Article

Proliferative and Apoptotic Pathways in the Testis of Quail *Coturnix Coturnix* during the Seasonal Reproductive Cycle

Sara Falvo [1,†], **Luigi Rosati** [2,†] , **Maria Maddalena Di Fiore** [1] , **Federica Di Giacomo Russo** [1],
Gabriella Chieffi Baccari [1] **and Alessandra Santillo** [1,*]

[1] Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Università degli studi della Campania "Luigi Vanvitelli", 81100 Caserta, Italy; sara.falvo@unicampania.it (S.F.); mariamaddalena.difiore@unicampania.it (M.M.D.F.); federica.digiacomorusso@unicampania.it (F.D.G.R.); gabriella.chieffi@unicampania.it (G.C.B.)

[2] Dipartimento di Biologia, Università degli Studi di Napoli "Federico II", 80138 Napoli, Italy; luigi.rosati@unina.it

[*] Correspondence: alessandra.santillo@unicampania.it

[†] First two authors equally contributed.

Simple Summary: The quail *Coturnix coturnix* exhibits an annual cycle of testis size, sexual steroid production, and spermatogenesis. The testicular levels of both 17β-estradiol (E_2) and androgens are higher during the reproductive period compared to the non-reproductive period, suggesting that estrogens act in synergy with the androgens for the initiation of spermatogenesis. Therefore, the present study aimed to investigate the estrogen responsive system in quail testis in relation to the reproduction seasons, with a focus on the molecular pathways activated in both active and regressive quail testes. The results indicated that estrogens participated in the activation of mitotic and meiotic events during the reproductive period by activating the ERK1/2 and Akt-1 pathways. In the non-reproductive period, when the E_2/ERα levels are low, ERK1/2 and Akt-1 pathways remain inactive and apoptotic events occur. Our results suggest that the activation or inhibition of these molecular pathways plays a crucial role in the physiological switch "on/off" of the testicular activity in male quail during the seasonal reproductive cycle.

Abstract: The quail *Coturnix coturnix* is a seasonal breeding species, with the annual reproductive cycle of its testes comprising an activation phase and a regression phase. Our previous results have proven that the testicular levels of both 17β-estradiol (E_2) and androgens are higher during the reproductive period compared to the non-reproductive period, which led us to hypothesize that estrogens and androgens may act synergistically to initiate spermatogenesis. The present study was, therefore, aimed to investigate the estrogen responsive system in quail testis in relation to the reproduction seasonality, with a focus on the molecular pathways elicited in both active and regressive quail testes. Western blotting and immunohistochemistry analysis revealed that the expression of ERα, which is the predominant form of estrogen receptors in quail testis, was correlated with E_2 concentration, suggesting that increased levels of E_2-induced ERα could play a key role in the resumption of spermatogenesis during the reproductive period, when both PCNA and SYCP3, the mitotic and meiotic markers, respectively, were also increased. In the reproductive period we also found the activation of the ERK1/2 and Akt-1 kinase pathways and an increase in second messengers cAMP and cGMP levels. In the non-reproductive phase, when the E2/ERα levels were low, the inactivation of ERK1/2 and Akt-1 pathways favored apoptotic events due to an increase in the levels of Bax and cytochrome C, with a consequent regression of the gonad.

Keywords: quail; testis; estrogens; estrogens receptor α; ERK; Akt; PCNA; Bax; cytochrome c

1. Introduction

In seasonal breeding vertebrates, steroidogenesis and spermatogenesis undergo fluctuations during the transition between the stages of the annual cycle [1–3]. Sex steroid hormones, such as testosterone and estrogens, are the key factors in the hormonal interplay that governs seasonal breeding [1–3]. Estrogens, which have historically been considered female hormones, have exhibited potential functions in the male reproductive system as well, such as in the processes of spermatogenesis and spermiogenesis [4]. The estrogen 17β-estradiol (E_2) is synthesized by the irreversible conversion of testosterone in the presence of the P450 aromatase enzyme [5]. The action of E_2 is mediated by two intracellular estrogen receptors (ERs), namely, ERα and ERβ [6]. The information regarding the ERs in avian testis is scarce. Earlier studies have reported the mRNA expression of testicular ERs in the embryos of chicken [7,8] and duck [9], and the adults of gander [10] and Japanese quail [11,12]. The immunoreactivity of ERs has been detected in the testes of chickens [13,14], Japanese quail [15,16], and domestic goose [17]. Meanwhile, a seasonal pattern of ER expression and E2 concentration has only been demonstrated in male domestic goose [17].

Several studies suggested that estrogen activity within the testis might be mediated by the regulation of the activity of mitogen-activated protein kinases (MAPKs) [18,19], which are known to occupy a focal point in signal transduction [20]. The most extensively studied members of the MAPK family are: (1) the extracellular signal-regulated kinases (ERK1 and ERK2), which are involved in the regulation of spermatogonia proliferation and the meiotic progression of spermatocytes [21–23], and (2) the serine/threonine kinase Akt-1, which mediates the growth factor-dependent cell survival in various cell types through the inactivation of several pro-apoptotic molecules [24,25]. In the testes of mammalian as well as non-mammalian seasonal breeders, cell proliferation and apoptosis occur at different time points during the annual cycle [26–28]. In several bird species, the transition from the breeding to non-breeding stage leads to significant testicular atrophy, which has been associated with the increased incidence of cell death through apoptosis and the decreased rate of cell proliferation [29–32]. However, the intracellular signal transduction mechanisms underlying these changes occurring in the avian testicular activity during the reproductive cycle remain unknown so far. In this regard, the quail *Coturnix coturnix* represents an excellent avian model, as it demonstrates an annual cycle of testis size, sexual steroid production, and spermatogenesis [33–35]. A previous study by our research group revealed that in quail testis, the levels of both E_2 and androgens were higher during the reproductive period compared to the non-reproductive period, suggesting that estrogens act in synergy with androgens for the initiation of spermatogenesis [35]. Therefore, the present study was aimed to investigate the estrogen responsive system in quail testis in relation to reproduction seasonality. In order to achieve this objective, an analysis of the ERα signaling and the potential participation of ERK1/2 and Akt-1, in both active and regressive quail testes was performed, with particular focus on the proliferative and apoptotic pathways.

2. Materials and Methods

2.1. Animals

Adult males of quails, *C. coturnix*, (3-month-old) were collected in the surroundings of Naples during January–February (non-reproductive period) and July–August (reproductive period), according to the Ministry of Health guidelines as previously reported [36]. For each period, 10 animals were used. For each animal, the testes were dissected out, weighed and rapidly immersed either in Bouin's fluid (Sigma-Aldrich, Milan, Italy) and then embedded in paraffin, for histological investigations, or in liquid nitrogen and then stored at $-80\,^\circ$C for biochemical analysis.

2.2. 17β-Estradiol Assay

17β-estradiol levels were determined in the testis of quails from both non-reproductive and reproductive periods using an enzyme immunoassay kit (DiaMetra, Milan, Italy).

Testes (*n* = 5 from each period) were homogenized 1:10 (*w/v*) with PBS ×1. The homogenate, obtained from each testis, was then mixed vigorously with ethyl ether (1:10 *v/v*) and the ether phase was withdrawn after centrifugation at 3000× *g* for 10 min. The upper phase (ethyl ether) was transferred to a glass tube and was left to evaporate on a hot plate at 40–50 °C under a hood. The residue was dissolved in 0.25 mL of 0.05 M sodium phosphate buffer, pH 7.5, containing bovine serum albumin at a concentration of 10 mg/mL, and then utilized for the ELISA assay [37–39]. All samples (three replicates for each sample) ran in the same ELISA. The used 17β-estradiol ELISA kit has the following characteristics: sensitivity of 8.68 pg/mL, an intra-assay variability < 9% and an inter-assay variability < 10%.

