Seasonal changes in reproductive anatomy and gonadal hormone concentrations of African penguins (*Spheniscus demersus*)

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Highlights

•Reproductive macro-anatomy, histology and hormone levels of African penguin.
•Gonadal macro- and micro-anatomy reflect African penguin breeding status.
•Male and female African penguins have matching reproductive steroid levels.
•Confirmation of two distinct breeding periods during an annual cycle.

Abstract

Several standard descriptions of the avian male and female reproductive tract have been reported, including effects of age, stage of reproductive maturity and gonadal hormone concentrations. Limited information on penguin reproductive biology and a lack of information on the African penguin (*Spheniscus demersus*) necessitated a detailed description of salient structural features of this species and provided an opportunity to evaluate seasonal changes in gonadal steroid hormone concentrations. Tissues from 36 males (adults and juveniles) and 29 females (adults and juveniles) were used for macro-anatomical descriptions and histology of the testes and ovaries. In addition, concentrations of gonadal steroid hormones for eight captive African penguins (four females and four males) were quantified during two breeding and one non-breeding season. The testes were asymmetrical, with the right testis having smaller dimensions compared to the left testis. Marked spermatogenic cellular associations and spermatid developmental stages were present in adult testes only during the breeding season. There was variation in the dimensions of the single ovary during follicular development related to the age and breeding status of the females. Testosterone, dihydrotestosterone, and estradiol concentrations fluctuated during the breeding and non-breeding periods, with males and females having similar steroid concentrations. The results from this study confirm that the breeding status in African penguins can be deduced based on testicular and ovarian histological structures. The results from the present study focused on African penguin reproductive biology should be considered in management strategies for the conservation of the species.

Keywords: African penguin; Dihydrotestosterone; Estradiol; Ovary; Testis; Testosterone

1. Introduction

The avian order *Sphenisciformes* (penguins), along with species of the order *Procellariiformes* (petrels and albatrosses), are considered the most threatened groups of seabirds (Croxall et al., 2012; O'Brien et al., 2015). There are 11 of the 19 species included in *Sphenisciformes* that are currently listed as threatened with extinction (vulnerable or endangered; International Union for Conservation of Nature IUCN, 2013; O'Brien et al. 2015; 2016), including the African penguin (*Spheniscus demersis*) that is endemic to the Southern African region (Crawford et al., 2011).

During the previous century (1900–2000), the population size of the African penguin decreased by 90 %, from 1.5 to 3 million in the early 1900s to less than 18,000 breeding pairs in 2019 (Crawford et al., 2011; Sherley et al., 2020), due to factors such as loss of breeding habitat and commercial harvesting of eggs (Frost et al., 1976). Additional significant threats to this species include a reduction in prey availability as a result of industrial fishing, changes in the local marine environment (van der Lingen et al., 2006; Weller et al., 2014), penguin disease (e.g. avian malaria), and consequently reduced reproductive outcomes due to the lack of capacity to rapidly adapt to these threats (Trathan et al., 2015).

African penguins are monogamous and pairs breed throughout the year; however, the egg-laying period differs depending on the island the pairs have their habitat (Borboroglu and Boersma, 2013). The peak egg-laying periods are in November/January, and May/July and the egg laying interval is on average 3 days (Conway et al., 1999). The clutch size of African penguins is usually two eggs and incubation is shared equally between the parents (Crawford et al., 1999). The breeding interval for birds that reproduce is 10.5 months and there is a period of reproductive quiescence of 4.0 months if breeding does not occur (Crawford et al., 1999). Physiological maturity is probably the primary factor determining at what age individuals first breed (Boswell and MacIver, 1975; Blendea et al., 2012), while successful breeding relates to environmental factors affecting reproductive maturity. At 4 years of age, *S. demersus* are reproductive mature and will breed for the first time when residing in their natural habitat. In captivity, however, birds start selecting a breeding partner at 2 years of age (Crawford et al., 1999).

Even though there have been numerous studies on penguin breeding ecology and sperm cryopreservation, only limited information is available on reproductive biology of penguins, in particular studies related to macro- and micro-anatomy of the reproductive organs (Boersma, 1978; Gee et al., 2004; Ancel et al., 2013; O'Brien et al., 2016). In terms of reproductive endocrinology, several research groups have reported seasonal changes in plasma concentrations of testosterone and 17β-estradiol for various penguin species, but respective data for the African penguin are lacking (Williams, 1992; Cherel et al., 1994; Cockrem and Seddon, 1994; Fowler et al., 1994; McQueen et al., 1998; Otsuka et al., 1998; Ninnes et al., 2011). The aim of the present study was to address this scarcity of information by investigating the structural aspects of the male and female reproductive tracts of African penguins. Emphasis was placed on the basic histology of the gonads during the breeding and non-breeding seasons. A second objective was to characterize seasonally associated variability in plasma testosterone, dihydrotestosterone, and 17β-estradiol concentrations, and relate these to the gonadal development phases and breeding seasons of *S. demersus*.

2. Materials and methods

2.1. Study site and ethical approval

The study was conducted by collecting organ, tissue, and blood samples from both live and dead (not killed to conduct the present study) African penguins, as well as penguins in captive colonies, at the Southern African Foundation for the Conservation of Coastal Birds (SANCCOB, Cape Town, South Africa). All procedures were conducted using procedures consistent with the ethical guidelines and approval of the University of the Western Cape (ScPGC2013/06/10), Cape Nature (RES201/41), and the National Zoological Garden (NZG/P13/07).

2.2. Animals used for macro- and micro-anatomy assessments

African penguins which had to be euthanized or that were accidentally killed were dissected within 15 min after the animal died. The testes and ovaries were removed and fixed in Bouin's fixative (comprising of picric acid, formalin, and acetic acid). Tissues from 36 males (31 adults and five juveniles) and 29 females (23 adults and six juveniles) were collected during both the breeding and non-breeding periods (2014–2016) and used for providing macro-anatomical descriptions and histology evaluations.

2.3. Macro-anatomical description

Testis and ovary structures were assessed by reporting on location, color, shape, weight and dimensions (length, width and volume) using a Digital Calliper KTV150 (Lasec, Cape Town, South Africa) accurate to 1 μm. Epididymis measurements did not occur due to the difficulty incurred when attempting to separate the epididymis from the testis. The number and size of follicles protruding from the ovaries were also recorded.

