



ELSEVIER

Applied Animal Behaviour Science 66 (2000) 335–349

APPLIED ANIMAL
BEHAVIOUR
SCIENCE

www.elsevier.com/locate/applanim

Neonatal handling of Amazon parrots alters the stress response and immune function

J.C. Collette^a, J.R. Millam^{a,*}, K.C. Klasing^a, P.S. Wakenell^b

^a Department of Avian Sciences, University of California, Davis, CA 95616-8521, USA

^b Department of Population, Health and Reproduction, University of California, Davis, CA 95616-8521, USA

Accepted 24 September 1999

Abstract

The influence of neonatal handling on behavior and immune function was assessed in Orange-winged Amazon parrots (*Amazona amazonica*). Chicks ($n = 11$) were gently handled daily from 25 days of age until 38 days post-fledging, while control chicks ($n = 9$) were not handled. At 10 days post-fledging (~ 66 days of age), chicks were given tests to evaluate tameness (e.g., willingness to perch on an offered finger). They were then restrained for 10 min, either by being held while perching (handled group) or, because they would not perch, by being restrained in a towel (nonhandled group). Serum corticosterone levels were measured and immune status was assessed by: the delayed-type hypersensitivity (DTH) response to phytohemagglutinin-P (PH-P) injection; the humoral response to a killed Newcastle disease virus (NDV) challenge; and heterophil:lymphocyte ratio (H:L). Handled chicks were tamer by all measures of tameness. DTH was greater in nonhandled chicks ($P \leq 0.002$), as were serum corticosterone levels (Wilcoxon, $P \leq 0.05$), while NDV antibody titers were possibly reduced ($P \leq 0.09$). H:L ratios did not differ. We conclude that handling conditioned the birds to be held in a manner that appeared not to be stressful. The greater DTH response of nonhandled chicks suggests that either their DTH response was enhanced by the acute stress of being restrained in a towel, and/or the DTH response of handled chicks was suppressed as a result of the repeated physiologic stress from handling during the neonatal period. In either event, handling produced marked differences in response to types of

* Corresponding author. Department of Animal Science, University of California, One Shields Avenue, Davis, CA 95616-8521, USA. Tel.: +1-530-752-1149; fax: +1-530-752-0175; e-mail: jrmillam@ucdavis.edu

restraint that would be typically encountered in the husbandry of Amazons in captivity. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Orange-winged Amazon parrots; *Amazona amazonica*; Neonatal handling; Immune system; Delayed-type hypersensitivity; Taming

1. Introduction

Adult behaviors of parrots reared in captivity vary considerably, depending on method of rearing. The most naturalistic method of rearing is to permit cavity-nesting parent parrots to feed and rear their altricial young in nest boxes, much as they would do in the wild. Parent-reared chicks can be expected to have normal reproductive performance as adults (Myers et al., 1988), but they may never become as tame toward humans as chicks reared by other methods.

In contrast to parent-rearing, artificial methods of rearing altricial species typically consist of removing chicks from their parents at, or shortly after, hatching. All requirements for feeding and maintaining the correct thermal environment are then provided by humans rather than parent birds. While such human-reared parrots typically become very tame, they may have impaired reproductive performance as adults (Myers et al., 1988).

Other methods of rearing that provide intermediate degrees of human intervention may combine desirable features of both parent-rearing and human-rearing. Supplemental feeding of chicks by humans of otherwise parent-reared chicks can produce tame parrots (Low, 1991). Similarly, occasional human handling of parrot chicks that are otherwise parent-fed and -reared produces tame cockatiels (Arksy, Aengus, Wakenell, Kass and Millan, unpublished observation) and Orange-winged Amazon parrots (Aengus and Millam, in press). The latter method most closely resembles neonatal handling protocols used in studies of altricial rats, which similarly have a period of parental dependency.

Neonatal handling typically consists of temporarily removing recently born or hatched animals from the parents. Neonatal handling permanently alters the hypothalamic–pituitary–adrenal (HPA) response to stress in adult rats (Ader and Grotta, 1973). Handled adults have a lower basal level of corticotropin-releasing factor (CRF) than nonhandled rats and their CRF depletion rate during stress is much lower (Plotsky and Meaney, 1993). Likewise, their serum adrenocorticotrophic hormone levels are lower and the corticosterone response to stress is reduced (Levine, 1957, 1962; Hess et al., 1969). The attenuated HPA response is due to increased sensitivity to the negative feedback of glucocorticoids on CRF neurons (Meaney et al., 1989) and is associated with a greater number of type II glucocorticoid receptors in the hippocampus (Meaney et al., 1985, 1989).