2.3. Immunohistochemistry

Quail testes from reproductive (*n* = 5) and non-reproductive (*n* = 5) periods were embedded in paraffin. Each paraffin-embedded reproductive and non-reproductive quail testis was cut (5 μm-thick), and the serial sections mounted on poly-L-lysine slides were processed for immunohistochemistry [40,41]. Briefly, deparaffinized sections were washed in 0.1 M phosphate-buffered saline (PBS; pH 7.6), and then incubated in 2.5% H_2O_2. To reduce no-specific background the sections were incubated in normal goat serum (Pierce, Rockford, IL, USA) for 1 h at room temperature. Next, sections were treated overnight at 4 °C with the primary antibodies diluted in normal goat serum: (i) rabbit polyclonal antibody ERα (1:400, Abcam, Cambridge, MA, USA), and (ii) mouse monoclonal antibody PCNA (1:300, Sigma-Aldrich, Milan, Italy). After washing in PBS, the sections were incubated for 1 h at room temperature with a biotin-conjugated goat anti-rabbit/mouse secondary antibody (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and an avidin-biotin-peroxidase complex (ABC immune peroxidase kit, Pierce, Rockford, IL, USA), using DAB (Sigma Aldrich, Milan, Italy) as chromogen. Sections were counterstained with Mayer's hematoxylin. For negative controls, the primary antibody was omitted. Immunohistochemical signal was observed using a Zeiss Axioskop microscope; images were acquired by using AxioVision 4.7 software (Zeiss, Oberkochen, Germany). The immunostaining for both ERα and PCNA was carried out at the same time. Three investigators individually blindly rank the intensity of staining using a light microscope; afterwards, one researcher took digital photographs.

2.4. cAMP and cGMP Enzyme Immunoassay

The second messengers, cAMP and cGMP, play a key role in the signal transduction implicated in the regulation of spermatogenesis [42,43]. Frozen testes from reproductive (*n* = 5) and non-reproductive (*n* = 5) quails were weighed and homogenized in 10 volumes of cold 5% TCA. Then, the homogenate obtained from each testis was centrifuged at 600× *g* for 10 min; the supernatant was extracted with 3 volumes of water-saturated ether and aqueous extract was dried. Samples were reconstituted with 0.6 mL of assay buffer 2, a buffer containing proteins, detergents and sodium azide as preservative (A5219, Sigma Aldrich, St. Louis, MO, USA) and then utilized for cAMP (CA201) and cGMP (CG201) enzyme immunoassay (Sigma Aldrich, St. Louis, MO, USA). The acetylated version of both kits was running and the sensitivities were 0.039 pmol/mL for cAMP and 0.088 pmol/mL for cGMP.

2.5. Protein Extraction and Western Blot Analysis

Western blot analysis has been performed to investigate the ERα protein expression as well as ERK1/2 and Akt-1 phosphorylations, in both active and regressive quail testes. To determine the testicular proliferative rate in the quail during both reproductive and non-reproductive periods we investigated the protein expression of proliferating cell nuclear antigen (PCNA), a marker of DNA synthesis [44]. SYCP3, encoding the synaptonemal complex proteins and expressed at the meiotic prophase, has been used as meiotic marker [45,46]. Finally, to study the apoptosis pathway, protein levels of Bax and cytochrome c were assayed [47].

Each testis from reproductive ($n = 5$) and non-reproductive ($n = 5$) quails were homogenized directly in lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100 (1:2 *w/v*), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate, pH 7.4 (Sigma Chemical Corporation, St. Louis, MO, USA), then clarified by centrifugation at $14{,}000 \times g$ for 10 min [35]. Protein concentration was determined by the Bradford assay (Bio-Rad, Melville, NY, USA). Fifty micrograms of total protein extracts for each sample (reproductive and non-reproductive testes) were boiled in Laemmli buffer for 5 min at 95 °C before each electrophoresis. Three electrophoresis sodium dodecyl sulfate–PAGE (12% polyacrylamide) were performed at same time. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The complete transfer was assessed using prestained protein standards (Bio-Rad, Melville, NY, USA). Each membrane was first treated for 1 h with blocking solution (5% no-fat powdered milk in 25 mM Tris, pH 7.4; 200 mM NaCl; 0.5% TritonX-100, TBS/Tween) and then incubated overnight at 4 °C with one of the following primary antibodies: anti-ERα, raised in rabbit, diluited 1:1000 (Abcam, Cambridge, MA, USA), anti-P-ERK1/2, raised in rabbit, diluited 1:1000 (Cell Signaling, Danvers, MA, USA), anti-ERK1/2, raised in rabbit, diluited 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-P-Akt-1, raised in rabbit, diluited 1:1000 (Cell Signaling, Danvers, MA, USA), anti-Akt-1, raised in rabbit, diluited 1:1000 (Cell Signaling, Danvers, MA, USA), anti-PCNA, raised in mouse, diluited 1:1000 (Sigma-Aldrich, Milan, Italy), anti-SYCP3, raised in mouse, diluited 1:250 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) anti-bax, raised in rabbit, diluited 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-cytochrome complex, raised in rabbit, diluited 1:1000 (Cell Signaling, Danvers, MA, USA). After washing with TBS-tween, membranes were incubated with horseradish-peroxidase anti-rabbit IgG or anti-mouse IgG secondary antibodies for 1 h at room temperature, followed by signal detection using enhanced chemiluminescence (ECL) (Amersham Bioscience, Bath, UK). The number of proteins was quantified using Image J software (National Institutes of Health, Bethesda, Rockville, MD, USA) and normalized with respect to β-actin protein (housekeeping protein), whose expression did not change between two examined periods.

2.6. Statistical Analysis

The values obtained were compared by Student's *t*-test for between-group comparisons. The differences were considered statistically significant at $p < 0.05$ (*) and $p < 0.01$ (**). All data were expressed as the mean ± standard deviation (SD).

3. Results

3.1. E_2 Levels and ERα Protein Expression

E_2 concentration and ERα protein expression in quail testis were analyzed during reproductive as well as non-reproductive periods (Figure 1). In particular, E_2 levels were observed to be significantly higher in the testes during the reproductive period compared to the values detected in the gonads of non-reproductive quails (130 ± 9 pg/g tissue and 62 ± 7 pg/g tissue, respectively) (Figure 1B).

Western blot analysis revealed that the ERα expression in the testis was significantly higher in the reproductive period compared to the non-reproductive period (Figure 1A,B). These data were supported by the findings of immunohistochemical studies, which revealed an increased signal of ERα in quail testis during the reproductive period (Figure 1C). Specifically, it was observed that during the reproductive period, ERα was localized mainly in Leydig and germ cells, such as spermatocytes I and II, spermatids, and spermatozoa, with the strong positivity detected in spermatocytes I (Figure 1Ca,b). No signal was detected in Sertoli cells and spermatogonia. In the non-reproductive period, a faint positivity was observed only in Sertoli cells (Figure 1Cc). No immunohistochemical signal was detected in the control sections (Figure 1Cd).
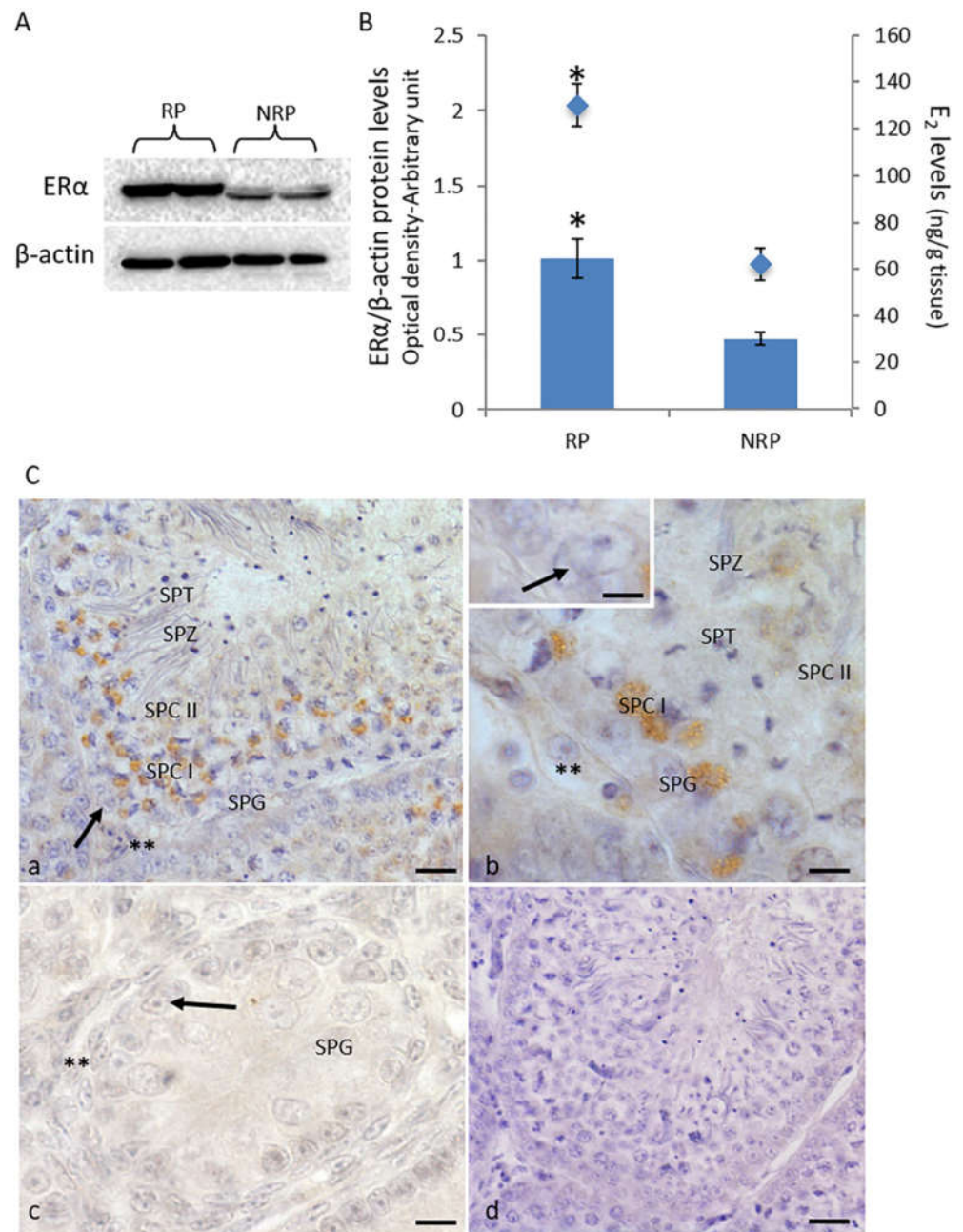
Figure 1. (**A**) Western blot detection of ERα in quail testis during reproductive and non-reproductive periods. A specific band of 66 kDa was detected. (**B**) The amount of protein was quantified using the ImageJ program and normalized with respect to β-actin protein. The values presented represent the means ± S.D. of the values obtained for five animals (two bands were shown). * $p < 0.05$. RP, reproductive period; NRP, non-reproductive period. (**C**) Immunohistochemical localization (brown areas) of ERα. (**a,b**) Reproductive period. The signal is evident in Leydig cells (******), spermatocytes I (Spc I) and II (Spc II), spermatids (Spt), and spermatozoa (Spz). No signal is evident in Sertoli cells (arrow) and spermatogonia (Spg). (**c**) Non-reproductive period. The signal is evident in Sertoli cells (arrow). No positivity was observed in Leydig cells (******) and spermatogonia (Spg). (**d**) Control section. Scale bars correspond to 5 μm in (**a,c,d**) and 10 μm in (**b**).

