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# Changes in Circulating LH, Sex Steroid Hormones, Thyroid Hormones and Corticosterone in Relation to Breeding and Molting in Captive Humboldt Penguins (*Spheniscus humboldti*) Kept in an Outdoor Open Display

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**ABSTRACT**—Penguins are highly adapted to marine life. Their hydrodynamic efficiency depends on feathers which wear with age and need to be replaced regularly. During molting, penguins can not enter the sea to forage and are forced to fast. Therefore the duration of molting is necessarily brief. To better understand molting in penguins, we collected plasma samples from 16 (8 pairs) Humboldt penguins kept in an open display pen at Tokyo Sea Life Park from May to September, 1994 and estimated circulating concentrations of LH, testosterone, estradiol, thyroxine (T4), triiodothyronine (T3) and corticosterone. Body mass was also measured at each blood sampling. Throughout the year, reproductive activities (egg laying, incubation, hatching and rearing) and molting were observed and recorded. Humboldt penguins maintained reproductive activity from January to December except during molting. Each pair started molting between the end of July and early August; usually males started earlier. The duration of molting was  $13.4 \pm 0.8$  days for males and  $12.9 \pm 0.3$  days for females. Body masses were highest just before the start of molting in both sexes. Plasma concentrations of LH were high, ( $> 2$  ng/ml) in May in both sexes, then gradually decreased, to  $0.53 \pm 0.38$  ng/ml in males and  $0.72 \pm 0.11$  ng/ml in females by the end of July. Testosterone and estradiol concentrations in plasma decreased and were lowest during molting. On the other hand, plasma concentrations of T4 were low until early July (ca. 20 ng/ml) and then doubled within 10 days; the high levels were maintained for one month and then decreased greatly in males and slightly in females. When the plasma concentrations of T4 started to decrease, plasma concentrations of LH increased. Changes in plasma T3 were not consistent with molting. These results indicate that the decrease of plasma levels of sex steroid hormones and the sharp increase of T4 induced molting, which lasted only for a short period.

## INTRODUCTION

Molting, a process of replacing old worn feathers with new ones, is an important event in the life cycle of birds (see for review Payne, 1972). This is an energy-demanding process (see for review King, 1981) and in most avian species, especially birds living in temperate regions, it does not overlap significantly with other energy-demanding events, such as migration and breeding. Molting is also a highly adaptive event, varying in form, duration, frequency and timing (Ginn and Melville, 1983).

Penguins are highly adapted to marine life. The penguins' feathers protect the skin against water and serve as insula-

tion (Stonehouse, 1967). Partial wear of body feathers decreases swimming ability and insulation. Thus penguins cannot enter the sea to forage and are forced to fast during molting. To achieve an energy-demanding molt during a period of forced starvation, penguins have developed a unique strategy. They take in a lot of food during the pre-molt period (Groscolas *et al.*, 1986) and renew the whole plumage over a very short period relative to other bird species. In their natural environment, most species of penguin molt after breeding (postnuptial molt) during summer (December–April in the Southern Hemisphere).

Humboldt penguins (*Spheniscus humboldti*) distribute between Foca island ( $5^{\circ} 12' S$ ) in Peru to the north and Punihiuil islands ( $42^{\circ} S$ ) in Chile to the south. However, the number of wild Humboldt penguins has decreased by 70% in 15 years to about 7,500 in 1996 (Araya and Bernal, 1996), and the spe-

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cies is listed as endangered. On the other hand, Humboldt penguins adapt well to artificial conditions and have been bred successfully in Japan and are one of the most popular penguin species in zoos and aquariums (Hori, 1996). In captivity, they molt during the local summer of the Northern Hemisphere. Despite their successful breeding in captivity, Humboldt penguins are not well studied. Meritt and King (1987) and Scholten (1987, 1992) conducted studies using captive birds in America and Europe, respectively, but to date, there have been no physiological studies concerning breeding and molting. Successfully breeding captive Humboldt penguins in Japanese zoos and aquariums provide a good opportunity for such studies.

