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Behavioral and adrenocortical responses to mate separation and reunion in the zebra finch

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Abstract

The adaptive value of social affiliation has been well established. It is unclear, however, what endogenous mechanisms may mediate affiliative behavior. The Australian zebra finch (*Taeniopygia guttata*) breeds colonially and adults maintain lifelong pair bonds that may be disrupted in the wild due to high mortality rates. Many of its natural, social behaviors are maintained in laboratory conditions, making this species well suited for studying the mechanisms of affiliation. This study examines the behavioral and neuroendocrine responses to pair mate separation and reunion in zebra finches. We measured plasma corticosterone (CORT) and behavioral changes following separation from a pair bonded mate, and again upon reintroducing the mate or an opposite-sex cagemate. Plasma CORT concentrations were: (1) elevated during pair mate separation, even in the presence of other same-sex individuals, and (2) reduced to baseline upon reunion with the pair mate but not upon re-pairing with a new opposite-sex partner. These findings show that zebra finches exhibit hormonal responses to separation and reunion specifically with a bonded pair mate and not with other familiar conspecifics. In addition, alterations in behavior during separation and reunion are consistent with monogamous pair bond maintenance. This study presents evidence for adrenocortical involvement in avian pair bonding, and for hypothalamic–pituitary–adrenal activation in response to an ecologically relevant social stressor.

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Social attachment between mating partners is widely distributed among vertebrates. Reproductive pairing is most prevalent in birds, which may pair for the breeding season or longer. The zebra finch is a highly social species with lifelong monogamous pair bonds, and extensive laboratory and field studies of zebra finches have established a suite of behaviors associated with pair bonding which can be readily identified in a colony of captive breeders (Zann, 1996). Butterfield (1970) identified the selective pressures, both ecological and social, that may give rise to lifelong pair bonds in finches. However, the underlying physiological mechanisms that mediate pair formation and maintenance are unknown in any bird species.

In mammals, recent studies of monogamous pair bonding

species show that both peptide and steroid hormones regulate social attachment. Pair formation is facilitated by oxytocin in female prairie voles (Williams et al., 1994) while vasopressin mediates pairing in males (Insel et al., 1998). In both rodents and primates, plasma glucocorticoids are reduced after pairing (Ginther et al., 2001; Reburn and Wynne-Edwards, 1999), and increase in response to experimental separation from bonded partners (Castro and Matt, 1997; Mendoza and Mason, 1986; Norcross and Newman, 1999). These and other studies have demonstrated that pair bonding is associated with changes in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis and other neuroendocrine systems in mammals.

Separating male–female pairs of zebra finches elicits changes in behavior, suggesting an accompanying shift in circulating hormones. Separating males increases their song rates (Immelmann, 1959) and activity (Butterfield, 1970),

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and separation increases defecation rates in both males and females (Butterfield, 1970). These findings suggest that isolation from a pair mate, such as would naturally occur if the partner died, may be a significantly stressful event in zebra finches. We hypothesized that pair mate separation in zebra finches elevates baseline corticosterone (CORT, the primary avian glucocorticoid, Holmes and Phillips, 1976), and this hormonal change may be linked to behavioral alterations, including adaptive changes that would promote locating the partner or re-pairing.

In a series of four experiments, we tested the behavioral and adrenocortical responses to pair mate separation in zebra finches. First, we visually separated male–female pairs, housing them either alone or in same-sex groups, and measured basal CORT concentrations before and after separation. Second, we separated pairs, but then reunited some pairs while forming other artificial pairs to test if reunion exclusively with the pair mate would attenuate CORT release following separation. Third, we asked if both acoustic and visual separation from the pair mate would result in elevated CORT. Last, we separated pairs but kept individuals in same-sex dyads during separation to test whether social contact alone could attenuate CORT release associated with pair separation.

