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Increases in apoptosis and declines in Bcl-X_L protein characterise testicular regression in American crows (*Corvus brachyrhynchos*)

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Abstract

The present study investigated the cellular changes observed during testicular regression in American crows. Testes from adults caught during the early (March), progressing (April), peak (early May), transitional (late May), and post- (June) breeding season were examined. Apoptosis was assessed by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) and Bcl-X_L protein immunolabelling. Testis mass increased two-fold from March to early May ($P < 0.05$), then declined 19-fold by June ($P < 0.001$) without corresponding changes in body mass ($P > 0.05$). Testicular activity, evaluated using a spermatogenic index, increased nearly two-fold from March to early May and declined nine-fold in June ($P < 0.001$). Seminiferous tubule diameter declined four-fold in June compared with earlier months ($P < 0.001$). In all testes, TUNEL-positive germ cells were detected at low levels, with the highest levels observed in late May ($P < 0.001$). In contrast, TUNEL-positive Sertoli cells were maintained at low levels in March–April and increased nine-fold in early May ($P < 0.001$). The Bcl-X_L immunostaining was detected in Sertoli cells in March–early May; however, staining was most intense in March–April and substantially weaker by early May. These data suggest that the seasonal rise in testicular competence occurs slowly in American crows; however, testis function is terminated rapidly after the breeding season. Furthermore, it is likely that Sertoli cell apoptosis followed by massive germ cell loss is responsible for the rapid reduction in testis mass.

Keywords

avian; seasonal reproduction

Introduction

American crows (*Corvus brachyrhynchos*) are known vectors for West Nile virus and other pathogens, with a rapidly increasing population in urban centres such as Southern California

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(Marzluff *et al.* 2001); however, little is known about their breeding biology at the level of the gonad. Understanding reproductive function in crows not only allows a comparison with other passerines, but also helps to elucidate information about a species that impacts both human and domestic animal populations.

Crows start to breed around 3 years of age (Wilmore 1977) and typically limit their breeding season to January–May, with the bulk of reproductive activity occurring between March and May (Marshall and Coombs 1957; Kilham 1985). In many non-tropical avian species, including crows, photoperiod (hours of light per day) appears to be the primary temporal cue for controlling seasonal reproduction (Wilson and Donham 1988). Photoperiod acts upon the hypothalamic–pituitary–gonadal (HPG) axis in birds, as well as in other species, to induce changes in gonadal function; photoperiod can induce testicular atrophy or regression, as well as stimulate testicular recrudescence or regrowth. In many species, testis size increases during the breeding season as a result of increases in the length, diameter and activity of the seminiferous tubules, in addition to increases in the number and activity of Leydig cells (Birkhead and Moller 1992). In the non-breeding season, declines in testis size correlate with decreased spermatogenesis, reduced testosterone production and, ultimately, a loss of reproductive function. Although it was known that substantial changes in gonadal size and function occur during the transition between the breeding and non-breeding season, neither the timing of the morphological changes nor the intracellular mechanisms responsible for testicular regression in American crows had been fully determined.

In many seasonally breeding species, including Passeriformes, testicular regression involves increases in germ cell apoptosis (Furuta *et al.* 1994; Young and Nelson 2001; Young *et al.* 2001). Apoptosis, or programmed cell death, is a mechanism that regulates normal tissue development and homeostasis (Sinha Hikim and Swerdloff 1999). Apoptotic activation is initiated by extra- or intracellular pathways that lead to a cascade of signalling events that ultimately result in DNA fragmentation, cell shrinkage and a disassembled nuclear envelope. Neighbouring cells then phagocytose the dying cell, generally without triggering a systemic immune response (Le Grand 1997). Apoptosis is the preeminent form of cell death occurring during seasonal testicular regression in most species studied to date, with death limited to the renewable germ cell population (Young and Nelson 2001). However, both germ cells and spermatogenesis-supporting Sertoli cells are observed to undergo apoptotic cell death in European starlings. Because these birds undergo testicular regression in response to photoperiod that is more rapid than that observed in many mammals, Sertoli cell death is believed to serve as a mechanism to allow expeditious testis atrophy to occur (Young *et al.* 2001). It was not known whether this mechanism was limited to starlings or whether Sertoli cell death also occurred in other seasonally breeding passerines.