3.2. cAMP and cGMP Levels

The levels of cAMP and cGMP in quail testes were analyzed in both reproductive and non-reproductive periods. Concentrations of both cAMP and cGMP were observed to be higher in the reproductive period compared to the non-reproductive period (Table 1).

Table 1. cAMP and cGMP levels in reproductive and non-reproductive testes of quail *C. coturnix*.

	Reproductive	Non-Reproductive
cAMP (pmol/g tissue)	45.4 ± 3.6 *	33.6 ± 2.7
cGMP (pmol/g tissue)	31.7 ± 1.2 **	14.5 ± 1.9

** $p < 0.01$ and * $p < 0.05$, reproductive vs. non-reproductive period.

3.3. ERK1/2 and Akt-1 Activities

ERK1/2 activity, expressed as phosphorylated-ERK1/2 (P-ERK1/2) levels, was significantly higher in the reproductive period compared to the non-reproductive period (Figure 2A). Similarly, during the reproductive period, the quail testes exhibited a significant increase in the Akt-1 activity (Figure 2B).
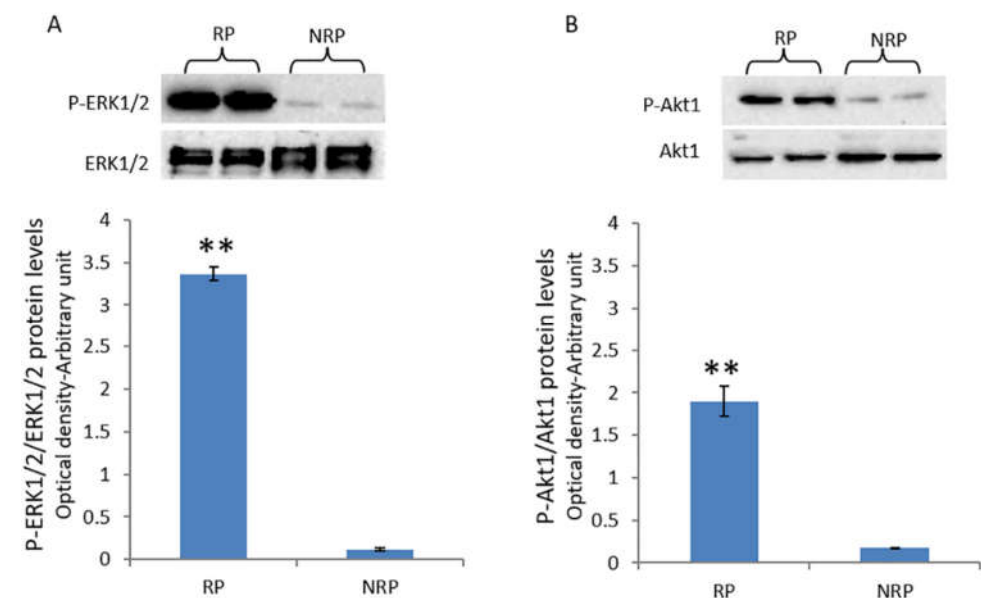

Figure 2. Expressions of P-ERK1/2 and P-Akt-1 in quail testis during reproductive and non-reproductive periods. (**A**) Detection of P-ERK1/2 protein using Western blot analysis. A specific band of 42 kDa was detected. The amount of phosphorylated ERK1/2 was quantified using the ImageJ program and normalized with respect to ERK1/2. The values presented represent the means $\pm$ S.D. of the values obtained for five samples (two bands were shown in the upper panel). ** $p < 0.01$. (**B**) Western blot analysis for Akt-1 protein in quail testis during reproductive and non-reproductive periods. A specific band of 60 kDa was detected. The amount of phosphorylated Akt-1 was quantified using the ImageJ program and normalized with respect to Akt-1. The values presented represent the means $\pm$ S.D. of the values obtained for five samples (two bands were shown). ** $p < 0.01$. RP, reproductive period; NRP, non-reproductive period.

3.4. PCNA Protein Expression and Immunolocalization

Western blot analysis revealed that during the reproductive period, the PCNA protein levels in the testis were higher than those in the non-reproductive period (Figure 3A,B). Immunohistochemical analysis also revealed a more intense immunopositivity for PCNA in the reproductive period with respect to the non-reproductive period (Figure 3). In both periods, a strong immunohistochemical signal for PCNA was detected in spermatogonia (Figure 3Ca–c), while no signal was detected in Leydig cells (Figure 3Ca–c). In the