Thus the purpose of the present experiments is to obtain basic physiological information on molting and breeding in Humboldt penguins in Japan. We observed the behavior of Humboldt penguins kept in an open aquarium display and collected blood samples for measurement of circulating hormones, luteinizing hormone (LH), testosterone (T), estradiol (E2), thyroxine (T4), triiodothyronine (T3) and corticosterone (B), as well as body weight.

## MATERIALS AND METHODS

### Animals

The study site was Tokyo Sea Life Park located in Kasai, Edogawa-ku, Tokyo (35° 38' N, 139° 53' E). The park has an outdoor open field pen (1095 m<sup>2</sup>) with a pool (314.5 m<sup>2</sup> with 400 t of water) which holds a mixed colony of Humboldt penguins and Rockhopper penguins (the number of Humboldt penguins was 92 in 1994). Individual penguins are identified by color wing bands. We selected 8 pairs of adult Humboldt penguins (Pair 1: No. 25 and 5, Pair 2: No. 6 and 7, Pair 3: No. 11 and 24, Pair 4: No. 35 and 59, Pair 5: No. 8 and 53, Pair 6: No. 56 and 58, Pair 7: No. 27 and 43, Pair 8: No. 68 and 67) for the study. Their ages were from 3 to 18 years. Four birds were born wild and the rest born in zoos. All pairs had showed reproductive activity in preceding years using artificial rock nests in the pen.

The birds are fed fresh live horse mackerels and silver stripe round herrings given in the pool every day except Monday.

### Observation of breeding and molting

Most pairs use the same rock nests for many years. We observed breeding behavior such as nest building, egg laying, incubating and chick rearing through a whole year (1994). Dates of hatching or removal of eggs were recorded for each nest. The clutch size in Humboldt penguins is two but the data shown below is always for the first egg. Eggs which were accidentally broken or did not hatch were removed after the incubation period. In some cases, however, unhatched eggs remained beyond the incubation period to adjust the population in the pen.

Molting is a process whereby new feathers grow under the skin, extruding the old feathers as they emerge. However, it is impossible to detect the whole process from external observation, and we defined a molt as the period between the dates the first and last old worn-out feathers are lost. During molting, the penguins did not enter the pool or eat.

### Sample collection

Since previous observations have indicated molting of the Humboldt penguins occurs during summer on the conditions mentioned above, we collected blood samples from May to September (1994), by which time all had completed molting except Pair 8.

Since we could not collect blood samples from 16 individuals in one day, we divided the birds into 3 sampling groups and collected samples every 3 weeks from each group between 1300 and 1800. Eight to 9 samples were obtained from each bird. A 2-ml blood sample was taken from a leg vein with a 25 gauge needle and a syringe into a heparinized tube. The samples were centrifuged and the plasma samples stored frozen until the assays. At blood sampling, the birds were weighed to the nearest 0.01 kg and the stage of molt evaluated.

### Hormone assay

Each hormone was assayed in a single run to avoid interassay variation. Plasma concentrations of luteinizing hormone (LH) were measured in 50- $\mu$ l sample volumes, in duplicate, using the radioimmunoassay method described by Hattori and Wakabayashi (1979). Chicken LH (fraction IRC-2, Gunma) was used for reference preparations and preparations of chicken LH (fraction AGCHDS112312A) were used for iodination. The antiserum (AH-MH No. 1) was raised against chicken LH (fraction IRC-2, Gunma). Results are expressed in nanograms per milliliter of a chicken LH fraction IRC-2, Gunma. Intraassay variation was 6.5%.

Plasma concentrations of testosterone (T), estradiol (E2) and corticosterone (B) were estimated with specific radioimmunoassays in 10  $\mu$ l plasma sample volumes in duplicate using antisera supplied from Prof. K. Wakabayashi with intraassay variations of 5.7, 5.8 and 12.2%, respectively. Plasma concentrations of thyroxine (T4) and triiodothyronine (T3) were measured in 5  $\mu$ l samples in duplicate according to Tasaki *et al.* (1986) with slight modifications using antisera obtained from Endocrine Sciences Products (Calabasas Hills, California) and intraassay variations were 15.5 and 14.6%, respectively.

