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EFFECTS OF BLOOD SAMPLING STRESS ON HORMONE LEVELS IN THE SEMIPALMATED SANDPIPER

CHERI L. GRATTO-TREVOR[1], LEWIS W. ORING[2], AND ALBERT J. FIVIZZANI

Department of Biology
University of North Dakota
Grand Forks, North Dakota 58202 USA

Abstract.—We examined the effects of handling and blood sampling stress on circulating levels of prolactin, corticosterone, testosterone, progesterone, and estradiol in Semipalmated Sandpipers (*Calidris pusilla*). Experimental birds were captured in nest traps, immediately bled once, then restrained for 15 or 30 min and bled again. Effects of restraint and bleeding on hormone levels were variable. Plasma corticosterone levels increased significantly in all experimental birds. In experimental birds, elevated testosterone and prolactin levels were decreased, while low values of testosterone increased. Such a response to handling would only suppress the detection of slight differences in mean hormone levels between breeding stages, and would not create artifactual differences where none actually exist. There was no relationship between sampling time and levels of prolactin, testosterone, progesterone, and estradiol in nonexperimental (less stressed) birds.

EFECTO DE LA TENSIÓN, EN LOS NIVELES HORMONALES CAUSADA POR EL MANEJO Y SANGRAMIENTO DE INDIVIDUOS DE *CALIDRIS PUSILLA*

Sinopsis.—Estudiamos en individuos de *Calidris pusilla* el efecto de la tensión, en los niveles circulatorios de prolactina, corticosterona, testosterona, progesterona y estradiol causada por el manejo y sangramiento de pájaros. Las aves fueron capturadas colocando trampas en sus nidos, de inmediato eran sangradas, luego sostenidas por períodos de 15 ó 30 mins. y sangradas nuevamente. El efecto de sostener a las aves y sangrarlas, en los niveles hormonales, resultó ser variable. Los niveles de corticosterona aumentaron significativamente en todas las aves experimentales. Por su parte, los niveles elevados de testosterona y prolactina se redujeron. En individuos donde los niveles de testosterona fueron bajos de primera instancia, éstos aumentaron. Dicha respuesta a la manipulación tan solo suprime la detección de pequeñas diferencias en los niveles promedios de las hormonas durante etapas reproductivas, y no establece diferencias ficticias en donde éstas no existen. No se encontró relación entre la hora en que se toman las muestras y los niveles de las hormonas antes mencionadas en aves no-experimentales (menos expuesta a tensión).

In any field study involving measurement of hormones, it is important that stresses such as capture, handling, and blood sampling do not themselves affect circulating hormone levels. Previous studies have noted that even relatively short periods of handling stress may affect plasma concentrations of some hormones. In particular, corticosterone levels are known to increase rapidly in response to stress: sometimes in as little as 1 min (Harvey et al. 1980, Klingbeil 1985). Plasma levels of other hormones may change more slowly in response to stress, but testosterone and prolactin levels have been shown in some studies to decrease significantly

[1] *Current address: Canadian Wildlife Service, Prairie and Northern Research Centre, 115 Perimeter Road, Saskatoon, Saskatchewan 57N 0X4 Canada.*
[2] *Current address: Ecology, Evolution and Conservation Biology Program, University of Nevada, Reno, Nevada 89512 USA.*

within 15 min to an hour (e.g., Wilson et al. 1979, El Halawani et al. 1985). However, results are not always consistent, particularly among different species, and types of stress.

The objective of this paper is to examine the effects of stress from blood sampling on hormone levels of Semipalmated Sandpipers (*Calidris pusilla*) used in a field study of the relationship between reproductive behavior and endocrinology. We examined the effect of blood sampling on behavior and survival of the birds themselves in a previous paper (Colwell et al. 1988). Semipalmated Sandpipers are monogamous, and both parents incubate the clutch equally.

METHODS

We studied Semipalmated Sandpipers at La Pérouse Bay (58°24'N, 94°24'W) on the coast of Hudson Bay, 40 km east of Churchill, Manitoba in the summers of 1985, 1986, and 1987. The 3 km^2 study area is situated in the Mast River Delta, and consists primarily of low islets of *Salix brachycarpa* or *Betula glandulosa* and mixed sedges and grasses in fresh water.

Gratto-Trevor carried out population studies of Semipalmated Sandpipers in this area from 1980 to 1987, so most birds were already color-banded, and the breeding system of the species was well-known. We captured incubating birds in walk-in nest traps, and nonincubating birds in mist nets (banding permits 10363X and 10201). For Semipalmated Sandpipers, the largest member of a pair as determined by bill length normally was assumed to be the female (Prater et al. 1977). We often verified sexing by behavior (flight displays, copulation), which indicated that sexing by size was very accurate (Gratto and Cooke 1987).