Neonatal handling also alters the immune response. Nonhandled adult rats subjected to a variety of stressors (heat, handling, restraint, food deprivation, etc.) show decreased numbers of lymphocytes and leukocytes in the blood (Keller et al., 1981; Steplewski and Vogel, 1986; Dhabhar et al., 1994). Not only are immune cell numbers decreased, but apoptosis of thymocytes occurs when endogenous corticosterone levels are high (Gross and Siegel, 1982). Whether the antibody response to antigens is suppressed or enhanced

by neonatal handling is not clear. In some studies, handling rat pups from 0–28 days suppressed production of plaque-forming cells and reduced the antibody response to a sheep red blood cell (SRBC) challenge at 7–8 weeks of age (Raymond et al., 1986; Von Hoersten et al., 1993). In contrast, handling rat pups from birth to weaning increased antibody titer to a flagellin challenge when measured at 10–12 weeks (Solomon et al., 1968), and antibody production to a human serum albumin challenge was enhanced in neonatally handled rats tested at 100 days of age (Persinger and Falter, 1992). However, there is no difference between neonatally handled mice and control mice if the antigen challenge is administered immediately after the handling period at weaning. In this instance, differences were not seen until animals were 60 days of age (Lown and Dutka, 1987).

Neonatal handling of precocial domestic fowl chicks similarly attenuates the HPA response to capture and restraint (Hemsworth et al., 1994). As little as 30 s of handling, two or three times a day, can produce significant differences in behavior by 4 days of age (Jones, 1993). Young chicks can similarly imprint on humans and other surrogate objects with very limited exposure in the first few days of life (Bolhuis, 1991). However, fear was equally reduced in 20-day-old chicks, whether they were handled from 0–9 days or from 10–18 days, suggesting that a sensitive period for fear reduction is not confined solely to the first few days of life but may exist for the first few weeks (Jones and Waddington, 1993). Chicks show reduced avoidance of humans, even if their previous experience with humans (being briefly suspended by their legs) is probably unpleasant (Jones, 1993). Even allowing chicks to observe handling of other chicks or simply having a human stand near their cage is as effective as physical handling in reducing fear of humans (Jones, 1993). At 6 weeks of age, handled chicks begin to approach the researcher's hand eagerly, face the researcher and do not flee or become frightened while being removed from the cage (Gross and Siegel, 1982).

As with rats, neonatal handling also alters the immune response of birds. Neonatally handled chicks have higher antibody titers to red blood cell challenge than nonhandled controls (Gross and Siegel, 1982), and they are more resistant to *Mycoplasma gallisepticum* and *Escherichia coli* challenges than nonhandled chicks (Gross and Siegel, 1979).

Neonatal handling produces tameness, alters the stress response and improves immune competence in both altricial rats and precocial chickens. The present study was designed to determine whether neonatal handling would have similar effects in a highly altricial avian species, the Orange-winged Amazon parrot (*Amazona amazonica*). If so, neonatal handling could potentially improve captive welfare, companionability and reproductive performance of members of this highly endangered genus of parrots. To measure immunocompetence after neonatal handling we used a battery of tests. Serum corticosterone levels were measured because high glucocorticoids are considered immunosuppressive; similarly heterophil:lymphocyte ratios (H:L) were measured, as they correlate better with some types of stress in chickens than do serum corticosterone levels (Gross and Siegel, 1983). Also, two foreign protein challenge-tests were used: Newcastle disease virus (NDV) administered subcutaneously to characterize the humoral response and phytohemagglutinin-P (PH-P) administered intradermally to characterize the delayed-type hypersensitivity (DTH) response. Both of these foreign proteins are potentially antigenic in birds.

2. Methods

2.1. *Animals and housing*

Sixteen pairs of wild-caught (not tame) Orange-winged Amazons were stimulated to breed by exposure to long daylengths and provision of nest boxes (Millam et al., 1995). Pairs were housed in suspended welded-wire cages (1 m × 1 m × 2 m), one pair per cage, eight cages per room. Each cage had two 1 m × 4 cm × 8.5 cm wooden perches, one located at the front and the other closer to the nest box at the back of the cage. All birds were maintained according to approved UC Davis' Animal Use and Care Protocols.

The birds were fed Roudybush maintenance pellets (Roudybush, Sacramento, CA) ad libitum during nonbreeding times and Roudybush breeding pellets during the breeding, laying and rearing stages. The birds were switched to the breeding diet for this experiment on April 2, 1995. Water was available ad libitum from nipple waterers.

