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# Age-related and photoperiodic variation of the *DAZ* gene family in the testis of the Syrian hamster (*Mesocricetus auratus*)

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## Summary

The *D*eleted in *AZ*oospermia (*DAZ*) gene family regulates the development, maturation and maintenance of germ cells and spermatogenesis in mammals. The *DAZ* family consists of two autosomal genes, *Boule* and *Dazl* (*Daz-like*), and the *Daz* gene on chromosome Y. The aim of this study was to analyze the localization of DAZL and BOULE during testicular ontogeny of the seasonal-breeding Syrian hamster, *Mesocricetus auratus*. We also evaluated the testicular expression of *DAZ* family genes under short- or long-photoperiod conditions. In the pre-pubertal and adult testis, DAZL protein was found mainly in spermatogonia. BOULE was found in the spermatogonia from 20 days of age and during the pre-pubertal and adult period it was also detected in spermatocytes and round spermatids. DAZL and BOULE expression in spermatogonia was strictly nuclear only in 20-day-old hamsters. We also detected the novel mRNA and protein expression of BOULE in Leydig cells. In adult hamsters, *Dazl* expression was increased in regressed testis compared with non-regressed testis and DAZL protein expression was restricted to primary spermatocytes in regressed testis. These results show that DAZL and BOULE are expressed in spermatogonia at early stages in the Syrian hamster, then both proteins translocate to the cytoplasm when meiosis starts. In the adult regressed testis, the absence of DAZL in spermatogonia might be related to the decrease in germ cell number, suggesting that DAZ gene family expression is involved in changes in seminiferous epithelium during photoregression.

Keywords: BOULE, DAZL, Photoperiod, Syrian hamster, Testis

## Introduction

*D*eleted in *AZ*oospermia (*DAZ*) family genes are essential during spermatogenesis and required for fertility in nearly all animals (Saunders *et al*., 2003; Van Gompel & Xu, 2010; Van Gompel & Xu, 2011). Cloning and characterization of *DAZ* gene sequence in several species has led to the identification of three family members: two autosomal genes, *BOULE* and *DAZ-L* (*DAZ-Like*), and the *DAZ* gene cluster in the Y chromosome (Foresta *et al*., 2001; Jiao *et al*., 2002; Yen, 2004; Kostova *et al*., 2007). Evolutionary studies have identified *BOULE* as the ancestor of the *DAZ* gene family and orthologous genes have been found in *Caenorhabditis elegans*, *Drosophila*, mice and humans (Karashima *et al*., 2000; Xu *et al*., 2001). *DAZ-L* orthologues arose from a duplication of *BOULE* and are found only in vertebrates (Van Gompel & Xu, 2011; Smorag *et al*., 2014). The *DAZ* gene arose from an ancestral *DAZ-L* gene and is present only in Old World monkeys and great apes (Saxena *et al*., 1996; Gromoll *et al*., 1999).

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In all the species analyzed so far, *DAZ* gene family members encode for germ cell-specific proteins and are essential for the development and maturation of germ cells and regulation of spermatogenesis (Saunders *et al*., 2003; Van Gompel & Xu, 2010; Van Gompel & Xu, 2011). The proteins encoded by the *DAZ* gene family contain an RNA-binding domain suggesting a functional role in mRNA stability and regulation of protein synthesis (Maines & Wasserman, 1999; Venables *et al*., 2001; Jiao *et al*., 2002; Cheng *et al*., 2006). Recently, multiple putative targets of the *DAZ* gene family have been proposed. Particularly, the DAZL and BOULE mRNA substrates include gametogenesis regulators such as SYCP3 and many cell cycle regulators factors such as CDC25 phosphatases (Cheng *et al*., 2006; Luetjens *et al*., 2004; Smorag *et al*., 2014).

Golden (Syrian) hamsters, *Mesocricetus auratus*, breed seasonally and show circa-annual changes in the function of the hypothalamic–pituitary–gonadal axis (Bartke, 1985). Changes in reproductive function are modulated by photic stimuli. During the non-breeding season, male hamsters exhibit a profound atrophy of the reproductive system (Bartke *et al*., 1999). When these animals are exposed to less than 12.5 h daylight [short photoperiod; i.e., 6 h light/18 h dark, short day (SD)] for 14–16 weeks, a marked regression of the testis is observed (Bartke, 1985; Simonneaux *et al*., 2009; Mason *et al*., 2010). The exposure of male hamsters to SD causes testicular morphological alterations that include a reduction in testicular weight and a decrease in the volume of both the interstitial and the tubular compartment (Frungieri *et al*., 1999; Bartke & Russell, 1988). The content of germ cells decreases and the seminiferous tubules only show spermatogonia and primary spermatocytes (Bartke & Russell, 1988).