Methods

Birds

All zebra finches were in established pairs taken from a large breeding colony (housed on a 14L:10D photoperiod) prior to beginning each experiment. Zebra finches establish and maintain pair bonds in large social groups, and the presence of a pair bond was confirmed when male–female pairs met the following behavioral criteria, (1) clumping, (2) allopreening, and (3) cohabitation of a single nest box, in addition to other pairing behaviors, including mate guarding and copulation (after Butterfield, 1970; Silcox and Evans, 1982). New pairs were used for each experiment and all pairs had successfully reared chicks to fledging before the start of the experiments (an indication of bond strength, Immelmann, 1959). Nest material was removed 2 weeks prior to each experiment to prevent egg laying and chick rearing from interfering with behavioral and physiological measurements. The Institutional Animal Care and Use Committee at Cornell University (Protocol 95-73-98 and 01-78) approved all procedures reported in this study.

Sampling and hormone analysis

To facilitate capture and sampling no more than four birds in each study were housed in the same room. Throughout each study subjects were housed in visual and acoustic contact with other birds of the colony that were not part of the study. Blood samples were taken within 3 min of en-

tering the room to determine basal CORT concentration (after Wingfield et al., 1982), and experiments were completed between 1100 and 1300 to control for diurnal variation. Approximately 50 μ l of whole blood was collected in heparinized microhematocrit tubes following alar venipuncture. Plasma was separated under centrifugation, stored frozen, and analyzed for CORT using radioimmunoassay following dichloromethane extraction as described previously (Wingfield et al., 1992). Within- and between-assay variability were 7.5 and 6.4%, respectively.

Pair separation

Experiment 1

In the first experiment, we tested whether pair mate separation would elevate plasma CORT, and whether the presence of same-sex birds during separation would affect the response. Ten pairs (20 birds) were assigned to two groups: *pair housed* ($n = 12$) or *group housed* ($n = 8$). *Pair housed* birds were placed in individual pair cages ($60 \times 35 \times 35$ cm), while *group housed* birds were placed in a single group of four pairs in a larger cage ($80 \times 60 \times 60$ cm). All birds were acclimated following cage transfer for 24 h prior to the first baseline blood sample (“baseline”). This was to compensate for any stress associated with moving birds to and from cages through the rest of the experiment. Following a 24-h rest period, *pair housed* birds were separated from their pair mates and housed alone in cages, while remaining in vocal and acoustic contact with their pair mates and other zebra finches of the colony. *Group housed* birds were separated from their pair mates and housed in same-sex groups during the period of separation, resulting in one cage of separated males ($n = 4$) and another of separated females ($n = 4$), both in vocal contact with the other. A second blood sample was taken from all birds 24 h after separation (“separation”). This 24-h separation period was chosen as the minimum sampling interval for the entire study, since a shorter interval between consecutive blood samples may cause undue physiological stress in small passerine birds.

Experiment 2

In experiment 2, we tested whether pair mate reunion would attenuate CORT elevation following a separation period used in experiment 1. Fourteen pairs (28 birds) were assigned to one of three groups: *reunite* ($n = 12$), *new pair* ($n = 12$), or *control* ($n = 4$). All birds were transferred from the breeding colony to individual cages in pairs, and a blood sample was taken 24 h after this initial move (baseline). Following a 24-h rest period, pairs in both *reunite* and *new pair* groups were separated from their pair mates and housed in new individual cages, while *control* pairs were subject to equivalent handling but not separated. Plasma samples were taken from all birds after 24 h in the new housing conditions (separated). Following a 24-h rest period, *reunite* birds were placed in a new cage with their

respective pair mate, while *new pair* birds were placed in a new cage with a novel, opposite-sex bird. *Control* pairs were again subject to equivalent handling. For all pairs one blood sample was taken each at 24 h (“re-pair 24”) and 48 h (“re-pair 48”) after the final cage transfer.