2.4. Histological assessment

After fixation in Bouin's fixative, representative samples of the reproductive tissues were placed in labeled cassettes and processed using standard histological procedures and routine staining with hematoxylin and eosin (Bancroft and Stevens, 1996) as well as toluidine blue (Kay, 1965). Sections of the gonads were viewed using a Basler A312fc digital camera (Microptic S.L., Barcelona, Spain), mounted on a Nikon Eclipse 50i microscope (IMP Solutions, Cape Town, South Africa) with 10x and 40x objectives. The diameters of testes and ovaries were determined using the measurement instrument of the Toolbox of the Sperm Class Analyser (SCA) computer-aided sperm analysis (CASA) system (Microptic S.L., Barcelona, Spain), version 6.1. Values for cross-sections from 12 ovaries and 14 testes were recorded, as well as 18 randomly selected seminiferous tubules in the testes of each male. The diameter of the seminiferous tubules was determined for the minor and major axes to obtain the mean diameter of these tubules. The number of follicles in the ovaries was manually counted.

2.5. Animals used for determining steroid hormone concentrations

Healthy African penguins that were classified as not having the capacity to survive if returned to their natural habitat were housed outdoors at SANCCOB in an enclosure where there were natural photoperiodic conditions and natural fluctuations in air temperature and wind. Blood

samples were collected for monitoring alterations in plasma steroid concentrations from four captive penguin pairs (four males and four females) that had developed to the adult moulting stage by the time there was initiation of sample collection.

2.6. Blood sample collection and processing

Blood sampling was conducted once a month between March 2014 and August 2015. A total of 110 blood samples (1 mL) were collected from the penguin's foot vein using 18-gauge needles and heparinized micro-hematocrit capillary tubes (BR749311−1000EA, Sigma, Cape Town, South Africa). All blood samples were collected within 30 min after a bird was restrained. Blood samples were centrifuged (micro-hematocrit centrifuge, Hawksley) at 200 x *g* for 5 min within 1 h of collection and resulting plasma was stored at -80 °C for subsequent hormone analyses. Of the 110 samples collected, only from 45 samples (23 samples from males and 22 samples from females) were there sufficient plasma volumes for steroid hormone analyses.

2.7. Steroid quantitation

Immunoreactive plasma testosterone (iT), dihydrotestosterone (iDHT), and 17β-estradiol (iE2) concentrations were determined using commercial enzyme-immunoassays (iT: EIA-1559, iDHT: EIA-4132, iE2: EIA-2693; BIOCOM-Biotech, DRG Instrument GmbH, Germany) and the protocol provided by the manufacturing company. Sensitivity of the assays were 83.0 pg/mL (iT), 6.0 pg/mL (iDHT), and 10.6 pg/mL (iE2), respectively. Further assay characteristics including intra- and inter-assay coefficient of variation (CV) as well as antibody cross-reactivities are provided in the manufacturer's pamphlet. All samples were quantified in duplicate following EIA specific protocols provided by the manufacturing company and conducted at the Comparative Spermatology Laboratory at the University of the Western Cape. Plasma concentrations of iT, iDHT, and iE2 are expressed in pg/mL.

2.8. Statistical analysis

Descriptive statistics were mainly used to describe the differences in male and female reproductive macro-anatomy, testicular and ovarian histology, as well as the variation in individual monthly median plasma iT, iDHT, and iE2 concentrations. MedCalc version 17.2 (MedCalc Software, Mariakerke, Belgium) was utilized for statistical analysis of values related to seasonal differences of anatomical features. Values determined using comparative measurements of macro-anatomical structures as well as histological characteristics are expressed in mean ± SD as well as the range of the respective measurement, using suitable dimensions. Comparisons of plasma steroid concentrations were expressed in fold differences of overall monthly medians, with the range of respective values also provided.

3. Results

3.1. Male reproductive macro-anatomy

The African penguin testes are located in the abdominal cavity, ventral to both the vertebral column and the kidneys, and are surrounded by a well-developed fibrous capsule that includes connective tissue. The bean-shaped left testis and the egg-shaped right testis (Fig. 1) are positioned towards the midline and the caudal vena cava lies in the narrow space between the dorsomedial surfaces of the two testes. Differences in testicular size and weight were

noticeable depending on age, with juvenile testes being comparatively smaller than that of adults (Table 1), with an average length and width of 7.31 ± 0.61 and 2.43 ± 0.97 mm, respectively. Furthermore, juvenile testes have no clearly visible superficial blood vessels, are cream in color and cylindrical in shape (Fig. 1A). The testes of adult penguins are creamy-white in color (Fig. 1B).

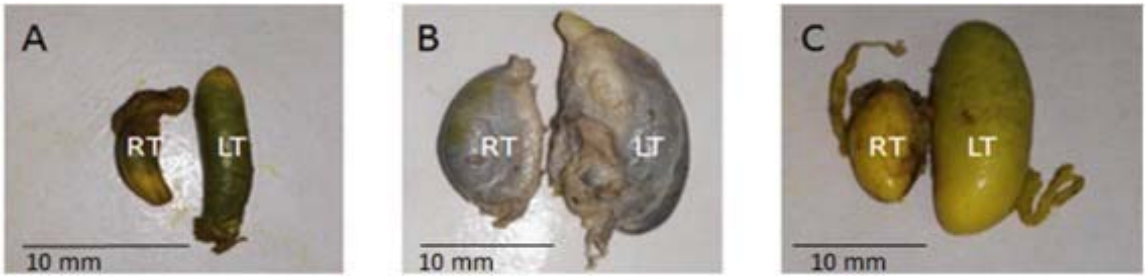

Fig. 1. Examples of excised testes of (A) juvenile and (B,C) reproductively mature African penguins (ventral view); Note the difference in size and shape between the right (RT) and the left (LT) testes; Large LT is bean-shaped and smaller RT is round to oval in shape; Epididymis is not clearly distinct on either testis; Yellow color observed in Figure C) is due to the picric acid in Bouin's fixative that stains the testes yellow in color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1. Values for left and right testis dimensions (mean ± SD, range) for adult African penguins (*n* = 31).

	Right testis		Left testis	
	Mean ± SD	Range	Mean ± SD	Range
Length (mm)	16.80 ± 4.37	8.21 – 25.13	25.39 ± 5.85	14.10 – 39.09
Width (mm)	7.93 ± 2.63	2.95 – 15.25	11.48 ± 4.09	5.11 – 23.10
Volume (ml)	0.75 ± 0.62	0.05 – 3.45	2.46 ± 2.23	0.22 – 10.26
Weight (g)	1.15 ± 0.75	0.10 – 4.00	2.71 ± 1.46	0.39 – 7.41

The testes are asymmetrical in African penguin (Fig. 1) with the right testis on average tending to be smaller, but not significantly so, in length, width, volume and weight compared to the left testis. There, however are large individual ranges in values for each variable (Table 1). During the breeding season, the adult testes tend to be larger. There was no distinct difference for testis dimensions when measurements were taken in the breeding and non-breeding seasons (Table 2). There were smaller ranges in values for testis dimensions of both the right and left testis during the non-breeding than breeding season.

