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The Adrenocortical Response to Stress in Male Bush Warblers, *Cettia diphone*: A Comparison of Breeding Populations in Honshu and Hokkaido, Japan

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ABSTRACT—We compared the adrenocortical responses to acute stress in two free-living populations of bush warblers, *Cettia diphone* by measuring the increase in circulating levels of corticosterone in response to capture, handling and restraint over one hour. We wished to test the hypothesis that populations living in more severe habitats with shorter breeding seasons are likely to suppress the adrenocortical response to acute stresses during the nesting phase. We chose the bush warbler because males show no parental care which also has been shown to be an ecological correlate of stress modulation. Male bush warblers responded to playback of songs and long calls with aggressive behavior that was identical at the two sites (Furano, Hokkaido and Chichibu, Honshu). Plasma levels of testosterone were also similar in the two populations suggesting that they were in the same reproductive state when tested. Males of both populations showed marked increases in circulating corticosterone levels during the capture stress protocol. We assumed that at the northern site, Furano, bush warblers would have a reduced adrenocortical response to stress compared with Chichibu (the southern site). Males at Furano, however, showed higher initial plasma levels of corticosterone and showed a greater response compared with males at Chichibu. Relatively less individual variation in the adrenocortical response to stress was seen at Chichibu compared with Furano, and less than in other passerine species studied to date. There was no relationship of body mass or fat score to initial corticosterone level, maximum level generated over one hour of capture stress, or the rate and percent increases of corticosterone. These data indicate that stress modulation may not be a simple consequence of shorter breeding season and more severe climate. Other ecological bases, as yet unknown, are involved.

INTRODUCTION

There is growing evidence that unpredictable events in the environment such as severe inclement weather, predation attempts and habitat disturbance, may result in temporary modification of behavior and physiology of free-living birds. These facultative, or "emergency", behavioral patterns re-direct an individual's behavior and physiology toward survival and away from the normal homeostatic state relevant for the time of year (e.g. breeding, migration, winter etc.). As soon as the disturbance passes, or alternate suitable habitat is found, then the individual returns to the normal behavioral and physiological state appropriate for the time of year [12, 13]. There is also expanding evidence that these facultative behavioral patterns and accompanying changes in physiology are similar to those observed during the initial responses (i.e. within minutes to hours) to a variety of acute stress stimuli [1]. Furthermore, rises in circulating levels of corticosterone occur in a fashion identical to those induced by stress in most vertebrates, and appear to orchestrate the rapid transition to the emergency state [13, 17]. We acknowledge that other hormones may be involved, especially if the facultative behavioral pattern is triggered in different habitats and contexts. However, the evidence thus far indicates that rises in corticosterone are a common and central component of this response.

Although the triggering of a facultative behavioral pattern in response to unpredictable events in the environment may be adaptive insofar as the individual avoids becoming severely stressed, it appears that at least the acute responses of the hypothalamo-pituitary adrenal axis to stress can be modulated. In some avian species the rate of corticosterone increase in blood, and the maximum corticosterone level generated over one hour of capture, handling and restraint can vary as functions of population and time of year, as well as among individuals within a population [9, 13]. It has been proposed that decreased sensitivity of the adrenocortical response to acute stress during the breeding season may be widespread in environments in which local conditions are frequently severe (e.g. deserts, [20]) and especially when the breeding season is very short (e.g. high latitude, [18]). Such

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modulation of the adrenocortical response to stress may allow birds breeding in such habitats to withstand potentially stressful conditions so that they can breed successfully [12, 13].

Field endocrinology has the potential to provide an experimental basis for ecological aspects of endocrine function and explain why the adrenocortical response to stress is modulated. Investigation of adrenocortical responses of individuals in different environmental settings and at different times of year is one useful approach. Another is to compare several species in similar and varied habitats in an attempt to identify ecological bases for differences in endocrine function. A study of Sonoran Desert birds during the breeding and non-breeding season showed clearly that those species living in the driest desert had a lower rate of increase, and reduced maximum plasma levels of corticosterone when breeding. Moreover, these adrenocortical responses to capture stress were lower than in those species breeding in riparian habitats with permanent water and extensive shade [20]. Investigations of several avian species that breed in the Arctic showed that some had relatively low adrenocortical responses to capture and handling stress, whereas others actually increased their sensitivity to stress [14, 17, 19]. Thus, although short breeding seasons in a severe environment may have selected for reduced sensitivity of the hypothalamo-pituitary-adrenal axis to stress in some species, other ecological factors may also be in effect.