Because American crows are intimately tied to urban environments and because of their link to the spread of viruses, such as West Nile, we sought to examine the question of Sertoli cell death in crows during seasonal changes in testis function, as well as the role of an apoptotic protein, namely Bcl-X_L, during this process. Members of the Bcl-2 family are involved in regulating apoptosis and can either induce (Bax, Bcl-X_S, Bak, Bad) or inhibit (Bcl-2, Bcl-X_L, Bcl-w) apoptotic cell death, depending on their expression levels and balance between proteins (Korsmeyer 1995; Sakamaki 2003). The Bcl-X_L protein is a prosurvival member of

this family that reduces testicular apoptosis to the point of spermatogenesis disruption when overexpressed (Rodriguez *et al.* 1997) and increases testicular apoptosis when downregulated (Arriola *et al.* 1999). However, the degree to which apoptosis plays a part in testicular regression in American crows, which apoptotic proteins are present and which cells specifically are being affected by apoptosis during the transition between breeding and non-breeding seasons was not known. We hypothesised that testicular regression would be preceded by a decrease in testis activity, increases in apoptotic cell death of both germ and Sertoli cells and a decrease in the cell survival protein Bcl-X_L. To investigate this, we established a timeline of morphological testicular changes and examined key biochemical indicators of testis function. Morphological and functional changes were quantified by assessing testis mass, seminiferous tubule diameter and spermatogenic index in wild crows caught before, during and following the breeding season. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) was used to determine the extent of apoptotic cell death and testicular cell survival was assessed by immunostaining for the anti-apoptotic protein Bcl-X_L.

Materials and methods

Animals

Adult male American crows (*Corvus brachyrhynchos*; $n = 26$) were wild-caught during March–June 2004, in collaboration with Predator Management of California Department of Fish and Game and Orange County Vector Control. Southern California collection sites included Seal Beach, Bolsa Chica, Terminal Island, Venice Beach and Huntington Beach; these sites are a maximum of 52.5 km (32.6 miles) apart. Testis tissues, along with age and body mass data, were collected in the field from healthy crows. All crows were divided into five groups based on 2004 catch date: March, April, early May, late May and June ($n = 3–6$ per group), with these months representing the early, progressing, peak, transition and post-breeding seasons. The crows collected in May were split into early and late May groups because substantial changes in testis mass and activity were observed within this transitional month. The crows collected in June displayed full testicular regression and, thus, were considered to be in a non-breeding state.

Tissue processing and histological analysis

Following removal of connective tissue, testes were weighed and then fixed in 10% neutral buffered formalin for 1 week, washed in three changes of 0.05 M phosphate-buffered saline (PBS), dehydrated in a series of ethanol washes and embedded in paraffin. Tissues were cut using a rotary microtome and 6- μ m sections were collected from every 60 μ m of tissue and serially mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA). For each assessment, except seminiferous tubule diameter and TUNEL, a total of six cross sections, taken from across the testis, was analysed for each crow. A total of three cross sections was analysed for seminiferous tubule diameter and TUNEL.

To assess seminiferous tubule diameter and spermatogenic index, sections were deparaffinised, rehydrated through a graded series of alcohol and xylenes, and stained with hematoxylin and eosin. Because seminiferous tubule diameter is correlated with testis

function (Amann 1986), the diameter of 18 seminiferous tubules in each testis cross section was averaged for each bird. Spermatogenic activity was assessed using a spermatogenic index adopted by Grocock and Clarke (1974). For each crow, 30 seminiferous tubules were evaluated across six cross sections and given a score (0–5) based on testicular competence. A value of 0 was given to small or regressed tubules that contained only Sertoli cells, spermatogonia and a few spermatocytes; a value of 5 was given to large tubules displaying complete spermatogenesis with abundant spermatozoa production.