Aimed to increase our understanding on the involvement of *DAZ* gene family in experimental mammals, we investigated the expression of DAZL and BOULE in newborn, pubertal and adult Syrian hamster. We specially evaluated how *DAZ* gene family members are expressed under short- or long-photoperiod conditions. We observed that DAZL and BOULE translocate from the nucleus to the cytoplasm at the time of meiosis initiation. Moreover, we showed that DAZL and BOULE varied during photoperiod suggesting that both proteins could be involved in the transition from active to inactivating testicular stages in the adult hamster.

## Materials and Methods

### Animals

Male Syrian hamsters (*Mesocricetus auratus*) were raised in the local Animal Care Unit (Charles River descendants, Animal Care Laboratory, Instituto de Biología y Medicina Experimental, IBYME-CONICET, Buenos Aires, Argentina). Animals were kept from birth in long day (LD) conditions (14 h light/10 h dark; lights on between 0700–2100 h) at 23 ± 2°C with food and water supply *ad libitum*. The pre-pubertal hamsters used in this study were grouped according to age: 10 ($n = 5$), 20 ($n = 5$), 35 ($n = 5$) and 45 ($n = 5$) days old. Adult hamsters (90 days old) were kept under LD (non-regressed group, $n = 5$) or SD (regressed group, $n = 5$) conditions for 16 weeks. Hamsters from our colony reached the maximal gonadal regression after 16 weeks under SD conditions (6 h light/18 h dark; lights on between 0900–1500 h). Hamsters were euthanized by asphyxiation with $CO_2$ according to the protocols approved by the Institutional Animal Care Unit and Use Committee (Institute of Biology and Experimental Medicine, National Council for Scientific and Technical Research; Buenos Aires, Argentina) and in compliance with the National Institutes of Health guidelines. Testes were removed for immunohistochemistry studies or kept at –80°C for molecular analyses.

### Immunohistochemistry

Paraffin-embedded sections were cut into 5-μm sections, mounted onto cleaned slides, dewaxed in xylene, rehydrated in decreasing graded alcohols and finally washed with tap water. Endogenous peroxidase activity was inhibited using 0.5% v/v $H_2O_2$/methanol for 20 min at room temperature. Sections were then blocked for 30 min with 1.5% normal goat serum in phosphate-buffered saline (PBS) and incubated overnight at 4°C with primary antibody (1:100 diluted rabbit anti-DAZ-L, Ab34139, Abcam, UK; 1:50 diluted rabbit anti-BOULE, H-89, sc-67371, Santa Cruz Biotechnology, Inc., USA). After three rinses in PBS, sections were incubated for 1 h at room temperature with the appropriate 1:200 diluted biotinylated secondary antibody (Vector Labs, UK). After further washing in PBS, sections were incubated for 30 min with 1:100 diluted streptavidin–peroxidase complexes (ABC kit, Vector Labs, UK), finally washed twice with PBS and development of peroxidase activity was achieved with 0.05% w/v 3,3-diaminobenzidine and 0.1% v/v $H_2O_2$ in Tris–HCl. Lastly, sections were washed with distilled water. Negative controls were processed simultaneously by omitting the primary antibody or pre-absorbing the primary antibody with specific synthetic peptides.

### Purification of Leydig cells

Leydig cells were isolated from a pool of five testes obtained from adult hamsters (90 days old) kept in LD (non-regressed testes) and five testes from animals exposed to SD for 16 weeks (regressed testes). Leydig