### Statistics

Where sample size was adequate and equality of variance criteria could be met, parametric tests of analysis of variance (ANOVA) and Student's *t* test were preferentially used. Differences were considered significant when  $P < 0.05$ . Throughout the text, hormone concentrations are the mean  $\pm$  SEM.

## RESULTS

### Breeding and molting

In the outdoor display ground at Kasai, the Humboldt penguins showed breeding activity throughout the year except for the period of molting (Fig. 1). Four pairs laid 3 clutches, 2 laid two clutches and 2 laid one clutch.

Seven pairs laid the first clutch during March while Pair 3 laid early in February. Five pairs laid a second clutch in May after an unsuccessful breeding and Pair 1 laid a late first clutch in May. Two pairs (6 and 7) successfully hatched the first clutch and reared chicks to fledglings. Two pairs (2 and 8) out of the five which had the second clutch succeeded in rearing chicks to fledglings.

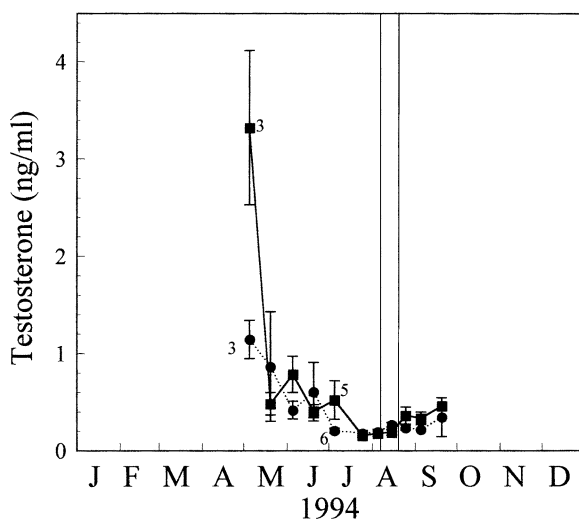
Irrespective of the success or failure of the preceding breeding, all the birds molted during late July to mid-August. One pair (8) laid the second clutch late on the last day of May and the eggs hatched on July 17. They reared the hatchling and molted very late, the male in August and the female in early October.

In all the pairs, the males began to molt several days earlier than the females ( $10.8 \pm 4.3$  days). For the males, the mean date of the beginning and end of molt was August 2 ( $\pm 4.0$  days) and August 15 ( $\pm 3.9$  days), whereas in the females it was August 12 ( $\pm 8.0$  days) and August 25 ( $\pm 7.6$  days),

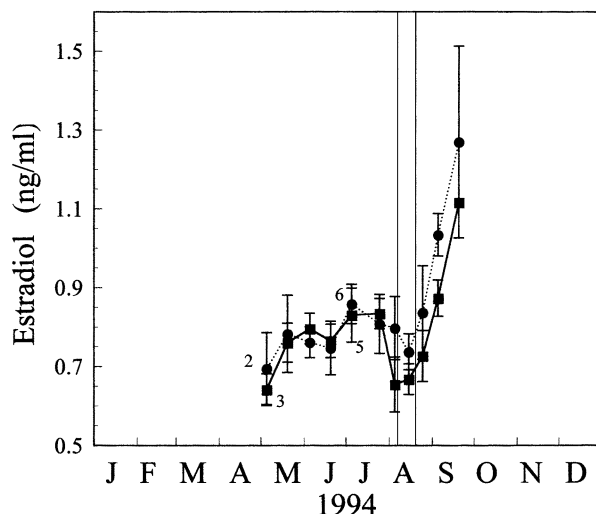


kg) until early July and then increased to a maximum value of  $4.94 \pm 0.11$  kg ( $n = 6$ ) in early August ( $4.30$  vs  $4.94$  kg,  $t = -3.85$ ,  $df = 10$ ;  $P < 0.01$ ) before decreasing significantly after the beginning of molting ( $4.94$  vs  $4.04$  kg,  $t = 4.70$ ,  $df = 15$ ;  $P < 0.01$ ).

