

## STRESS, CORTICOSTERONE, AND HETEROPHIL TO LYMPHOCYTE RATIOS IN FREE-LIVING ADÉLIE PENGUINS<sup>1</sup>

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**Abstract.** We measured corticosterone in plasma collected from free-living Adélie Penguins (*Pygoscelis adeliae*) and made blood smears to count the ratio of heterophils to lymphocytes (H/L). Our objective was to categorize how these two measures of stress vary with potential stressors in the birds' environment. In penguins that were repeatedly sampled from three to eight times, repeated handling did not affect corticosterone levels or H/L, but there were significant differences among individuals. Nesting stage did not affect corticosterone level, but H/L was significantly lower during the chick stage than in the courting or incubation stages. Sex and handling times of less than 5 min had no effect on either corticosterone or H/L. In birds that had fasted up to 40 days during the courtship and early incubation stages, there was no increase in corticosterone or H/L with length of fasting, but in birds that had fasted more than 50 days, corticosterone levels increased. Birds with obvious injuries had significantly higher H/L than birds that had recently engaged in fights or those caring for chicks, but corticosterone levels did not differ in these groups. In free-living birds, H/L ratios provide a measure of stress that may be more useful than a single measure of plasma corticosterone in assessing response to chronic stressors like injury or crowded conditions in the breeding colony.

**Key words:** Adélie Penguin, corticosterone, heterophil, lymphocyte, *Pygoscelis adeliae*, stress.

### INTRODUCTION

Free-living animals face a variety of potential stressors including inclement weather, food deprivation, agonistic social interactions, human disturbance, predators, injury, and disease. An estimation of the level of "stress," especially chronic stress, that animals face would be valuable to biologists, conservationists, and wildlife managers. Stress has been relatively well studied in laboratory and domestic animals (reviewed in Broom and Johnson 1993). Physiological responses to acute stress include the rapid secretion of catecholamines from the adrenal medulla and glucocorticoids from the adrenal cortex (Sapolsky 1992), and their levels have often been used to assess the level of stress (Harvey et al. 1984, Wingfield 1994). In birds, epinephrine levels can increase within seconds of exposure to stress, and glucocorticoids rise within minutes (Le Maho et al. 1992). In the field, plasma levels of glucocorticoids can increase by an order of magnitude within 20–30 min of capture, so un-

less handling time is very short, the effect of these investigator-imposed stressors may swamp other inputs. Elevation of corticosterone, the major glucocorticoid in birds, leads to a series of events that can enhance short-term survival, including redirected behavior and mobilization of energy reserves (Wingfield et al. 1998). The half-life of these hormones is short (minutes to hours), so their levels drop and their effects disappear if the stressor is removed. This is functionally important because chronic stress and chronically elevated glucocorticoids can result in stress-related disease (Sapolsky 1992).

Another index to stress is the ratio of heterophils to lymphocytes (H/L) in blood (Gross and Siegel 1983, Maxwell 1993). Stressors, including food or water deprivation, temperature extremes, constant light, and exposure to novel social situations elevate the number of heterophils and depress the number of lymphocytes (Gross and Siegel 1986, Gross 1989, McFarlane and Curtis 1989). Leukocyte numbers change more slowly (30 min to 20 hr) in response to stress than does corticosterone (Dein 1986, Maxwell 1993, Cunnick et al. 1994). These changes are less variable and longer lasting than the corticosterone response, and multiple stressors usually have an additive affect (McFarlane and Curtis 1989, McKee and Harrison 1995). The mech-

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anisms mediating these cellular changes are poorly defined in birds (Dohms and Metz 1991), but may include changes in adrenal corticotropic hormone and corticosterone (Gross and Siegel 1983, Gross 1989), and/or altered production of and responsiveness to cytokines (Cunnick et al. 1994).