We sampled blood by puncturing the brachial vein with a small (25 G) needle and collecting blood in heparinized micro-hematocrit tubes. Pressure was applied to the wound if bleeding continued after sampling. For each sample we recorded handling time (time from capture to completion of blood sampling). Samples were kept on ice in the field, and transported to camp later in the day. At camp, samples were immediately centrifuged. Plasma was drawn off with a microsyringe and placed in labelled plastic tubes, then stored and transported in liquid nitrogen. At the University of North Dakota, samples were frozen at −20°C until analyzed.

We examined effects of stress on circulating hormone levels in two ways. First, 14 Semipalmated Sandpipers (seven males and seven females) were captured during incubation in 1987 and bled immediately, then bled again shortly afterwards. Half (three females, four males) were bled 15 min later, and the remainder (four females, three males) 30 min after first bleeding. Fifteen min was considered the maximum time allowed for normal blood sampling, as a previous study found a substantial decline in plasma prolactin levels after more than 15 min handling time in Wilson's Phalaropes (Colwell, pers. comm.). The experimental birds in this study sampled after 30 min were used to determine if effects of handling stress on hormone levels existed, or became more noticeable at

this longer time period. For convenience, birds restrained for 15 min were held in the hand, while those held for 30 min were placed in small cloth bags.

All blood samples were taken by Gratto-Trevor, who was experienced in avian blood sampling. In 10/14 instances, only one wing was bled initially. In all cases the other wing was bled later. Both wings were bled during collection of the second sample in 5/14 birds. Samples from ten of the experimental birds were assayed for four hormones (corticosterone, testosterone, prolactin, and progesterone). Due to small plasma volumes, two birds were assayed only for prolactin, and two for all hormones except prolactin.

Second, we examined the effect of handling time (in a much larger data set) by correlating circulating levels of hormones with handling time. These samples were originally collected in 1985 to 1987 to examine the relationship between hormone levels and breeding behavior. In order to obtain the largest data set possible, while standardizing capture techniques and hormone levels so that only sampling time varied, we used only incubating birds (n = 140). Hormones examined for nonexperimental birds were prolactin, testosterone, progesterone and estradiol.

We analyzed steroid samples at the University of North Dakota, using the radioimmunoassay initially described by Wingfield and Farner (1975) and revised by Fivizzani et al. (1986). Separate samples, or those previously subdivided, were used to obtain prolactin values in the laboratory of Dr. M. El Halawani at the University of Minnesota. The turkey radioimmunoassay of Burke and Dennison (1980) and Burke and Papkoff (1980) was used. (For more details on assays, and validation of the prolactin assay for this species, see Gratto 1989, Gratto-Trevor et al. 1990).

We used both parametric and non-parametric statistical tests for all comparisons. Only parametric tests are presented here, since there were no differences in results between types of tests. Statistical tests used were: t-test (and Mann-Whitney U-test), paired t-test (and Wilcoxan matched-pairs signed-ranks test), Pearson correlation (and Spearman rank correlation).

RESULTS

In all 12 experimental birds bled twice in 30 min or less, corticosterone levels increased significantly between the first and second samples (Table 1). There was no significant difference between hormone level changes in males versus females (n = 6,6, t statistic = -0.03, df = 10, P = 0.98, t-test), nor between the two time periods (Table 2).

Six experimental birds had higher testosterone levels during the second sampling, while six had lower levels. The overall mean difference was not significant (Table 1). There also was no significant difference in mean change between sexes (n = 6,6, t statistic = 1.65, df = 10, P = 0.13, t-test), or time periods (Table 2). However, there was a significant negative correlation between initial testosterone level and the change in testosterone during sampling (Pearson correlation, n = 12, r = $-$ 0.69, P

TABLE 1. Changes in hormone levels of incubating Semipalmated Sandpipers sampled twice within 30 min or less. Mean change refers to the average difference between each pair of samples. Values are in ng/ml for prolactin, and pg/ml for all other hormones. All comparisons are paired *t*-tests ($n = 12$ for each hormone, df = 10).

Hormone	Mean initial value	Mean change (SE)	*t*	*P*
Corticosterone	3352	+5016 (686)	7.31	0.0001
Testosterone	386	+3 (68)	0.04	0.97
Prolactin	220	−62 (10)	−6.15	0.0001
Progesterone	587	+194 (78)	2.50	0.03

< 0.01). Testosterone levels that were initially high, decreased; and those originally low, increased. This was not related to sex differences in initial testosterone levels, as initial values in females ranged from 158 to 735 pg/ml and males from 169 to 899 pg/ml. In both females and males, three testosterone values increased between first and second sampling, and three decreased.