**Changes in LH.** The plasma concentrations of LH in both sexes showed similar profiles and showed significant changes throughout the sampling period (ANOVAs by sex,  $F(9, 53) = 5.40$  for males,  $F(9, 52) = 3.18$  for females,  $P < 0.01$  in both cases; Fig. 3). The circulating levels of LH in both sexes were high, above  $2.0$  ng/ml, at the start of sampling. Then the plasma concentrations of LH decreased gradually to a minimum level of  $0.53 \pm 0.04$  ng/ml ( $n = 9$ ) in the males and  $0.72 \pm 0.16$  ng/ml ( $n = 7$ ) in the females in late July just before the



**Fig. 4.** Changes in plasma concentrations of testosterone in male and female Humboldt penguins during the sampling period. Details are as for Fig. 3.



**Fig. 5.** Changes in plasma concentrations of estradiol in male and female Humboldt penguins during the sampling period. Details are as for Fig. 3.

start of molting. The decrease found between late June and late July was significant in both sexes ( $1.58$  vs  $0.53$  ng/ml,  $t = 2.79$ ,  $df = 4$ ;  $P < 0.05$  in male,  $1.70$  vs  $0.72$  ng/ml,  $t = 3.88$ ,  $df = 10$ ;  $P < 0.01$  in female). Circulating concentrations of LH in the males showed a clear increase to reproductive levels after the onset of molt ( $0.53$  vs  $1.30$  ng/ml,  $t = -2.21$ ,  $df = 15$ ;  $P < 0.05$ ). There was no significant increase in LH levels of the females after late July (ANOVA,  $F(4, 30) = 2.69$ , NS).

**Changes in T.** Plasma concentrations of T in the males showed significant changes throughout this sampling period (ANOVA,  $F(10, 53) = 19.02$ ,  $P < 0.01$ ). The maximum level was  $3.32 \pm 0.79$  ng/ml ( $n = 3$ ) at the start of the experiment. Then the concentrations of T were  $0.5$ – $1.0$  ng/ml until early July. In late July, the levels became the lowest ( $0.16 \pm 0.02$  ng/ml,  $n = 9$ ). The low concentration in males was maintained until the middle of August, then it increased to  $0.3$ – $0.5$  ng/ml (ANOVA,  $F(5, 32) = 2.57$ ,  $P < 0.05$ ) (Fig. 4).

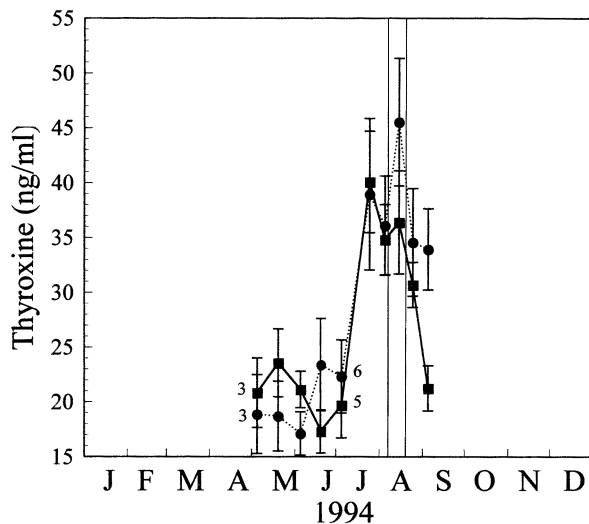
Plasma concentrations of T in the females showed the highest levels,  $1.15 \pm 0.20$  ng/ml ( $n = 3$ ), in early May and significantly declined to  $0.21 \pm 0.03$  ng/ml ( $n = 6$ ) in early July ( $1.15$  vs  $0.21$  ng/ml,  $t = 4.73$ ,  $df = 2$ ;  $P < 0.05$ ). Thereafter, the levels remained low. There were no significant changes in the female T levels after July (ANOVA,  $F(6, 37) = 1.26$ , NS) (Fig. 4).