Change in H/L ratio has rarely been used as a measure of stress in free-living birds, even though it is an easy measurement to make. Leukocyte counts in two species of gulls differed between healthy and abnormal individuals (Averbeck 1992). The H/L ratio was about 0.6 in normal gulls and increased to over 2.9 in gulls that were oiled, emaciated, infected with endoparasites, or injured. Recently the H/L ratio was used to compare stress effects following different procedures to translocate birds (Work et al. 1999). Leukocyte counts also have been used as a measure of immune function in studies on sexual selection in birds (Dufva and Allander 1995, Zuk et al. 1995, Johnsen and Zuk 1998). Baseline data on cell counts exist for some species (ISIS 1995), but the manner in which H/L varies with species, sex, reproductive stage, and environment has not been investigated systematically.

As part of a larger project on the hormonal control of reproductive behavior in free-living Adélie Penguins (*Pygoscelis adeliae*), we obtained blood samples from a large number of penguins, some repeatedly, throughout the course of their nesting season (Vleck et al. 1999). Potential stressors for these birds included repeated handling and blood collection, long-term fasting while in the nesting colony, and intense fights with other penguins. Our objective was to use these samples to measure H/L ratios and corticosterone levels across the breeding season, to examine the correlation between them, and to categorize how they vary with potential stressors in the birds' environment.

## METHODS

We studied Adélie Penguins at breeding colonies on Torgersen Island, near Palmer Station on Anvers Island, Antarctic Peninsula (64°46'S latitude, 64°05'W longitude) between 12 October 1996 and 31 January 1997. All birds were individually marked with a metal flipper band for identification, and the sex and breeding status of most were known. We censused the colonies daily, weather permitting, to determine which

birds were present and the stage of the nesting cycle.

We collected blood samples of 1–2 ml ( $n = 118$ ) from the jugular vein using a heparinized syringe and 20 gauge needle and transferred blood immediately to heparinized containers. We defined handling time as the elapsed time from first approaching the bird to obtaining the blood sample. Approach and capture usually took only a few seconds when birds were tending nests, but could take longer for birds that were not on nests and had to be pursued. Mean ( $\pm$  SE) handling time was  $1.8 \pm 0.1$  min, range = 0–7 min. In a separate experiment, we collected serial blood samples over 30 min to examine the time course of corticosterone change in individuals in response to handling stress. Handling for 30 min routinely resulted in an order of magnitude increase in plasma corticosterone, but no increase was detectable in the first 5 min after approaching a penguin (Vleck, unpubl. data). Our focus here is on corticosterone levels prior to the acute stress of handling, not on the response to acute stress. Consequently, for corticosterone analyses we used only samples with handling times less than 5 min ( $n = 113$ ). A preliminary statistical analysis indicated no effect of the handling times  $< 5$  min in any of our analyses ( $P > 0.1$ ), so handling time was not included as a factor in any further analyses.

We kept blood samples cold, but protected from freezing until we returned to the laboratory (1–6 hr after collection). Each sample was then vortexed to resuspend the cells. A subsample was immediately taken for determination of hematocrit and hemoglobin content, and a small drop ( $\sim 2 \mu\text{l}$ ) was used to make a smear on a clean glass slide. Hematocrit was determined by centrifuging resuspended whole blood in a capillary tube and measuring the ratio of packed red cell volume to total blood volume to the nearest 0.5 mm with a ruler. Hemoglobin concentration was determined with the cyanmethemoglobin method (Sigma Diagnostics Procedure No. 525, St. Louis, Missouri). For 28 samples, a hematocrit tube was filled with blood immediately after collection in the field, and later, a second tube was prepared in the lab as described above. There was no difference between hematocrit measured in freshly collected ( $0.50 \pm 0.01$ ) and resuspended blood ( $0.49 \pm 0.01$ ) (paired  $t_{27} = 1.9$ ,  $P = 0.07$ ). To further verify the lack of resuspension effect on cell counts, we collected