cells were isolated under sterile conditions using a discontinuous Percoll density gradient according to Frungieri *et al*. (2005). Both testes from each animal were decapsulated, immersed in Medium 199 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) at pH 7.2 containing 0.1% bovine serum albumin (Sigma, St. Louis, MO, USA) and 0.2 mg/g tissue collagenase (Worthington Biochemical, Freehold, NY, USA) at a ratio of 1:1 (w/v), and incubated for 10 min at 30–32°C with gentle shaking. The incubation was stopped by the addition of 50 ml cold Medium 199 without collagenase. The supernatants were filtered (cell strainer, BD Falcon, Becton Dickinson and Co., Franklin Lake, MA, USA) and centrifuged at 800 *g* for 7 min. The pellet was resuspended, subjected to a discontinuous Percoll density gradient, and centrifuged. Cells that migrated to the 1.06–1.12 g/ml density fraction were collected and resuspended in fresh Medium 199. An aliquot was incubated for 5 min with 0.4% w/v Trypan blue stain and used for cell counting and viability assay using a light microscope. The viability of all Leydig cell preparations was around 97.5–98.5%. To evaluate the purity of Leydig cells, 3β-hydroxysteroid dehydrogenase activity was measured according to Levy *et al*. (1959). The cell preparations contained 85–90% hamster Leydig cells.

### RNA isolation and real-time-polymerase chain reaction

Total testicular RNA from non-regressed or regressed testis was extracted with TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Total RNA from purified hamster Leydig cells was extracted using a Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, MO, USA) according to the manufacturer's instructions. RNA (1 μg) was treated with DNase I (Invitrogen, USA) and used for reverse transcription in a 20 μl final volume containing M-MLV reverse transcriptase (Promega, 200 U/μl, USA) and random hexamer primers (Biodynamics, USA). Reverse-transcribed cDNA was employed for quantitative polymerase chain reaction (PCR) using SYBR Green PCR Master Mix and specific forward (F) and reverse (R) primers in a Stratagene MPX500 cycler (Stratagene, USA). The F and R primers were: *Dazl* (NM_010021): F: 5′AATGTTCAGTTCATGATGCTGCT-3′ and R: 5′TGTATGCTTCGGTCCACAGACT-3′; *Boule* (NM_029267) F: 5′TATAAGGATAAGAAACTCAA CATTGGT-3′and R: 5′GAAGTTACTTCTGGAGTATG AAAATA-3′and *Gapdh* (NM_008084) F: 5′CCAGA ACATCATCCCTGCAT-3′and R: 5′GTTCAGCTCTG GGATGACCTT-3′. Primers were used at a concentration of 0.3 μM in each reaction. Cycling conditions were as follow: step 1, 10 min at 95°C; step 2, 30 s at 95°C; step 3, 30 s at 55°C; step 4, 30 s at 60°C, repeating from step 2 to step 4, 40 times. Data from the reaction were collected and analyzed using complementary computer software (MxPro3005P v4.10 Build 389, Schema 85). Relative quantitation of gene expression was calculated using standard curves and normalized to *Gapdh* in each sample (Pfaffl, 2001). The amplified cDNA products were size-fractioned in a 2% agarose gel and visualized by staining with GelRed nucleic acid gel stains (Biotium Inc., USA).

### Statistical analysis

Mean and standard error of the mean (SEM) were calculated and the InfoStat Software (version 2011, developed by Statistics Department, National University of Córdoba) was used to calculate differences between two groups using Student's *t*-test; a *P*-value of less than 0.05 was considered statistically significant.

## Results

### Different localization of DAZL during testicular ontogeny and photoperiod

In the developing seminiferous tubules of 10-day-old hamsters, DAZL localized in the cytoplasm of spermatogonia mostly with a perinuclear distribution covering half the cytoplasm (Fig. 1*A*). In 20-day-old animals, DAZL protein translocated to the nucleus of spermatogonia and no cytoplasmic staining was further observed (Fig. 1*A*). Once meiosis started (35 days old), DAZL re-located in the cytoplasm displaying a wide distribution in the majority of germ cells (Fig. 1*A*). From 45 days old to adulthood, DAZL was mainly distributed in the perinuclear region of spermatogonia and some occasional primary spermatocytes in active testis (Fig. 1*A*, *B*). DAZL expression in adult regressed testis was mainly detectable in the perinuclear region of primary spermatocytes and no DAZL expression was observed in spermatogonia (Fig. 1*B*).

### BOULE immunolocalization in germ cells and novel expression in Leydig cells

BOULE protein became detectable exclusively in the nuclei of spermatogonia from 20 days of age (Fig. 2*A*). From 35 days old to adulthood, BOULE translocated to the cytoplasm and was mainly detected in primary spermatocytes and in successive stages until round spermatid (Fig. 2*A*, *B*). A few BOULE positive spermatogonia were still observed in testis from 35 days old to adulthood (Fig. 2*A*, *B*). The adult regressed testis showed a strong perinuclear staining of BOULE in spermatocytes and no expression was detected in spermatogonia (Fig. 2*B*).