reproductive period, a faint signal was also detected in Sertoli cells and spermatocytes I (Figure 3Ca,b). No immunohistochemical signal was detected for the control sections (Figure 3Cd).
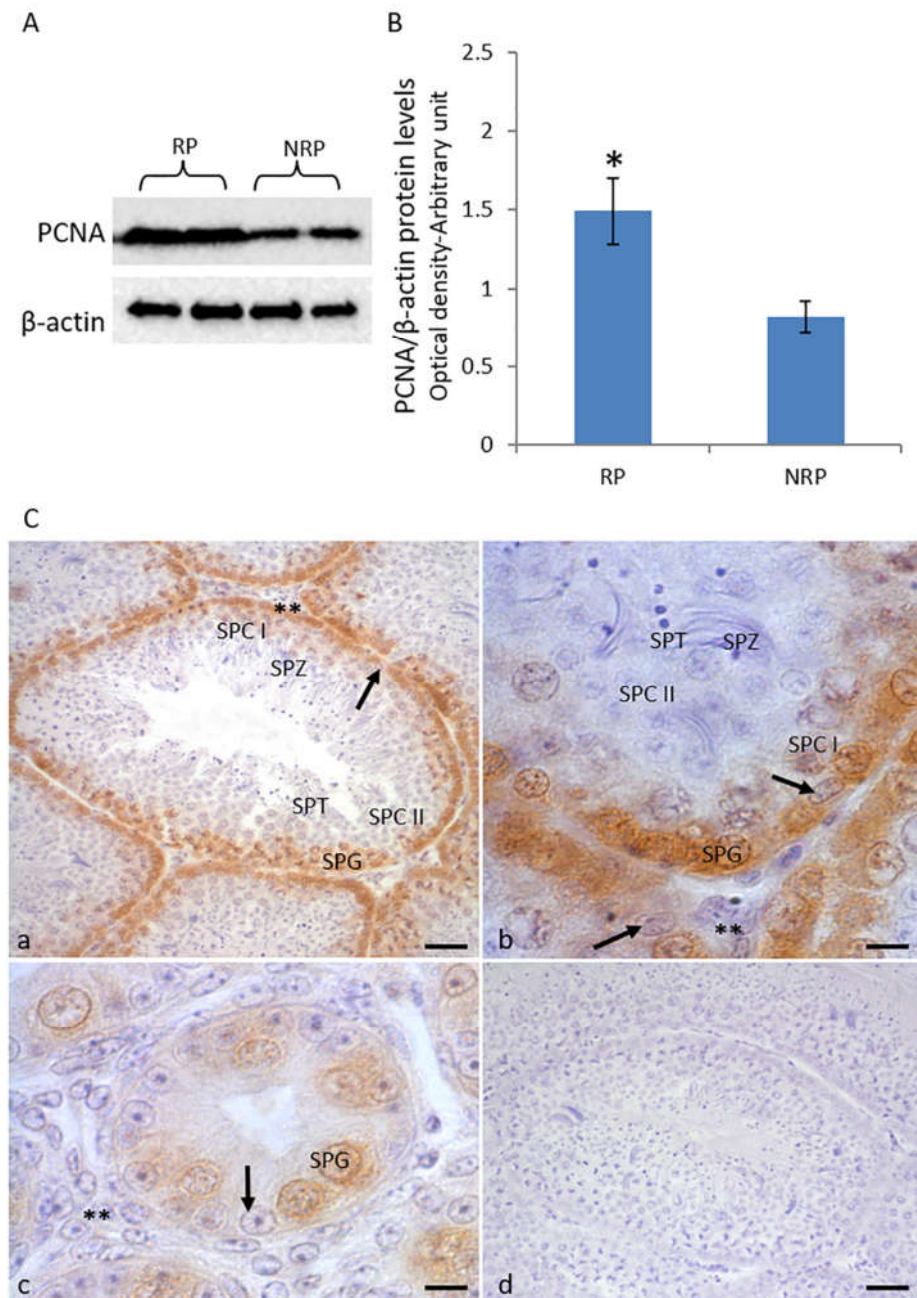

Figure 3. (**A**) Western blot analysis of testicular PCNA expression in quail during reproductive and non-reproductive periods. A specific band of 36 kDa was detected. (**B**) The amount of PCNA was quantified using the ImageJ program and normalized with respect to β-actin. The values presented represent the means ± S.D. of the values obtained for five samples (two bands were shown). * $p < 0.05$. RP, reproductive period; NRP, non-reproductive period. (**C**) Immunohistochemical localization (brown areas) of PCNA. (**a,b**) Reproductive period. The signal is evident in spermatogonia (Spg), spermatocytes I (Spc I), and Sertoli (arrow) cells. No signal is evident in Leydig cells (******), spermatocytes II (Spc II), spermatids (Spt), and spermatozoa (Spz). (**c**) Non-reproductive period. The signal is evident only in spermatogonia (Spg). No positivity was detected in Leydig cells (******) and Sertoli cells (arrow). (**d**) Control section. Scale bars correspond to 5 μm in (**a,c,d**) and 10 μm in (**b**).

3.5. SYCP3 Protein Expression

The expression levels of SYCP3 protein in quail testis during the reproductive period were approximately two-fold higher than those during the non-reproductive period (Figure 4).
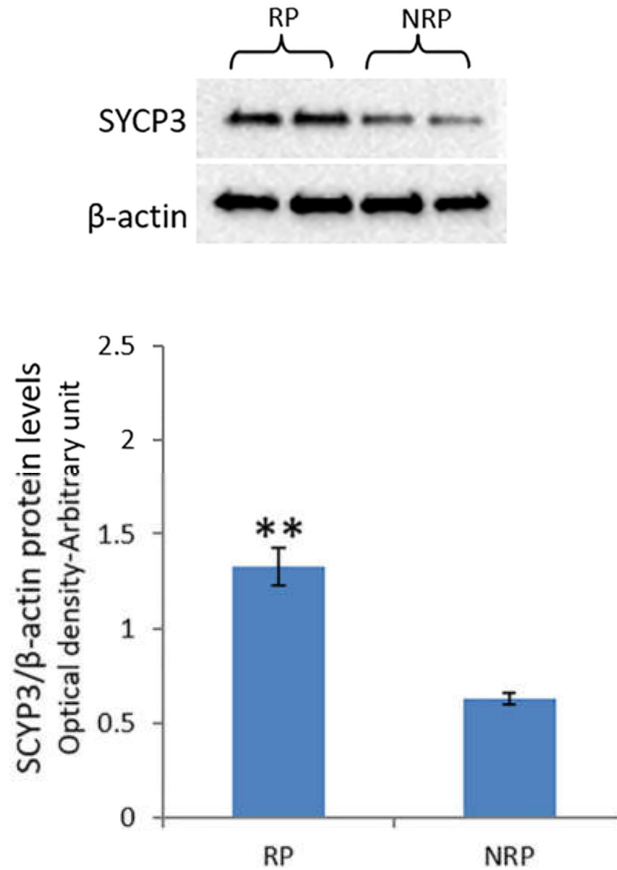

Figure 4. Western blot detections for SYCP3 in quail testis during reproductive and non-reproductive periods. A specific band of 33 kDa was detected. The amount of protein was quantified using the ImageJ program and normalized with respect to β-actin protein. The values presented represent the means ± S.D. of the values obtained for five animals (two bands were shown). ** $p < 0.01$ for reproductive period vs. non-reproductive period. RP, reproductive period; NRP, non-reproductive period.

3.6. Bax and Cytochrome C Protein Expressions

The present study revealed that the levels of both Bax and cytochrome c proteins were significantly higher in the testis during the non-reproductive period with respect to the reproductive period (Figure 5A–C).
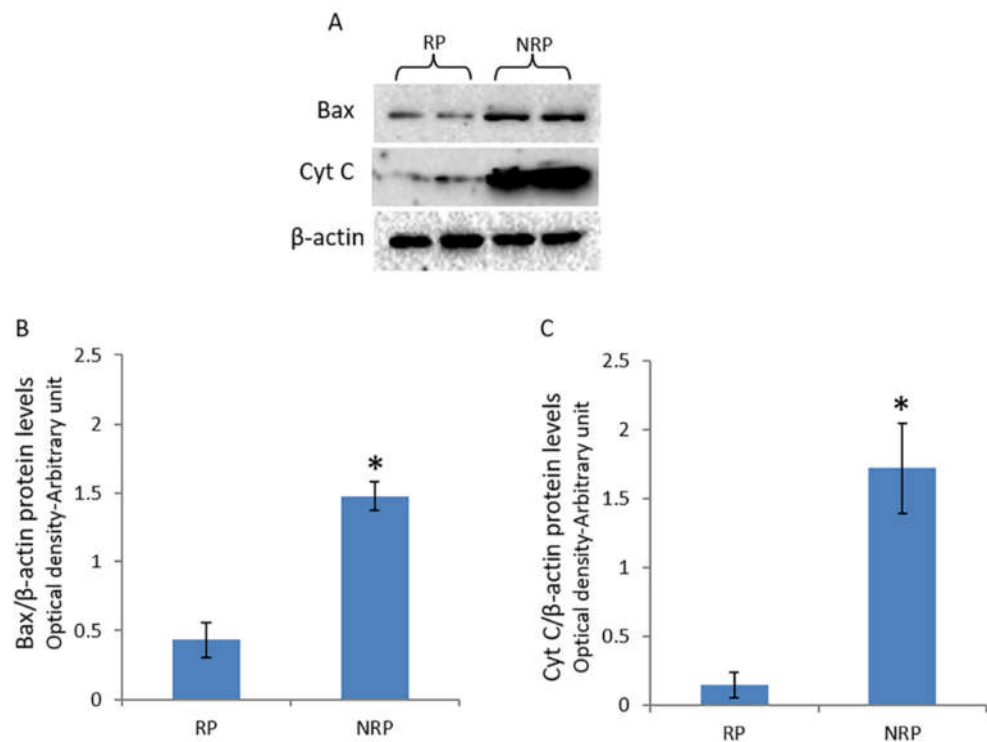
Figure 5. Bax and cytochrome c protein levels in quail testes during reproductive and non-reproductive periods. (**A**) Western blot analysis for Bax (23 kDa) and cytochrome c (14 kDa) proteins in quail testis during reproductive and non-reproductive periods. The amounts of Bax (**B**) and cytochrome c (**C**) were quantified using the ImageJ program and normalized with respect to β-actin. The values presented represent the means $\pm$ S.D. of the values obtained for five samples (two bands were shown). $*$ $p < 0.05$ for reproductive period vs. non-reproductive period. RP, reproductive period; NRP, non-reproductive period.