On April 6, nest boxes were hung on the outside rear of the hanging cages, and photoperiod was increased from 9 h light:15 h dark (9L:15D) with onset at 8:00 a.m. to 15L:9D with onset at 6:00 a.m. The boxes were constructed of stainless steel sheet metal in a "grandfather clock" style (40 cm × 38 cm × 76 cm). Partitions could be inserted horizontally from the back to divide the nest box into three vertically separate sections. This facilitated the removal of chicks or eggs from the nest box by allowing separation of the parents from the chicks or eggs. Two metal doors at the back of the nest box allowed separate access to the lower two sections. The nest box entrance was 6 cm from the top of the nest box. A plywood insert with a 9-cm diameter hole was placed on the outside of the nest box over the nest hole, slightly reducing the diameter of the nest box opening, permitting the birds to enlarge the entrance by chewing. Wire mesh cloth (1.25 cm × 2.5 cm) was attached to the inside of the box to provide the birds with a ladder to facilitate movement from the entrance of the box to its bottom. Pine shavings were piled 8–12 cm high in the bottom of each box at the beginning of the experiment.

During the breeding period, the nest boxes were checked daily for eggs, and all eggs were date-marked. Eggs that did not hatch were later inspected to determine the cause (infertility, maldevelopment, etc.). Chicks were banded with closed bands at approximately 3 weeks of age.

2.2. *Reproductive results*

Twelve of the 16 Amazon pairs laid a total of 45 eggs for a mean egg production of 3.75 eggs per laying pair (excluding one pair of birds that laid 15 eggs which candled infertile; this pair was later found to consist of two females). Thirty of the 45 eggs were candled fertile for a fertility rate of 66.6%. Twenty-three of the 30 fertile eggs hatched, for a hatch-of-fertile rate of 76.6%. Two of the hatched chicks were cannibalized in the first 2 weeks of life by their parents, another became ill and was removed from the analysis leaving 20 chicks for the handling treatments.

2.3. *Handling treatment*

Whole clutches of chicks were sequentially assigned to either the neonatally handled (six clutches, 11 chicks) or nonhandled control groups (five clutches, 10 chicks).

All of the chicks of a clutch were removed and handled at the same time by either of two handlers. When the oldest chick in the clutch reached 25 days of age, chicks in clutches assigned to the handled group were removed from the nest box for daily handling. During handling, chicks were kept in a shallow tub which was lined with a towel and positioned under a heat lamp to prevent chilling. Chicks were handled for 15–20 min before being returned to the nest box. This was done until the end of the experiment, which was 38 days after fledging for each bird. Fledging age (when birds were first discovered outside of the nest box) ranged from 50 to 62 days. Handling involved stroking the head, neck, beak and back, talking softly and occasionally offering food (pieces of Cheerios brand breakfast cereal, General Mills, Minneapolis, MN). Assessment began 10 days after fledging and ended 28 days later when the final blood sample was drawn for the humoral response test. Until the immune testing, birds of the control group were not exposed to any handling except to be banded.

2.4. Tameness testing

Birds were observed daily to determine the day of fledging. Ten days after fledging, the birds were subjected to a battery of behavioral and physiological tests to characterize tameness. The tests consisted of determining: whether a chick would attempt to avoid (flinch) or permit being touched on the dorsal aspect of the head, or cheek; whether a chick would reject a food offering; whether a chick would approach the handler's hand from a perch and the latency of this response; and the respiration rate after being placed on the perch. For each behavior, scores of the bird's responses were recorded. The food acceptance, head touch, and cheek touch each received a score from 0 to 2 points. Two points indicated that the bird readily accepted the food or allowed the researcher to touch it. One point meant that food or contact was accepted with some coaxing and zero points meant that the actions were avoided completely or that chicks aggressively bit and/or flew away. The latency to approach was measured by the amount of time that elapsed before the bird would perch on the researcher's hand. The latency test was limited to 200 s. Two-hundred seconds were assigned if the bird bit and/or flew to avoid contact. Respiration rate was scored as the number of respirations per minute. Subjective evaluations were also made as to whether chicks resisted being removed from the nest box for handling.

2.5. Restraint of handled vs. nonhandled birds

Immunoresponse testing for each bird began at 7:00 p.m., 10 days after fledging (day 1). At this time each bird was subjected to a handling/restraint period of 10 min. For the handled birds this included removing the bird from the cage and allowing the bird to perch on the researcher's hand while gently holding it in place with the other hand for the allotted time period. This treatment is consistent with the type of minimal restraint a tame bird would experience with its owner, veterinarian, caretaker, etc., and was similar to the daily treatment handled birds had been experiencing. In contrast, nonhandled control birds were captured, by necessity, with a net and restrained in a towel for the 10-min time period. This treatment is also consistent with the restraint an untamed bird

would undergo when removed from its cage for inspection by a veterinarian. The measurements of immune response were performed subsequent to this episode of handling/restraint.