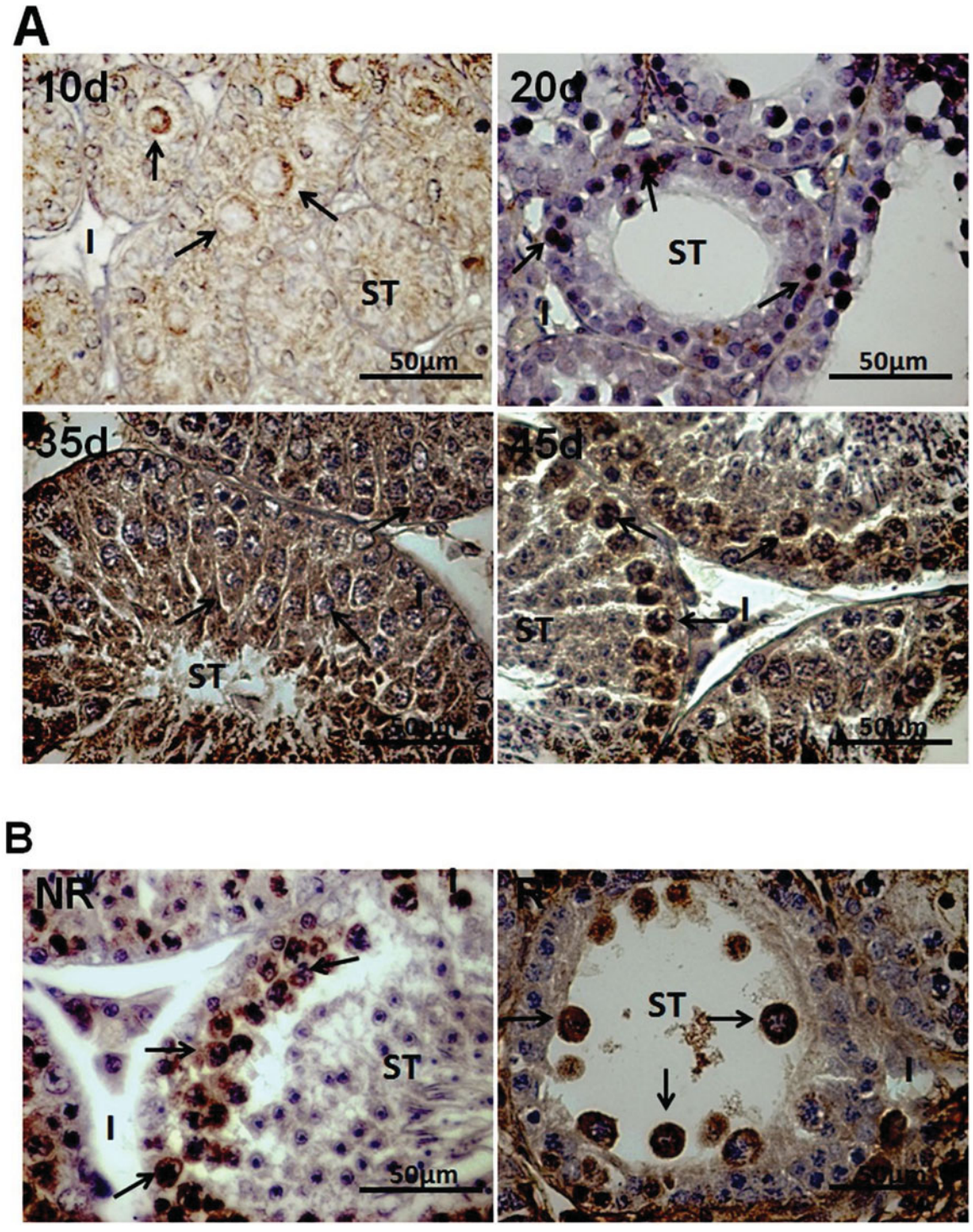


**Figure 1** Immunolocalization of DAZL in pre-pubertal and adult non-regressed and regressed testis in the Syrian hamster. DAZL immunohistochemistry in the testis of 10, 20, 35 and 45-day-old hamsters (*A*) and in the testis of non-regressed and regressed adult hamsters (*B*). Black arrows indicate positive germ cells. I: interstitium; ST: seminiferous tubules; d: days; NR: non-regressed; R: regressed.