4. Discussion

It is well established that estrogen signaling is required for the maintenance of male reproductive function in vertebrates [17,21,22,48]. The present study is the first to investigate the E_2/ERα-activated molecular pathways underlying the testicular activity changes in *C. coturnix* during its seasonal reproductive cycle. Several studies have reported ERα as the predominant form in quail testis [11,15,49], although there is no study on the seasonal pattern of ERα expression in the current literature. In line with our previous study, the present study demonstrated that quail testis exhibits the highest titers of E_2 during the reproductive period, when P450 aromatase, the enzyme that converts testosterone into E_2, was expressed at the highest levels [35]. Accordingly, during this period, the levels of both cAMP and cGMP were increased. It is well recognized that both cAMP and cGMP play key roles in spermatogenesis, including the regulation of the blood-testis barrier [43,50]. Furthermore, several studies have demonstrated cAMP to be the main intracellular messenger mediating the expression of P450 aromatase [50], which induces E_2 production, along with a consequent upregulation of its receptor. The Western blotting and immunohistochemistry analyses conducted in the present study revealed that the expression of the ERα protein was correlated with E_2 concentration. Specifically, higher levels of ERα protein were detected in quail testis during the reproductive period compared to those in the non-reproductive period. ERα immunopositivity was observed in Leydig cells as well as germ cells (spermatocytes I and II, spermatids, and spermatozoa) during the reproductive period, while a weak immunohistochemical signal was detected only in Sertoli cells during the non-reproductive period. These findings suggest that testicular E_2 concentration could affect the expression of ERα and that E_2/ERα levels have a role in the testicular seasonal activity in *C. coturnix*. In particular, the increased levels of E_2-induced ERα could have

a key role in the resumption of spermatogenesis during the reproductive period, when both mitosis and meiosis, as well as the differentiation process of germ cells, are activated. Therefore, we have hypothesized that estrogens might act in synergy with androgens, with the levels of the latter also highest during the reproductive period. In contrast, during the non-reproductive period, when both E_2 titers and ERα protein expression were significantly reduced, spermatogenesis was blocked. Seasonal fluctuations have also been described in gander testis [16], although in this species the seasonal expression pattern of ERs is different from those of quail. In fact, in gander testis E_2 levels and its receptors showed an opposite trend, with the highest E_2 levels in the breeding stage and the highest gene and protein expressions of ERs during the non-breeding stage [16]. The authors hypothesized that excessive doses of estrogens could disrupt testis physiology, therefore a downregulation of ERs protein by E_2 as a physiological mechanism decreasing the sensitivity of testicular tissue to estrogens has been proposed for gander testes during the breeding season, when the intratesticular E_2 concentration was very high [16]. A positive correlation between ERα protein expression and spermatogenic activity was reported in the testes of immature, mature, as well as aged chickens [13,51]. Furthermore, in quail, a decrease in both oxidative stress-induced plasma estradiol and ERα testicular expression was reported to reduce spermatogenesis [15]. Different from birds, in the amphibian *Pelophylax esculentus* and reptile *Podarcis sicula*, the estrogenic pathway is essential during the post-reproductive period. It has been proposed that in these species, estradiol mediates the interruption of the reproductive processes inhibiting androgen biosynthesis [39,52–54].

Several studies have suggested that estrogen action in the testis might be mediated by the regulation of the activity of mitogen-activated protein kinases (MAPKs) [18,19]. In the present study, the activation of ERK1/2 and Akt-1 pathways in quail testis was investigated. Both ERK1/2 and Akt-1 pathways are reported to play crucial roles in the testis, including the processes of spermatogonia proliferation and meiotic progression of spermatocytes [23,55–60]. In the present study, high levels of P-ERK1/2 were detected in quail testis during the reproductive period, while the non-reproductive period testis exhibited considerably low levels of phosphorylated ERK1/2 protein. Concomitantly, the Akt-1 phosphorylation levels were positively correlated with the P-ERK1/2 levels, resulting high in the reproductive period and low during the non-reproductive phase. These findings suggested that during the reproductive period, both ERK1/2 and Akt-1 pathways could be induced by E_2 via binding to ERα; conversely, in the non-reproductive period, when both E_2 levels and ERα expression are low, the ERK1/2 and Akt-1 pathways remain inactive. These data are consistent with the other studies, which have reported that E_2 regulates ERK1/2 and Akt-1 activation in the male germ cells of the frog *Rana esculenta* and lizard *P. sicula* via ERs during the respective annual cycles [21,22,61,62]. A positive correlation between E_2/ERs signaling and ERK/Akt-1 pathways has also been reported in mammals [63,64]. In vitro experiments have demonstrated a direct role of E_2/ERs signaling in the stimulation of proliferation in both somatic [18,65] and germ cells [19,66–69] through the activation of ERK1/2 and Akt-1. Interestingly, it was observed in the present study that the changes in the activation status of both ERK1/2 and Akt-1 were well-correlated with the quail spermatogenetic activity, indicating a key role of these proteins in the regulation of testicular epithelium proliferation and meiotic progression of germ cells in quail testis. In this regard, an increase in the testicular protein levels of PCNA, a mitotic marker, was observed during the reproductive period compared to the non-reproductive period. These findings were supported by the immunohistochemical analysis, which revealed an increased immunohistochemical signal for PCNA in quail testis during the reproductive period. In particular, PCNA was observed to be localized in both somatic and germ cells, with a more intense immunopositivity in spermatogonia. Interestingly, several studies have evidenced that PCNA is regulated by ERK1/2 and Akt-1 signaling pathways. Specifically, the inhibition of ERK1/2 and Akt-1 pathways downregulates the expression of PCNA in the Sertoli cells of chicken [69] and piglets [70], thereby suppressing their proliferation. Recently, it has been demonstrated that treatment

with excitatory D-amino acids, which are known to promote spermatogenesis, increases ERK1/2 and Akt-1 activation, as well as the PCNA protein levels, in both frog [71] and rat testis [57] as well as in the spermatogonial cell line GC-1 [58–60,72,73]. Chieffi et al. [21,22] reported that the E_2-induced ERK1/2 activation was associated with the increased PCNA protein expression in the spermatogonia of *P. esculentus* and *P. sicula* during their respective reproductive periods. Therefore, the findings of the present study suggest that in quail testis, PCNA protein expression could be enhanced through the E_2-induced ERK1/2 and Akt-1 activation during the reproductive period, while in the non-reproductive period, when the ERK1/2 and Akt-1 pathways are inactive, the PCNA protein levels are decreased.

A positive correlation between ERK1/2 and Akt-1 pathway activities and the levels of SYCP3 protein, a meiotic marker, observed in quail testis in the present study indicated a key role of ERK1/2 and Akt-1 phosphorylation in the meiotic progression of germ cells during the reproductive cycle. Similarly, a positive correlation between ERK1/2 activation and the increased number of cells positive for SYCP3 was observed in mouse testis in a previous study [45], which also demonstrated that Akt-1 inhibition abolished SYCP3 induction and the meiotic entry of postnatal mouse male germ cells [56]. Furthermore, inhibition of ERK1/2 was reported to suppress the genic expression of SYCP3 in cultured fetal germ cells [74]. Therefore, the findings of the present study are evidence for the local regulation of quail spermatogenesis via ERK/Akt-1 activation or inhibition during the reproductive cycle.

Studies have reported that reduced PCNA levels in quail testis are associated with testicular regression or atrophy via apoptotic induction [29,44,75–77]. Consistent with this, the present study revealed an increase in the expression levels of pro-apoptotic proteins, Bax and cytochrome c, in quail testis during the non-reproductive period; in addition, decreased levels of PCNA and SYCP3 were observed, suggesting that apoptosis might be a factor in the regression of quail testicular activity during the non-reproductive phase of the annual cycle. Interestingly, several studies have reported that apoptosis could be induced by the inhibition of ERK1/2 and Akt-1 signaling pathways [78–80]. Therefore, we hypothesized that the inactivation of ERK1/2 and Akt-1 signaling pathways in quail testis during the non-reproductive period could have a key role in the induction of apoptotic events and the consequent regression of the gonad.