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labelling

The TACS XL Blue Label *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA) was used to detect apoptotic cells in the TUNEL assay. Sections were deparaffinised, rehydrated and treated with proteinase K before labelling with the 3' OH termini-labelling terminal deoxynucleotidyl transferase (TdT). Biotinylated nucleotides were visualised using streptavidin–horseradish peroxidase and TACS Blue Label. Sections were counterstained with Nuclear Fast Red for 4 min, mounted and visualised with a light microscope. Negative controls were processed without TdT and showed no staining. Positive controls were processed with TACS-Nuclease (Trevigen) and high levels of apoptosis were observed, confirming the performance of the assay. Quantification of apoptotic activity was conducted by dividing the number of apoptotic cells by the number of seminiferous tubules per cross section. This method controls for a reduction in testis size (Young *et al.* 1999, 2001).

Immunohistochemical analysis

To examine the extent of expression for an anti-apoptotic factor during testicular growth and regression, Bcl-X_L protein immunostaining was also assessed. Tissue sections were deparaffinised by three washes in xylenes and then rehydrated in a series of ethanol solutions and PBS. Sections were then placed in an antigen-retrieving solution (Citra; Vector Laboratories, Burlingame, CA, USA) and heated in a pressure cooker for 10 min. To block endogenous peroxidases, sections were placed in 3% H₂O₂ in methanol solution. Following treatment with normal mouse serum, sections were incubated with a 1 : 75 dilution of Bcl-X_L antibody (Laboratory Vision, Fremont, CA, USA) and left overnight at room temperature. Sections were washed and then incubated with secondary antibody and avidin–biotin peroxidase solution (mouse IgG Vectastain ABC kit; Vector Laboratories). Sections were then visualised using Vector Red solution (Vector Laboratories) for 10 min. Sections were analysed qualitatively by comparing the intensity and localisation of the staining within each group. Negative controls were processed without primary antibody and showed no staining.

Statistical analysis

One-way ANOVA was used to compare body mass, paired testis mass, seminiferous tubule diameter, spermatogenic activity and TUNEL-positive cells across groups. Owing to unequal variances, TUNEL-positive germ cells were log-transformed before ANOVA. The Student–Newman–Keul's test was used for all pairwise comparisons. Differences were considered significant if $P < 0.05$. Statistical analyses were conducted using the Prism software package (Graphpad, San Diego, CA, USA).

Results

Body mass and testis mass

Body mass remained constant throughout the breeding and non-breeding season (Table 1; $P > 0.05$). In contrast, paired testis mass increased two-fold, peaking in early May, compared with March values collected at the start of breeding season (Table 1; $P < 0.05$). Paired testis mass then decreased 19-fold during the transition into the June non-breeding season (Table 1; $P < 0.001$).

Histological analysis and reproductive competence

Hematoxylin and eosin-stained testes were used to assess testicular function (Fig. 1). Sperm production was evident in March, but became abundant in early May (Fig. 1a–c). With the absence of spermatids and spermatozoa, testes function declined in late May (Fig. 1d). In June, the testes were inactive, with only Sertoli cells, spermatogonia and few spermatocytes present (Fig. 1e).

Testicular function during the breeding and non-breeding seasons was also examined by measuring seminiferous tubule diameter and assessing spermatogenic index for all crows. Seminiferous tubule diameter remained constant throughout the breeding season, before decreasing four-fold in June (Table 2; $P < 0.001$). The spermatogenic index, a measure of reproductive competence, increased nearly two-fold from March values to peak in early May, and then declined nine-fold with transition into the non-breeding season (Table 2; $P < 0.001$).