Table 2. Values for seasonal testis dimensions (mean ± SD, range) in adult African penguins during the breeding (*n* = 22) and non-breeding (*n* = 9) season.

	Breeding				Non-breeding			
	Right testis		Left testis		Right testis		Left testis	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Length (mm)	18.11 ± 4.07	8.21–25.13	28.10 ± 4.63	22.68 – 39.09	15.51 ± 8.48	9.98 –18.82	21.14 ± 3.33	14.40 – 26.32
Width (mm)	9.10 ± 2.03	5.58 –15.25	13.24 ± 3.66	7.43 – 23.10	6.12 ± 1.82	2.95 – 9.03	8.64 ± 1.53	5.11 – 9.94
Weight (g)	1.41 ± 0.77	0.43 – 4.00	3.27 ± 1.36	1.11 – 7.41	0.57 ± 0.23	0.10 – 0.91	1.34 ± 0.50	0.39 – 2.22

3.2. Testicular histology

The testicular capsule is comprised of an outer thin tunica serosa, a thick tunica albuginea under the tunica serosa and the innermost, extremely thin, tunica vasculosa. Smooth muscle cells in the testicular capsule are presumably responsible for the spontaneous contractions of

the capsule. Histological characteristics of the testes vary with the age and sexual activity/breeding cycle, with maximal development of tissues during the breeding season (Fig. 2).

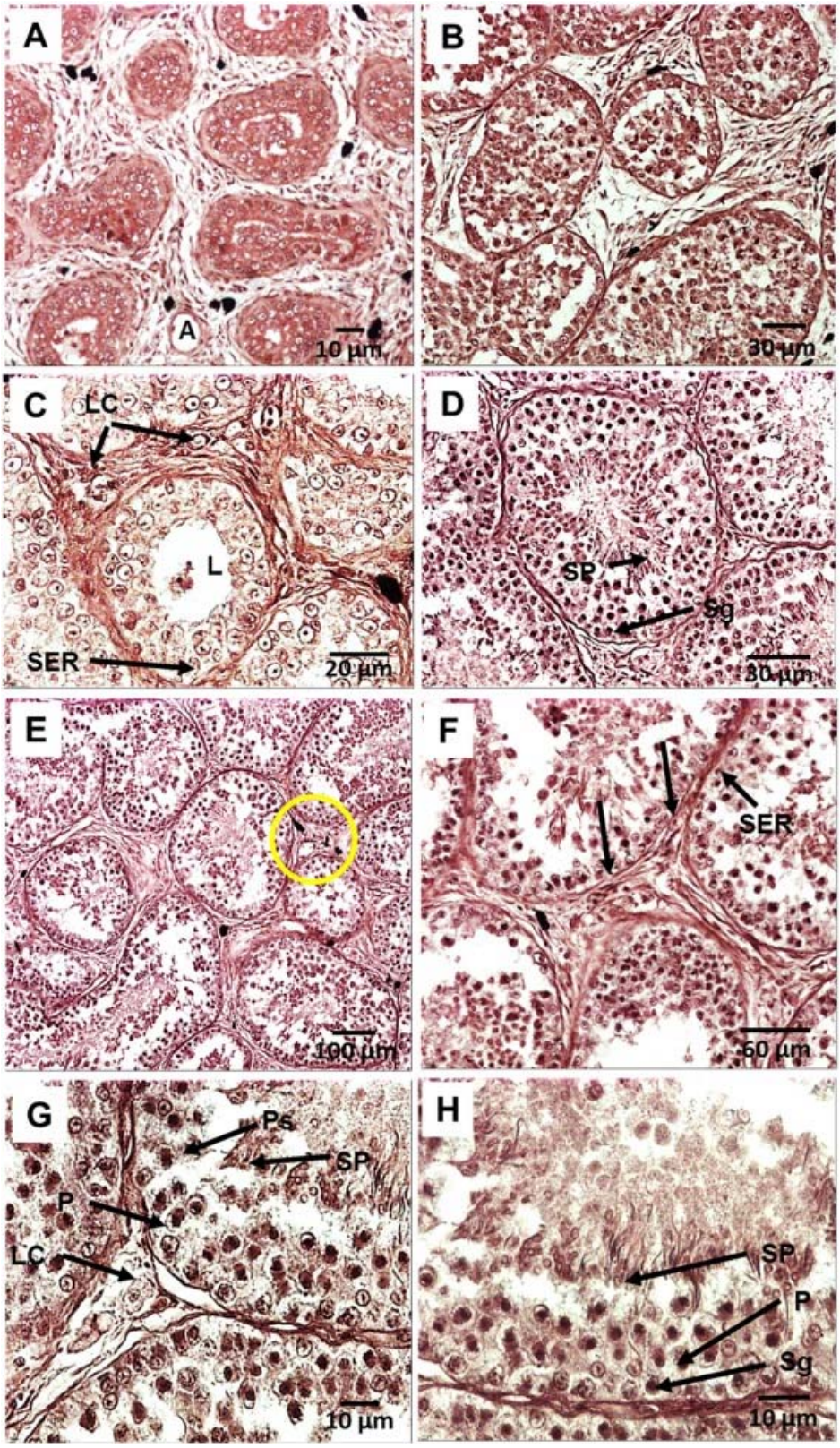

Fig. 2. A-D. (A, B) Micrographs of juvenile testis cross sections at X100 initial magnification with different shapes of the seminiferous tubules, with arteries being prevalent (A); (C, D) Micrographs of non-breeding adult African penguin with testis having dense seminiferous tubule tissue separated by narrow alignments of interstitial tissue; Lumen of seminiferous tubule (L), Sertoli cells (SER), spermatogonia (Sg), spermatids (SP) and Leydig cells (LC). **E–H.** Seminiferous tubules of adult African penguins collected during the breeding season; E) Note the interstices between groups of three seminiferous tubules (yellow circled), where the interstitial tissue is initially observed; F) Dense seminiferous tubule tissues containing Sertoli cells (SER) and separated by narrow alignments of interstitial tissue (arrows); GH). Organization of the seminiferous epithelium at greater magnification with seminiferous tubules having various germ cells in the epithelium, namely spermatogonia (Sg), primary spermatocytes (P), secondary spermatocyte (Ps), spermatids (SP) and Leydig cells (LC).

