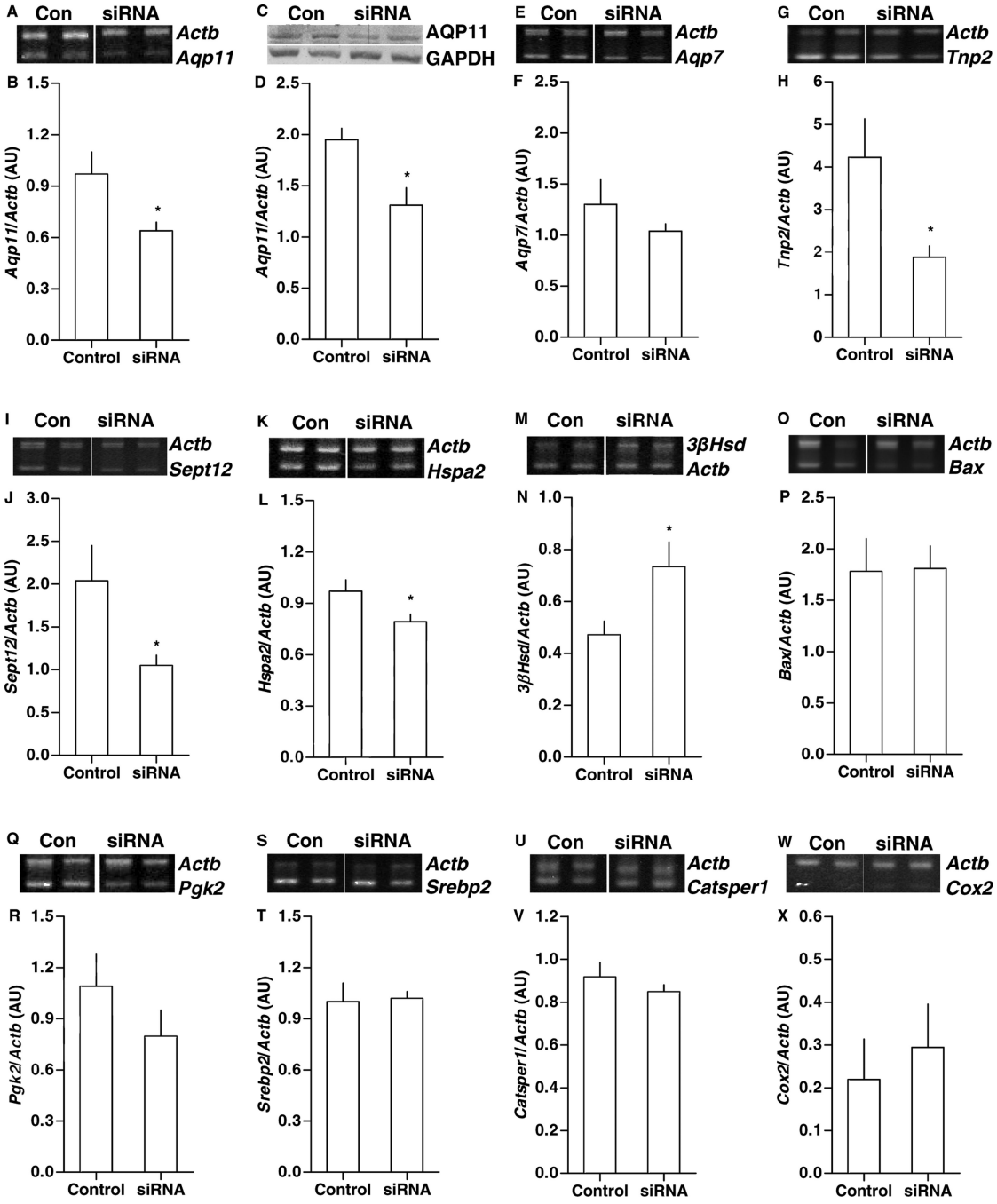

Figure 2. *Aqp11* Knockdown effects on testicular expression

Hamsters were injected with vehicle control (n = 8) or *Aqp11* siRNA (n = 16), and assessed for experimental and reference mRNA or protein levels by RT-PCR (with beta-actin (*Actb*) co-amplification) or Western blot (normalized to GAPDH protein), respectively. Representative bands are shown for agarose or polyacrylamide gel analyses. **(A)** *Aqp11* and *Actb*. **(B)** *Aqp11*/*Actb* mRNA levels decreased. **(C)** Western blot analysis of AQP11 and GAPDH. **(D)** AQP11/GAPDH protein levels decreased. **(E)** *Aqp7* and *Actb*. **(F)** *Aqp7*/*Actb* mRNA levels did not change. **(G)** *Tnp2* and *Actb*. **(H)** *Tnp2*/*Actb* mRNA levels decreased.