blood samples from six House Sparrows (*Passer domesticus*). We measured hematocrit and made a blood smear with these samples immediately after the sample was collected, and again 6 and 18 hr after the samples had been stored in heparinized vacutainer tubes at 0°C, following the same resuspension protocol used for penguin blood. Hematocrit in fresh blood ( $0.50 \pm 0.01$ ) did not differ from that in resuspended samples at 6 hr ( $0.50 \pm 0.01$ ) (paired  $t_5 = 2.3$ ,  $P = 0.07$ ), but by 18 hr hematocrit had decreased slightly ( $0.49 \pm 0.01$ ) (paired  $t_5 = 4.4$ ,  $P < 0.01$ ). However, heterophil counts (see below) did not differ over time (paired  $t_5 = -1.1$ ,  $P = 0.33$ , for change between 0 and 6 hr; paired  $t_5 = 0.5$ ,  $P = 0.65$ , for change between 0 and 18 hr). Mean heterophil counts were  $44 \pm 3$ ,  $46 \pm 3$ , and  $43 \pm 3$  in the sets of six sparrow smears prepared 0, 6, and 18 hr, respectively, after collection. Consequently, we assumed that vortexing produced a uniform suspension of penguin cells, and that smears of resuspended blood made several hours after blood collection provided an accurate reflection of cell numbers.

Each resuspended blood sample was centrifuged and plasma was separated and stored at -80°C. We measured plasma corticosterone concentration by radioimmunoassay following the technique of Wingfield and Farner (1975), but without chromatography. Briefly, we extracted 30  $\mu$ l of plasma with 5 ml of freshly distilled dichloromethane. The resuspended extract was assayed in triplicate using an antibody to corticosterone (B3-163, Endocrine Science Products, Calabasas Hills, California). We equilibrated about 1,000 cpm of tritiated corticosterone with each plasma sample before extraction to measure the amount recovered after extraction. Extraction efficiency averaged over 80% for all assays. We monitored inter- and intra-assay variability in the six assays used by repeatedly measuring the level of corticosterone in two penguin plasma pools (a high pool of  $\sim 27.4$  ng ml<sup>-1</sup> and a low pool of  $\sim 3.6$  ng ml<sup>-1</sup>). Mean intra-assay CV was 0.11 and 0.32 and inter-assay CV was 0.14 and 0.41 for the high and low pools, respectively. Lowest detectable concentration averaged about 1.5 ng ml<sup>-1</sup>.

Blood smears were air-dried, then stained with Wright-Giemsa stain, and examined under a compound microscope at a magnification of 100 $\times$  with oil immersion. We identified cells using standard avian guidelines (Dein 1986,

Campbell 1988, Zinsmeister 1988). In each field, heterophils and lymphocytes were counted until the cumulative total (heterophils + lymphocytes) was 100 cells. All cell counts within a species were done by a single observer. Five slides were counted three times. The 95% confidence interval for the mean heterophil count on a given slide was  $\pm 1.6$  cells, indicating that our cell identification and count gave a repeatable measure of the relative number of heterophils and lymphocytes.

#### STATISTICAL ANALYSIS

Plasma corticosterone concentrations were not normally distributed, so for statistical analyses we used the logarithms of corticosterone concentrations (log corticosterone). The ratios of heterophils to lymphocytes also were not normally distributed, so for analyses we used the number of heterophils counted in the total sample of 100 heterophils + lymphocytes. Both log corticosterone and heterophil count (H) were normally distributed. Our heterophil counts can be converted to H/L ratios as  $H/L = H/(100 - H)$ . In analyses of variance, we did not include interaction terms. In no case were interaction terms significant, and where available, lack-of-fit tests showed that adding interactions would not significantly increase the explanatory power of our statistical models. For multiple comparisons following significant ANOVA results, we used the Tukey-Kramer honestly significant difference as our criterion for significance. We used linear regression to describe the relationship between correlated variables. We considered differences significant if  $P < 0.05$ , and report values as mean  $\pm$  SE.

#### RESULTS

Corticosterone concentrations averaged  $5.8 \pm 0.4$  ng ml<sup>-1</sup> plasma ( $n = 113$ , range 0.8 to 26.1), and H counts averaged  $50.4 \pm 1.4$  ( $n = 105$ ), ranging from 14 to 89 heterophils per 100 cells counted (corresponding to H/L ratios from 0.16 to 8.09). Within those samples, we examined repeated handling, duration without food (penguins feed at sea and must fast as long as they remain in the breeding colony), sex, nesting stage, fights with conspecifics, and external injuries as sources of variation.