In the juvenile testis, a larger area is covered by interstitial tissue and seminiferous tubules (ST) are less closely associated (Fig. 2A-B) compared with what occurs in the adult testis (Fig. 2C-F). The different cell types in juvenile males are not as distinctly organized as in the adult testis. Various cell types typical of spermatogenesis could be identified in the seminiferous epithelium during the breeding and non-breeding seasons of the adults. The spermatogenic cells include, from the periphery to the lumen of the tubules, spermatogonia, primary and secondary spermatocytes as well as elongated, immature and mature spermatids. The different stages of spermatogenesis are arranged in well-defined concentric layers within the epithelium. During the non-breeding season, the cellular associations of spermatogenic cells observed were not as clearly defined as in penguins during the breeding season. There were numerous spermatid developmental stages only during the breeding season (Fig. 2E-H).

The ST are bound together by interstitial tissue, which consists of blood vessels, connective tissue, and Leydig cells (LC) containing lipid droplets (Fig. 2A and C). The LC are polymorphic cells with a spherical, polyhedral shape and have large nuclei with distinct nucleoli (Fig. 2C and G). The LC are located in the angular spaces among the ST and blood vessels are closely associated with these cells (Fig. 2E). The ST opens directly into the rete testis as in other avian species.

The cross sections of the ST had a round to oval shape due to the complex folding of the tubules (Fig. 2 E–F). The mean diameter of ST of adult males was 133.05 ± 64.12 μm, containing various types of spermatogenic cells (Fig. 2G–H). The ST diameter ranged from 48.27 μm (non-breeding) to 303.42 μm (breeding), with the most frequently observed ST diameters being between 60 and 140 μm (Table 3).

Table 3. Values for morphological variables (mean ± SD, range) of seminiferous tubules for African penguins during the breeding and non-breeding season (n = 6).

	Breeding		Non-breeding	
	Mean ± SD	Range	Mean ± SD	Range
Total diameter (μm)	184.62 ± 52.54	93.3 – 303.42	74.72 ± 14.86	48.27 – 111.36
Lumen diameter (μm)	96.03 ± 36.07	42.32 – 151.54	43.67 ± 13.20	23.01 – 88.44
Epithelium thickness (μm)	57.61 ± 18.91	16.93 – 94.48	21.23 ± 12.47	14.09 – 90.05

Breeding males tend to have larger diameters of ST than in males during the non-breeding season (two-fold larger on average); however, there were no differences in values when assessed using statistical procedures. The combined ST lumen and epithelium thickness means were 62.34 ± 32.58 μm and 33.99 ± 23.21 μm, respectively. In an adult penguin, the testes have a large amount of fluid in the lumen of the ST (Table 3).

3.3. Female reproductive macro-anatomy

The female African penguin reproductive structures include a left ovary, an oviduct, a uterus and a vagina. The ovary is attached to the bodies of the lumbar vertebrae by the mesovarium and located dorsomedial to the spleen. Even though the ovary of a juvenile female is flattened and resembles a pad of fat tissue (Fig. 3A), it is comparable in size to that of an adult penguin. There were no externally visible follicles in juvenile ovaries (Fig. 3A), which makes it difficult to differentiate testis and ovarian structure in juveniles. In breeding adult females, the ventral surface of the ovary has a grape-like cluster of small but noticeable follicles that are easy to identify (Fig. 3C and D). There were large variations in the ovarian shape and size among the adult penguins included in this study, with a mean length of 25.72 ± 5.37 mm and a mean width of 9.02 ± 3.87 mm (n = 23). After the breeding season, the ovary of an adult penguin is quiescent from a functional perspective with there being a lesser length and width and less macroscopically visible follicles during the non-breeding season, as compared with the breeding season (Fig. 3B). Ovaries during the non-breeding period, therefore, are classified as being mature, however, are functionally inactive during the non-breeding season. The mean weight of an adult ovary during the breeding season was 1.84 ± 1.19 g and during the non-breeding season the adult ovary averaged 0.65 ± 0.17 g in weight, with no significant differences between values for females during the breeding and non-breeding season.

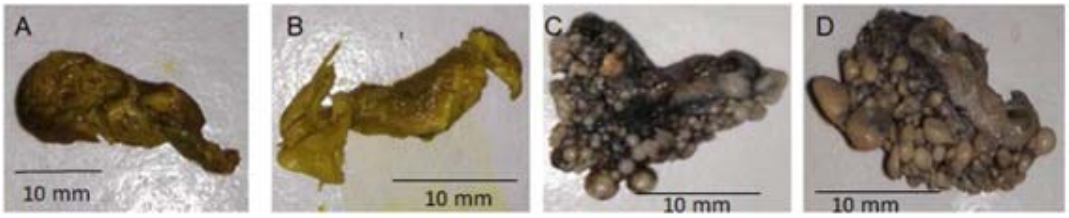

Fig. 3. Macroscopic view of selected ovaries of (A) juvenile, (B) non-breeding adult penguin and (C,D) breeding adult penguin; Numerous follicles are present with the appearance of a collection of grapes in adult female ovaries during the breeding period.

Determinations form macroscopic observation of the follicles indicated there were changes in size and color during follicular development. During the breeding season follicles ranged from small, white follicles with a diameter of <0.01 mm to large, mature follicles with a maximum diameter of 4.2 mm (Fig. 3C and D). The mean follicle diameter was 2.52 ± 1.58 mm. The number of follicles and stages of development are indicative of ovarian functions and can be used to classify a female penguin's status as breeding or non-breeding. The well-developed, yolk-filled follicles can be described as mature and are indicative that a penguin is reproductively active. The number of follicles for breeding penguins ranged from 30 to 160 per ovary (n = 19), with most ovaries containing 50–80 follicles.

