The stress series protocol

Responses to modifying information can be assessed by measuring changes in plasma levels of glucocorticosteroids secreted by the adrenal gland (Wingfield 1994). This is also known as the 'adrenocortical response to stress' and is common to virtually all gnathostome vertebrate classes. A simple comparison of baseline plasma levels of glucocorticosteroids in free-living versus captive populations could indicate quickly whether non-breeding captive birds had higher plasma levels of corticosterone than free-living birds sampled at the same time. If so, then captive conditions may be 'stressful.' One well-known effect of prolonged (chronic) high levels of glucocorticosteroids is complete inhibition of reproductive function. Certainly, if captive populations are stressed, then reproduction will be compromised — information that is vital to captive breeding programs. Recent work suggests that fecal and urinary levels of steroids also can be used to indicate responsiveness to modifying factors, especially in cases when reproduction is impaired (e.g., Creel et al. 1992, 1996; Wasser 1995). Fecal and urine samples usually can be collected without disturbing the subjects, so the technique may prove especially useful for monitoring critically endangered species.

The hormonal response to modifying factors is common to a wide spectrum of stimuli, including capture and handling. Capture and restraint results in a rapid increase of glucocorticosteroids, usually within 5–10 minutes, and reaches a maximum within 30–60 minutes (Wingfield 1994). Serial bleeding adds to the cumulative effects of handling stress. As an example, a very small (20–30 μl) sample of blood from the wing vein of a bird may be collected as soon as possible after capture (e.g., Wingfield & Farner 1976). The bird is then held in a cloth bag for 60 minutes and further samples collected at 5, 10, 30, and 60 minutes after capture for measurement of changing glucocorticosteroid levels. Under field conditions, blood samples can be stored on ice until return to the laboratory where it must be centrifuged, at about 2000 rpm for 5 minutes, and the plasma then harvested (Wingfield & Farner 1976). Plasma can be stored at −20°C for several months until assayed for glucocorticosteroid content. If field sites are remote, then blood can be centrifuged in the field using an automobile battery as a power source, or a hand operated centrifuge if power is not available. Plasma can be stored on dry ice in a cooler (2–3 kg will last about three days in a regular cooler) or in a liquid nitrogen container (some are designed for field use and can keep plasma samples frozen for several weeks, depending upon size of the container).

Examples of the pattern of glucocorticosteroid secretion during the capture stress protocol are given in Fig. 5.1. Note that plotted baseline levels, such as at the time of capture, versus time of day indicate whether natural changes in glucocorticosteroid secretion could confound our results. This is as close to a true control as we can obtain for this procedure in the field. Extensive data thus far show that diurnal changes in baseline levels of glucocorticosteroids are not responsible for the marked elevations following capture and restraint (Wingfield 1994; Wingfield et al. 1994a,b). The collection of capture stress series in free-living birds and captive populations can, therefore, tell us a great deal about sensitivity to modifying factors and current conditions. Changes in circulating levels of glucocorticosteroids are sensitive to human disturbance as well as other types of disturbances, such as natural environmental conditions, disease, or exposure to pollution. Data in the field indicate that sensitivity to the capture stress protocol may change with season and reproductive state as well as with the body condition of individuals (e.g., Wingfield et al. 1994a,b).

In general we feel that a rapid and steep increase in plasma levels of glucocorticosteroids during the capture stress protocol indicates greater sensitivity of the adrenocortical axis to stress than does a shallow and slow increase. We have found, for example, that although baseline (i.e., within 1 minute of capture) glucocorticosteroid levels may be similar in captive and free-living birds, captives tend to have a greatly enhanced elevation of glucocorticosteroids following restraint. The collection of stress series from males and females in breeding and non-breeding seasons would also be an essential start in determining sensitivity to modifying factors at two important stages in the life cycle.