#### EFFECTS OF REPEATED HANDLING

We sampled six female and seven male Adélie Penguins from three to eight times each during

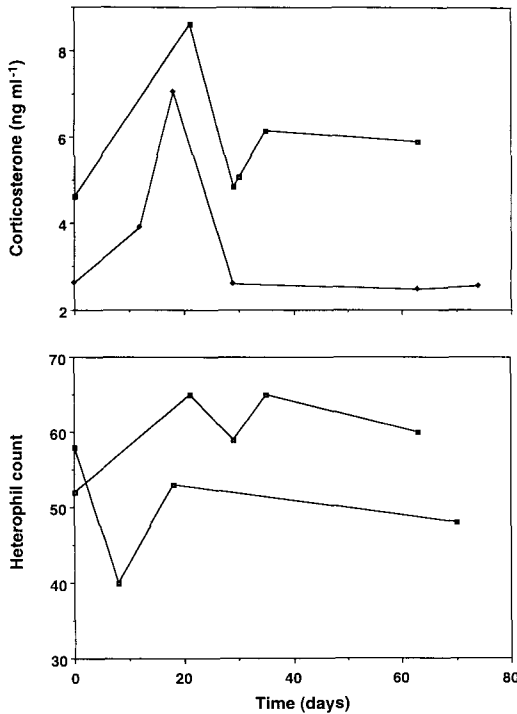


FIGURE 1. The plasma level of corticosterone (top panel) and number of heterophils in a count of 100 heterophils plus lymphocytes (bottom panel) in individual Adélie Penguins measured repeatedly throughout the breeding season.

the courtship, incubation, and chick stages of reproduction. The average interval between samples was  $13.4 \pm 2.0$  days ( $n = 51$ , range 1 to 59). None of these birds showed signs of obvious injury or experienced fasts  $> 40$  days. Analyses of variance using sampling interval, sex, nesting stage, and bird as main effects accounted for a significant portion of the variance in both corticosterone ( $F_{15,30} = 4.9$ ,  $P < 0.001$ ) and H count ( $F_{15,24} = 2.4$ ,  $P < 0.03$ ). The interval since the bird was last handled had no significant effect on either corticosterone or H count ( $P > 0.7$ ), nor did sex ( $P > 0.7$ ). However, the effect of individual bird accounted for a significant fraction of the sample variance for both corticosterone ( $F_{11,30} = 4.4$ ,  $P < 0.001$ ) and H count ( $F_{11,24} = 2.7$ ,  $P < 0.02$ ). That is, repeat samples from a given bird tended to be either relatively low or high compared to other birds (Fig. 1). There was a positive correlation between heterophil count and corticosterone ( $H = 38 + 16.6$  log corticosterone,  $r = 0.30$ , two-tailed  $t_{47} =$

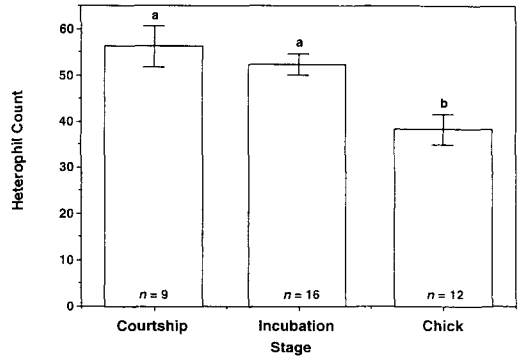


FIGURE 2. The mean ( $\pm$  SE) number of heterophils in a count of 100 heterophils plus lymphocytes measured during the courtship, incubation, or chick stage of reproduction in Adélie Penguins. Shared letters indicate values that do not differ ( $P > 0.40$ ) from each other.

$2.16$ ,  $P = 0.03$ ). Nesting stage had a significant effect on corticosterone ( $F_{2,30} = 6.8$ ,  $P < 0.01$ ), but not on H count ( $P = 0.1$ ).