A recent analysis of several arctic species by Wingfield *et al.* [17] showed that among about 15 species the major correlate of reduced adrenocortical sensitivity to acute stress was the degree of parental care provided. The rate of corticosterone increase, and the maximum corticosterone level generated over one hour of capture and handling stress, was lower in individuals that were incubating or feeding young. This trend was apparent regardless of sex. Thus there may be multiple interdependent ecological factors that have selected for modulation of the adrenocortical response to acute stress. Careful selection of species in which one or several of these possible ecological factors can be eliminated or controlled could provide valuable insight into this approach to environmental endocrinology.

We have chosen the bush warbler, *Cettia diphone*, as a model for comparisons of latitude and climate on the adrenocortical response to stress. The bush warbler is an abundant member of the family Sylviidae that breeds in mountain forests throughout much of Japan. We compared two populations: one in central Honshu with diverse broad leaf forest, and the second in central Hokkaido with taiga-type forest of mixed coniferous and deciduous trees and less diversity. The breeding season in central Hokkaido is shorter than in Honshu. We focused on male bush warblers because they are territorial throughout the breeding season and show no parental care [4]. In this way we were able to eliminate one ecological factor-parental behavior, and focus more on population and individual differences in the adrenocortical response to stress with habitat.

MATERIALS AND METHODS

Study sites, capture techniques and collection of samples

Male bush warblers were captured in Japanese mist nets at two field sites: the University of Tokyo Forest at Furano in central Hokkaido (43°20'N, 142°30'W), and the University of Tokyo Forest at Chichibu in central Honshu (35°55'N, 138°52'W). Although the Furano Field Station is not at high latitude, the climate is typical of northern continental Asia with cold winters and cool summers (Table 1). Bush warblers frequented thick undergrowth, particularly bamboo grass, *Sasa senanensis*, between 600 and 1,500 m elevation. The forest here is a mixture of deciduous and coniferous trees, but dominated by conifers. The climate at Chichibu is typical of central-southern continental Asia with cool winters and warm humid summers. Bush warblers frequented luxuriant undergrowth of the mountain forests (1,000–1,900 m elevation), particularly areas of bamboo grass (*Pseudosasa purpurascens* below 1,700 m and *Sasa nipponica* above 1,700 m). The forests at Chichibu are diverse with over 250 species of trees. Bush warblers breeding in central Hokkaido are migrants and have a shorter breeding season than in central Honshu and are also exposed to lower average temperatures (Table 1). At the Chichibu site, bush warblers breeding at higher altitudes also migrate, but only a short distance to the adjacent lowlands.

Table 1. Weather data for Furano* field station (Hokkaido) and Chichibu** field station (Honshu)

Year round average	Precipitation mm	Temperature °C	Minimum temperature	Maximum temperature
Furano	1,300	6.8°	1.1°	12.0°
Chichibu	1,393	10.9°	6.3°	14.2°

June 1993	Precipitation mm	Minimum temperature °C	Maximum temperature °C
Furano	94.5	2.5°	24.0°
Chichibu	221.5	8.9°	27.7°

July 1993	Precipitation mm	Minimum temperature °C	Maximum temperature °C
Furano	18.5	9.0°	30.0°
Chichibu	265.5	10.3°	29.0°

* Weather data from Furano were collected at Yamabe, 43°15'N, 142°23'E, and at 220 m altitude.

** Weather data from Chichibu were collected Tochimoto, 35°56''N, 138°52'E, at 770 m altitude for the year round averages. In June and July 1993, weather data were collected at Yatakezawa (the nearest station to our study site in Chichibu), 35°56'N, 138°49'E, at 1050 m altitude.