2.6. Humoral response to vaccination

At 7:00 a.m. the day following the restraint period (day 2; to permit the potential effects of restraint on the humoral response to be fully exerted, the birds received 0.5 ml of Layermune Newcastle disease vaccine (NDV, Biomune, Lenexa, KS) subcutaneously in the inguinal region. Fourteen days after this primary injection, a booster of the same dose was given. Fourteen days following the booster, 1 ml of blood was taken from the ulnar or jugular vein for the evaluation of NDV antibodies. A hemagglutination inhibition test using chicken red blood cells was performed to determine the presence of NDV antibodies in serially diluted samples (Hanson, 1980).

2.7. Corticosterone and H:L ratio determinations

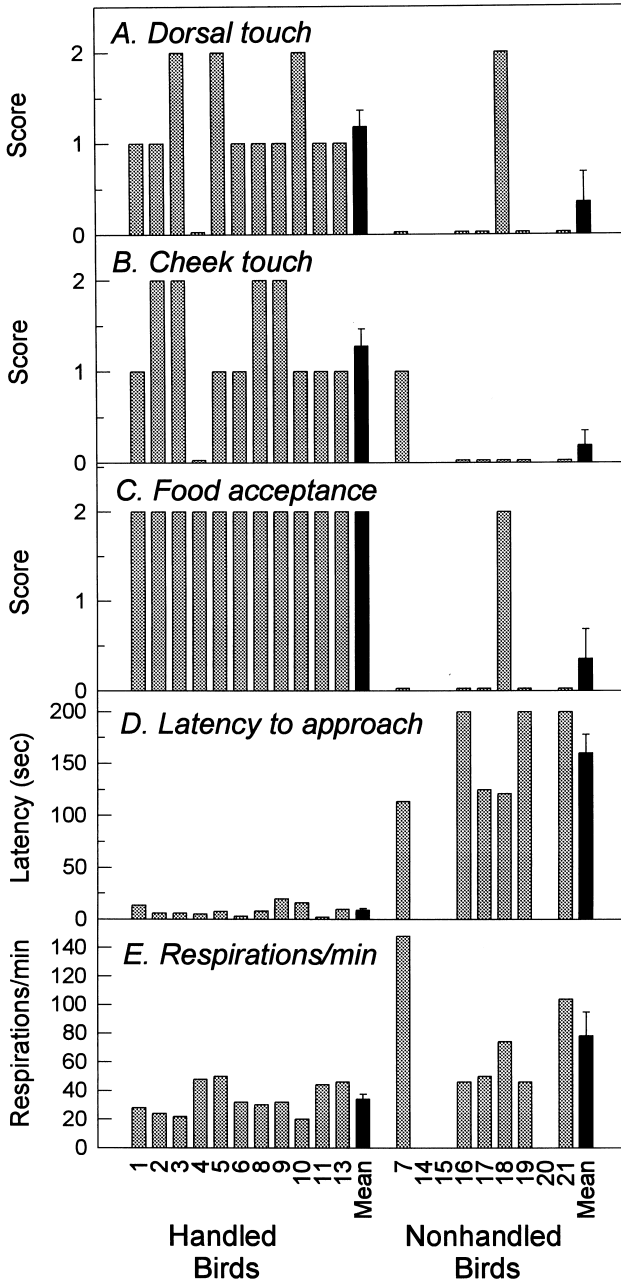
Also on day 2 at 7:00 a.m. (just prior to the vaccination described above and before the wing-web measurements described below), approximately 2 ml of blood was collected from the right ulnar vein. One portion of the blood ($\sim 50 \mu\text{l}$) was used to make a blood smear (slide and cover slip method) for H:L ratio evaluation, while the remaining blood was centrifuged at $\sim 1350 \times g$ for 10 min and the serum decanted and stored at -20°C until assay for corticosterone. All serum samples were assayed in duplicate in a single batch using a commercial radioimmunoassay (Immunochem Double Antibody Corticosterone ^{125}I RIA kit, ICN Biomedicals, Costa Mesa, CA).

The blood smears were fixed by pipetting just enough 100% ethanol onto the slide to maintain surface tension, yet still cover the entire surface of the slide. The alcohol remained on the slides for 10 min before being poured off, and the slides were then allowed to dry for 24 h under cover. After the drying period, the slides were covered with sodium phosphate buffer (0.1 M; pH 7.4) in a manner similar to that used with the alcohol. Ten drops of Giemsa stain were added to the buffer on the slide and left to incubate for 50 min. The slides were then rinsed thoroughly with excess phosphate buffer and allowed to dry 24 h under cover. The blood smears were evaluated using an Olympus BH2 microscope at $500 \times$ and/or $1000 \times$ magnification. References were used for cell recognition (Campbell and Dien, 1984; Hawkey, 1989). A total of 250 white blood cells from each of two slides were counted per bird, and the ratio between lymphocytes and heterophils (H:L) was then calculated. Poor staining precluded obtaining results from three birds.