#### EFFECTS OF NESTING STAGE

The courtship, incubation, and chick stages of the reproductive cycle in Adélie Penguins impose different behavioral constraints and potential stresses on the birds. We can examine those effects with more power by including more birds. In this analysis we use only one sample per individual, so that all samples are independent. Where we had more than one sample per bird, we chose the sample that provided the best balanced sample of sexes in each reproductive stage without regard for corticosterone level or H count. In most cases we used the first sample for each bird. We excluded birds that were obviously injured or had been fighting recently. A model including sex and nesting stage as main effects did not account for a significant portion of the variance in corticosterone ( $P > 0.9$ ), but did for H count ( $F_{3,32} = 4.7$ ,  $P < 0.01$ ). Nesting stage was the source of that significance ( $F_{2,32} = 6.8$ ,  $P < 0.01$ ); sex had no significant effect in the model ( $P > 0.7$ ). There was no difference in H count between the courtship and incubation stages of reproduction, but H count was significantly lower after the eggs had hatched and birds were feeding chicks than during the two earlier stages (Fig. 2). H count and corticosterone were not correlated ( $P > 0.3$ ) within this sample.

TABLE 1. Linear regressions describing physiological variables in Adélie Penguins as functions of fasting time in days. Corticosterone concentration is in  $\text{ng ml}^{-1}$ .

Dependent variable	Intercept	Slope	<i>r</i>	<i>n</i>	<i>P</i>
log corticosterone, $\text{ng ml}^{-1}$	0.4	0.01	0.52	18	0.03 <sup>a</sup>
Heterophil count	36.1	0.46	0.42	15	0.11
Male mass, kg	5.11	-0.043	0.91	13	<0.001
Hemoglobin, $\text{g dl}^{-1}$	15.4	0.09	0.59	16	0.02
Hematocrit	0.45	0.002	0.62	17	<0.01

<sup>a</sup> Significance depends on inclusion of two birds with fasts over 55 days.

#### EFFECT OF FASTING

Once they return to the breeding colonies in the spring, Adélie Penguins do not leave to feed until after the eggs are laid, which on Torgersen Island is usually about 3–4 weeks after their arrival. An incubating bird can leave to forage only after its mate returns. Males arrive, on average, about 5 days earlier than females, and usually take the first bout of incubation, so male fasts average 13–17 days longer than female fasts (Bucher and Vleck 1998). We knew the date on which each bird ended its fast, but we did not know the beginning (arrival) date for some birds because most were not banded when they arrived. We estimated the length of time that these birds had been fasting when sampled using the mean arrival dates for males (20 October 1996) and for females (25 October 1996) as the first day of the fast. Birds that we sampled had fasted from 1 to 57 days. For this analysis, we used one sample per bird, chosen without regard to corticosterone level or H-count, to distribute samples as evenly as possible over the observed range of fast lengths.

Corticosterone concentration increased significantly ( $P = 0.03$ ) with the length of the fast (Table 1). That effect, however, was due to two individuals that had fasted for more than 55 days because their mates never returned; no other bird had fasted more than 40 days. When those two outliers were not included, the effect of fasting length on corticosterone was not significant ( $P > 0.4$ ). There was also a positive relationship between H count and fasting duration when the two longest-fasting birds were included, but it did not reach statistical significance ( $P = 0.11$ ). H count was positively correlated with corticosterone in these fasting birds ( $H = 33 + 23.4 \log \text{corticosterone}$ ,  $r = 0.58$ , two-tailed  $t_{13} = 2.57$ ,  $P = 0.02$ ).

There were other physiological effects of fasting in Adélie Penguins. Body mass decreased,

and hematocrit and hemoglobin increased with the length of the fast (Table 1). We included only male data in the body mass regression because males are larger than females, and because our sample size for females was too small to provide adequate power. There was no correlation between body mass and either corticosterone or H count for these males ( $P = 0.1$ ). Over a typical 37 day courtship/incubation fast (Bucher and Vleck 1998), a 5.1 kg male penguin lost about 31% of its body mass and experienced a 23% increase in hemoglobin concentration and 18% increase in hematocrit. The two birds that had fasted  $> 50$  days had lost more than 40% of their initial body masses.