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labelling analysis

Apoptotic labelling of germ cells was observed in relatively low levels in March, tapering to low to no cell death during April and early May, and increasing substantially in the late May group (Fig. 2). Apoptotic activity was also observed in Sertoli cells in American crows undergoing testicular regression. Sertoli cells were considered TUNEL positive if the specific staining extended from the basement membrane to the lumen of the seminiferous tubule (Fig. 2c). The TUNEL-positive Sertoli cells were expressed at high levels in early May (Fig. 2c), with low to no Sertoli cell death throughout the rest of the months (Fig. 2a, b, d, e). Negative control sections, processed without TdT, showed no staining (Fig. 2d, late May inset).

The number of labelled germ cells was quantified by counting the number of TUNEL-positive germ cells per seminiferous tubule within each cross section. Germ cell death peaked by 32-fold in late May compared with the other groups ($P < 0.001$) and levels remained constant throughout the rest of the months (Fig. 3a). Sertoli cell death was quantified by counting the number of TUNEL-positive Sertoli cells per seminiferous tubule within each cross section. In contrast with peak germ cell death, peak numbers of TUNEL-positive Sertoli cells were observed in early May, with levels nine-fold higher than levels observed in other months (Fig. 3b; $P < 0.001$).

Immunohistochemical localisation of Bcl-X_L

Immunohistochemistry using a Bcl-X_L antibody localised this anti-apoptotic protein to Sertoli cells. Staining was relatively strong in March and April; however, expression of Bcl-X_L was substantially weaker in early May (Fig. 4). Following the early May group, staining intensity returned in the late May and June groups (Fig. 4*d, e*). Negative control slides, processed without primary antibody, showed no staining (Fig. 4*a*, March inset).

Discussion

The timeline of reproductive activity of male American crows in Southern California appears to increase during March, peaks in April to early May, transitions into the non-breeding season during late May and becomes fully inactive by June. In addition, data from the present study suggest, for the first time, that apoptosis is a likely factor in the regression of testicular activity in crows, because levels of apoptotic cell death increased during testicular atrophy, concomitant with declines in the expression of the anti-apoptotic factor Bcl-X_L in Sertoli cells. Furthermore, the present study substantiates Sertoli cell death in another passerine with rapid testicular regression, namely American crows.

Owing to limitations of animal collection, American crows were only collected during the prebreeding, breeding and immediately post-breeding seasons (March–June). It is likely that crows remain reproductively inactive throughout the remaining months, because only one annual peak of breeding behaviour has been reported (Verbeek and Caffrey 2002). It is therefore probable that testicular regrowth begins before March, based on the differences observed in testis mass in March compared with June (Table 1; $P < 0.05$). Examination of crow testes from year-round samples would confirm this speculation, as well as identify an extended timeline of testicular regrowth in this species.

In male American crows, the transition into the non-breeding season is characterised by testicular regression and a decrease in testis activity. The mechanism by which testicular regression is induced appears to be through significant increases in apoptotic cell death. In American crows, *in situ* TUNEL labelling was restricted to germ cells and Sertoli cells. During the start of the breeding season (March), there was a slight increase in germ cell death; however, this was not significantly different from the other months during the breeding season (Fig. 3*a*; $P > 0.05$). Apoptotic activity observed in this month is a likely result of prebreeding re-organisation of the recrudescing testis. Although increases in apoptosis are limited to the regression period in many species, apoptotic cell death increases during recrudescence, as well as during regression, in ground squirrels (Tsvetkov and Takeva 1989). Indeed, testicular apoptosis does not completely disappear during the breeding season, because a baseline level has been noted in the present study, as well as in stallions and European starlings (Young *et al.* 2001; Heninger *et al.* 2004).

Sertoli cells are the supportive cells in the seminiferous epithelium, regulating germ cell development, as well as death (Ritzen *et al.* 1981). In general, Sertoli cells are considered to exist as a stable population, surviving when subjected to stimuli that would induce cell death in other testis cell types (Tanaka and Yasuda 1980; Pudney 1995); hypophysectomy of adult rats does not induce morphological signs of apoptosis within Sertoli cells (Ghosh *et al.*

1992), nor does gonadotrophin-releasing hormone antagonist treatment affect the Sertoli cell population in adult rats (Billig *et al.* 1995). Despite the central belief that Sertoli cells do not die, in some seasonal breeders, such as viscachas (Munoz *et al.* 2001) and another passerine species, namely European Starlings (Young *et al.* 2001), the number of Sertoli cells can decrease during testicular regression. The results from the present study suggest that Sertoli cell death, in addition to germ cell death, occurs in American crows during testicular regression.