Fig. 1. Tameness of neonatally handled and nonhandled chicks at 10 days post-fledging, as determined by behavioral tests and respiratory rate. Handled chicks were tamer by all measures. (A) Flinch response to touching the chick on top of the head ($P \leq 0.027$). (B) Flinch response to touching the chick on the cheek ($P \leq 0.002$). (C) Rejection or acceptance of an offered food item ($P \leq 0.016$). A, B, and C were evaluated on a point system from zero to two. (D) Latency to approach and perch on the handler's hand ($P \leq 0.002$). (E) Respirations per minute ($P \leq 0.047$).

2.8. DTH testing for cell-mediated immunity

The DTH response was also determined on day 2 by injecting a foreign protein, PHA-P (Sigma, St. Louis, MO; 0.25 mg dissolved in 0.05 ml sterile saline), into the



bird's wing web area at 7:00 a.m. and measuring with a micrometer the thickness of the area before and 12 h after the injection.

2.9. Statistics

Variance of days to fledge between the two groups was analyzed by the *F*-test for equality of variance. Individual tameness tests were analyzed by the Satterthwaite test using equal (dorsal touch, cheek touch) or unequal (latency to approach, respirations/min) variance. Food treat acceptance was analyzed by Student's *t*-test of the means. DTH, serum corticosterone, H:L ratios, and humoral data were analyzed using Student's *t*-test.

3. Results

3.1. Age at fledging

Mean age at fledging in both groups was similar (handled group, 55.5 days, nonhandled controls, 57.3 days), but the variance in fledging was quite different.

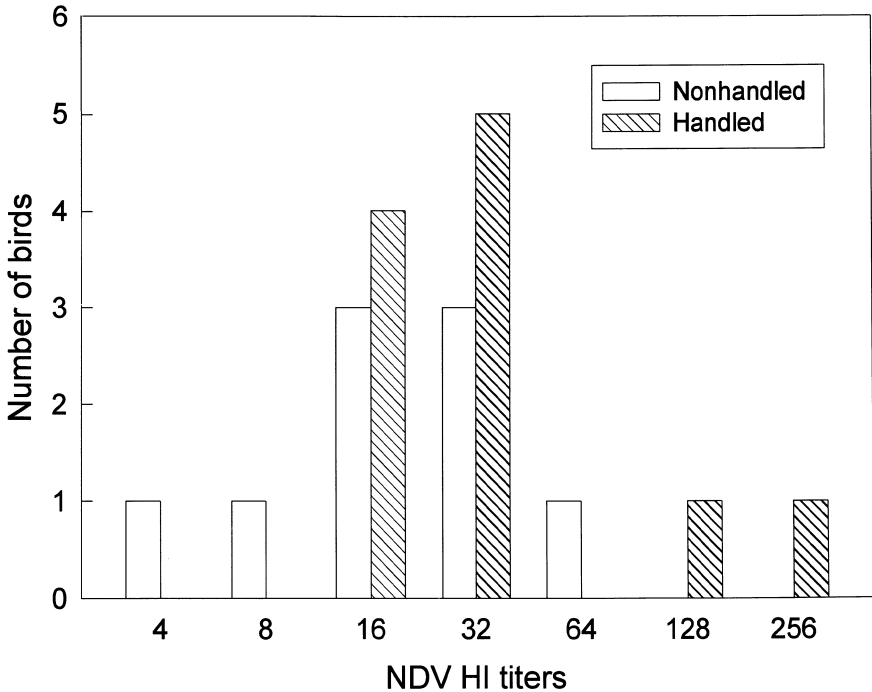


Fig. 2. Humoral response of neonatally handled and nonhandled Amazon chicks to killed Newcastle disease vaccine. The vaccine was administered subcutaneously and a booster was administered 14 days later. Serum was taken 14 days following the booster for detection of NDV antibodies using hemagglutination inhibition. The difference between mean titers was not statistically significant ($P \leq 0.09$).

Fledging in the nonhandled control birds ranged from 55 to 59 days, while fledging in the handled birds ranged from 50 to 62 days. This difference was significant (F -test for equality of variance, $F = 5.90$; $p \leq 0.05$).

3.2. Tameness

The groups showed distinct differences in all tameness tests (Fig. 1). Handled birds allowed human touch and accepted the food items from the researcher much more readily than nonhandled controls (dorsal head touch, $P \leq 0.027$; cheek touch, $P \leq 0.002$; food acceptance, $P \leq 0.016$). The handled group consistently approached and perched on the hand of the researcher, whereas the nonhandled control birds tended to avoid the handler ($P \leq 0.002$). The handled group also had a lower average respiration rate than the nonhandled control group ($P \leq 0.05$), although some of the individual respiration rates of the birds in the handled group overlapped with birds in the nonhandled control group.