**Changes in E2.** The temporal patterns of E2 plasma concentrations in both sexes were similar (Fig. 5). There were significant changes in the males throughout the sampling period (ANOVA,  $F(10, 53) = 2.12$ ,  $P < 0.05$ ), however, no significant variations were found in the females (ANOVA,  $F(10, 53) = 1.64$ , NS). Circulating E2 levels were low ( $< 0.9$  ng/ml) until early September in the males and late August in the females. The E2 plasma concentrations increased, maximizing in both sexes after molting. In the males, it was  $1.12$  ng/ml ( $n = 1$ ) and in the females,  $1.27 \pm 0.24$  ng/ml ( $n = 3$ ), at the end of this experimental period, late September. In males, the plasma concentrations decreased significantly from late July to early August ( $0.83$  vs  $0.65$  ng/ml,  $t = 2.34$ ,  $df = 15$ ;  $P < 0.05$ ) after which low levels were maintained during molting. Plasma E2 increased significantly after the molting ( $0.67$  vs  $0.87$  ng/ml,  $t = -2.46$ ,  $df = 8$ ;  $P < 0.05$ ).

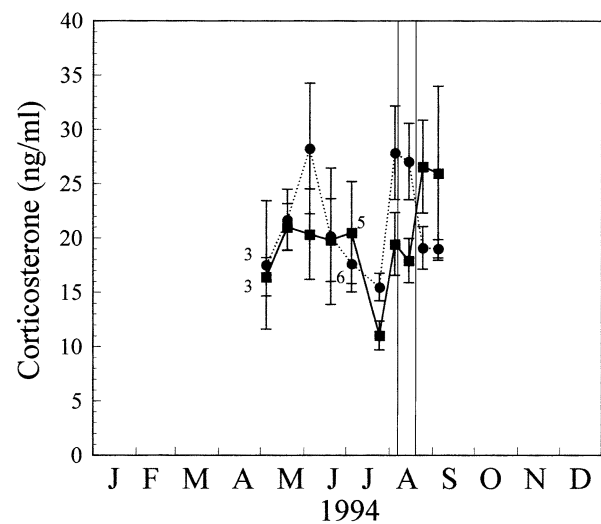
**Changes in T4.** Both sexes had a biphasic pattern of circulating levels of T4 (ANOVAs by sex,  $F(9, 53) = 5.24$ ,  $P < 0.01$  for males,  $F(9, 52) = 4.04$ ,  $P < 0.01$  for females; Fig 6). In both sexes, plasma concentrations of T4 were stable and low during the early portion of the sampling period and they rapidly elevated from early to late July in the males ( $19.71$  vs  $40.08$  ng/ml,  $t = -3.05$ ,  $df = 12$ ;  $P < 0.01$ ) and from early July to early August in the females ( $22.33$  vs  $36.10$  ng/ml,  $t = -2.45$ ,  $df = 10$ ;  $P < 0.05$ ). These high levels were maintained during molting in both sexes. After the molting period, T4 plasma concentrations decreased in the males ( $36.40$  vs  $21.25$  ng/ml,  $t = 1.54$ ,  $df = 8$ ; NS) but the high levels were maintained in the females.

**Changes in T3.** There was no significant variation throughout the experimental period in either sex (ANOVAs by sex,  $F(9, 53) = 1.64$ , NS for males,  $F(9, 52) = 1.06$ , NS for fe-

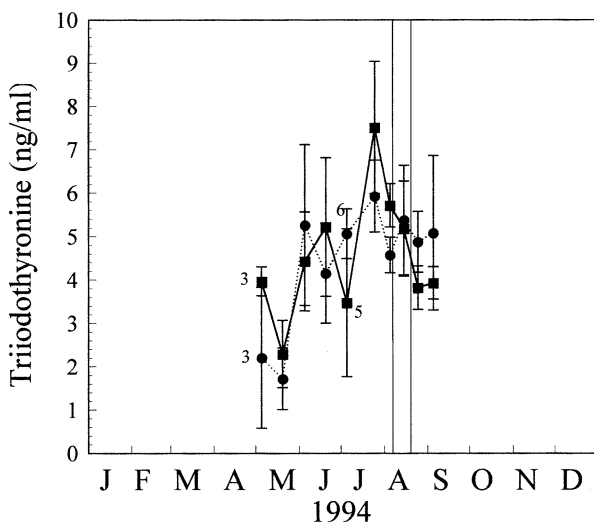




**Fig. 6.** Changes in plasma concentrations of thyroxine in male and female Humboldt penguins during the sampling period. Details are as for Fig. 3.