(I) *Sept12* and *Actb*. **(J)** *Sept12*/*Actb* mRNA levels decreased. **(K)** *Hspa2* and *Actb*. **(L)** *Hspa2*/*Actb* mRNA levels decreased. **(M)** *3βHsd* and *Actb*. **(N)** *3βHsd*/*Actb* mRNA levels increased. **(O)** *Bax* and *Actb*. **(P)** *Bax*/*Actb* mRNA levels did not change. **(Q)** *Pgk2* and *Actb*. **(R)** *Pgk2*/*Actb* mRNA levels did not change. **(S)** *Srebp2* and *Actb*. **(T)** *Srebp2*/*Actb* mRNA levels did not change. **(U)** *Catsper1* and *Actb*. **(V)** *Catsper1*/*Actb* mRNA levels did not change. **(W)** *Cox2* and *Actb*. **(X)** *Cox2*/*Actb* mRNA levels did not change. Means ± SEM are shown in graphs. For siRNA vs control, *$p < 0.05$.

Table 1

PCR Pre-amplification Conditions

cDNA	Primers	T_a (°C)	Preamp Cycles
		A. Experimental cDNA Clones	
Aqp11	Fwd: ACTGGCTTGCTCCTTCTCTAGGT	59.5	2 Gapdh
	Rev: TTGAAACAGTGACGAGTCCAGTGC		1 Actb
At7l1B	Fwd: ATCCCGTTAACAGCACCACCTCT	59.5	1 Gapdh
	Rev: AAAGGAACGAAGGAACTCACAGCGTC		4 Actb
Corolc	Fwd: ACAGGGAYCTCAAGGTNGTCAAGAAGA	59.5	8 Actb
	Rev: AAGAGTGGATCTGGGAGCAGTTCA		
Dusp3	Fwd: GTGCARGATCTCAACGACCTGCT	59.5	5 Gapdh
	Rev: CATAAGCTCCCTAAGGGTGGAGTGA		
Jlsl	No primers		N/A
Niam	Fwd: ATGCARCTATGAGCCTTGAAGCCTT	59.5	2 Gapdh
	Rev: AAGCTTATCCCAGGGTACACTGTCA		
Undl		N/A	N/A
Und2	N/A	N/A	N/A
Zfp639	Fwd: TGTGCAGTTCTCCTCAAGCAGTGA	59.5	3 Gapdh
	Rev: TTCCCAAACCTCATCAGGCAGTCA		
		B. Reference cDNA Clones	
Bax	Fwd: GCTGCAGAGGATGATTGCTA	55.0	7 Actb
	Rev: GATGGTGAGTGAGGCAGTGA		
Bcl-2	Fwd: CCTTCCAGCTTGAGAGCAAC	56.0	8 Actb
	Rev: CAGATGCCGGTTCAGGTACT		
Catsper1	Fwd: ATGGTCATGGCAGTGCTGGACTT	59.0	8 Actb
	Rev: CCAGGTTGAGGAAGATGAAGTACTGGAT		
Cox1	Fwd: CAACTCCATCTTTGGGGAGA	54.0	12 Actb
	Rev: AACATACGGGCAGGTCTTTG		
Cox2	Fwd: CAACTCCCTTGGGTGTGAAAGGAA	59.0	15 Actb
	Rev: TGAGTGTCATTGACTGTGGGAGGA		
3P-Hsd	Fwd: CCTGAAAAATGGTGGCACTT	54.0	10 Actb
	Rev: TGTGACCAAGTATCGGGTGA		
Hspa2	Fwd: CCATGGTCCTCACTAAGATGAAGGAGAT	59.0	3 Actb
	Rev: GTTGGGCCCAATGTCCTTCTTGT		
Pgk2	Fwd: GCTATCCTTGGTGGAGCCAAAGT	59.0	3 Actb
	Rev: GTTCCTTTAGCAAAGGCATCCCATTCA		
Sept12	Fwd: TGGTGAACACGCTGTTCAAGTCCAA	59.0	2 Actb
	Rev: CTCAGGATAGGGTCCCAGCACTT		
Tnp2	Fwd: AGACCTTTGAAGGGAAAGTGAGCAAGA	59.0	1 Actb
	Rev: ATTGCTGCAGTGACATGTTCCTGT		
		C. Housekeeping cDNA Clones	
Actb	Fwd: TCGTACCACAGGCATTGTGATGGA	N/A	N/A