To check weather conditions at each site, we tabulated total precipitation as well as average, maximum and minimum temperatures year round (Table 1). Additionally, for June and July 1993 we include the total precipitation at each site, and the average (taken at 09.00 h each day), maximum and minimum temperatures. Weather data from Furano were collected at Yamabe, 43°15'N, 142°23'E, and at 220 m altitude. Weather data from Chichibu were collected

Tochimoto, 35°56"N, 138°52'E, at 770 m altitude for the year round averages. In June and July 1993, weather data were collected at Yatakezawa (the nearest station to our study site in Chichibu), 35°56'N, 138°49'E, at 1050 m altitude.

All bush warblers were captured from mid-June to early July. They were highly territorial and attracted into mist nets by playing tape-recorded songs through a speaker placed next to an erected net. Warblers were removed from the net and a blood sample collected as soon as possible after capture (within 2 min). In some cases blood samples were collected into 4–6 heparinized capillary tubes (ca. 200 μl) from the wing (alar) vein for measurement of circulating levels of testosterone. In other cases, birds were sampled as part of the capture stress protocol described below. After blood sampling all birds were weighed to the nearest 0.1 g, and length of the wing, culmen and tarsus measured to the nearest 0.1 mm. Fat score in the furculum and abdomen was assessed on an arbitrary scale of 0–5 in which 0=no fat and 5=gross bulging fat depots (see [15]). After sampling all birds were released on, or close to, the same territory from which they were captured.

Capillary tubes containing blood were sealed at one end with molding clay and then secured in small plastic canisters on ice until returned to the laboratory 2–6 hr later. Here the blood was centrifuged, plasma harvested and stored frozen until assay. Plasma samples were transported on dry ice to Seattle.

Capture stress protocol

To assess the sensitivity of the hypothalamo-pituitary-adrenal axis to acute stress we collected a single capillary tube (about 30 μl) from blood from the wing vein of male bush warblers as soon as possible after capture (usually within 1–2 min). We call this sample Min-1 for convenience. Further samples (one capillary tube each) were then collected at 5, 10, 30 and 60 min after capture. In the interim, birds were weighed and measured, and held in cloth holding bags. This procedure has been described in detail by Wingfield *et al.* [14, 19, 20]. Since capture, handling and restraint have been shown to result in an elevation of circulating corticosterone in several species of birds (e.g. [6–8], we feel that this protocol is the most useful way of comparing adrenocortical responsiveness to stress among individuals within a population as well as comparing different populations (see [13, 14, 17, 19]). Because it is not possible to collect unstressed samples as a control in the field, we plotted the initial plasma level of corticosterone (i.e. Min-1 sample) as a function of time of day of capture to show that no diurnal changes in baseline levels of corticosterone could have confounded the adrenocortical response to stress (see [13, 20] for details).

Behavioral observations

During each period of song playback we observed the following behaviors over a 10 min interval or longer if the response time was greater: 1. "Latency to Respond", i.e. the time taken for a male bush warbler to approach or sing in response to the playback of tape-recorded song on his territory. 2. "Number of Songs" given during the playback period. 3. "Number of Long Calls" (a long series of loud notes associated with territorial behavior typical of this species, 5). 4. "Closest Approach", estimated in meters from the source of the playback, i.e. the speaker.

The bush warbler is highly territorial and apparently forms no pair bond with females. There may be more than one female nesting on a male's territory, but males provide no parental care [4]. Therefore all samples from males can be compared as they were collected at the same time of year and in the same stage of breeding. There is no potential confounding issue of whether males were parental or not.

Hormone assays

Plasma levels of testosterone and corticosterone were measured by radioimmunoassay after extraction of the plasma with 5 ml of redistilled dichloromethane. Approximately 2,000 cpm of respective tritiated steroid were added to the plasma prior to extraction for calculation of percent losses during processing. Corticosterone was measured directly in dried extracts taken up in phosphate-buffered saline whereas testosterone was measured after partial purification on diatomaceous earth/glycol columns. All samples were assayed within a single assay to avoid interassay variation. Intra-assay variation in these procedures is less than 12%. Details of these assays have been published elsewhere [2, 20].