There was considerable variation in behavior, even within the handled group. Some of the handled individuals exhibited consistently high degrees of tameness, e.g., #02, #03, while others displayed more aggressive and fearful behaviors (biting, contact avoidance), e.g., #04, #13. Bird #10 was quite tame according to the behavioral tests but was subjectively noted by both handlers to be the least behaviorally adapted to

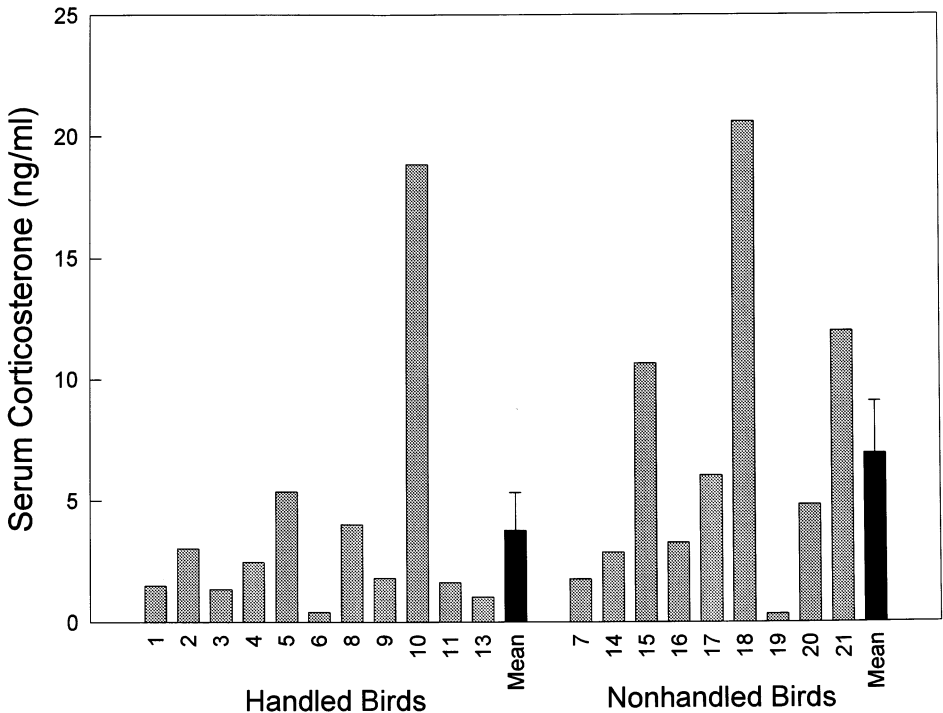


Fig. 3. Serum corticosterone (ng/ml) 12 h after the restraint stress ($P \leq 0.05$, Wilcoxon t -test; $P \leq 0.28$, Student's t -test).

handling of all the handled birds. Despite the variability within the group, handled birds were significantly tamer overall than any of the nonhandled control birds.

Three of the handled birds (#05, #06, #11) consistently resisted being removed from the nest box or cage. Their resistance was often associated with defensive behavior by one or both of the parent birds, which screamed at and/or bit at the handler. The chicks would also occasionally display these behaviors. Once away from the parents, however, they appeared much calmer. As these chicks matured, their resistance to the initial removal from the cage increased.

3.3. Humoral response

The geometric mean titers for the hemagglutination inhibition tests for the handled and nonhandled control groups were 34.08 ± 2.36 and 18.66 ± 2.19 (mean \pm SE), respectively. The difference in antibody response was significant at $P \leq 0.09$ using a two-tailed Student's *t*-test (Fig. 2).

3.4. Corticosterone

The corticosterone levels of handled birds were significantly lower than nonhandled control birds using a nonparametric analysis (Wilcoxon *t*-test, $P \leq 0.05$), but using Student's *t*-test, the groups were not statistically different ($3.76 \text{ ng/ml} \pm 1.57$ vs. $6.92 \text{ ng/ml} \pm 2.15$; $P \leq 0.28$; Fig. 3).