Unexpectedly, specific staining of BOULE was also detected in the cytoplasm of Leydig cells in testis from 35-day-old animals to adulthood (Fig. 2). The mRNA of BOULE was successfully isolated from hamster Leydig cells by real-time PCR; size-fractioning in a 2% agarose gel confirmed the size of the product (169 bp). The commercial cell line GC-1 spg that shows characteristics of type B spermatogonia

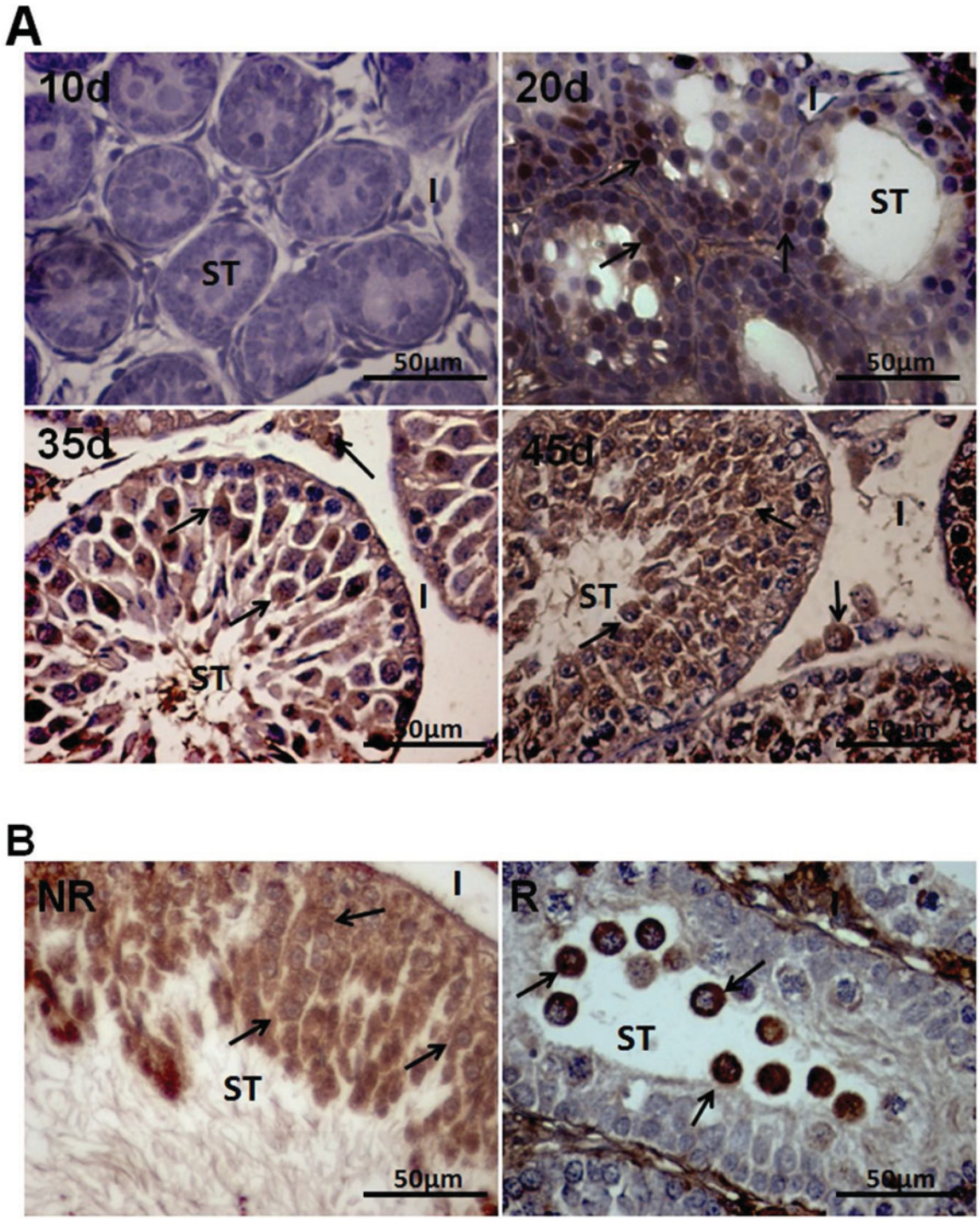


**Figure 2** Immunolocalization of BOULE in pre-pubertal and adult non-regressed and regressed testis in the Syrian hamster. BOULE immunohistochemistry in the testis of 10, 20, 35 and 45-day-old hamsters (*A*) and in the testis of non-regressed and regressed adult hamsters (*B*). Black arrows indicate positive cells. I: interstitium; ST: seminiferous tubules; d: days; NR: non-regressed; R: regressed.