#### EFFECT OF FIGHTING AND EXTERNAL INJURY

We sampled several birds within minutes after an observed fight with another penguin and a few birds that had obvious recent injuries (wounds on face, body, or flippers). Only during the chick stage did we have sufficient sample size to compare values from fighting or obviously injured birds with those from normal, nesting birds at the same time. None of the injured or fighting birds had an active nest at the time. For some, we knew their nest had been lost, but for others we did not know their nesting history. There was no difference in corticosterone among birds that were fighting, injured, or caring for chicks ( $F_{2,20} = 0.2$ ,  $P = 0.8$ ), but there were differences in H count ( $F_{2,20} = 12.8$ ,  $P < 0.001$ ). The H count was significantly higher in injured birds than in the fighting birds or normal birds, which did not differ from each other (Fig. 3). There was no correlation between H count and corticosterone in these data ( $P > 0.7$ ).

#### DISCUSSION

Handling Adélie Penguins at intervals of several days during the breeding season did not produce

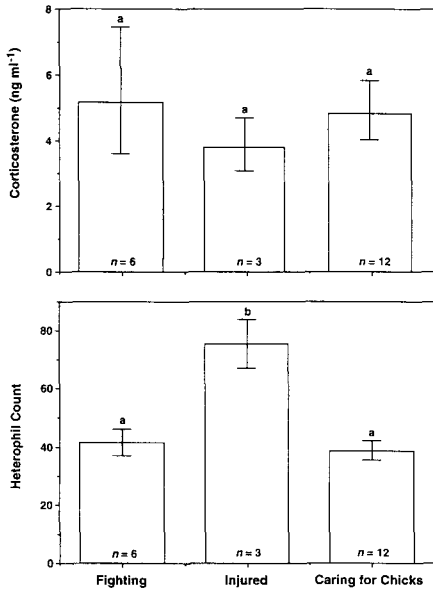


FIGURE 3. The mean ( $\pm$  SE) corticosterone concentration (top panel) and number of heterophils in a count of 100 heterophils plus lymphocytes (bottom panel) in Adélie Penguins that were fighting immediately before capture, had fresh injuries, or were caring for chicks. The error bars for corticosterone are asymmetrical because they are from the log-transformed values. Shared letters indicate values that do not differ ( $P > 0.50$ ) from each other.

a rise in corticosterone or heterophil/lymphocyte ratio that would evidence a chronic stress effect. In birds that we handled multiple times over the course of the season, we could detect no effect of our sampling protocol, which included capturing, taking a blood sample, weighing, and measuring the birds, on either corticosterone levels or H/L ratio. The volume of blood taken from these birds at each sampling period was less than 1% of their calculated blood volume of over 300 ml. In smaller species that are repeatedly bled and in which a larger fraction of blood volume is removed, repeated handling could have a more pronounced effect.

The mean and range of H/L ratios that we report are similar to those found in another much smaller study of free-living Adélie Penguins (Zinsmeister and VanDerHeyden 1987). Likewise, the level of corticosterone is similar to that measured in other species of penguins measured immediately after capture (Holberton et al. 1996, Hood et al. 1998). We could detect no effect of handling times of less than 5 min on corticoste-

rone level, although workers with other avian species have reported that corticosterone can increase within 2 min after capture (Schwabl et al. 1991, Le Maho et al. 1992, Hood et al. 1998). Plasma corticosterone does begin to increase in samples taken more than 5 min after approach and capture of Adélie Penguins (Vleck, unpubl. data). Our data suggest that in Adélie Penguins, it is possible to collect blood samples rapidly enough that capture and handling stress do not affect corticosterone levels.