Experiment 3

In the first two experiments separated birds were housed in the same room as their pair mates, such that pairs were visually but not acoustically separated. This artificial social environment may have been a source of frustration, which has been shown to elicit HPA activation in mammals (Lyons et al., 2000; Romero et al., 1995). In the third experiment, we asked if birds that were both acoustically and visually isolated from their pair mates exhibited elevated CORT during separation. Birds from the breeding colony were set up in individual pair cages and acclimated for 1 week. Eight pairs (16 birds) were assigned to two groups: *control* ($n = 4$) or *separated* ($n = 12$). An initial blood sample was taken from all birds (baseline). *Separated* birds were then individually housed in rooms separate from their pair mates, while remaining in visual and acoustic contact with other zebra finches of the colony. *Control* birds were subject to equivalent handling and relocation but not separated. After 24 h of separation, a blood sample was taken from all birds (separation), and pairs were reunited following a 24-h rest. All birds were subsequently sampled after 48 h of reunion (“reunion”).

Experiment 4

In the final experiment, we sought to test whether the presence of a same-sex cage mate during pair mate separation would alleviate the effects of isolation (accompanying mate separation) on HPA activation. Social isolation increases glucocorticoid concentrations in some mammalian (Boissy and LeNeindre, 1997; Sapolsky et al., 1997) and nonmammalian (Ruis et al., 2001) species, and therefore housing a bird alone may be a sufficiently stressful stimulus causing CORT release in experiments 1–3, masking any effect of separation from the pair mate. Five pairs ($n = 10$) were moved from the breeding colony into individual pair cages, and a blood sample was taken after a week of acclimation (baseline). All pairs were then acoustically and visually separated and housed in same-sex dyads (male–male or female–female) during the entire period of separation, and a blood sample was taken 24 h after the separation (separation). Each bird was then separated from its same-sex cagemate and reunited with its original, opposite-sex pair mate, and a blood sample was taken after 48 h of reunion (“reunion”). For display purposes, *dyad* refers to birds in experiment 4.

Behavior

Birds were behaviorally monitored on days when blood sampling did not occur. Birds were observed individually or

in pairs for 15 min from behind opaque blinds to minimize disturbance of normal behavior. In experiment 2, four males from the *reunite* group were observed during both separation and reunion periods for: (1) activity (number of perch hops) and (2) number of song bouts. In experiments 3 and 4, all pairs (*control* $n = 4$; *separated* $n = 12$; *dyad* $n = 10$) were observed for the first 15 min of reunion for: (1) directed song by males; (2) allopreening; (3) billing (greeting ceremony linked to pair bond maintenance, Morris, 1954); (4) clumping; and (5) nest box cohabitation.

Analysis

Tests revealed that both hormone and behavior data were distributed nonnormally ($P < 0.05$ for Shapiro–Wilks tests of normality). Since this study was a repeated-measures design all hormone data were analyzed on a within-subject basis with Wilcoxon signed-rank tests. Behavioral scores were analyzed between groups with Mann–Whitney *U*-tests. All significant differences are reported for $P \leq 0.05$. No sex differences in plasma CORT concentrations were observed in samples taken either at baseline and in response to mate separation/reunion, and therefore all data for male and female birds were analyzed together for each experiment. In addition, plasma CORT concentrations were not correlated within male–female pairs (i.e., one partner’s CORT level did not significantly predict that of the other; $P > 0.4$ for Spearman rank correlations) at baseline or in response to separation/reunion, and were therefore treated as independent samples for analysis.

Results

Experiment 1

Plasma CORT increased in response to pair separation only in one of the two groups in experiment 1. *Pair* housed birds were separated from their pair mates into individual cages and exhibited an increase in plasma CORT in response to separation (Fig. 1; $P = 0.01$). *Group* housed birds were separated from their pair mates but housed in same sex groups and did not exhibit a significant increase in plasma CORT in response to separation (Fig. 1; $P = 0.11$).