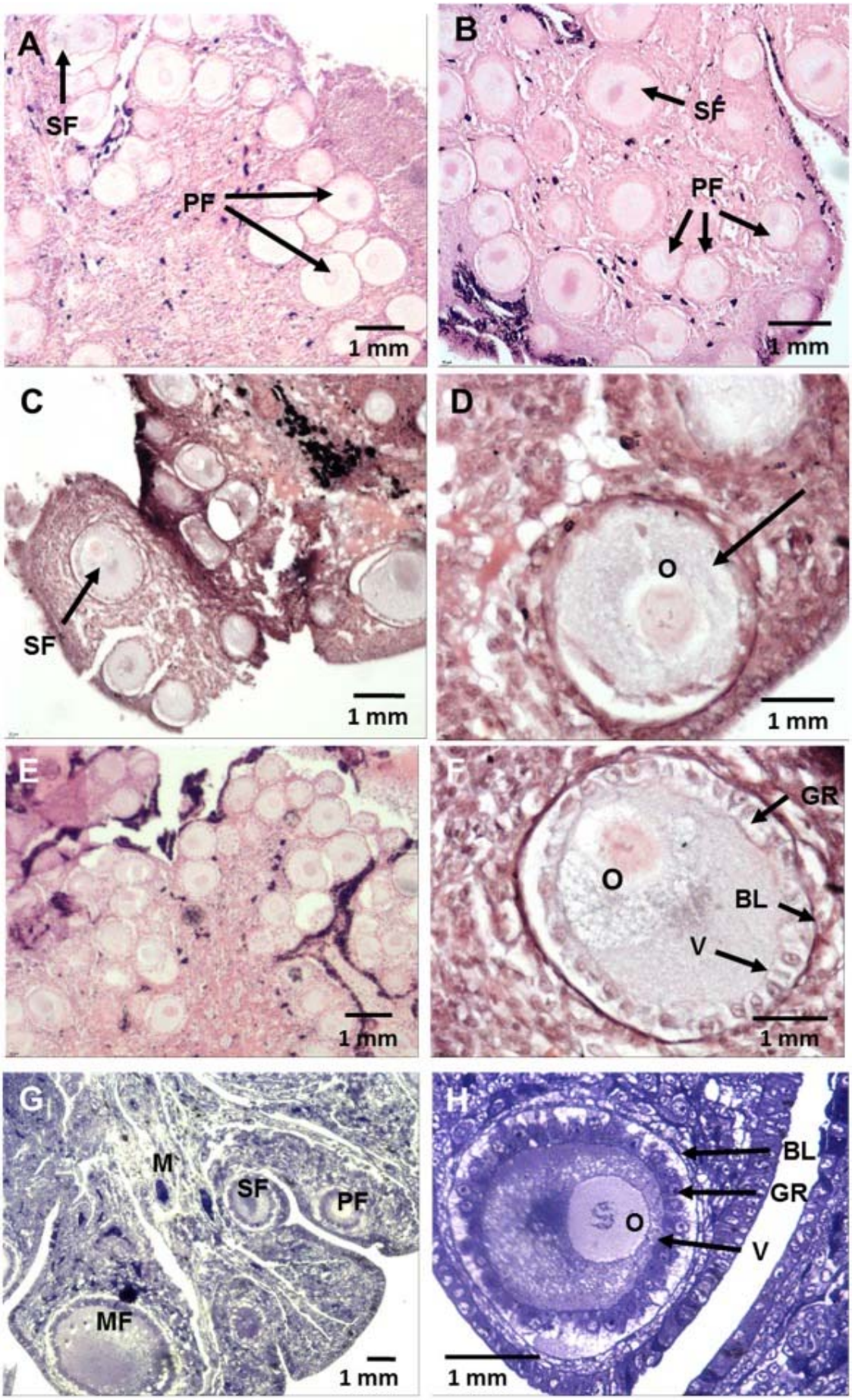
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Fig. 4. A-D. Micrographs of juvenile (A, B) and non-breeding adult (C, D) African penguin ovaries, when there are different stages of follicular development; (A, B) Primary follicles (PF) and secondary follicles (SF) in the cortical stroma of the ovary; (C, D) Large secondary follicles containing an enlarged oocyte (O). **E-H.** Micrographs of adult African penguin ovaries collected during the breeding season, stained with H & E (E-F) and toluidine blue (G-H), at different stages of follicular and membrane development; (E) Primary and secondary follicles in the cortex; (F, H) Enlarged follicle and oocyte (O), containing nucleus, surrounded by a thin vitelline membrane (V), proliferated granulosa cells (GR) and basal lamina (BL); G) Primary follicle (PF), secondary follicle (SF) and mature follicle (MF) in the cortex and blood vessels in the medulla (M).

3.4. Ovarian histology

Adult African penguin ovaries had an uneven surface topography, with prominences separated by grooves. The ovary is covered by a thin continuous mesothelium composed of a single layer of cuboidal epithelium. Internally, the ovary typically consists of an outer cortex (*zona parenchymatosa*) surrounding a vascular medulla (*zona vasculosa*). The outer cortex contains ova while the medulla is composed of primarily connective tissue with nerves and blood vessels being attached. In the cortex of the ovary, only primordial, primary and secondary follicles are present in juvenile and non-breeding penguins (Fig. 4A-D, Table 4), while in breeding females there are numerous follicles that are in different developmental stages, ranging from primordial follicles to mature follicles (Fig. 4E-H). Inside an ovisac, the mature follicles are present in the deeper area of the cortex, while the primordial and primary follicles are located peripherally (Fig. 4B, E, G). The primordial follicles are small (0.5–1.2 mm diameter) compared to the mature (vitelline) follicles (1.9–4.3 mm diameter) as indicated by values reported in Table 4. There were follicles with the largest diameter during the breeding season. Primordial follicles containing primary oocytes remain embedded within the stromal tissue in the cortex and lack an epithelial cell layer (Fig. 4E). The granulosa and theca layers of mature follicles are separated by an acellular basement membrane, namely the basal lamina. Granulosa cells of the follicles are cuboidal and situated in between the basal lamina and the vitelline membrane (Fig. 4F and H). The vitelline membrane is an acellular fibrous layer and homologous to the mammalian zona pellucida. Further follicular growth leads to follicular fluid accumulating in the antrum and proliferation of the granulosa cells, while the fully-developed oocyte is surrounded by the vitelline membrane.

Table 4. Values (mean ± SD, range) for diameter of different classes of follicles (n = 22) and follicle diameter for juvenile (n = 6) and adult non-breeding (n = 10) and breeding (n = 6) African penguin females.

	Number of follicles	Mean ± SD (mm)	Range (mm)
Primary follicles	32	1.12 ± 0.15	0.71 – 1.35
Secondary follicles	65	1.63 ± 0.20	1.31 – 2.08
Tertiary follicles	22	2.52 ± 0.57	1.9 – 4.3
Juvenile	29	1.60 ± 0.44	0.71 – 2.49
Non-breeding adult	39	1.54 ± 0.53	0.91 – 13.1
Breeding adult	35	1.86 ± 0.52	1.18 – 4.25
All	103	1.66 ± 0.55	0.71 – 4.25

3.5. Seasonal changes in reproductive steroid concentrations

Median iT concentrations were between ~140-fold and ~350-fold greater in males and females during the breeding season compared to the non-breeding season, except for October when iT concentrations were similar to those during the non-breeding season for both sexes (Table 5). When comparing iT concentrations during the breeding seasons, there were the

greatest concentrations in January (Breeding Season 1). Individual variation was large (two to six-fold) during seasonal and monthly periods, except for October.