There is a significant effect of individual on corticosterone level and cell counts, and this probably occurs in other species. Others have noted wide variance between individuals within a species for corticosterone (Wingfield et al. 1998) and cell counts (Averbeck 1992, Hawkey et al. 1993). Much of this variance can be attributed to variation in input from environmental stressors, but some is probably due to individual variance in the response to that stress. This individual variation means that to detect effects of stressors, it is probably necessary to have repeated measures from the same bird before and after a stressor, or large enough sample sizes to have the power to detect population-level effects.

#### EFFECT OF NEST STAGE

In reproductive adult Adélie Penguins, heterophil cell counts were significantly lower relative to the lymphocyte counts when birds were caring for chicks than during courtship or incubation stages of reproduction. This lower H/L ratio should be indicative of lower stress levels. Such variation with reproductive stage (or season, confounded with reproductive stage in penguins), should be considered in assessing the importance of stressors that can vary independently of stage or season. It also raises the biologically interesting question of why chick care should be less stressful than the earlier stages of reproduction in penguins. We can suggest three non-exclusive hypotheses: first, after chicks hatch, adults experience declining necessity to defend their nest site, eggs, and chicks from conspecifics and predators (skuas, *Catharacta* sp. on Torgersen Island). After chicks grow to a size at which they can thermoregulate independently and risk of skua predation is low, parents leave the colony, returning only to feed the chicks. Density of birds in the colony drops, reducing agonistic interactions. We did not detect an acute

effect of fighting on corticosterone level or H count (except for birds that were badly injured; see below), but chronically elevated levels of agonistic interactions within a nesting stage could affect stress levels. Second, seasonal changes in weather may reduce stress. The chick stage coincides with the warmest part of the Antarctic summer, when energy costs of thermoregulation are minimal (Chappell et al. 1990). That hypothesis could be tested using data from within or between years given greater variation in weather than we experienced. Third, after chicks hatch, parents leave the colony to forage much more frequently, so their ability to maintain nutritional and water homeostasis may improve. Our study of fasting birds (discussion below), however, supports the hypothesis that corticosterone levels or H/L ratios do not vary during fasting in penguins until a bird's energy reserves are nearly exhausted. Natural or manipulative field experiments designed to identify the variables responsible for these long term and population-level changes in stress indices would be valuable.

#### EFFECTS OF FASTING, FIGHTING, AND INJURY

Corticosterone levels increased in Adélie Penguins fasting during the courtship and early incubation stage of reproduction, but only when the fast extended for over 40 days. The average length of this fast for Adélie Penguins on Torgersen Island is 37 days for males and 22 days for females (Bucher and Vleck 1998). During these fasts, birds progressively lose body mass and become dehydrated as indicated by increases in hematocrit and hemoglobin concentration. Lengthy fasts are a normal part of the biology of penguins. In fasting King (*Aptenodytes patagonicus*) and Magellanic (*Spheniscus magellanicus*) Penguins, corticosterone levels do not increase until the birds have nearly depleted fat stores and begun using protein as a primary energy store, which may take months in large species (Cherel et al. 1988a, 1988b, Hood et al. 1998). In contrast, corticosterone levels rise steadily through long incubation shifts (11–15 days) in Grey-headed (*Diomedea chrysostoma*) and Black-browed (*D. melanophoris*) Albatrosses that do not have the physiological capacity to fast for as long as penguins do (Hector and Harvey 1986).

The male Adélie Penguin with the longest fast

that we measured abandoned his nest after 57 days of fasting, at a body mass of only 2.8 kg. He had the highest corticosterone level (18.8 ng ml<sup>-1</sup>) and H count (77 cells; H/L = 3.35) of any bird in our fasting samples. Interestingly, this bird returned to the colony 11 days later at a body mass 47% higher than when he had abandoned his nest. His corticosterone level had decreased to 2.5 ng ml<sup>-1</sup>. He did, however, have a fresh wound on his side and an H-count that was even higher than after his long fast (88 cells; H/L = 7.33). When he was recaptured about a month later, the wound had healed, and his corticosterone level (4.3 ng ml<sup>-1</sup>) and H-count (55 cells; H/L = 1.22) were in the normal range for birds at that time.