Statistical analysis

Plasma levels of testosterone, initial levels of corticosterone (i.e. Min-1 samples), body mass, fat score and latency to respond to playback were compared with locality by Student's t-test (unpaired, 2 tailed). Changes in corticosterone levels following capture and during handling and restraint were compared by two way analysis of variance (ANOVA) for repeated measures and locality. Post hoc tests were by Fisher's least significant difference tests (PLSD).

We also compared individual characteristics of the adrenocortical response to stress as follows (see also [13, 14, 17, 19] for full details). Individual variation in the dynamics of corticosterone levels following capture and handling can be great. We used Spearman rank correlation to look for possible relationships between body mass and fat score of individuals with initial corticosterone level (Min-1 sample), maximum corticosterone level generated during the capture stress protocol, rate of increase and percent increase of corticosterone. Similarly, we also used Spearman rank correlation to compare testosterone levels as a function of time of exposure to song playback because it has been shown in other passerines that territorial challenges can result in an elevation of circulating testosterone levels in males [11]. Finally, we also used this technique to look for possible relationships of behavioral responses to the playback protocol and baseline plasma levels of corticosterone.

RESULTS

Weather Data

The yearly average precipitation is similar at Chichibu and Furano (Table 1). However, the average temperature is about 4°C cooler at Furano. This is apparently due to a lower average high temperature (Table 1). For the months of June and July 1993, precipitation was 2–14 fold higher at Chichibu, but temperatures were similar-especially in July (Table 1). However, in June (i.e. early breeding season), minimum temperature was 6°C cooler at Furano and close to the freezing level.

Plasma levels of testosterone and corticosterone in relation to territorial behavior of male bush warblers

Behavioral observations of male bush warblers during the protocol of song playback showed territorial behaviors typical of most passerine species. Over a 10-min-observation period males responded with a mean of 21.17±

4.54 songs, 5.5±2.9 long calls (n=6). They approached an average of 17.5±5.7 M (n=6) to the speaker during the playback observation period. Latency to respond to the playback protocol was the same at Furano and Chichibu field sites (2.42±0.42 min (n=17) at Furano and 4.41±1.2 min (n=19) at Chichibu; t=−1.47, p=0.075). Similarly, average plasma levels of testosterone were also identical in each group of males, 3.12±0.31 (n=9) at Furano, and 2.85±0.43 (n=11) at Chichibu, (t=−0.487, p=0.632). These data suggest that the two populations of males were in comparable reproductive and territorial states. However, if we compared the relationship of circulating testosterone levels and length of time exposed to the playback protocol, there was a significant negative correlation in males sampled at Furano (Fig. 1, upper panel, z=−2.35, p=0.0186) but not at Chichibu (Fig. 1, lower panel, z=0.66, p=0.5089). Duration of playback protocol prior to capture had no significant effect on baseline corticosterone levels (Spearman Rank Correlations, z=1.412, p=0.158 at Furano, and z=−0.439, p=0.661 at Chichibu).