Experiment 2

As in experiment 1, plasma CORT increased above baseline during pair mate separation in both *reunite* and *new pair* groups (Fig. 2; $P = 0.02$ for separation vs baseline) while CORT remained at baseline in *control* birds during the equivalent separation period (Fig. 2; $P = 0.35$ for separation vs baseline). Following separation, in male–female pair mates that were reunited (*reunite*) plasma CORT returned to baseline after 48 h of reunion (Fig. 2; $P = 0.04$ for re-pair 48 vs separation). When novel opposite-sex birds

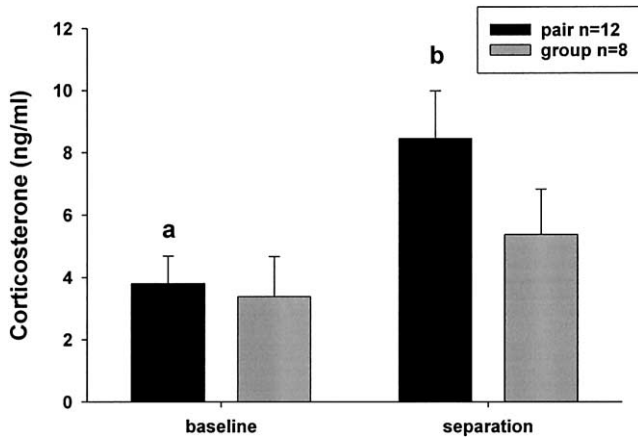


Fig. 1. Plasma corticosterone increased in response to mate separation in birds housed as individual pairs, who were housed alone during the period of separation, but not in group housed birds, who were housed in same-sex groups during the period of separation. Values are plotted as means \pm SEM; different letters indicate within-group differences for $P \leq 0.05$.

were co-introduced in a cage after pair mate separation (*new pair*) circulating CORT did not drop significantly from elevated levels by 48 h after re-pairing (Fig. 2; $P = 0.45$ for re-pair 48 vs separation, $P = 0.02$ for re-pair 48 vs baseline). Corticosterone in *control* pairs remained unchanged through the equivalent period (Fig. 2; $P = 0.32$ for re-pair 48 vs separation).

Experiment 3

Acoustic communication in zebra finches is important for individual recognition (Zann, 1996) and maintaining contact when members of a pair are visually separated

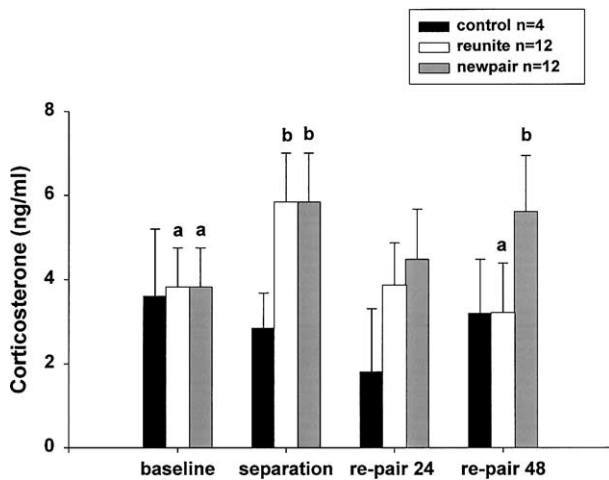


Fig. 2. Plasma corticosterone responses to mate separation and reunion under different experimental conditions. Corticosterone was elevated in separated pairs and returned to baseline in pairs that were reunited (reunite) but remained elevated in pairs of novel individuals created artificially following separation (new pair). Values are plotted as means \pm SEM; different letters indicate within-group differences for $P \leq 0.05$.