We could detect no effect of recent fighting (bird captured within minutes of end of fight) on corticosterone level or H counts. The lack of an elevation in corticosterone seems surprising in view of how violent these fights appear to human observers, although visible injury (or nest loss) during these fights is the exception rather than the rule. The lack of significance may be due to small sample size and the high variance in these data. Some fighters did have unusually high values of corticosterone. We saw a pair of penguins attempt to usurp the nest of an incubating bird, and after about 10 min of fighting, in which we initially thought the incubating bird had been killed, we obtained blood samples from all three birds. Corticosterone levels were not elevated (2.7 and 5.5 ng ml<sup>-1</sup>) in the two usurpers, but corticosterone in the loser and former owner of the nest was 22.4 ng ml<sup>-1</sup>. In another case in which we could identify the owner of a territory and an invader, the owner's corticosterone level was elevated (8.1 ng ml<sup>-1</sup>) relative to the loser's corticosterone value (1.8 ng ml<sup>-1</sup>). For both of these birds with elevated corticosterone, we could see blood in the nostrils, indicating injury from the fight. Whether or not a bird mounts a physiological stress response to a fight may depend on the importance of the outcome (nest owners risk losing their reproductive investment) and the intensity of the fight.

Adélie Penguins with recent external injuries had elevated heterophil counts, but not elevated corticosterone levels. The H/L ratio of injured birds (3.1) was higher than that of recently fighting penguins (H/L = 0.71) or birds tending their nests (H/L = 0.62). The heterophils may be elevated because they participate in combating in-

fection and/or the lymphocytes may have decreased as part of the general response to stress (Dein 1986). These results suggest that the H/L ratio is a longer lasting response to the stress of injury than is corticosterone level. Similar hematological findings were reported in a study on captive Gentoo Penguins (*Pygoscelis papua*) (Hawkey et al. 1985). In that study, H/L in clinically normal birds (H/L = 2.3) was lower than that found in birds with "bumblefoot" (H/L = 4.4), a common condition in captive birds involving lesions, inflammation, and infection in the feet. Other hematological measures that have been used to assess condition in free-living birds include hematocrit (Morton 1993), sedimentation rate of red blood cells (Svensson and Merila 1996), and glycosylated hemoglobin (Andersson and Gustafsson 1995). The H/L ratio should also prove a useful tool for assessing condition in field-caught birds.

#### CORTICOSTERONE AND CELLS COUNTS AS MEASURES OF STRESS IN FREE-LIVING BIRDS

Elevations in corticosterone and H/L ratios are both responses to stress in birds. These two measures were moderately correlated with each other in two of our data sets (i.e., repeat sample birds and fasting birds, but not in injured or fighting birds, or in birds considered across the nesting stages). High correlations between these two measures have been found in chickens (*Gallus gallus*), but only when working with well-socialized groups that had a stable social hierarchy before any experimental procedures began (Gross and Siegel 1983). In Adélie Penguins, neither corticosterone levels nor H/L differ between the sexes, or with days since the bird was last handled. The relative merits of which measure of stress to use depends on the circumstances and the question being asked. Preparing blood smears and counting cells requires little equipment or expense compared to radioimmunoassay of hormones and could probably be accomplished in nearly any field study in which birds are caught. Corticosterone levels change rapidly after capture, so blood samples have to be collected immediately after capture to measure environmental stress as opposed to the stress of capture and handling. Handling times for Adélie Penguins, and for many other bird species (Wingfield 1994), can be short enough to meet that constraint. Corticosterone levels may be a more sensitive indicator of short term stressors

such as food deprivation, inclement weather, or agonistic interactions (Wingfield 1994), whereas H/L ratio may be a more persistent indicator of stress associated with injury or with reproductive cycles and seasonal changes. Individual birds differ significantly in corticosterone level and H/L ratios. Those differences persist over time and are not correlated with obvious differences in external stressors. Thus demonstrating changes in stress using these measures requires either large sample sizes or longitudinal studies with repeated measures from the same individuals.

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