Table 5. Immunoreactive plasma testosterone (iT), dihydrotestosterone (iDHT) and 17β-estradiol (iE2) concentrations of African penguins (n = 4 males; n = 4 females) during breeding and non-breeding seasons.

Status			BR1	NBR		BR2		
Month			Jan	Apr	May	Jun	July	Oct
iT (pg/mL)	Male	Median	1411.3	2.1	7.0	1103.8	686.9	5.2
		Range	763.9 – 1875.9	1.8 – 8.0	6.3 – 25.5	704.8 – 1428.1	545.3 – 3142.6	4.3 – 5.7
	Female	Median	1725.2	5.1	5.6	728.6	860.0	5.1*
		Range	835.2 – 3881.1	2.4 – 9.2	3.8 – 10.1	448.1 – 2676.4	594.4 – 1849.1	4.4 – 5.8
iDHT (pg/mL)	Male	Median	2808.9	620.6	2835.2	1263.6	11.6	387.4#
		Range	275.3 – 7050.5	488.4 – 1213.6	2272.6–3934.8	394.3–1889.2	7.1–44.5	364.1–2056.0
	Female	Median	3161.0	613.8	2209.8	1666.7	22.3	1370.4*
		Range	842.3 – 7525.7	364.6 – 2596.2	1294.0–3927.8	524.4–8406.4	10.5–36.8	938.5–1802.2
iE2 (pg/mL)	Male	Median	4665.8	11664.0	188.5	NM	6792.8	402.0#
		Range	185.8 – 26231.6	7168.0 – 16356.4	148.1 – 236.3	NM	4436.4 – 14167.7	320.1 – 676.1
	Female	Median	3358.1	15796.0	245.0	NM	6970.4	547.4*
		Range	1060.5 – 6291.4	7294.9 – 18873.4	136.8 – 4874.8	NM	26.3 – 14049.6	424.5 – 670.3

BR = breeding season (Jan, Jun, Jul, Oct), NBR = non-breeding season (Apr, May), NM = not measured.

*n = 2, #n = 3.

Monthly median iDHT concentrations were similar between the sexes with no distinct pattern between the seasons (Table 5). In January (Breeding Season 1), median iDHT concentrations were the greatest, however, in a comparable range to that in May (Non-breeding season), followed by slightly lesser overall median iDHT concentrations in June (beginning of Breeding Season 2). There were intermediate median iDHT concentrations in April (Non-breeding season), which are comparable to respective values in October (Breeding Season 2). There were markedly lesser concentrations of iDHT in July (Breeding Season 2). Individual penguin variations in concentrations of iDHT were greatest (nine to 25-fold) during January.

Median iE2 concentrations were similar between the sexes (Table 5), with greatest and least median iE2 concentrations during the non-breeding season (median iE2 concentrations were ~60-fold greater in April compared to May). There were similar median iE2 concentrations in January (Breeding Season 1) and July (Breeding Season 2), with respective values in October (Breeding Season 2) being ten-times less. As with iT and iDHT, there were wide-ranging concentrations of iE2 among individuals during seasons and months when samples were collected.

4. Discussion

4.1. Gonadal dimensions and histology

The asymmetry in African penguin testes is consistent with similar findings in most bird species (Birkhead et al., 1998; Yu, 1998; Aire, 2007; Gunn et al., 2008; du Plessis and Soley, 2012), except for quail and sparrow which did not have consistent asymmetry (Yu, 1998). One explanation for the difference in testis size originates from an embryological study by Witschi (1935), in which the cortex of the right undifferentiated gonad ceases to have chemotactic attraction for primordial germ cells as does the cortex of the left gonad. Although the left organ is larger than the right, it is believed that these testes contribute equally to the production of androgens and, possibly, also spermatozoa (Malecki et al., 1998).

The size of the avian testis differs considerably with season of the year (Aire, 2007). Møller (1998) reported that during the active breeding season, testis size is positively correlated with sperm production. The size and the weight of the testes can also be positively correlated with the age of a bird, leading to the hypothesis that older birds are more likely to be sexually active than younger birds. Testicular volume is another important criterion in the evaluation of male reproduction and these data are necessary for precise monitoring of testicular growth (Lin et al., 2009). Similar to other bird species (Marshall, 1961), adult African penguin testes increased in size and weight during the breeding season compared with testes of juvenile birds and in penguins during the non-breeding season, which is thought to indicate males become sexually active when the testis enlarge (Bull et al., 2007). It, therefore, is our understanding that the accurate measurement of male penguin reproductive organs that are collected during random times of the year can be informative in evaluating the reproductive status and in determining associations with sperm production.

The avian testicular interstitial tissue contains blood vessels and Leydig cells and is moderately dense in structure (Aire, 1997). This compact structural feature is common in birds, except for the ostrich interstitial tissue where there is loosely organized connective tissue (Al-Tememy, 2010). Avian Leydig cells are functionally and structurally similar to those of mammals, because these cells are dispersed in the interstitial tissue in small groups within the larger inter-tubular spaces in most birds (Aire, 1997). In the present study, Leydig cells contained lipid droplets when adult penguins were in breeding stages with these droplets probably being associated with secretion of testosterone.

The ST of most avian species are dissimilar from those of mammals because of the highly complex, anastomotic, ending network of tubules that have an open end and that are tightly packed making up a large percentage of the testicular parenchyma (Hodges, 1974; Aire, 2000). In the current study, most of the volume of the adult penguin testes consisted of ST, while in juvenile males the ST were less predominant compared with adults. Results from the present study are consistent with those of Aire (1997) in which the ST composition of the testis of non-breeding birds was smaller in size. During the non-breeding season, the diameter of the penguin ST was two-fold smaller compared to the breeding season, albeit all testicular cell types were present in adult male penguins irrespective of the season of the year. The lack of spermatids in juvenile African penguins is consistent with results from a study with pukeko (*Porphyrio porphyrio melanotus*) in which there was a correlation between a larger testicular size and extent of development of the seminiferous tubules (Gunn et al., 2008). Results from the present study indicate that spermatogenesis in birds, as in mammals, involves a process of division from spermatogonia to mature spermatids, during which sperm undergo a final meiotic division. In the seminiferous epithelium of African penguins the germ cells are not arranged at random but are structured into well-defined cellular associations and spermatogenesis is helical as occurs in most birds and mammals (Jones and Lin, 1993). There, however, are important differences compared with mammals relating to rapid production and passage of sperm through the reproductive tract to the site where sperm can be released. In sexually active birds, it takes 1–4 days for spermatozoa to be formed and be transported through the epididymal ducts (Nickel et al., 1977); accordingly, sperm production in birds occurs four times more rapidly than in mammals. The vas deferens of the quail contains the equivalent of 26 ejaculates which can be replaced in 24 h (Jones and Lin, 1993). Furthermore, the relatively shorter period of time required to produce large numbers of sperm in these penguins is probably important for mating and reproduction to occur during the very short breeding period in the African penguin (January and February).