Sertoli cell death remained low throughout the early and progressing breeding season, and increased significantly during the brief peak of breeding season (Fig. 3b). It may be that the high level of apoptotic activity observed in early May prepares the testes for rapid transition into the non-breeding season, because massive germ cell death followed in the late-May group (Fig. 3a). The time window from which peak breeding is observed in American crows to the time the testes are fully inactive is within a few weeks. Because Sertoli cells are the supportive cells within the seminiferous epithelium, changes in Sertoli cell function result in germ cell loss (Lee *et al.* 1997). Indeed, the number of Sertoli cells can limit the population of germ cells (Orth *et al.* 1988). By targeting the Sertoli cells to die, widespread and rapid germ cell death can occur, which, in turn, may accelerate testicular regression, allowing the functioning of the testes of American crows to shut down within a matter of 2–3 weeks. Although rapid cell death in a limited area is suggestive of necrotic cell death, hallmarks of necrosis, such as cell swelling and infiltration of leucocytes, were not observed during testicular regression in crows. Therefore, it is likely that, in this species, apoptosis plays a key role in testicular regression. The mechanism of apoptotic Sertoli cell death followed by germ cell death may be limited to species with rapid testicular atrophy, or it may play a larger role in photoresponsive passerines.

Because they are known to live up to 12 years in captivity, American crows likely live for many years in the wild (Wilmore 1977). With each subsequent breeding cycle, the testis regenerates and becomes fully active. However, because our results suggest that Sertoli cells undergo apoptosis as they transition into the non-breeding season, Sertoli cells may be mitotically replenished in response to a seasonal cue before the next breeding season. Alternatively, Sertoli cell numbers may continue to decline with age, a decrease that may be reflected in changes in fertility, similar to the age-related reduction in Sertoli cells in humans (Kimura *et al.* 2003). Further studies examining the number of Sertoli cells between two consecutive breeding cycles would address this uncertainty.

Cell death was assessed indirectly in the present study by examining the prosurvival protein Bcl-X_L within the testis. The Bcl-X_L protein plays an important role in balancing cell death within the testis by regulating germ cell survival (Print and Loveland 2000). Within human adult testes, Bcl-X_L is localised to the cytoplasmic region of spermatogonia (Oldereid *et al.* 2001). However, in male crows, Bcl-X_L was localised to Sertoli cells (Fig. 4). In the present study, Bcl-X_L staining in Sertoli cells was specific and intense during March and April, months when TUNEL labelling was low. In contrast, staining was substantially weaker in early May, a time period when TUNEL staining indicating Sertoli cell death was significantly increased (Fig. 3b). This inverse correlation may demonstrate a partial mechanism for seasonal testis cell death and the reduction in Sertoli cell Bcl-X_L may be

critical to allow rapid testicular regression to occur. Indeed, one way apoptosis can be triggered is through the removal of certain prosurvival signals, ultimately leading to a shift in prosurvival to prodeath signals (Young and Nelson 2001). This suggested shift may be triggered by changes in reproductive hormones, as a reduction in testosterone and gonadotropins promotes testicular apoptosis (Henriksen *et al.* 1995; Billig *et al.* 1996; Nandi *et al.* 1999; Young and Nelson 2001). The decreased gonadotropin concentrations that occur during the transition from breeding to non-breeding conditions in many species may lead to the downregulation of prosurvival proteins and upregulation of apoptosis in gonadotropin-dependent Sertoli cells, as observed in the present study (Almiron and Chemes 1988; Dawson *et al.* 2001; Dawson 2002).