5. Conclusions

In conclusion, our results indicate that estrogens might be acting in synergy with the androgens in quail testis during the reproductive period. In particular, high levels of $E_2/ER\alpha$ induce mitotic and meiotic events in germ cells through the activation of ERK1/2 and Akt-1 pathways. On the contrary, during the non-reproductive period, when the $E_2/ER\alpha$ levels are low, ERK1/2 and Akt-1 pathways are inactive and apoptotic events occur. Therefore, it is suggested that the activation or inhibition of these molecular pathways might have crucial roles in the physiological switch "on/off" of the testicular activity in male quails during their seasonal reproductive cycle.

Author Contributions: Conceptualization, A.S., M.M.D.F. and L.R.; Investigation, S.F., F.D.G.R. and L.R.; data curation, A.S., L.R. and S.F.; writing—original draft preparation, A.S. and S.F.; writing—review and editing, A.S., G.C.B., M.M.D.F. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The study was conducted according to the ethical provisions imposed by the European Union and permitted by the National Committee of the Italian Ministry of Health on in vivo experimentation before 2013. All treatments were performed before 2013, the samples were collected and stored at the Department of Biology and analyzed to obtain present results.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: Authors declare no conflicts of interest or competing interest.

References

1. Andò, S.; Ciarcia, G.; Panno, M.L.; Imbrogno, E.; Tarantino, G.; Buffone, M.; Beraldi, E.; Angelini, F.; Botte, V. Sex steroids levels in the plasma and testis during the reproductive cycle of lizard *Podarcis s. sicula*. *Raf. Gen. Comp. Endocrinol.* **1992**, *85*, 1–7. [CrossRef]
2. Deviche, P.; Hurley, L.L.; Fokidis, H.B. Avian testicular structure, function, and regulation. In *Hormones and Reproduction in Vertebrates: Birds*; Norris, D.O., Lopez, K.H., Eds.; Academic Press: San Diego, CA, USA, 2011; pp. 27–70.
3. Rastogi, R.K.; Pinelli, C.; Polese, G.; D'Aniello, B.; Chieffi Baccari, G. Hormones and reproductive cycles in anuran amphibians. In *Hormones and Reproduction of Vertebrates*; Norris, D.O., Lopez, K.H., Eds.; Elsevier: Boulder, CO, USA, 2011; Volume 2, pp. 171–186.
4. Hess, R.A.; Cooke, P.S. Estrogen in the male: A historical perspective. *Biol. Reprod.* **2018**, *99*, 27–44. [CrossRef] [PubMed]
5. Cooke, P.S.; Nanjappa, M.K.; Ko, C.; Prins, G.S.; Hess, R.A. Estrogens in male physiology. *Physiol. Rev.* **2017**, *97*, 995–1043. [CrossRef]
6. Osz, J.; Brelivet, Y.; Peluso-Iltis, C.; Cura, V.; Eiler, S.; Ruff, M.; Bourguet, W.; Rochel, N.; Moras, D. Structural basis for a molecular allosteric control mechanism of cofactor binding to nuclear receptors. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E588–E594. [CrossRef] [PubMed]
7. Andrews, J.E.; Smith, C.A.; Sinclair, A.H. Sites of estrogen receptor and aromatase expression in the chicken embryo. *Gen. Comp. Endocrinol.* **1997**, *108*, 182–190. [CrossRef] [PubMed]
8. Nakabayashi, O.; Kikuchi, H.; Kikuchi, T.; Mizuno, S. Differential expression of genes for aromatase and estrogen receptor during the gonadal development in chicken embryos. *J. Mol. Endocrinol.* **1998**, *20*, 193–202. [CrossRef]
9. Koba, N.; Ohfuji, T.; Ha, Y.; Mizushima, S.; Tsukada, A.; Saito, N.; Shimada, K. Profiles of mRNA expression of FOXL2, P450arom, DMRT1, AMH, P450 c17, SF1, ERa and AR, in relation to gonadal sex differentiation in duck embryo. *J. Poult. Sci.* **2008**, *45*, 132–138. [CrossRef]
10. Opalka, M.; Leska, A.; Kamińska, B.; Dusza, L. Oestrogen receptor α and β mRNA expression in testis of ganders fed diets containing different levels of phytoestrogens. *J. Anim. Feed. Sci.* **2008**, *17*, 600–607. [CrossRef]
11. Ichikawa, K.; Yamamoto, I.; Tsukada, A.; Saito, N.; Shimada, K. cDNA cloning and mRNA expression of estrogen receptor a in Japanese quail. *J. Poult. Sci.* **2003**, *40*, 121–129. [CrossRef]
12. Lakaye, B.; Foidart, A.; Grisar, T.; Balthazart, J. Partial cloning and distribution of estrogen receptor beta in the avian brain. *Neuroreporter* **1998**, *9*, 2743–2748. [CrossRef]
13. Gonzàlez-Moràn, M.G. Changes in the immunohistochemical localization of estrogen receptor alpha and in the stereological parameters of the testes of mature and aged chickens (*Gallus domesticus*). *Biochem. Biophys. Res. Commun.* **2019**, *510*, 309–314. [CrossRef]
14. Oliveira, A.G.; Dornas, R.A.P.; Mahecha, G.A.B.; Oliveira, C.A. Occurrence and cellular distribution of estrogen receptors ERα and ERβ in the testis and epididymal region of roosters. *Gen. Comp. Endocrinol.* **2011**, *170*, 597–603. [CrossRef]
15. Baghel, K.; Niranjan, M.K.; Srivastava, R. Water and food restriction decreases immunoreactivity of oestrogen receptor alpha and antioxidant activity in testes of sexually mature *Coturnix coturnix japonica*. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 1738–1747. [CrossRef]
16. Baghel, K.; Srivastava, R. Photoperiod dependent expression of estrogen receptor alpha in testes of Japanese quail: Involvement of Withania somnifera in apoptosis amelioration. *Biochem. Biophys. Res. Commun.* **2021**, *534*, 957–965. [CrossRef]
17. Leska, A.; Kiezun, J.; Kaminska, B.; Dusza, L. Estradiol concentration and the expression of estrogen receptors in the testes of the domestic goose (*Anser anser f.domestica*) during the annual reproductive cycle. *Domest. Anim. Endocrinol.* **2015**, *51*, 96–104. [CrossRef] [PubMed]
18. Lucas, T.F.; Royer, C.; Siu, E.R.; Lazari, M.F.; Porto, C.S. Expression and signaling of G protein-coupled estrogen receptor 1 (GPER) in rat sertoli cells. *Biol. Reprod.* **2010**, *83*, 307–317. [CrossRef] [PubMed]
19. Sirianni, R.; Chimento, A.; Ruggiero, C.; De Luca, A.; Lappano, R.; Ando, S.; Maggiolini, M.; Pezzi, V. The novel estrogen receptor, G protein-coupled receptor 30, mediates the proliferative effects induced by 17beta-estradiol on mouse spermatogonial GC-1 cell line. *Endocrinology* **2008**, *149*, 5043–5051. [CrossRef]
20. Cobb, M.H. MAP kinase pathways. *Prog. Biophys. Mol. Biol.* **1999**, *71*, 479–500. [CrossRef]
21. Chieffi, P.; Colucci-D'Amato, G.L.; Staibano, S.; Franco, R.; Tramontano, D. Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. *J. Endocrinol.* **2000**, *167*, 77–84. [CrossRef]
22. Chieffi, P.; Colucci D'amato, L.; Guarino, F.; Salvatore, G.; Angelini, F. 17b-estradiol induces spermatogonial proliferation through mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2) activity in the lizard (*Podarcis s. sicula*). *Mol. Reprod. Dev.* **2002**, *61*, 218–225. [CrossRef]
23. Sette, C.; Barchi, M.; Bianchini, A.; Conti, M.; Rossi, P.; Geremia, R. Activation of the mitogen-activated protein kinase ERK1 during meiotic progression of mouse pachytene spermatocytes. *J. Biol. Chem.* **1999**, *274*, 33571–33579. [CrossRef]
24. Datta, S.R.; Brunet, A.; Greenberg, M.E. Cellular survival: A play in three Akts. *Genes. Dev.* **1999**, *13*, 2905–2927. [CrossRef] [PubMed]

25. Kandel, E.S.; Hay, N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp. Cell. Res.* **1999**, *253*, 210–229. [CrossRef] [PubMed]

26. Blottner, S.; Hingst, O.; Meyer, H.H.D. Inverse relationship between testicular proliferation and apoptosis in mammalian seasonal breeders. *Theriogenology* **1995**, *44*, 320–328. [CrossRef]