Eleven of 12 experimental birds had higher prolactin levels at second sampling, and the difference was highly significant (Table 1). Again, there was no difference in mean change between sexes ($n = 6,6$, *t* statistic = −0.80, df = 10, $P = 0.44$, *t*-test), or time periods (Table 2).

Progesterone levels in nine of 12 experimental birds increased between first and second sampling, and this difference was significant (Table 1). Of the three birds with decreasing progesterone levels, two had the highest initial levels, but the third had the lowest initial level of all 12 birds. There was no significant difference between sexes ($n = 6,6$, *t* statistic = −0.43, df = 10, $P = 0.68$, *t*-test), or time periods (Table 2). In the ten experimental birds sampled twice for all four hormones, there were no significant correlations between change in one hormone and change in another ($P > 0.30$, Pearson correlations).

Overall correlations between handling time and hormone levels were examined with non-experimental birds. This allowed us to indirectly examine the effect of sampling stress in a much larger number of birds. For example, we tested the relationship between total handling time and prolactin level for all female Semipalmated Sandpipers sampled in 1985. Similar correlations were performed on all other incubating (non-experimental) birds for each hormone, sex, and year. Handling time ranged from 1 to 23 min (average 9), although less than 5% were greater than 15 min. Only one of 24 correlations was significant (Table 3). Since one of 20 would be expected to be significant at alpha 0.05 by chance alone, and there were no consistent trends among years and sexes for any hormone, there was apparently no consistent effect of handling time on circulating prolactin, testosterone, progesterone, or estradiol levels in these birds.

TABLE 2. Comparison of changes in hormone levels of incubating Semipalmated Sandpipers sampled twice 15 min apart versus 30 min apart. Mean change refers to the average difference between each pair of samples. Values are in ng/ml for prolactin and pg/ml for all other hormones. All comparisons were via *t*-tests ($n = 6$ for each time and hormone, df = 10).

Hormone	15 minutes apart		30 minutes apart	
	Mean change (SE)	*P*	Mean change (SE)	*t*
Corticosterone	4662 (398)	0.64	5371 (1364)	−0.50
Testosterone	109 (100)	0.12	−103 (78)	1.67
Prolactin	−56 (19)	0.59	−68 (7)	0.56
Progesterone	179 (82)	0.85	210 (140)	−0.19

DISCUSSION

Corticosterone.—Stress in birds, as in other vertebrates, characteristically results in increased circulating corticosterone levels (e.g., Scanes et al. 1980, Klingbeil 1985). In fact, Harvey et al. (1980) found that levels of corticosterone in the domestic Mallard (*Anas platyrhynchus*) could double within 1 min of handling and brachial vein bleeding. In the present study, corticosterone levels increased dramatically in all bled and restrained (experimental) individuals within 30 min, thus demonstrating the extreme responsiveness of the adrenal cortex to stress.

Testosterone.—Stress can result in a large decrease in plasma testosterone levels (Sossinka et al. 1980). Repeated sampling at 15 min intervals depressed circulating testosterone levels within an hour in adult male domestic fowl (*Gallus domesticus*) (Wilson et al. 1979). However, eight of the 20 roosters showed an increase in testosterone 15 to 30 min after initial blood sampling. It is possible that these data reflect the same results as in our study. In Semipalmated Sandpipers, the direction of change in testosterone appears to have been dependent upon the initial testosterone level. Levels in birds with initially high testosterone decreased, whereas levels in those with initially low values increased. Moore and Zoeller (1985) noted differing responses of plasma androgen levels to injections of corticosterone in male rough-skinned newts (*Taricha grandulosa*) at different times of the year. When androgen levels were low in September, there was no effect of corticosterone injections. However, in February, when initial androgen values were high, injections of corticosterone resulted in a decrease in androgen levels within 30 min. This result may reflect the same phenomenon of differing responses to stress, depending upon initial hormone levels.

Prolactin.—Many researchers have observed a significant increase in circulating levels of prolactin when mammals are stressed (e.g., Neill 1970, Horrobin 1973, Seggie and Brown 1975, Döhler et al. 1977). Few studies appear to have considered stress and prolactin in birds, and these apparently only in the domestic turkey (*Meleagris gallopavo*). Opel and Proudman (1982) found significant increases in serum prolactin levels

TABLE 3. Relationships between handling time and hormone level of incubating Semipalmated Sandpipers (Pearson correlations).