Understanding crow breeding biology is a timely subject because crows are specific vectors for the West Nile virus and because populations are increasing in urban areas, such as Southern California. In addition, crows are targeted for removal each year by the California Department of Fish and Game owing to their predation impact on eggs of endangered coastal birds. Overall, our results identify significant changes in testicular morphology and activity, determining for the first time the functional breeding and non-breeding season in American crows. In addition, we found that peak breeding season increases in Sertoli cell death are immediately followed by high levels of germ cell death and that this combination likely induces the rapid testicular regression observed in American crows. Although the inversely correlated staining pattern of anti-apoptotic Bcl-X_L with TUNEL-positive Sertoli cells complements these data, other apoptotic proteins should be analysed to further understand the cellular mechanisms involved in regulating testicular activity in male American crows. Because these morphological and cellular data yield precise timing information about testicular activity, they can potentially be used to develop effective reproductive management of urban crow populations.

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Fig. 1.

Hematoxylin and eosin staining of 6- μ m testis cross sections from crows caught in March, April, early May, late May and June (*a–e*, respectively) demonstrates an increase in spermatogenesis during March–early May. In contrast, June shows no spermatogenic activity. sc, spermatocytes; sg, spermatogonia; sz, spermatozoa. Photographs were taken at an original magnification of $\times 20$; insets were at an original magnification of $\times 60$.

Fig. 2.

In situ terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) of 6- μ m cross sections from crows caught in March, April, early May, late May, and June (*a–e*, respectively). These sections illustrate TUNEL-positive Sertoli cell staining in early May (*c*), followed by high levels of TUNEL-positive germ cell staining in late May (*d*). No staining was evident in sections processed without terminal deoxynucleotidyl transferase (TdT) enzyme (inset). Photographs were taken at an original magnification of $\times 20$.

Fig. 3. Mean ($\pm$ s.e.m.) terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL)-positive (a) germ cell and (b) Sertoli cell death per seminiferous tubule in American crows over the period March–June. Groups with different superscript letters are significantly different ($P < 0.05$).

Fig. 4.

Bcl-X_L staining of 6- μ m cross sections from crows caught in March, April, early May, late May, and June (*a-e*, respectively). High levels of staining were observed in March–April (*a*, *b*), with low staining in early May (*c*), followed by increase staining in late May (*d*, *e*). S, staining in Sertoli cells. Photographs were taken at an original magnification of $\times 20$; insets were at an original magnification of $\times 40$.

Table 1

Average body and paired testis mass in American crows during the breeding (March–May) and non-breeding (June) seasons

Values represent the mean $\pm$ s.e.m.

Month	Body mass (g)	Paired testis mass (g)
March	425.7 $\pm$ 14.6	1.1 $\pm$ 0.2 ^a
April	413.3 $\pm$ 7.2	2.0 $\pm$ 0.2 ^b
Early May	425.2 $\pm$ 28.2	2.3 $\pm$ 0.6 ^b
Late May	438.3 $\pm$ 68.3	0.4 $\pm$ 0.2 ^{ac}
June	416.7 $\pm$ 8.3	0.1 $\pm$ 0.0 ^c

^{a-c} Values with different superscript letters differ significantly ($P < 0.05$).

Table 2

Reproductive competence in male American crows during the breeding (March–May) and non-breeding (June) seasons

Values represent the mean $\pm$ s.e.m.

Month	Tubule diameter (μm)	Sperm index
March	177.8 $\pm$ 12.9 ^{ab}	2.7 $\pm$ 0.2 ^a
April	194.2 $\pm$ 10.3 ^{ab}	3.7 $\pm$ 0.2 ^b
Early May	225.7 $\pm$ 18.4 ^a	4.5 $\pm$ 0.1 ^c
Late May	130.3 $\pm$ 45.5 ^b	1.3 $\pm$ 0.3 ^d
June	51.1 $\pm$ 6.2 ^c	0.5 $\pm$ 0.2 ^e

^{a–e} Values with different superscript letters differ significantly ($P < 0.05$).