cDNA	Primers	T_a (°C)	Preamp Cycles
	Rev: ACTCCTGCTTGCTGATCCACATCT		
Gapdh (1)	Fwd: TCACCATCTTCCAGGAGCGAGAYCC	N/A	N/A
	Rev: CCTGCTTCACCACCTTCTTGATGT		
Gapdh (2)	Fwd: TCCTGCACCACCAYCTGCTTAG	N/A	N/A
	Rev: CCTGCTTCACCACCTTCTTGATGT		

Preamp cycles: # of PCR cycles performed on target before adding primers for internal standard. Gapdh and β-actin were pre-amplified before adding Aqp11 primers, due to aquaporin 11 abundance in testis.

Table 2

Rat Orthologs of Candidate Hamster cDNA Clones

Hamster	Rat	Size (bp)	Accession #
cDNA1	Aquaporin-11 (Aqp11)	476	NM_173105
cDNA2	Ataxin-7-like protein 1 (At7l1)	414	XM_234056
cDNA3	Coronin actin-binding protein 1c	233	NM_001109327
cDNA4	Dual specificity phosphatase (Dusp3)	135	NM_028207
cDNA5	Nuclear interactor of Arf and Mdm2 (Niam)	151	NM 001009344
cDNA6	Unique Sequence (Jls1)	218	N/A
cDNA7	Undetermined 1 (Und1)	N/A	N/A
cDNA8	Undetermined 2 (Und2)	N/A	N/A
cDNA9	Zinc finger protein-639 (Zfp639)	752	NM_001080907

Dusp3 and Niam were identified by relaxing BLAST search parameters, and their amino acid sequences were not deduced, as their clones fell outside the coding regions. Two attempts to clone Und1 and Und2 cDNA were unsuccessful.

Table 3

ACP-DD-PCR Analysis of Candidate Hamster cDNA Clones

cDNA	Size (bp)	Change	Accession #
Aquaporin-11 (Aqp11)	393	↓in SD-S	GQ254789.1
Ataxin-7-like protein-1 (At7l1)	331	↓in SD-S	GQ254792.1
Coronin actin-binding protein-1c (Coro1c)	152	↑in SD-S	GQ254791.1
Dual specificity phosphatase-3 (Dusp3)	640	↓in SD-S	GU170206.1
Unknown Sequence (Jls1)	218	↓in SD-S	TBD
Nuclear interactor of Arf and Mdm2 (Niam)	323	↓in SD-S	GU170205.1
Undetermined 1 (Und1)	N/A	N/A	N/A
Undetermined 1 (Und1)	N/A	N/A	N/A
Zinc Finger protein-639 (Zfp639)	639	↑in SD-S	GQ254790.1

Testicular mRNA levels were assessed by differential display PCR for hamsters (n = 2?) exposed to 16 weeks of long days (LD, 14L:10D), 16 weeks of short days during photosensitivity (SD-S, 10L:14D), or 22 weeks of SD during photorefractoriness (SD-R, 10L:14D).