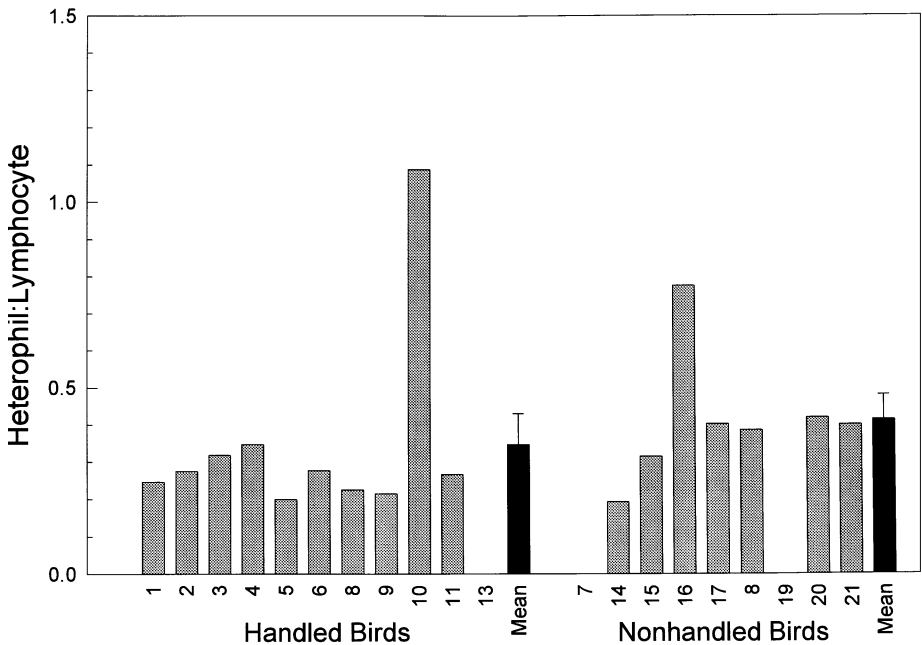


Fig. 4. H:L ratios of neonatally handled and nonhandled chicks. The groups were not significantly different.

3.5. Heterophil:lymphocyte ratio

Birds in the handled group had slightly lower H:L ratios than nonhandled control birds (0.36 ± 0.15 vs. 0.41 ± 0.06), but the difference was not significant (Student's *t*-test; $P \leq 0.13$; Fig. 4). However, one bird (#10) from the handled group had an extremely high ratio that increased the mean for the handled group. This bird also had a much higher corticosterone level than other birds in the group. If the data from this bird is removed, the means of the two groups are statistically different using a parametric test (corticosterone, $P \leq 0.04$; H:L ratio, $P \leq 0.03$; Student's *t*-test).

3.6. Cell-mediated immunity

The handled group had a dramatically reduced DTH response to the PHA-P challenge as compared to nonhandled controls: mean change in tissue thickness of the handled birds was significantly less than that of the nonhandled control birds ($0.28 \text{ mm} \pm 0.06$ vs. $1.12 \text{ mm} \pm 0.11$; Student's *t*-test, $P \leq 0.002$; Fig. 5). This difference was also reflected in the appearance of the injection sites, which were typically discolored and swollen in nonhandled control birds but distinctly less so in handled birds.

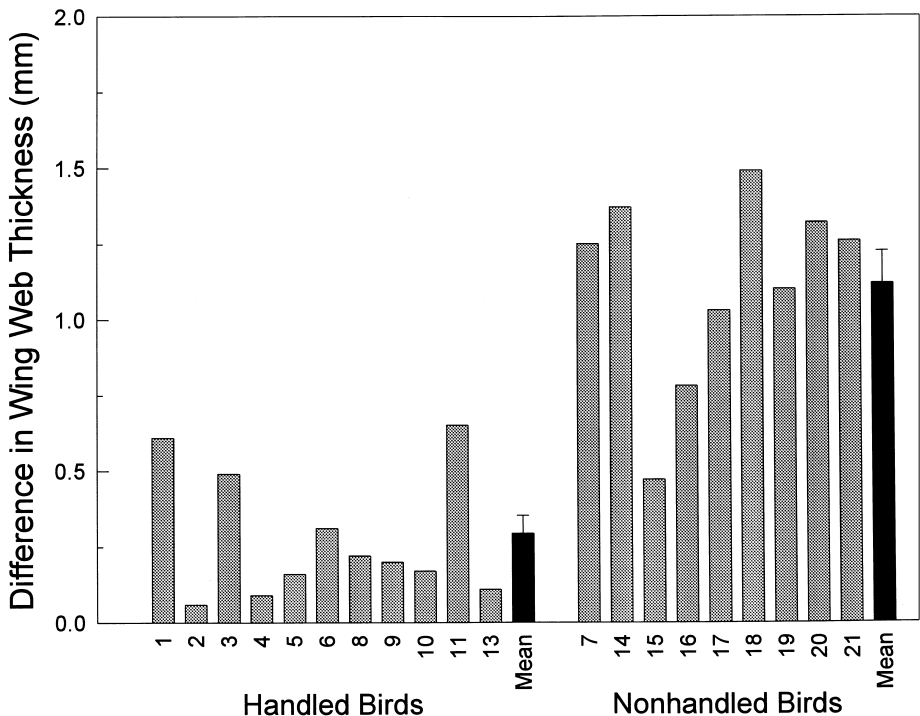


Fig. 5. DTH response to PH-P injected in the wing web of Amazon chicks. Difference in thickness of wing web was measured before and 12 h after injection of PHA-P. The injection sites of the nonhandled chicks at 12 h were visibly red and swollen as opposed to the less visible reactions of the handled chicks. The difference between the groups is highly significant ($P \leq 0.0024$).