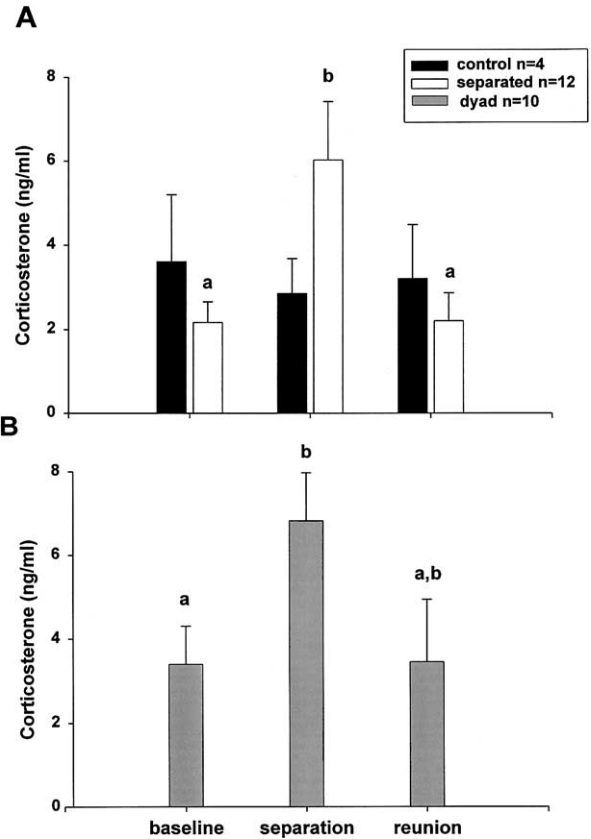


Fig. 3. Plasma corticosterone responses to mate separation and reunion under different housing conditions. Corticosterone was elevated in pairs that were acoustically and visually separated and returned to baseline upon reunion (A, separated). When separated individuals were kept in same-sex dyads during separation corticosterone was elevated and returned to baseline upon pair mate reunion (B, dyad). Values are plotted as means \pm SEM; different letters indicate within-group differences for $P \leq 0.05$.

(Silcox and Evans, 1982). In experiments 1 and 2, separated birds were visually but not acoustically isolated from their pair mates. In the third experiment, *separated* pairs were isolated acoustically as well as visually from their pair mates, while *control* pairs were subject to equivalent handling. For *separated* pairs, acoustic and visual separation elicited an increase in plasma CORT from baseline (Fig. 3A; $P = 0.004$ for separation vs baseline) and CORT returned to baseline following 48 h of pair mate reunion (Fig. 3A; $P = 0.003$ for reunion vs separation). Plasma CORT in *control* pairs remained unchanged through all three sampling periods (Fig. 3, $P > 0.3$ for all comparisons).

Experiment 4

Housing separated individuals in same-sex dyads did not prevent the elevation of plasma CORT above baseline during separation (Fig. 3B; $P = 0.02$ for separation vs baseline). Although not statistically significant, when male-female pairs were reunited plasma CORT tended to return to baseline by 48 h after reunion (Fig. 3B; $P = 0.07$ for reunion vs separation).

Table 1
Pairing behavior during reunion

Behavior	Control (<i>n</i> = 4)	Separated (<i>n</i> = 12)	Dyad (<i>n</i> = 10)
Directed song by males	0.2 ± 0.3	2.8 ± 0.7**	2.8 ± 1.7
Billing	1.5 ± 0.7	6.5 ± 1.7**	0.3 ± 0.3
Allopreening	0.2 ± 0.2	1.5 ± 0.3*	0.1 ± 0.2
Clumping	2.0 ± 1.4	3.8 ± 1.6	2.1 ± 0.7
Nest box cohabitation	1.5 ± 2.1	0.5 ± 0.3	0.3 ± 0.1

* $P < 0.05$; ** $P < 0.005$ compared with control. Values are number of events (mean ± SEM) observed per 15 min. Note: Sample sizes for directed song by males are half the number given.