27. Young, K.A.; Nelson, R.J. Mediation of seasonal testicular regression by apoptosis. *Reproduction* **2001**, *122*, 677–685. [CrossRef] [PubMed]

28. Scaia, M.F.; Czuchlej, S.C.; Cervino, N.; Ceballos, N.R. Apoptosis, Proliferation and Presence of Estradiol Receptors in the Testes and Bidder's Organ of the Toad *Rhinella Arenarum* (Amphibia, Anura). *J. Morphol.* **2016**, *277*, 412–423. [CrossRef]

29. Banerjee, S.; Chaturvedi, C.M. Apoptotic mechanism behind the testicular atrophy in photorefractory and scotosensitive quail: Involvement of GnIH induced p-53 dependent Bax- Caspase-3 mediated pathway. *J. Photochem. Photobiol. B. Biol.* **2017**, *176*, 124–135. [CrossRef]

30. Islam, M.N.; Tsukahara, N.; Sugita, S. Apoptosis-mediated seasonal testicular regression in the Japanese Jungle crow (*Corvus macrorhynchos*). *Theriogenology* **2012**, *77*, 1854–1865. [CrossRef]

31. Jenkins, L.K.; Ross, W.L.; Young, K.A. Increases in apoptosis and declines in Bcl-XL protein characterize testicular regression in American crows (*Corvus brachyrhynchos*). *Reprod. Fertil. Dev.* **2007**, *19*, 461–469. [CrossRef]

32. Young, K.A.; Ball, G.F.; Nelson, R.J. Photoperiod-induced testicular apoptosis in European starlings (*Sturnus vulgaris*). *Biol. Reprod.* **2001**, *64*, 706–713. [CrossRef] [PubMed]

33. Baraldi-Artoni, S.M.; Orsi, A.M.; Carvaljo, T.L.L.; Lopes, R.A. The Annual Testicular Cycle of the Domestic Quail (*Coturnix coturnix japonica*). *Anat. Histol. Embryol.* **1997**, *26*, 337–339. [CrossRef] [PubMed]

34. Baraldi-Artoni, S.M.; Orsi, A.M.; Carvaljo, T.L.L.; Vicentin, C.A.; Stefanini, M.A. Seasonal morphology of domestic quail (*Coturnix coturnix japonica*) testis. *Adv. Anat. Embryol. Cell. Biol.* **1999**, *28*, 217–220. [CrossRef]

35. Rosati, L.; Di Fiore, M.M.; Prisco, P.; Di Giacomo Russo, F.; Venditti, M.; Andreuccetti, P.; Chieffi Baccari, G.; Santillo, A. Seasonal expression and cellular distribution of star and steroidogenic enzymes in quail testis. *J. Exp. Zool. B Mol. Dev. Evol.* **2019**, *332*, 198–209. [CrossRef] [PubMed]

36. Prisco, M.; Rosati, L.; Agnese, M.; Aceto, S.; Andreuccetti, P.; Valiante, S. Pituitary adenylate cyclase-activating polypeptide in the testis of the quail *Coturnix coturnix*: Expression, localization, and phylogenetic analysis. *Evol. Dev.* **2019**, *21*, 145–156. [CrossRef] [PubMed]

37. Falvo, S.; Di Fiore, M.M.; Burrone, L.; Chieffi Baccari, G.; Longobardi, S.; Santillo, A. Androgen and oestrogen modulation by D-aspartate in rat epididymis. *Reprod. Fertil. Dev.* **2016**, *28*, 1865–1872. [CrossRef]

38. Rosati, L.; Prisco, M.; Di Fiore, M.M.; Santillo, A.; Sciarrillo, R.; Valiante, S.; Laforgia, V.; Coraggio, F.; Andreuccetti, P.; Agnese, M. Sex steroid hormone secretion in the wall lizard *Podarcis sicula* testis: The involvement of VIP. *J. Exp. Zool. A Ecol. Genet. Physiol.* **2015**, *323*, 714–721. [CrossRef] [PubMed]

39. Rosati, L.; Prisco, M.; Di Fiore, M.M.; Santillo, A.; Valiante, S.; Andreuccetti, P.; Agnese, M. Role of PACAP on testosterone and 17β-estradiol production in the testis of wall lizard *Podarcis sicula*. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2016**, *191*, 180–186. [CrossRef] [PubMed]

40. Rosati, L.; Santillo, A.; Di Fiore, M.M.; Andreuccetti, P.; Prisco, M. Testicular steroidogenic enzymes in the lizard *Podarcis sicula* during the spermatogenic cycle. *C. R. Biol.* **2017**, *340*, 492–498. [CrossRef]

41. Rosati, L.; Agnese, M.; Verderame, M.; Aniello, F.; Venditti, M.; Mita, D.G.; Andreuccetti, P.; Prisco, M. Morphological and molecular responses in ovaries of *Mytilus galloprovincialis* collected in two different sites of the Naples Bay. *J. Exp. Zool. A Ecol. Integr. Physiol.* **2019**, *331*, 52–60. [CrossRef]

42. Di Fiore, M.M.; Lamanna, C. , Assisi, L., Botte, V. Opposing effects of D-aspartic acid and nitric oxide on tuning of testosterone production in mallard testis during the reproductive cycle. *Reprod. Biol. Endocrinol.* **2008**, *6*, 28. [CrossRef]

43. Lee, N.P.Y.; Cheng, C.Y. Nitric oxide/nitric oxide synthase, spermatogenesis, and tight junction dynamics. *Biol. Reprod.* **2004**, *70*, 267–276. [CrossRef] [PubMed]

44. Banerjee, S.; Chaturvedi, C.M. Specific neural phase relation of serotonin and dopamine modulate the testicular activity in Japanese quail. *J. Cell. Physiol.* **2019**, *234*, 2866–2879. [CrossRef]

45. Grimaldi, P.; Orlando, P.; Di Siena, S.; Lolicato, F.; Petrosino, S.M.; Bisogno, T.; Geremia, R.; De Petrocellis, L.; Di Marzo, V. The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 11131–11136. [CrossRef]

46. Santillo, A.; Giacco, A.; Falvo, S.; Di Giacomo Russo, F.; Senese, R.; Di Fiore, M.M.; Chieffi Baccari, G.; Lanni, A.; de Lange, P. Mild exercise rescues steroidogenesis and spermatogenesis in rats submitted to food withdrawal. *Front. Endocrinol.* **2020**, *11*, 302. [CrossRef] [PubMed]

47. Shukla, K.K.; Mahdi, A.A.; Rajender, S. Apoptosis, spermatogenesis and male infertility. *Front. Biosci.* **2012**, *4*, 746–754. [CrossRef]

48. Carreau, S.; Bouraima-Lelong, H.; Delalande, C. Estrogens—New players in spermatogenesis. *Reprod. Biol.* **2011**, *11*, 174–193. [CrossRef]

49. Mattsson, A.; Brunström, B. Effects of selective and combined activation of estrogen receptor alpha and beta on reproductive organ development and sexual behaviour in Japanese quail (*Coturnix japonica*). *PLoS ONE* **2017**, *12*, e0180548. [CrossRef]

50. Stocco, C. Aromatase expression in the ovary: Hormonal and molecular regulation. *Steroids* **2008**, *73*, 473–487. [CrossRef]

51. Gonzàlez-Moràn, M.G.; Guerra-Araiza, C.; Campos, M.G.; Camacho-Arroyo, I. Histological and sex steroid hormone receptor changes in testes of immature, mature, and aged chickens. *Domest. Anim. Endocrinol.* **2008**, *35*, 371–379. [CrossRef]

52. Polzonetti-Magni, A.; Botte, V.; Bellini-Cardellini, L.; Gobbetti, A.; Crasto, A. Plasma sex hormones and post-reproductive period in the green frog, *Rana esculenta* complex. *Gen. Comp. Endocrinol.* **1984**, *54*, 372–377. [CrossRef]

53. Santillo, A.; Falvo, S.; Chieffi Baccari, G.; Di Fiore, M.M. Seasonal changes in gene expression of steroidogenic enzymes, androgen and estrogen receptors in frog testis. *Acta Zool.* **2017**, *98*, 221–227. [CrossRef]

54. Santillo, A.; Falvo, S.; Di Fiore, M.M.; Chieffi Baccari, G. Seasonal changes and sexual dimorphism in gene expression of StAR protein, steroidogenic enzymes and sex hormone receptors in the frog brain. *Gen. Comp. Endocrinol.* **2017**, *246*, 226–232. [CrossRef]

55. Hasegawa, K.; Namekawa, S.H.; Saga, Y. MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. *Stem Cells.* **2013**, *31*, 2517–2527. [CrossRef] [PubMed]