Further analysis in relation to body mass, condition, fat score, and other variables reveals additional information at the individual level (Wingfield 1994). Within a sampled cohort of individuals, there may be little or great variation in the pattern of glucocorticosteroid levels during the capture stress protocol (Fig. 5.2). Such data are accumulated over time and eventually provide critical evaluation at the individual level, especially as lower sensitivity to modifying factors (i.e., a flat pattern of glucocorticosteroid release following capture and restraint) is often correlated with good body condition, whereas a steep pattern of glucocorticosteroid release (Fig. 5.2) may be related to poor body condition (e.g., Smith et al. 1994; Wingfield et al. 1994a,b; Dunlap & Wingfield 1995).
Fig. 5.1. (a) Schematic representation of the cascade of events that follows an increase in corticotropin-releasing factor secretion triggered by a modifying factor such as habitat modification. Corticotropin levels rise a few seconds later followed by release of corticosterone from the adrenal gland (after Wingfield 1994). (b) The profile of corticosterone levels in the plasma of the Japanese white-eye (Zosterops japonica) during capture, handling, and restraint for 1 hour. Points are the means and vertical bars standard errors. Note how the level of corticosterone increases as suggested in (a).

Fig. 5.2. Individual variation in the corticosterone profiles during capture, handling, and restraint in male and female Common Amakihi (Hemignathus virens). Numbers in the figure key represent individual band numbers.
Measurement of glucocorticosteroid levels in plasma

An important initial step is to determine which glucocorticosteroid is found in the species of interest. Cortisol is the major glucocorticosteroid of humans and some other mammals and all teleost fish, whereas corticosterone is found in other mammals and all non-mammalian tetrapods (e.g., Gorbman et al. 1983). Cortisol and corticosterone differ by the addition of a single hydroxyl group, but their biological actions are identical. The antisera generated against corticosterone, however, usually do not cross-react with cortisol (and vice versa). Thus it is critical to determine which hormone is the major glucocorticosteroid in your subject species. Consulting Gorbman et al. (1983) as a standard textbook is one way to do this. More detailed information can be found in Idler (1972).

The two most sensitive methods to measure circulating levels of steroid hormones are by radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA). Both can use small samples (down to 5 µl in volume), but in our experience RIA is still a preferable method in terms of inter-assay variability. Certain items of equipment required, however, are expensive, and the assay itself requires some training. It is strongly advised that an investigator unfamiliar with the technique makes contact with an endocrinology laboratory and inquires about possible collaborations. Most larger research universities will have at least one laboratory of this type. Others may have to inquire further afield to make appropriate contact. The effort is well worth the initial outlay because the technique provides large quantities of information once it is established. Detailed descriptions of the RIA procedure for steroid hormones, and particularly glucocorticosteroids, can be found in Wingfield & Farner (1975) and Wingfield et al. (1994a,b).

Examples in free-living populations

Thus far, we have described the changes of plasma glucocorticosteroid levels observed during stress, the effects of these steroids on facultative behavioral and physiologic patterns, and the methods by which investigators can collect appropriate samples and measure hormone levels. We will next give several examples of how the patterns of glucocorticosteroid secretions following stress can be applied to monitor free-living populations. Where data exist, we point out their direct relevance to conservation biology.

Adrenocortical responses to stress in Hawaiian honeycreepers

From January to March 1993 we collected blood samples and data on reproductive function from endemic forest birds of the Island of Hawaii (Hawaiian honeycreepers, Drepanidinae, Fringillidae). This subfamily has many critically endangered species, and we hope these studies will provide useful baseline information on the regulation of their breeding cycles. The following taxa are the focus of investigations: Apapane (Himatione sanguinea); Common Amakihi (Hemignathus virens); and Iiwi (Vestiaria coccinea), in the Hakalau Forest National Wildlife Refuge, Hawaii. Our questions are: do these insular island birds have an adrenocortical response to stress that is similar to related continental species (i.e., testing the generality of the capture stress protocol), and is it indeed possible to collect these types of samples without seriously debilitating the free-living birds?

We use the stress series protocol as a measure of responsiveness to stress in general in pre-breeding populations of Drepanidinids. In the Common Amakihi there is a significant rise in corticosterone following capture and handling (Fig. 5.3a, $F = 16.161, p < 0.0001$, two-way ANOVA for repeated measures). There is no difference in the response between males and females ($F = 3.514, p = 0.0936$). Similarly in Iiwi, the increase of corticosterone levels following capture and handling (Fig. 5.3b, $F = 11.286, p < 0.0001$) is similar in males and females ($F = 2.477, p = 0.1466$) but rather less dramatic than in the other species. Very few female Apapanes were captured, and so both sexes are combined in Fig. 5.3c). Again, there is a highly significant increase in corticosterone following capture and handling ($F = 10.503, p < 0.0001$).