	Female			Male		
	1985	1986	1987	1985	1986	1987
Prolactin						
n	37	17	19	30	16	21
r	−0.06	0.08	0.04	−0.34	−0.43	−0.22
P	0.08	0.75	0.86	0.07	0.10	0.35
Testosterone						
n	25	15	19	22	17	21
r	0.35	0.23	0.22	−0.11	0.29	0.50
P	0.09	0.42	0.36	0.62	0.25	0.02
Progesterone						
n	18	15	19	17	17	20
r	−0.24	0.23	−0.21	0.03	0.35	−0.16
P	0.32	0.41	0.40	0.89	0.17	0.51
Estradiol						
n	23	9	13	22	13	15
r	−0.25	0.14	0.49	0.26	−0.22	0.40
P	0.25	0.72	0.09	0.24	0.48	0.14

with serial blood sampling at 5 or 30 min intervals in immature male, immature female, and ovariectomized adult female turkeys. Slight rises in prolactin occurred in laying females and semen-producing males. El Halawani et al. (1985) also found that stress (acute immobilization) resulted in elevated prolactin levels within 15 min in immature turkeys. However, serial blood collection significantly depressed plasma prolactin levels in *incubating* turkey hens in 5 to 30 min. Burke and Papkoff (1980) also found that stress such as changes in an incubating female turkey's environment caused high prolactin levels to decline to those in the range of laying birds, in 24 h or less. Nevertheless, they observed no changes in prolactin levels as a result of handling or repeated venipuncture over a period of 1–2 h.

Our results also showed a clear decrease in plasma prolactin levels in both sexes of incubating Semipalmated Sandpipers within 15 and 30 min. Since circulating prolactin is very high in these birds during incubation, it may represent another instance where high levels decrease with stress, whereas low levels of nonincubating birds might have increased, as appears to be the case in turkeys. Differences in the rate of prolactin level change might be related to the extent of the bird's previous experience with handling and/or blood sampling stress.

Progesterone.—It is difficult to explain the significant increase in plasma progesterone levels with stress in the Semipalmated Sandpiper. Camper and Burke (1977) demonstrated that an injection of luteinizing hormone elicited a rapid rise in progesterone in laying turkeys (within 5 min).

Injections of prolactin alone failed to result in a decline in plasma progesterone levels within 30 min. However, injections of 500 or 1000 μg of prolactin 30 min before treatment with luteinizing hormone completely blocked the expected rise in progesterone. It is possible that this indicates a mechanism whereby a decrease in circulating prolactin level with stress, as found in Semipalmated Sandpipers here, might result in a rapid rise in progesterone. This could occur if the lowered prolactin levels no longer block the effect of luteinizing hormone on the release of progesterone. There was no indication of a negative correlation between change in prolactin and change in progesterone for the ten experimental Semipalmated Sandpipers examined. However, changes in prolactin levels between the time of capture and end of first sampling are unknown. If there is a time-lag effect of changing prolactin levels on progesterone, early prolactin changes might more likely reflect eventual differences in progesterone.

Correlations with handling time.—When hormone levels in all non-experimental incubating birds were examined together, there was no significant or consistent effect of handling time. However, if the direction of change is primarily dependent upon initial hormone levels, significant correlations between hormone levels and handling time would not be likely. The fact that there was never a significant difference in the magnitude of hormone level change between the 15 and 30 min intervals in experimental birds also may preclude hormone level versus handling time correlations. This finding may indicate that any reaction of hormone levels to stress occurs very quickly, and changes little in the short-term. Alternately, it simply may reflect the types of stress inflicted. As noted previously, birds held for 15 min were kept in the hand, while 30 min individuals were placed in small cloth bags. Perhaps being tightly immobilized in view of one's captor is more stressful than being able to move around slightly in a dark place. Only additional studies, with such concerns originally in mind, can answer this question.

In any case, experimental birds were undoubtedly stressed considerably more than those sampled normally (non-experimentals), and thus should reflect more extreme reactions to stress than exist in normal samples. Most normal samples were collected in less than 15 min, and from previously uninjured birds. Test birds already had been bled once, before being restrained for 15 or 30 min and bled again.

Even if hormone levels of non-experimental samples were affected somewhat by stress, it seems that for the hormones examined in our study, stress merely suppresses differences between means of breeding stage groups (e.g., prelaying versus incubation versus brooding), but does not create or inflate such differences. This would be so if stress always causes a decrease in initially high values, and an increase in those initially low, as found for testosterone in this study (see also Moore and Zoeller 1985), and suggested for prolactin (see Opel and Proudman 1982). The effect of handling and bleeding on circulating levels of hormones obviously is not straightforward, yet has been for the most part ignored in the avian

literature, and deserves much further study. Subsequent experimental studies should standardize methods of holding birds, and include non-incubating as well as incubating individuals. Nevertheless, the preliminary investigation reported here illustrates some intriguing patterns, and suggests several more.

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