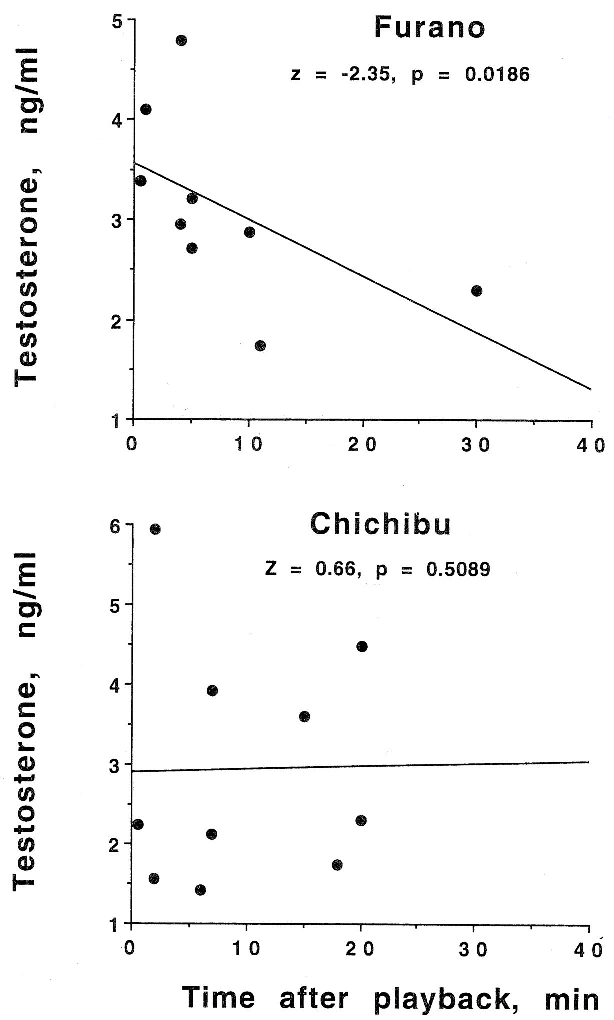

Fig. 1. Spearman Rank Correlations of plasma testosterone levels with time of exposure to the playback protocol in male bush warblers, *Cettia diphone*, sampled at Furano and Chichibu.

Adrenocortical responses to the capture stress protocol

Two way ANOVA for repeated measures showed a highly significant effect of time after capture on plasma levels of corticosterone (Fig. 2, F=18.565, p=0.0001, total Degrees of freedom=48) that was different at the two localities (Fig. 2, F=5.372, p=0.039). However, there was no interaction of locality and time after capture (F=0.535, p=0.711). At both localities (Fig. 2), plasma levels of corticosterone had increased by 10 min post-capture (p<0.05, Fisher's PLSD), and had reached a plateau by 30 min (p<0.05, Fisher's PLSD). Initial plasma levels of corticosterone (Min-1 and close to the baseline levels) remained at or below about 40 ng/ml throughout the day (Fig. 2, lower panel) suggesting that time of day was not a significant factor in the elevations of corticosterone levels during the capture stress protocol.

At the Furano field site, male bush warblers showed

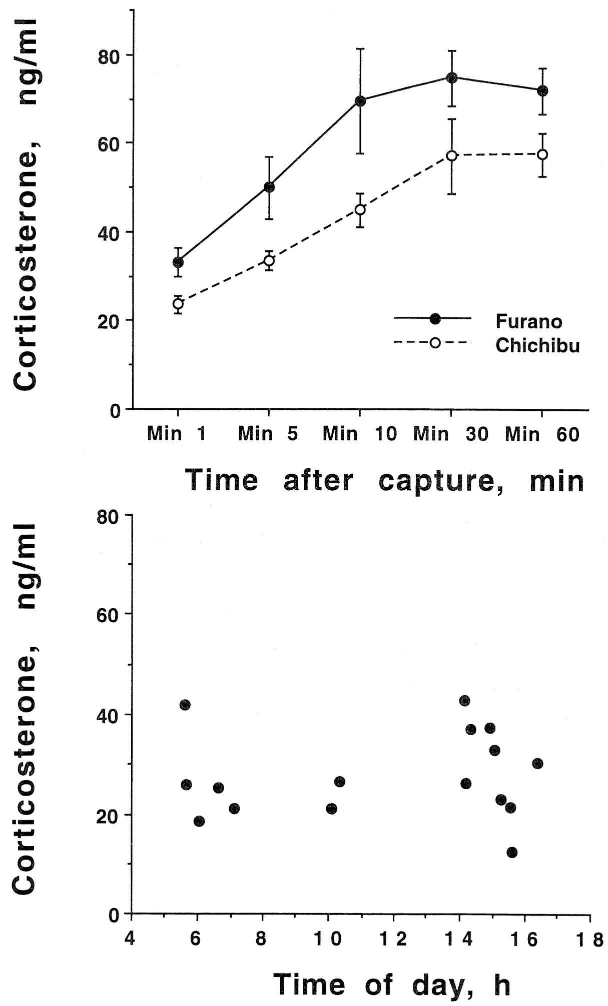

Fig. 2. *Upper panel.* Increase in plasma levels of corticosterone following capture, handling and restraint in male bush warblers, *Cettia diphone* at Furano and Chichibu. Points are means and vertical bars the standard errors. N=8 at Furano and n=10 at Chichibu. *Lower panel.* Initial plasma levels of corticosterone (Min-1) plotted as a function of time of day of capture for both field sites combined.