**Fig. 8.** Changes in plasma concentrations of corticosterone in male and female Humboldt penguins during the sampling period. Details are as for Fig. 3.



**Fig. 7.** Changes in plasma concentrations of triiodothyronine in male and female Humboldt penguins during the sampling period. Other details are same as in Fig. 3.

males) and the circulating T3 concentrations showed similar profiles in the males and females (Fig. 7). T3 levels were low in May, and maintained relatively high levels thereafter, especially in the females. But there were no associated changes in T3 with molting in females during the experimental period. In males, T3 levels were relatively elevated and became more variable through this period.

**Changes in B.** There were no significant changes throughout the experimental period either sex (ANOVAs by sex,  $F(9, 53) = 1.74$ , NS for males,  $F(9, 52) = 1.50$ , NS for females). The concentration of B was the lowest at the end of July in both sexes (Fig. 8).

## DISCUSSION

Our observations indicated that Humboldt penguins successfully manage their annual life cycle in captivity under outdoor conditions; they showed breeding activity throughout the year except during molting indicating that these two energy-demanding events do not overlap. It is reported that in their original habitat, Humboldt penguins can breed in any month of the year (Stonehouse, 1972 cited by Williams, 1995) and in captivity they nest all year round, with second and third clutches laid in the same year if the first one fails. Thus the birds used in the present experiment were not exceptional but rather a good model with which to study physiological mechanisms underlying the annual breeding and molting cycles of this species. To date, studies on captive penguins had focused only on morphology and behavior and no physiological studies had been undertaken.

Throughout the sampling period, the body weight of the males was always heavier than that of the females (Fig. 2). The body weight values for the males and females before the premolt period are comparable to those reported by Scholten (1987). Body weight increased significantly during the premolt period in both sexes. The birds fast for about 13 days during the molting. Therefore, they consume much more food than normal prior to fasting. This increased body weight was reduced during molting, returning to original values after the molt. In general, sparrows do not show significant variations in body weight between pre- and post-molt (e.g., Murphy *et al.*, 1988), because they can forage during molt. The female body weight peaked later than that of the males, as did T4. Also, the date of beginning of molt was later in females (Figs. 2 and 6).

The circulating levels of LH reached a minimum just before molting and the sex steroid hormones showed low levels during molting in both sexes suggesting the molting period is

a reproductively quiescent phase (Figs. 3-5). Annual events in birds come under the control of photoperiod as well as food availability (see for review Wingfield and Farner, 1994). Food availability before molting is especially important for the penguins since they can not forage during molting. In the wild, the period of premolt for Humboldt penguins overlaps with a time when food is abundant. The molting time is December and January (summer) in the Southern Hemisphere when anchovies approach the coast (Jordan, 1980). The fact that the Humboldt penguins, brought to or born in Tokyo Sea Life Park in the Northern Hemisphere and fed constantly all year round, molt in the local summer (July-August) indicates that molting is basically controlled by environmental factors.

As mentioned above, molting in Humboldt penguins does not overlap with breeding suggesting that hormones related to breeding activity suppress molting. Assenmacher and Jallageas (1980) described two precise temporal relationships between gonadal and thyroid cycles. 1) The annual gonadal cycle is inversely related to the thyroid cycle, i.e. the breeding season occurs when thyroid activity is low and vice versa. 2) The thyroid function displays a marked seasonal increase, peaking just before the end of the breeding season. Passerine birds and Indian finches are in group 1 and starlings, blackbirds, mallards and ducks are in group 2. Humboldt penguins, which show a postnuptial molt coinciding with a very rapid increase of T4 and decrease in sex steroid hormone secretion, fall into the latter group. This rapid increase of T4 is suggested to affect feather growth (Groscolas and Leloup, 1986). Their findings showed that the peak of the plasma concentrations of T4 coincide with the molt whereas that of T3 occurred later in emperor and adielie penguins. Also in the present study, the plasma concentration of T4 had a clearer correlation to molting than that of T3 (Figs. 6 and 7).