Relationships of the adrenocortical response to stress with body condition

Although capture and other stresses can result in marked increases in adrenocortical hormones such as corticosterone, there is also considerable variation in the degree to which individuals respond to the same stress. Hawaiian honeycreepers appear to show similar variation. In Common Amakihis (Fig. 5.2), both males and females exhibit large variations in not only the rate of increase in corticosterone following capture, but also the maximum corticosterone level generated by 60 minutes of capture and handling stress. Note that some (e.g., bird numbers M 92416 and M 92456, Fig. 5.2) have very little or no adrenocortical response to stress, whereas others (e.g., M 52824 and F 33198)
Fig. 5.3. Changes in mean (± SE) plasma levels of corticosterone following capture, handling, and restraint in free-living Drepanidinids at Hakalau National Wildlife Refuge, Hawaii.

Fig. 5.4. Individual variation in the corticosterone profiles during capture, handling, and restraint in male and female liwi (Vestiaria coccinea). Numbers in the figure key represent individual band numbers.

undergo a 5- to 20-fold increase in circulating corticosterone levels following similar treatment. Similarly, in the liwi (Fig. 5.4), both sexes show wide variation in rate of corticosterone increase and the maximum corticosterone level generated following stress (e.g., compare numbers 843 and 850 with 941 and 404).
Wingfield (1994) suggests that this variation may represent different degrees of resistance to stress possibly correlated with factors such as body condition, sex, and reproductive state. Accordingly, we have correlated different aspects of the adrenocortical response to stress during capture and handling with body condition (i.e., body mass and fat score) in Hawaiian honeycreeper species. In these species, the adrenocortical response to stress can be separated into distinct components, including initial level of corticosterone (i.e., at capture), rate of corticosterone increase over the 60 minute sampling period, percent increase in corticosterone, and maximum level generated during the stress series. These data can then be correlated with sex, body mass, fat score and reproductive state (see Wingfield 1994 for review of other species). As all birds in this study were pre-breeding, we cannot assess the effects of breeding status on responsiveness to stress, but we can investigate the effects of body condition.

The body masses of male and female Common Amakihi are similar (Fig. 5.5, $t = 1.233, p = 0.2364$, unpaired, DF = 15), whereas females have a higher fat score ($t = 2.483, p = 0.0253$, unpaired, DF = 15). In the Liiwi, males are heavier than females (Fig. 5.5, $t = 4.638, p = 0.0002$, unpaired, DF = 18), but females have a higher fat score ($t = 3.205, p = 0.0049$, unpaired, DF = 18). A comparison of fat score across species indicates that there is significant variation ($F = 16.896, p = 0.0001$, total DF = 46). Female Liiwi appear to have higher fat scores than all other species and sexes ($p < 0.05$, Fisher’s PLSD test). Male Common Amakihi have significantly less fat than all other species and sexes ($p < 0.05$, Fisher’s PLSD test). Correlations of body condition and stress responses are considered within species below.

**Common Amakihi**

There is a significant correlation of body mass and fat score in males, but this is not significant in females. When data for both sexes are combined, however, a significant relationship reappears (Table 5.2). The only correlations of body mass or fat score with components of the adrenocortical response to stress that are significant are the negative relationships of body mass to initial corticosterone level in males and females combined (Table 5.2). Birds with the least body mass appear to have the highest baseline levels of corticosterone, but the subsequent response to stress is not affected. Variation in the adrenocortical response to stress in this species appears to be independent of body condition.

**Liiwi**

In the Liiwi, there also is a positive correlation of body mass and fat score, but only in females (Table 5.2). In males there is a negative relationship of the rate of corticosterone increase following capture stress with body mass. Females show no tendency to modulate the adrenocortical response to stress in relation to body condition. If data from both males and females are combined, then a negative relationship of fat score and rate of
between a component of the stress response and body condition. In all other species there are negative relationships (e.g., Wingfield 1994). This point deserves further study in this species.