Table 4

Hamster Reference cDNA Clones

cDNA	Known Functions	Results
Aquaporin-11 *(Aqp11)*	Renal function	↓in SD-S, vs LD & SD-R
Ataxin-7-like protein-1 *(At7l1)*	SAGA histone acetylation	↓in SD-S, vs LD & SD-R
Coronin actin-binding protein-1c *(Coro1c)*	Wound healing, cytoskeleton in cellular projections	No detectable change
Dual specificity phosphatase-3 *(Dusp3)*	Jak/Stat pathway, cell cycle control	↓in SD-S, vs LD & SD-R
Unknown Sequence (Jls1)	N/A	N/A
Nuclear interactor of Arf & Mdm2 *(Niam)*	Cell cycle, chromosome stability	↓in SD-S, vs LD & SD-R
Undetermined 1 *(Und1)*	N/A	N/A
Undetermined 1 *(Und1)*	N/A	N/A
Zinc Finger protein-639 *(Zfp639)*	CRE binding	No detectable change
Bcl2-associated protein (Bax)	Pro-apoptosis	↓in SD-R, vs SD-S
B-cell lymphoma-2 *(Bcl2)*	Anti-apoptosis	↓in SD-R, vs SD-S
Cation channel sperm-associated protein-1 *(Catsper1)*	Sperm motility, spermatid-specific	↓in SD-S, vs LD & SD-R
Cyclooxygenase-2 *(Cox2)*	Prostaglandin synthesis, inflammation, steroidogenesis	↓in SD-S & SD-R, vs LD
Heat-shock-related protein-2 *(Hspa2)*	Spermatid-specific spermatogenesis	↓in SD-S & SD-R, vs LD
3Beta-hydroxysteroid dehydoxylase *(3β-Hsd)*	Steroidogenesis	↓in SD-S & SD-R, vs LD
Phosphoglycerate kinase 2 *(Pgk2)*	Spermatid-specific motility, spermatogenesis	↓in SD-S, vs LD & SD-R
Sterol regulatory element binding protein-2 *(Srepb2)*	Steroidogenesis	↓in SD-S, vs LD & SD-R
Transition protein-2 *(Tnp2)*	Spermatid-specific motility, DNA condensation, spermatogenesis	↓in SD-S, vs LD & SD-R

Testis mRNA levels for hamsters (n = 5-7) exposed to 16 weeks of long days (LD, 14L:10D), 16 weeks of short days during photosensitivity (SD-S, 10L:14D), or 22 weeks of SD during photorefractoriness (SD-R, 10L:14D) were assessed by RT-PCR.

*$p < 0.050$

**$p < 0.010$

Table 5

Photoperiodic Regulation of Testicular mRNA Levels

A. cDNA Clones from ACP-DD-PCR Screen

cDNA	LD	SD-S	SD-R	Samples	*F*-statistic	*p*-value
Aqp11	0.53 ± 0.05	0.25 ± 0.05**	0.45 ± 0.05†	n = 6-7	$F_{2,17}$ = 8.64	0.003
At7l1	1.46 ± 0.29	0.61 ± 0.11**	1.28 ± 0.23††	n = 5	$F_{2,12}$ = 4.04	0.046
Coro1c	1.13 ± 0.10	1.11 ± 0.17	0.99 ± 0.13	n = 5	$F_{2,12}$ = 0.31	0.740
Dusp3	1.92 ± 0.18	0.71 ± 0.19**	1.25 ± 0.10	n = 6-7	$F_{2,17}$ = 13.38	0.001
Jls1	N/D	N/D	N/D	n = 5	N/D	N/D
Niam	1.04 ± 0.07	0.66 ± 0.12**	1.01 ± 0.10†	n = 6-7	$F_{2,17}$ = 4.69	0.024
Und1	N/D	N/D	N/D	n = 5	N/D	N/D
Und2	N/D	N/D	N/D	n = 5	N/D	N/D
Zfp639	0.51 ± 0.05	0.51 ± 0.05	0.55 ± 0.08	n = 5	$F_{2,12}$ = 0.14	0.871