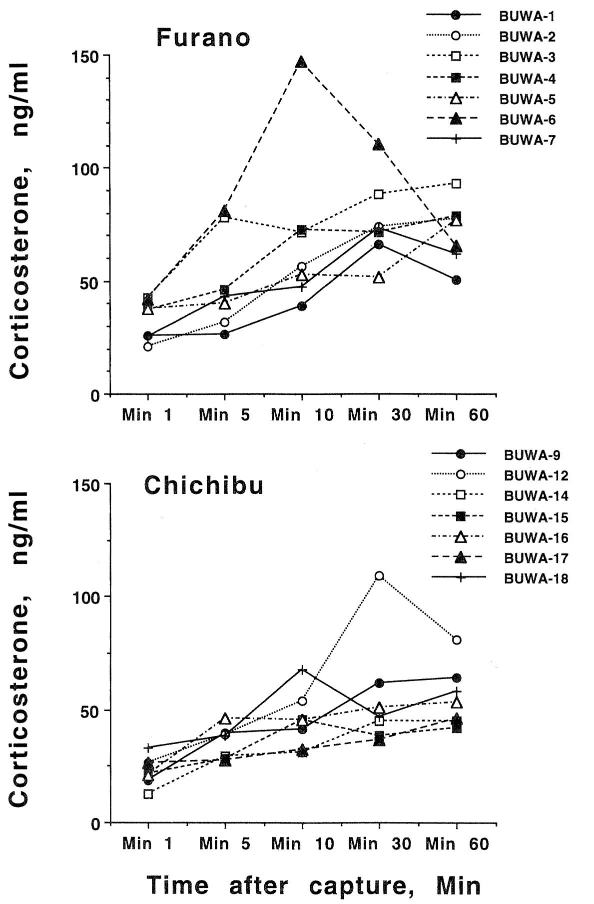

FIG. 3. Individual variation in the rise of corticosterone levels during capture, handling and restraint in male bush warblers, *Cettia diphone*, sampled at Furano (upper panel) and Chichibu (lower panel). BUWA refers to the standard abbreviation of species name (BUsh WArbler).

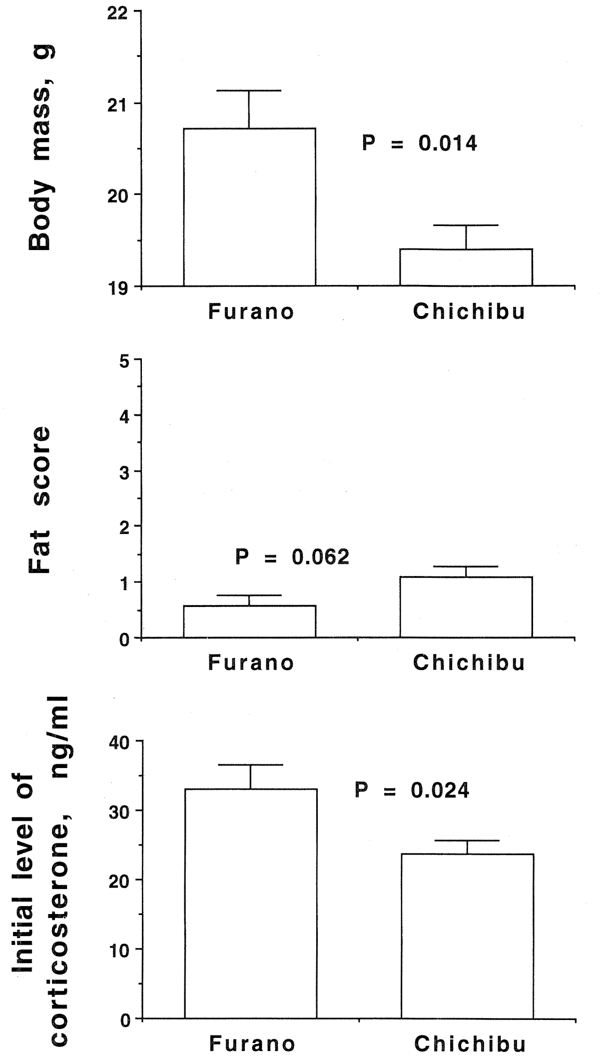

FIG. 4. Comparisons of body mass (upper panel), fat score (middle panel) and initial level of corticosterone (lower panel) in male bush warblers, *Cettia diphone*, sampled at Furano and Chichibu. Means ± standard errors.