The adrenocortical response to stress, as applied by capture and handling, in endemic Hawaiian honeycreepers appears to be similar to that seen in continental species and other vertebrates in general (see Wingfield 1988, 1994). In at least the Common Amakihi and Iiwi, there is no apparent sexual difference in the response, unlike many species from the North American mainland (e.g., Wingfield 1994a,b; Wingfield et al. 1995). This may be because virtually none of the birds was in breeding condition, when most sex differences in the response occurs in other species (Wingfield et al. 1995). Nevertheless, it is clear that Hawaiian honeycreepers are sensitive to the effects of stress, which could have extensive deleterious effects on reproductive success, resistance to disease, and endogenous reserves of protein and fat for survival during severe storms or other extreme modifying conditions. Chronic high levels of corticosteroids may also trigger facultative behavioral patterns that may force birds to leave the reserve and wander over a large area (Astheimer et al. 1992; Wingfield 1994). These possible effects will have important ramifications for management and monitoring populations in reserves.

Correlates of the stress response and body condition can be particularly revealing. In the Drepanidinids studied, sex differences in body mass are independent of fat score (Fig. 5.5). Although there appears to be great individual variation in responsiveness to stress, this variation is only partly correlated with body condition (Table 5.2, unlike in many northern temperate species, see Wingfield 1994). Particularly fascinating is the possibility that Apapane show a positive correlation of body condition and the stress response. As far as we are aware, this is a unique finding. Whether this is a phenomenon related to nomadism (in which flocks of birds wander looking for favorable food resources) remains to be seen. Note that high levels of corticosterone are known to promote irruptive behavior (see Astheimer et al. 1992). On the other hand, we must be cautious because with so many correlations, it is possible that some will be significant at the $p < 0.05$ level by chance. Further studies on variation in the stress response in relation to body condition, degree of infection, and reproductive state will shed further light on this potentially important phenomenon. If it turns out that variables such as body condition are reliable indicators of susceptibility to stress, then another potential tool can be applied to captive breeding programs and the management of free-

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### Table 5.2 Relationships of the adrenocortical response to stress and body condition in Hawaiian Drepanidinids

<table>
<thead>
<tr>
<th>Species</th>
<th>Correlation</th>
<th>$R^a$</th>
<th>$p$ value</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Common Amakihi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Body mass vs. fat score</td>
<td>0.803</td>
<td>0.001*</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Body mass vs. initial B level</td>
<td>-0.655</td>
<td>0.039*</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>Body mass vs. fat score</td>
<td>0.794</td>
<td>0.074</td>
<td>6</td>
</tr>
<tr>
<td>Male + female</td>
<td>Body mass vs. fat score</td>
<td>0.591</td>
<td>0.018*</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Body mass vs. initial B level</td>
<td>-0.492</td>
<td>0.049*</td>
<td>17</td>
</tr>
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<td><strong>Iiwi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>Body mass vs. fat score</td>
<td>-0.143</td>
<td>0.635</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Body mass vs. rate of B increase</td>
<td>-0.664</td>
<td>0.036*</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>Body mass vs. fat score</td>
<td>0.844</td>
<td>0.026*</td>
<td>8</td>
</tr>
<tr>
<td>Male + female</td>
<td>Fat score vs. rate of B increase</td>
<td>-0.459</td>
<td>0.058</td>
<td>18</td>
</tr>
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<td><strong>Apapane</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male + female</td>
<td>Body mass vs. fat score</td>
<td>0.416</td>
<td>0.212</td>
<td>10</td>
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<tr>
<td></td>
<td>Fat score vs. maximum B level</td>
<td>0.565</td>
<td>0.090</td>
<td>10</td>
</tr>
</tbody>
</table>

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*aSpearman Rank Correlation Coefficient.
*bDenotes significance at at least 0.05 level.

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corticosterone is suggested but is not significant (Table 5.2). Thus, in this species, body condition may influence the rate of adrenocortical responsiveness to stress, at least in males. Those birds with higher body mass show a slower increase in corticosterone secretion following application of a stressor stimulus.

**Apapane**

As very few females were subjected to the capture stress protocol, we combined the male and female data for multiple correlations with body condition. In this species there is no correlation of body mass and fat score (Table 5.2), unlike the case in many other mainland species studied (Wingfield 1994). Furthermore, there are no significant correlations among components of the adrenocortical response to stress and body condition, with the possible exception of fat score and maximum corticosterone levels generated during the stress series paradigm (Table 5.2). This relationship is not quite significant. Interestingly, the Apapane is the first species studied in which there is a possible positive relationship...