The single, left ovary and oviduct of female African penguins is consistent with these reproductive tissues in other birds (Mirhish and Nsaif, 2013; Sari et al., 2014; Vijayakumar et al., 2014; Essam et al., 2016; Alshammary et al., 2017). Both left and right ovaries are present during the early embryonic stages of presumably all birds (Jacob and Bakst, 2007), but only a functional left ovary develops post-hatching (Vijayakumar et al., 2014; Alshammary et al., 2017). In contrast, in some species (e.g. kiwi and vultures) both gonads develop, however, the size of the two gonads may be asymmetrical, with the left ovary characteristically being the larger organ (Kinsky, 1971).

The structural organization of the ovary of the African penguin (outer cortex with many follicles of different sizes and internal vascular medulla) are similar to descriptions reported for other avian species (Hodges, 1974; Alshammary et al., 2017). There was no distinct separation between the cortex and medulla in any of the penguin ovaries evaluated, with this possibly being the result of these two layers becoming non-distinct in structure during ovarian maturation (Hodges, 1974).

There are potentially a large number of primordial and primary follicles present in the avian ovary, but most never mature and there is only ovulation from a few, as occurs in mammals (Alshammary et al., 2017). In avian species, maturing follicles are generally categorized according to size and color of the yolk (Ottinger and Bakst, 1995). The ventral surface of the adult African penguin ovary had numerous ovarian follicles that varied in size. The change in number and size of follicles during the physical development and reproductive cycle indicates there is a transition from the juvenile to non-breeding and ultimately breeding stage in adult females, which leads to ovulation and oviposition. The most noticeable differences between the ovary in a reproductively inactive and active African penguins were the larger number of follicles developing to the different stages of maturation during the breeding season. Results in the present study of follicle size are consistent with the findings in ducks and some other fowl (Rahman, 2014) in which developing follicles were between 5 and 25 μm in diameter. The follicular structure of the African penguin is consistent with that of Pagagan ducks (Sari et al., 2014) where there are four layers of the follicle.

4.2. Fluctuations in plasma steroid hormones

In the present study, there were markedly greater plasma iT concentrations in both sexes during breeding (January) as well as during the transition from the non-breeding to the breeding period (June – July). This pattern probably relates to competition, territorial behavior, and aggression, as reported for many other penguin species during the nest-building and egg-laying period (Johnson, 2002; Ketterson et al., 2005). There was a similar tendency for greater testosterone concentrations in both the breeding male and female of Macaroni (*Eudyptes chrysolophus*), yellow-eyed (*Megadyptes antipodes*), Magellanic (*Spheniscus magellanicus*), and Humboldt (*Spheniscus humboldti*) penguins, respectively, (Otsuka et al., 1998; Williams, 1992; Cockrem and Seddon, 1994; Fowler et al., 1994). In the latter three species, however, testosterone concentrations were much less in females compared with males, which is in contrast to findings in the present study. It could be that African penguin breeding pairs have a greater competition for mates due to their two breeding seasons per annum, while most other penguin species have only one distinct breeding season, albeit this period is for a large portion of the year in some species (Cherel et al., 1994).

Because iE2 are similar to the pattern of iT concentrations in many respects, with comparatively greater iE2 concentrations during the breeding season in both sexes, there are

likely similar supportive actions of the estrogens on reproductive functions that have been previously described in this manuscript. These functions of estrogen would be consistent with previous findings for a number of penguin species, including King (*Aptenodytes patagonicus*), Magellanic, and Adelie penguins (*Pygoscelis adeliae*) (Cherel et al., 1994; Fowler et al., 1994; Ninnes et al., 2011). In male birds, many behavioral effects of testosterone are mediated through local conversion of androgens to estrogens (Goyman and Wingfield, 2014). Besides a plausible biological explanation, the conversion of testosterone to estradiol as a 'metabolic by-product' should also be considered.

The iDHT concentrations of both sexes of African penguins did not consistently fluctuate between the breeding and non-breeding season. Even though there was sporadic sample collection and no clearly defined function of DHT in avian species apart from pre-copulatory behavior (Balthazart et al., 1983; Dufty and Wingfield, 1986), it is possible that the comparatively greater iDHT concentrations detected in April, May, June, and October might be a result of conversion of testosterone to DHT because this conversion occurs in birds (Groothuis and Schwabl, 2008; Casagrande et al., 2011).

4.3. Anatomical and endocrinological links for seasonal reproduction in African penguins

Results from the present study on the annual seasonal changes in macro-anatomy and histology of the gonads of both male and female African penguins indicate that African penguins have two distinct breeding seasons and confirm findings of Borboroglu and Boersma (2013) based on number of eggs produced and clutch size. In the present study there was a close correlation between values of African penguin testicular size and spermatogenic cell type differentiation during both the breeding and non-breeding seasons, albeit with a smaller ST diameter during the second breeding season that starts in June. African penguin females may have ovulations throughout the entire breeding season, as indicated by the presence of follicles at all stages of the reproductive cycle in the current study during both annual breeding seasons.

There were relatively greater testosterone concentrations during the period when there were maximum seminiferous tubule diameters (Fig. 5) while there were markedly lesser testosterone concentrations during the non-breeding period which is consistent with the smaller seminiferous tubule diameter (on average 2.5-fold smaller) during the non-breeding season. In contrast, follicular development did not cease after the breeding period and there continued to be relatively greater concentrations of estrogen during the non-breeding period which may have occurred due to continued development of tertiary follicles that develop to the extent that ovulation occurs during the subsequent or second annual breeding season (Fig. 5). Furthermore, considering estrogens are still in relatively greater concentrations during April this could co-inside with chick rearing. On Robben Island near Cape Town, South Africa, (closest island to location where the penguins were located that were used in the present study), egg-laying occurs mainly between January to August, with most chicks (38 %) being produced in February and with 94 % of all chicks being produced by the end of May (Borboroglu and Boersma, 2013). Dihydrotestosterone was also in the greatest concentrations during the non-breeding season in the present study immediately preceding the second breeding period in the African penguin (Fig. 5) and thus, might have regulatory functions in preparation for the subsequent breeding period.