Behavior

In experiment 2, four males in the *reunion* group were behaviorally monitored during the periods of separation and reunion. Separation from a pair-bonded female has been shown to increase both locomotor activity and song rates in male zebra finches (Butterfield, 1970; Immelmann, 1959). Although the test comparing locomotor activity had low power, the decrease in locomotor activity (as measured by perch hops) in males reunited with their partners nearly reached significance (separation, 110.5 ± 64.7 ; reunion, 23.7 ± 8.6 ; $P = 0.06$). One male was observed singing during separation, and his song bouts were reduced upon reunion (separation, 24 bouts; reunion, 12 bouts).

In experiments 3 and 4, all pairs (*control*, *separated*, and *dyad*) were observed during the initial period of reunion for behaviors associated with pairing (Table 1). Upon reunion, pairs that had been acoustically and visually separated from one another (*separated*) had significantly greater frequencies than control pairs of male directed song ($P < 0.005$), incidents of within-pair billing ($P < 0.005$), and allopreening ($P = 0.03$), while clumping and nest box cohabitation were no different from control pairs ($P > 0.2$). Data for pairs that had been held in same-sex dyads during the period of separation (*dyad*, experiment 4) are presented in Table 1.

Discussion

The findings of this study are consistent with the hypothesis that pair mate separation and reunion modulate adrenocortical activity and behavior in zebra finches. In experiment 1, birds that were housed singly during the period of mate separation exhibited a twofold increase in resting plasma CORT concentration, while CORT in separated birds housed in same-sex groups was not significantly different from baseline. This suggests that the presence of same-sex individuals may reduce the stress of social isolation experienced during pair mate separation, a situation similar to that in primates, in which female–female pairs buffer the HPA activity associated with isolation (Saltzman et al., 1991). However, given the smaller sample size of the

group housed individuals, and the observation that group housed birds tended to have higher CORT concentrations during separation ($P = 0.11$), this result alone is inconclusive. Experiment 1 provided evidence for an adrenocortical response to mate separation, suggesting that a well-controlled study should be conducted.

In experiment 2, we reunited pairs after separation to test if CORT concentrations return to baseline when the partner vs a novel bird is introduced. Confirming our earlier findings, separated individuals had elevated CORT concentrations compared to baseline, while CORT in control birds remained at baseline after equivalent handling. When separated pairs were reunited, adrenocortical activity returned to baseline after 48 h, whereas when new pairs of opposite-sex birds were formed plasma CORT remained elevated after 48 h of re-pairing. Thus, the pattern of CORT response to re-pairing was specific to the identity of the individual that was introduced. Reunion with mating partners after brief separation has been shown to reduce HPA activity in marmosets (Smith et al., 1998), and behavioral responses differ during reunion with a mating partner vs a stranger in titi monkeys (Fernandez-Duque et al., 1997), both of which are monogamous pair-bonded species. In the current study, male zebra finches were behaviorally monitored during separation and reunion in experiment 2, and tended to show behavioral changes consistent with earlier findings (i.e., increased locomotor and vocal activity during separation as in Butterfield, 1970; Immelmann, 1959). Glucocorticoids have long been associated with heightened activity (Breuner et al., 1998; Sandi et al., 1996) and may provide a proximal link to increases in both locomotor and vocal activity during pair mate separation.

There are two alternative hypotheses that could explain the results of experiments 1 and 2: frustration and isolation. First, social frustration has been shown to elicit HPA activation in mammals (Lyons et al., 2000; Romero et al., 1995). The first two experiments utilized a protocol in which individuals were separated visually but not acoustically from their pair mates. Since zebra finches rely on acoustic communication for individual recognition (Zann, 1996) and nonvisual contact (Silcox and Evans, 1982), housing a pair such that they can hear but not see each other is a potential source of frustration. In this way, stress associated with frustration may explain elevated CORT concentrations during pair mate separation in experiments 1 and 2. However, when individuals were isolated visually as well as acoustically from their pair mates in experiment 3, plasma CORT was again elevated during separation and returned to baseline upon reunion. This result indicates that separation from social contact, and not frustration due to an unnatural social environment, accounts for heightened HPA activity in zebra finches.