B. Reference cDNA Clones

cDNA	LD	SD-S	SD-R	Samples	*F*-statistic	*p*-value
Bax	1.42 ± 0.15	1.66 ± 0.09	1.15 ± 0.12†	n = 5	$F_{2,12}$ = 4.34	0.038
Bcl2	0.82 ± 0.20	0.89 ± 0.07	0.61 ± 0.13†	n = 5	$F_{2,12}$ = 1.03	0.386
Catsper1	1.26 ± 0.17	0.42 ± 0.15*	1.58 ± 0.27††	n = 6-7	$F_{2,17}$ = 9.33	0.002
Cox2	0.41 ± 0.12	0.67 ± 0.15	0.63 ± 0.08	n = 5	$F_{2,12}$ = 1.36	0.294
3βHsd	0.59 ± 0.06	0.24 ± 0.05**	0.36 ± 0.05*	n = 5	$F_{2,12}$ = 11.04	0.002
Hspa2	0.70 ± 0.09	0.44 ± 0.07	0.43 ± 0.14	n = 6-7	$F_{2,17}$ = 2.39	0.122
Pgk2	1.14 ± 0.13	0.43 ±0.16**	0.99 ± 0.07††	n = 6-7	$F_{2,17}$ = 8.54	0.003
Srepb2	0.61 ± 0.07	0.35 ± 0.06*	0.64 ± 0.06†	n = 6-7	$F_{2,17}$ = 6.29	0.009
Tnp2	0.52 ± 0.11	0.17 ± 0.10*	0.50 ± 0.07†	n = 6-7	$F_{2,17}$ = 4.19	0.033

Testis mRNA levels were assessed by RT-PCR for hamsters (n = 5-7) exposed to 16 weeks of long days (LD, 14L:10D), 16 weeks of short days during photosensitivity (SD-S, 10L:14D), or 22 weeks of SD during photorefractoriness (SD-R, 10L:14D). (A) cDNA clones screened by ACP-DD-PCR and normalized after Actb co-amplification. ANOVA for mRNA expression levels between groups (*F*-statistic) are shown. (B) Reference cDNA normalized after Actb co-amplification. Means (± SEM) are shown. For SD-S or SD-R vs LD

For SD-S vs SD-R comparisons

* $p < 0.050$

** $p < 0.010$.

† $p < 0.050$

†† $p < 0.010$. N/D, not determined. Degrees of freedom (associated with *F*-statistics) represent treatments between groups (i.e., 2) and residuals within groups (i.e., 12 or 17).

Table 6

Relationship Between Testis Mass and mRNA Levels

cDNA	All, r^2	All, p	LD, r^2	LD, p	SD-S, r^2	SD-S, p	SD-R, r^2	SD-R, p
Aqp11	0.68**	0.001	0.010	0.976	0.640**	0.003	0.544	0.094
At7l1	0.14	0.165	0.298	0.205	0.018	0.829	0.440*	0.039
Corolc	0.01	0.908	−0.090	0.508	0.150	0.211	0.297**	0.009
Dusp3	0.52**	0.001	0.008	0.853	0.674*	0.024	0.035	0.722
Jls1	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Niam	0.45**	0.001	0.321	0.107	0.440	0.062	−0.119	0.531
Und1	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Und2	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Zfp639	0.34*	0.048	0.035	0.686	0.730**	0.008	0.55	0.091
Bax	0.14	0.096	0.053	0.620	0.004	0.918	0.019	0.796
Bcl2	0.01	0.718	0.102	0.485	0.311	0.329	0.673*	0.046
Catsper1	0.35**	0.004	0.471	0.053	−0.183	0.799	−0.146	0.580
Cox2	0.22*	0.037	0.042	0.658	0.806**	0.006	0.102	0.067
3βHsd	0.18	0.067	0.008	0.846	0.018	0.775	0.159	0.434
Hspa2	0.19*	0.030	−0.200	0.987	0.048	0.306	0.486	0.075
Pgk2	0.44**	0.001	−0.107	0.545	0.476	0.052	0.529	0.062
Srepb2	0.55**	0.001	0.251	0.252	0.746**	0.010	0.580	0.078
Tnp2	0.32**	0.006	−0.012	0.380	0.397	0.077	−0.234	0.829

Testicular mRNA levels were assessed by RT-PCR for hamsters (n = 5-7) exposed to long days (LD, 14L: 10D for 16 weeks), short days during photosensitivity (SD-S, 10L:14D for 16 weeks), or SD during photorefractoriness (SD-R, 10L:14D for 22 weeks). Linear regression analyses of mRNA levels versus testicular mass were performed over all three treatment groups and individual groups. The correlation coefficient (r^2) and p values are shown. N/D, not determined.

* $p < 0.050$

** $p < 0.010$