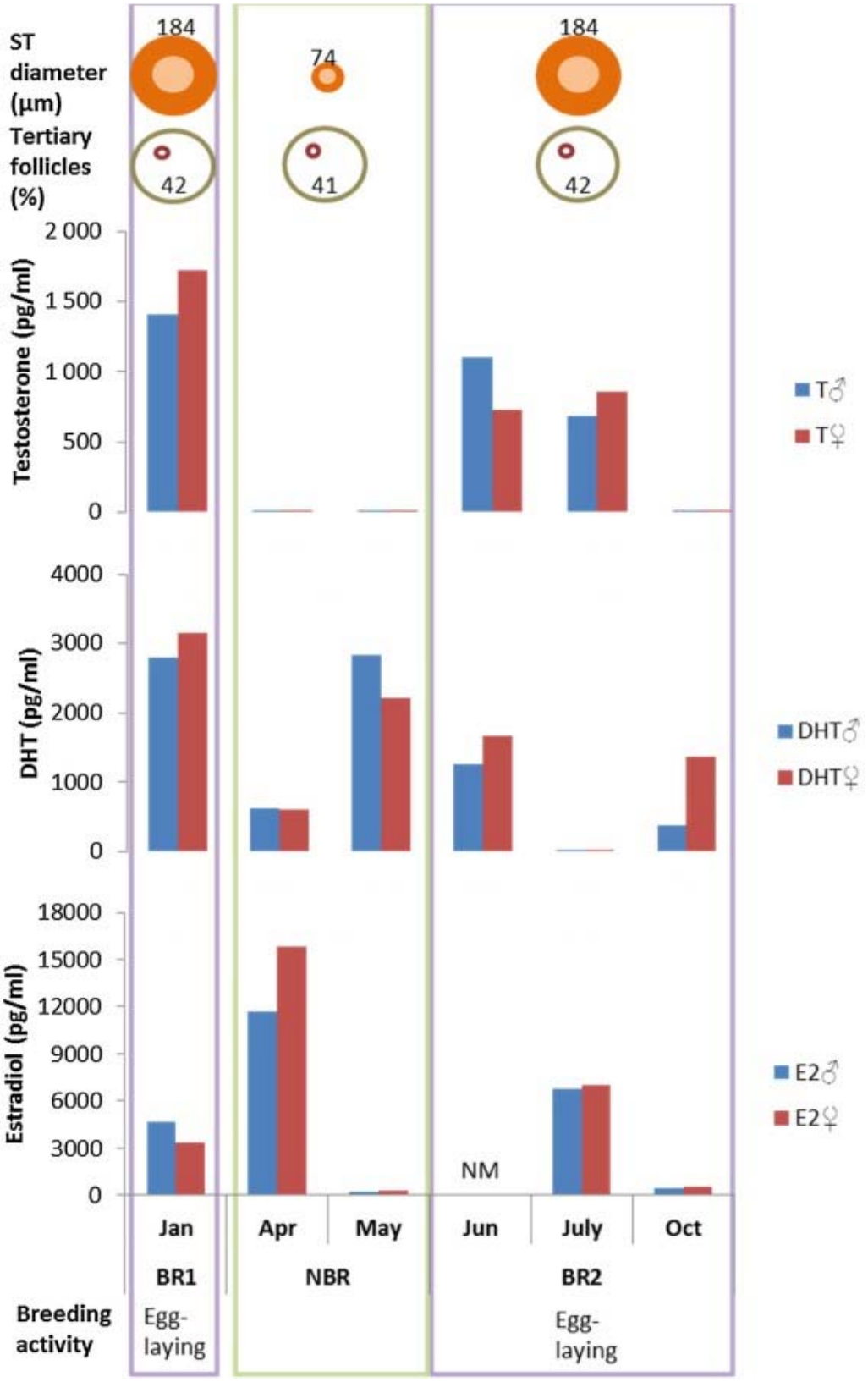

Fig. 5. Summary figure depicting the anatomical changes in gonads, three steroid hormone concentrations and reproductive functions of African penguin during their annual reproductive cycle, including short breeding (BR1), non-breeding (NBR) and long breeding (BR2) seasons ST, seminiferous tubules; NM, not measured.

These associations among the concentrations of the three hormones occur as a dynamic physiological process ensuring that there is continuous availability of steroid hormones for testosterone and estradiol synthesis. All male and female birds produce testosterone and/or androstenedione as the initial substrates in the steroidogenic pathway and there is conversion of these androgens to estradiol or DHT. Testosterone in males is synthesized in and secreted from Leydig cells in the testis and in Sertoli cells estrogen can be produced. Furthermore, the developing follicle is a source of all three steroids in birds with the thecal layers and granulosa cells of the follicle apparently being a source for a specific androgen (Groothuis and Schwabl, 2008). Whether the hypothalamic–pituitary–gonadal axis is involved in the regulation of short-term changes in testosterone in female birds still needs to be investigated because the adrenal gland or brain centers other than the hypothalamus could also be involved in regulation of these changes in testosterone concentration (Goyman and Wingfield, 2014).

5. Conclusion

Collective results from the present study on macro- and micro-anatomy and hormone concentrations confirm that African penguins have two distinct breeding periods during an annual cycle. The difference in gonadal surface anatomy and dimensions as well as detailed histological differences between juvenile and adult birds and also between breeding compared to non-breeding individuals highlights the changes in functions of these tissues in regulation of the breeding status of African penguins. Changes in steroid hormone concentrations between males and females during the breeding and non-breeding periods during the annual reproductive cycle of African penguins seem to be consistent with results from other penguin species and birds in general, albeit there are relatively greater concentrations of plasma testosterone in females that were unexpected. It is envisaged that the baseline results provided on reproductive biology can be integrated into management strategies for ensuring the long-term conservation of the African penguin as a threatened species in captivity and in their natural habitat.

Funding

This research was funded by the National Zoological Garden and the National Research Foundation through the Professional Development Programme of the Department of Science and Technology as well as the University of the Western Cape.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors express their gratitude to Ms Magaret Roestarf (former Chief Executive Officer), Dr. Stephen van der Spuy (Chief Executive Officer), Dr. Nola Parson (resident veterinarian) and Ms Nicky Stander (Seabird Rehabilitation Manager) at SANCCOB. Mrs Nolan Muller (Electron Microscopy Unit, National Health Laboratory Services, Tygerberg Hospital) is thanked for her preparation of the plastic-embedded material.

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