marked differences in individual adrenocortical responses to stress (Fig. 3, upper panel). Compare BUWA-7 that showed a very low amplitude change in plasma corticosterone levels with BUWA-6 that showed a dramatic increase by Min-10 and then a decrease. Individual variation in adrenocortical responses to the capture stress protocol were more uniform at Chichibu (Fig. 3, lower panel) with the possible exception of BUWA-12. However, this latter variant was due only to one high corticosterone level at sample Min-30.

Individual variations in adrenocortical responsiveness to the capture stress protocol as a function of body mass and fat depot

Male bush warblers at Furano were heavier than males at Chichibu (Fig. 4, upper panel, t=2.76, p=0.014, n=8 and 10 respectively), but had similar fat scores (Fig. 4, middle panel, t=−2.00, p=0.062, sample sizes same as for body mass). Initial levels of corticosterone were, however, lower in males at Chichibu (Fig. 4, lower panel, t=2.524, p=0.024, n=7 and 9 for Furano and Chichibu respectively). There were no differences in maximum corticosterone levels generated during the capture stress protocol (84.33±9.48 ng/ml at Furano and 66.14±8.17 ng/ml at Chichibu, t=1.46, p=0.16, n=8 and 10 respectively-note that these maxima were reached at different times among individuals and thus these means differ from the averages at 30 and 60 min in Fig. 2), rate of corticosterone increase (2.98±1.30 ng/ml/min at Furano and 1.83±0.34 ng/ml/min at Chichibu, t=1.00, p=0.33, n=7 and 10, respectively), or percent corticosterone increase (267.9±23.7 percent at Furano and 268.9±25.3 percent at Chichibu, t=−0.029, p=0.98, n=7 and 10, respectively).

There were no significant relationships of initial corticosterone level, maximum corticosterone concentration generated during the capture stress protocol, rate of corticosterone increase, or percent increase with body mass or fat score. This was true for Spearman rank correlations at both localities and when data for Furano and Chichibu were combined.

DISCUSSION

The bush warbler is a highly territorial passerine in which males are polygynous and provide no parental care. High plasma levels of testosterone in June and July are typical of polygynous males and higher than would be expected for other monogamous passerines in which males show parental care and have much lower levels of testosterone [16]. It would be of great interest to collect further samples from male bush warblers to determine whether testosterone levels remain high throughout the breeding season. The data presented here suggest that male bush warblers at Furano and Chichibu were in a similar reproductive phase and were territorial. Responses to playback of tape recorded songs were typical of other territorial male passerines (e.g. [11]). Latency to respond to the playback stimulus was the same at each locality.

Since some male bush warblers were exposed to the playback stimulus for up to 30 min before capture, we were concerned that this arousal might have an effect on endocrine state, e.g. plasma testosterone level, and circulating concentrations of corticosterone. Wingfield *et al.* [16] suggested that playback of conspecific songs, that mimic a territorial challenge, will result in an increase in plasma levels of testosterone. However, the degree to which species respond varies and appears to be related to mating system and breeding strategy. It was proposed that monogamous species in which males provided most parental care would be most responsive to playback and those males that show no parental care would be least responsive. Male bush warblers did not show any increase in plasma levels of testosterone in response to playback of tape recorded songs (Fig. 1). Indeed at Furano, males tended to show a decrease in testosterone as the duration of playback increased.