Second, a period of brief social isolation from familiar conspecifics elicits glucocorticoid release in a variety of species (Boissy and LeNeindre, 1997; Coe et al., 1978; Ruis et al., 2001). Since zebra finches are highly social, the

possibility exists that housing a bird alone in a cage may be sufficient to cause stress-induced CORT release, masking any effect of separation exclusively from the pair mate. However, in experiment 4, individuals were housed in same-sex dyads during the period of pair mate separation to provide a more socially relevant setting. Here, plasma CORT was again elevated in separated birds and tended to return to baseline concentrations following reunion. Though this result differs somewhat from our findings in the group housed birds in experiment 1, the results of experiment 4 are more conclusive since experiment 4 is a direct test of the isolation hypothesis and has higher power. Therefore, we conclude that the CORT response to separation from a bonded mate occurs in the presence of another individual and cannot be accounted for by social isolation in zebra finches. Similarly, infant squirrel monkeys separated from the mother exhibit cortisol responses even when cared for by other lactating females (Coe et al., 1978; Vogt and Hennessy, 1982). Furthermore, separating social partners causes a rise in plasma cortisol in marmosets, and the response occurs even in the presence of novel opposite-sex individuals (Norcross and Newman, 1999). Thus, in species with strong social bonds, the presence of other conspecifics during separation from the bonded partner does not significantly attenuate HPA activation.

When zebra finches were reunited after a period of separation they exhibited characteristic behaviors associated with greeting and pair maintenance. Upon reunion, males who were separated sang more directed song to their partners than control males. In zebra finches, vocalizing between mates has been linked to pair formation and maintenance, and auditory contact can maintain a bond during brief separation (Silcox and Evans, 1982). In addition, zebra finch pairs that were visually and acoustically separated showed a dramatic increase in billing upon reunion in relation to control pairs. Billing, or allofeeding (mock feeding), is associated with social bonding in siskins (Mundinger, 1979; Senar, 1984), and has been identified in zebra finches as part of a greeting ceremony between reunited pair mates (Morris, 1954). Furthermore, separated pairs of zebra finches exhibited higher rates of allopreening (an index of social bonding; Zann, 1996) than control pairs. In all, reunion following brief separation increased the incidence of several affiliative behaviors which may serve to reestablish or strengthen an existing bond, and this response to reunion may be a common feature of monogamous pair bonding in vertebrates (Matheson et al., 1996; Shepherd and French, 1999).

These experiments demonstrate that an ecologically relevant social setting can modulate adrenocortical activity and pairing behavior in a passerine (songbird). Previous studies have implicated a role for glucocorticoids in social bonding in birds: CORT increases during pair bond challenges (by unpaired challenger males) in monogamous geese (Hirschenhauser et al., 2000), and CORT is associated with parent–offspring bonds (Martin, 1978) and sibling contact

(Jones and Harvey, 1987) in chickens. To our knowledge, this is the first study to show that pair mate separation and reunion can regulate HPA activity in a bird. The function of glucocorticoid release during mate separation remains to be determined. It has been suggested, however, that elevated CORT may help maintain an existing pair bond during separation and delay the process of re-pairing in a monogamous mammal (Devries et al., 1995). Moreover, in many vertebrates social contact raises and social isolation reduces glucocorticoid concentrations (Hannes and Franck, 1983; Rotllant et al., 2000; Mench et al., 1986), but in those species with long-term monogamous pair bonds glucocorticoids are reduced upon pair formation and increase when partners are separated (Castro and Matt, 1997; Mendoza and Mason, 1986; Reburn and Wynne-Edwards, 1999). The current study provides further evidence that monogamy in birds is associated with neuroendocrine mechanisms similar to those found in mammals with long-term pair bonds.

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