and primary spermatocytes was used as a positive control. BOULE mRNA from isolated Leydig cells was detected in both non-regressed and regressed hamster (Fig. 3*A*).

### Testicular mRNA expression of *Dazl* and *Boule* in non-regressed and regressed testis

*Dazl* mRNA was significantly higher in regressed testis than in non-regressed ones. In contrast, the

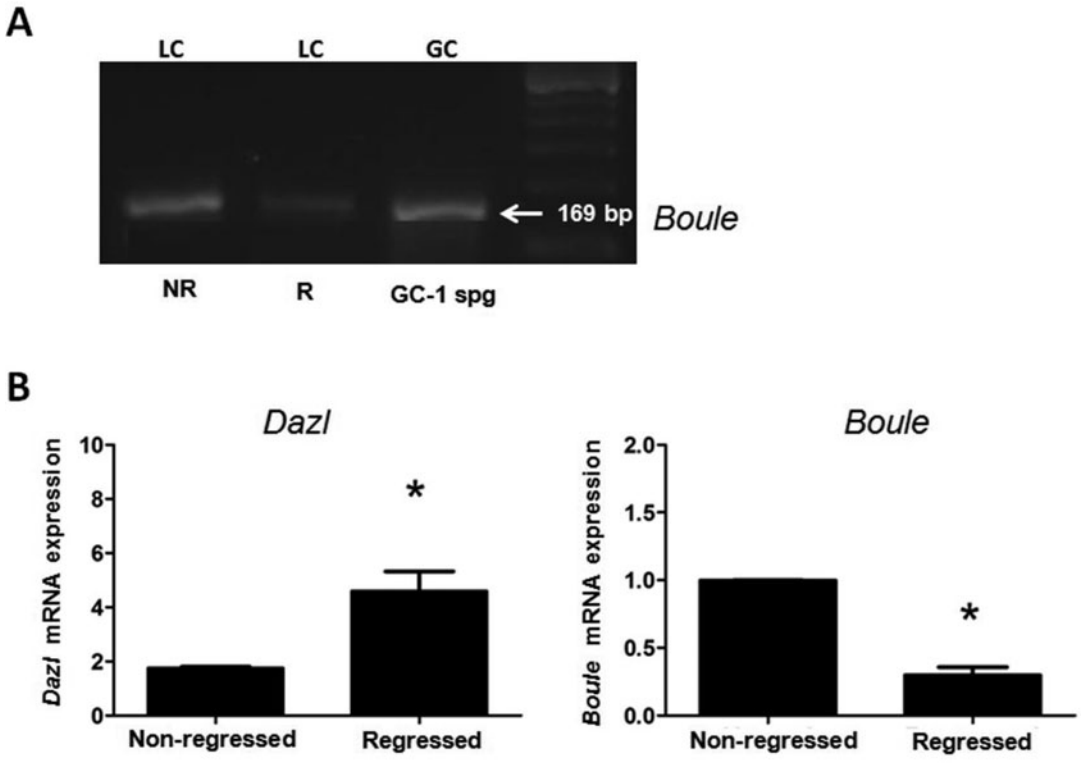


**Figure 3** Identification of *Boule* in purified hamster Leydig cells and basal testicular gene expression of *Dazl* and *Boule* in the Syrian hamster. (*A*) *Boule* mRNA was identified in purified Leydig cells from both non-regressed (NR) and regressed (R) testis, by real-time PCR and size-fractioning in a 2% agarose gel. The GC-1 spg cell line was used as a positive control. (*B*) Basal expression of *Dazl* and *Boule* mRNA in non-regressed and regressed testis measured by real-time PCR. All results are expressed relative to the housekeeping gene *Gapdh*. Data are plotted as the mean ± standard error of the mean (SEM) ($n = 5$). *Indicates statistical difference, $P < 0.05$. bp: base pairs.

expression of BOULE was significantly higher in non-regressed testis compared with regressed testis ($P < 0.05$) (Fig. 3*B*).