56. Pellegrini, M.; Di Siena, S.; Claps, G.; Di Cesare, S.; Dolci, S.; Rossi, P.; Geremia, R.; Grimaldi, P. Microgravity promotes differentiation and meiotic entry of postnatal mouse male germ cells. *PLoS ONE* **2010**, *5*, e9064. [CrossRef] [PubMed]

57. Santillo, A.; Falvo, S.; Chieffi, P.; Burrone, L.; Chieffi Baccari, G.; Longobardi, S.; Di Fiore, M.M. D-aspartate affects NMDA receptor-extracellular signal-regulated kinase pathway and upregulates androgen receptor expression in the rat testis. *Theriogenology* **2014**, *81*, 744–751. [CrossRef]

58. Santillo, A.; Falvo, S.; Chieffi, P.; Di Fiore, M.M.; Senese, R.; Chieffi Baccari, G. D-Aspartate Induces Proliferative Pathways in Spermatogonial GC-1 Cells. *J. Cell. Physiol.* **2016**, *231*, 490–495. [CrossRef]

59. Santillo, A.; Falvo, S.; Di Fiore, M.M.; Di Giacomo Russo, F.; Chieffi, P.; Usiello, A.; Pinelli, C.; Chieffi Baccari, G. AMPA receptor expression in mouse testis and spermatogonial GC-1 cells: A study on its regulation by excitatory amino acids. *J. Cell. Biochem.* **2019**, *120*, 11044–11055. [CrossRef]

60. Santillo, A.; Venditti, M.; Minucci, S.; Chieffi Baccari, G.; Falvo, S.; Rosati, L.; Di Fiore, M.M. D-Asp upregulates PREP and GluA2/3 expressions and induces p-ERK1/2 and p-Akt in rat testis. *Reproduction* **2019**, *158*, 357–367. [CrossRef]

61. Russo, M.; Troncone, G.; Guarino, F.M.; Angelini, F.; Chieffi, P. Estrogen-Induced Akt-1 Activity in the Lizard (*Podarcis s. sicula*) Testis. *Mol. Reprod. Dev.* **2005**, *71*, 52–57. [CrossRef]

62. Stabile, V.; Russo, M.; Chieffi, P. 17b-Estradiol induces Akt-1 through estrogen receptor-b in the frog (*Rana esculenta*) male germ cells. *Reproduction* **2006**, *132*, 477–484. [CrossRef] [PubMed]

63. Yao, Y.; Chang, X.; Wang, D.; Ma, H.; Wang, H.; Zhang, H.; Li, C.; Wang, J. Roles of ERK1/2 and PI3K/AKT signaling pathways in mitochondria-mediated apoptosis in testes of hypothyroid rats. *Toxicol. Res.* **2018**, *7*, 1214–1224. [CrossRef]

64. Zhang, Z.; Duan, L.; Du, X.; Ma, H.; Park, I.; Lee, C.; Zhang, J.; Shi, J. The proliferative effect of estradiol on human prostate stromal cells is mediated through activation of ERK. *Prostate* **2008**, *68*, 508–516. [CrossRef]

65. Meroni, S.B.; Galardo, M.N.; Rindone, G.; Gorga, A.; Riera, M.F.; Cigorraga, S.B. Molecular mechanisms and signaling pathways involved in sertoli cell proliferation. *Front. Endocrinol.* **2019**, *10*, 224. [CrossRef] [PubMed]

66. Vicini, E.; Loiarro, M.; Di Agostino, S.; Corallini, S.; Capolunghi, F.; Carsetti, R.; Chieffi, P.; Geremia, R.; Stefanini, M.; Sette, C. 17-beta-estradiol elicits genomic and non-genomic responses in mouse male germ cells. *J. Cell. Physiol.* **2006**, *206*, 238–245. [CrossRef]

67. La Sala, G.; Farini, D.; De Felici, M. Rapid estrogen signalling in mouse primordial germ cells. *Exp. Cell. Res.* **2010**, *316*, 1716–1727. [CrossRef]

68. Boscia, F.; Passaro, C.; Gigantino, V.; Perdona, S.; Franco, R.; Portella, G.; Chieffi, S.; Chieffi, P. High levels of GPR30 protein in human testicular carcinoma in situ and seminomas correlate with low levels of estrogen receptor-Beta and indicate a switch in estrogen responsiveness. *J. Cell. Physiol.* **2015**, *230*, 1290–1297. [CrossRef] [PubMed]

69. Xu, K.; Wang, J.; Liu, H.; Zhao, J.; Lu, W. Melatonin promotes the proliferation of chicken sertoli cells by activating the ERK/inhibin alpha subunit signaling pathway. *Molecules.* **2020**, *25*, 1230. [CrossRef]

70. Sun, Y.; Yang, W.; Luo, H.; Wang, X.; Chen, Z.; Zhang, J.; Wang, Y.; Li, X. Thyroid hormone inhibits the proliferation of piglet Sertoli cell via PI3K signaling pathway. *Theriogenology* **2015**, *83*, 86–94. [CrossRef]

71. Santillo, A.; Chieffi Baccari, G.; Falvo, S.; Di Giacomo Russo, F.; Venditti, M.; Di Fiore, M.M. Effects of D-Aspartate on sex hormone-dependent tissues in *Pelophylax esculentus*. In *Amphibians: Biology, Ecology and Conservation*; Cannon, L., Ed.; Nova Science Publishers: New York, NY, USA, 2018; pp. 21–37.

72. Di Fiore, M.M.; Boni, R.; Santillo, A.; Falvo, S.; Gallo, A.; Esposito, S.; Chieffi Baccari, G. D-aspartic acid in vertebrate reproduction: animal models and experimental designs. *Biomolecules* **2019**, *9*, 445. [CrossRef] [PubMed]

73. Venditti, M.; Santillo, A.; Falvo, S.; Di Fiore, M.M. , Chieffi Baccari, G.; Minucci, S. d-aspartate upregulates daam1 protein levels in the rat testis and induces its localization in spermatogonia nucleus. *Biomolecules* **2020**, *10*, 677. [CrossRef]

74. Kim, S.M.; Yokoyama, T.; Ng, D.; Ulu, F.; Yamazaki, Y. Retinoic acid-stimulated ERK1/2 pathway regulates meiotic initiation in cultured fetal germ cells. *PLoS ONE* **2019**, *14*, e0224628. [CrossRef] [PubMed]

75. Banerjee, S.; Tsutsui, K.; Chaturvedi, C.M. Apoptosis mediated testicular alteration in Japanese quail (*Coturnix coturnix japonica*) in response to temporal phase relation of serotonergic and dopaminergic oscillations. *J. Exp. Biol.* **2016**, *219*, 1476–1487. [CrossRef] [PubMed]

76. Banerjee, S.; Chaturvedi, C.M. Testicular atrophy and reproductive quiescence in photorefractory and scotosensitive quail: Involvement of hypothalamic deep brain photoreceptors and GnRH-GnIH system. *J. Photochem. Photobiol. B Biol.* **2017**, *175*, 254–268. [CrossRef] [PubMed]

77. Banerjee, S.; Chaturvedi, C.M. Simulated photoperiod influences testicular activity in quail via modulating local GnRHR-GnIHR, GH-R, Cnx-43 and 14-3-3. *J. Photochem. Photobiol. B Biol.* **2018**, *178*, 412–423. [CrossRef] [PubMed]
78. Liu, J.; Li, Q.; Liu, Z.; Lin, L.; Zhang, X.; Cao, M.; Jiang, J. Harmine induces cell cycle arrest and mitochondrial pathway-mediated cellular apoptosis in SW620 cells via inhibition of the Akt and ERK signaling pathways. *Oncol. Rep.* **2016**, *35*, 3363–3370. [CrossRef] [PubMed]
79. Wang, A.S.; Xu, C.W.; Xie, H.Y.; Yao, A.J.; Shen, Y.Z.; Wan, J.J.; Zhang, H.Q.; Fu, J.F.; Chen, Z.M.; Zou, Z.Q. DHA induces mitochondria-mediated 3T3-L1 adipocyte apoptosis by down-regulation of Akt and ERK. *J. Funct. Foods.* **2016**, 21517–21524. [CrossRef]
80. Li, L.; Wang, X.; Sharvan, R.; Gao, J.; Qu, S. Berberine could inhibit thyroid carcinoma cells by inducing mitochondrial apoptosis, G0/G1 cell cycle arrest and suppressing migration via PI3K-AKT and MAPK signaling pathways. *Biomed. Pharmacother.* **2017**, 951225–951231. [CrossRef]