Interestingly, this sudden increase in T4 seemed to be unconnected with any environmental factor but to coincide with the very sudden ending of the reproductive period as indicated by the decrease in circulating LH and sex steroid hormones (Figs. 3-6) as is the case in ducks and teals (Assenmacher and Jallageas, 1980). The idea that information regarding the end of breeding activity is a trigger for molting is further strengthened when the molting and changes in hormones are correlated in individual animals. For example, Pair No. 8 laid a second clutch late on the last day of May which hatched on July 17. The pair reared the hatchling to fledgling stage. Parallel to this delay in breeding activity, the plasma concentration of T4 of Pair 8 began to increase later in August in the male and did not increase in the female during the sampling period (data not shown). And they molted very late in August (male) and in early October (female). Scholten (1989) also suggested that the date of egg-laying is correlated with that of molting in this species. This is a highly adaptive trait; they cannot rear the chicks when they are molting, because they can not forage without the insulation of the feathers. After they complete rearing, Humboldt penguins eat as much as possible and gain fat for starvation during the molt ashore. These annual cycles are likely to be precisely

controlled by endocrine mechanisms. Seasonal maturation of the reproductive system is likely to be controlled by photoperiod even in sub-Antarctic and Antarctic species such as adielie penguins (Astheimer and Grau, 1985; Groscolas *et al.*, 1986), emperor penguins (Groscolas *et al.*, 1986) and in macaroni and gentoo penguins (Williams, 1992), before they arrive at the breeding ground. Reproductive activity in Humboldt penguins may also be controlled by photoperiod since the birds used in this experiment began to reproduce in the local winter (in most cases in November). As mentioned earlier, the beginning of molting was dependent on the end of reproduction indicating that molting is not necessarily controlled by photoperiod. However, the mechanism by which the reproduction is terminated in this species is unknown. The birds begin to breed in winter indicating the species a short-day breeder like sheep. Plasma concentrations of LH in both sexes began to increase during molting (Fig. 3) and E2 plasma concentrations increased after molting (Fig. 5). These results suggest the molting period is an interruption of breeding which is potentially sustained throughout the year.

The plasma concentrations of T and E2 were high during breeding, decreasing to minimal levels before molting. The results agree well with that obtained in emperor and adielie penguins (Groscolas *et al.*, 1986). The values of T and E2 in both sexes were almost the same for all sampling periods. This is reasonable since males and females share breeding (parental) duty (nest building, nest protecting, incubating and chick rearing). This was already observed in Western seagulls (Wingfield *et al.*, 1980, 1982) where there is no sexual dimorphism in morphology and behavior. The only difference was in the date molting began. We observed that the males molted before the female partners in 1994. Scholten (1989), Kojima (1978) and Warham (1972) have also reported that males molt before females in captive Humboldt penguins and wild Erect-crested penguins (*Eudyptes sclateri*). The reason for this is not clear at the present, but there may be some differences in parental care controlled by prolactin which suppresses T4 release between males and females.

Plasma concentrations of B were relatively high from May to June, decreased to the lowest values at the end of July and again increased upon molting (Fig. 8). It is difficult to draw a general conclusion as to the role of corticosterone in molting in this species, even though Peczely (1985) suggested its involvement in several species. Further studies are required.

Penguins are interesting because their breeding cycles markedly differ from those of most altricial passerines. However, all penguins used for physiological studies to date have been Antarctic or sub-Antarctic species. Penguins living in the mid-latitude, such as Humboldt penguins, are interesting to compare with passerines. We conducted a physiological study on captive Humboldt penguins and obtained results indicating that changes in sex steroids and thyroid hormones affect the short duration molt of the species. Further studies will clarify the actual mechanisms for molting in the species.

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