## Discussion

The importance of *Dazl* and *Boule* expression in germ cell development has been assessed from a wide variety of species from *Drosophila* and *C. elegans* to rodents and humans, leading to the establishment of the essential role that these genetic factors play in male fertility (Foresta *et al.*, 2001; Saunders *et al.*, 2003; Van Gompel & Xu, 2010; Van Gompel & Xu, 2011). Although *DAZ* gene family localization, function and regulation deserved a sustained research interest, there are still many unsolved concerns about their expression and regulation during the spermatogenic process. Here, we provide evidence that DAZL and BOULE proteins translocate from the nucleus to the cytoplasm of germ cells at the time of meiosis initiation in the testis of the Syriam hamster. Also, we observed that, under the influence of photoperiod, the expression of both proteins became undetectable in spermatogonia in regressed hamster testis.

Our analysis of the testicular localization of *DAZ* family members showed that in pre-pubertal and adult active testis DAZL protein was restricted mainly to spermatogonia. BOULE expression was first detected in spermatogonia from 20-day-old pups and from 35 days of age to adult active stage it began to be expressed from primary spermatocytes to round spermatids. Unexpectedly, BOULE protein and mRNA were detected in hamster Leydig cells. This finding is novel evidence for BOULE expression, which has not been described previously in other species. The biological meaning and function of BOULE in Leydig cells, as well as its occurrence in other species, remains to be investigated.

In mice, primates and human testis, DAZL was detected predominantly in the cytoplasm of spermatogonia and spermatocytes (Reijo *et al.*, 2000; Ruggiu *et al.*, 2000; Xu *et al.*, 2001; González *et al.*, 2016). In adult human and mouse testis, BOULE protein

was found to localize in the cytoplasm of pachytene spermatocytes and round spermatids but was absent at the time elongation begins (Xu *et al*., 2001; Van Gompel & Xu, 2011; González *et al*., 2015). Interestingly, we detected DAZL and BOULE proteins in the nuclei of spermatogonia in 20-day-old testis that then translocated to the cytoplasm/perinuclear region in 35-day-old testis, when meiosis is already active. This situation suggests that BOULE and DAZL might be involved in the regulation of the meiotic process as they are RNA-binding proteins that mainly modulate cell cycle regulator factors (Luetjens *et al*., 2004; Cheng *et al*., 2006; Smorag *et al*., 2014). In line with this aspect, studies in BOULE null mice have demonstrated that this protein participates in meiotic division and during the process of spermiogenesis (Van Gompel & Xu, 2010, 2011).

The Syrian hamster provides an excellent animal model by which to study the mechanisms involved in the control of reversible infertility, as it shows morphological and physiological testicular regression when exposed to SD photoperiod. Previously, no data have been available on DAZ gene family expression in seasonal breeders. During testicular regression, when the spermatogenic process is arrested, no DAZL protein was detected in spermatogonia. However, the remaining primary spermatocytes surprisingly showed DAZL and maintained BOULE expression. Higher levels of *Dazl* mRNA are seen in regressed testis, however DAZL protein is not expressed in spermatogonia. The absence of DAZL might be related to reduction in germ cell number during testicular regression as this absence has been shown to occur in animals that lack the *Dazl* gene (Saunders *et al*., 2003). In regressed testis, DAZL and BOULE proteins also showed a clearly strong perinuclear localization suggesting that both proteins concentrate at the chromatoid body. The chromatoid body is a germ cell-specific centre in which many mRNAs are transcribed and regulated by RNA-binding proteins (Kotaja & Sassone-Corsi, 2007). The perinuclear localization of DAZL and BOULE during short photoperiod exposure might indicate that they are involved in the regulation of the quiescent state of primary spermatocytes or in preparing them to recover the spermatogenic process at the time of testicular recrudescence.

In conclusion, these results contribute to enlarging basic knowledge on expression of DAZ gene family proteins and show that DAZL and BOULE translocate from the nucleus to the cytoplasm at the time of meiosis initiation in the male germ cells of the Syriam hamster. Moreover, we observed that variation in photoperiod induced changes in DAZL and BOULE expression, suggesting that *DAZ* genes could be involved in the shift between the active and inactive stage of the testes in the Syrian hamster.

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