It is also worth noting here that in Chichibu there appeared to be two groups of birds with high (>3.5 ng/ml) and low (<2.5 ng/ml) testosterone levels (Fig. 1, lower panel). In Furano, on the other hand, testosterone levels were mostly in the mid- to high range (1.4–6 ng/ml, Fig. 1, upper panel). It is possible that testosterone levels may fluctuate because of variations in male territorial behavior. Although males are polygynous and provide no direct parental care, they do mate-guard when a potential mate is receptive (there are always unmated males around the territory who could also mate with a receptive female). At least in Chichibu, after the female is incubating and no longer receptive, the male may shift his territory slightly and try to attract another female [5]. However, these males may provide some indirect parental care because they give long, loud alarm calls ("taniwatari") that females (and thus young) respond to (H. Momose, personal communication; [5]). Thus at this time testosterone levels in males may be lower. In Furano where the breeding season is shorter, onset of nesting may be more synchronous and there is less variation in testosterone among males. We have no explanation for this phenomenon at present, but further studies on the interrelationships of testosterone and aggression in this species would be needed to gain insight into this unique finding.

Plasma levels of corticosterone were not affected by the playback procedure suggesting that the general capture methods did not influence baseline levels of corticosterone in relation to the studies of adrenocortical responses to stress. Male bush warblers showed a marked increase in circulating corticosterone following capture, handling and restraint (Fig. 2). This is similar to many other studies on the effects of handling in birds (e.g. [6–8, 18]), including free-living species [14, 17–19]. The original hypothesis predicted that the adrenocortical response to stress at Furano-the northern site with the shortest breeding season and lower average temperature (Table 1)-would be less than at Chichibu. The opposite trend was found. Baseline levels of corticosterone (i.e. the Min-1 sample) and the increase in corticosterone following capture and handling were higher at Furano. Why this should be so is unclear. Many ecological factors could contribute to differences in the adrenocortical responses to stress in different populations. Here, high latitude and more severe climate do not appear to have resulted in suppressed adrenocortical responsiveness to acute stress. In arctic species, some appear to have reduced increases in corticosterone in response to stress, e.g. snow bunting, *Plectrophenax nivalis* and redpoll, *Acanthis flammea*, at Barrow Alaska [14, 19]. The Lapland longspur, *Calcarius lapponicus*, breeding on the North Slope of Alaska also appears to have a low rate of response to the capture stress protocol. However, after a three day snow storm in June the rate of increase in plasma levels of corticosterone following capture and handling was almost an order of magnitude higher than before the storm [1]. Clearly the responsiveness of the hypothalamo-pituitary-adrenal axis to acute stress can be modulated both up and down and within a short time period to allow maximum adaptation to unpredictable fluctuations in the environment.

Previous studies have shown that in addition to a population difference in responsiveness to acute stress, there may also be individual variation. At least in high latitude species, this variation may be related to body mass and fat depot. Individuals in better condition should be more resistant to the effects of acute stress on the hypothalamo-pituitary-adrenal axis [10, 13, 14, 19]. However, individual variation in the increases of corticosterone following the capture stress protocol were not great at Chichibu. At Furano, individual variation in the adrenocortical response to stress was greater, but this due primarily to one individual. The rest were more uniform compared with great variation in snow buntings and Lapland longspurs at Barrow, Alaska (Fig. 3, [19]). Further analysis revealed that in male bush warblers, there were no significant relationships of initial corticosterone levels, maximum corticosterone titer generated during the capture stress protocol, rate of corticosterone increase following capture and percent increase, with body mass or fat depot either at Furano or Chichibu. This may be because the climate at Furano is not sufficiently severe to select for mechanisms that allow breeding in the face of inclement weather in spring.

Certainly the fat scores of male bush warblers at both Furano and Chichibu are lower than those of male snow buntings and Lapland longspurs breeding at Barrow, Alaska, which suggests that male bush warblers do not need to store fat for potential contingencies of severe weather. Furthermore, individual variation of the adrenocortical response to stress in bush warblers appears less than in arctic birds suggesting that suppression of the adrencortical response to stress may indeed be a function of energy reserves in individuals. Further research is needed to tease apart these ecological bases of differences in endocrine function.

The higher adrenocortical response to acute stress in male bush warblers at Furano remains a mystery. It is possible that since male bush warblers show no parental care, then there is no selective pressure to resist effects of acute stress during the parental phase. If this is true, then females should show suppression of the adrenocortical response to stress at Furano. However, it should be noted that baseline levels of corticosterone were also higher in males at Furano. Whether this suggests a simple phylogenetic difference or a relationship to migratory distance remains to be determined.

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