4. Discussion

Neonatal handling clearly altered the manner in which young parrots could be held and examined: by fledging, handled birds willingly approached and perched when offered a finger, while nonhandled control birds required restraint in a towel. These extremes in handling/restraint reflect how tame and non-tame birds, respectively, are examined by veterinarians and other humans.

The variance in number of days to fledging was significantly greater in handled than nonhandled control birds. The increased variability in the handled group may indicate that neonatal handling was stressful. Variability in growth is interpreted as an index of stress in poultry (Siegel, 1995). Although corticosterone levels were not determined during the pre-fledging neonatal handling period, such an interpretation is consistent with the lower DTH response and possibly higher serum antibody response to NDV observed in handled birds.

The basis of the behavioral response to neonatal handling in altricial birds is not clear. Daily handling may have provided an opportunity for imprinting-like learning to occur in the handled birds. However, imprinting in chickens, a precocial species, is largely limited to the first week after hatch (Bolhuis, 1991). After imprinting, domestic fowl chicks avoid novel objects and often remain attached to the object on which they have imprinted, even if it is a human. In contrast, altricial species, such as Amazon parrots, can often become highly attached to their owner/feeder even if “tamed” well after fledging and weaning (personal observation). It is not clear whether attachment in both situations shares a common mechanism.

It is also not clear whether any components of the behavioral response to neonatal handling may reflect habituation to a neutral stimulus, habituation to a noxious stimulus, i.e., learned helplessness, or operant learning from a positive experience of being handled. Variability in age of fledging and the post-fledging immune tests may suggest learned helplessness, while willingness to approach and perch on an offered finger suggests positive reinforcement.

The clearest difference we observed in immune performance was in the DTH test of cell-mediated immunity (Fig. 5): nonhandled control chicks showed a greater cell-mediated response; however, their humoral response to the NDV challenge was reduced compared to handled chicks (Fig. 2). These results have conflicting support in the literature. Neonatally handled rats and mice show enhanced humoral and cell-mediated responses (Lown and Dutka, 1987; Persinger and Falter, 1992; Von Hoersten et al., 1993). Similarly, increased antibody production and enhanced resistance to *E. coli* and *M. gallisepticum* have been observed in neonatally handled chickens (Gross and Siegel, 1979, 1982).

In contrast, after rats are restrained by being placed in a glass tube, the number of immune cells (T cells, B cells, monocytes and natural killer cells) in the blood declines by as much as 50% to 80% (Dhabhar and McEwen, 1995). After 3 h, however, the cell numbers return to baseline levels. It was suggested that restraint stress caused the immune cells to exit the blood and enter the bone marrow, spleen, and lymph nodes to be primed to respond more potently to antigens. When 4-dinitro-fluorobenzene (DNFB) was dabbed onto the skin of rats' ears, it caused inflammation and initiated cell-media-

ted immunological memory (Dhabhar and McEwen, 1995). Six days later, rats from one of two groups were again restraint-stressed and again the immune cell numbers decreased in association with increased blood levels of corticosterone, epinephrine and norepinephrine. The restraint-stressed and control groups again had DNFB dabbed on the skin of their ears. The restraint-stressed group had an inflammatory response that was three times larger than the nonstressed group. The inflammatory response happened much more rapidly in the stressed group and remained more prominent for up to 6 days. Possibly, as a result of the stress, a portion of immune cells had gone to the skin of the ear where the cells had been “primed” and the stressed animal had thereby become better prepared for anti-inflammatory response. However, the amount or intensity of the stress can also affect the viability and reactivity of immune cells. If the stress is chronic or too severe, stress hormones that are released may ultimately cause destruction of leukocytes or the immune tissues themselves instead of enhancing the overall response (Brinkmann and Kristofic, 1995). For example, chronic restraint stress in mice causes thymic involution and T-lymphocyte apoptosis (Tarcic et al., 1998). Chronically elevated corticosteroid levels, either by chronic stress (Li et al., 1997) or by corticosteroid supplementation (Brinkmann and Kristofic, 1995), decrease T-helper type 1 responses (i.e., cell mediated) with minimal effects on T-helper type 2 responses (i.e., antibody mediated).

The DTH response of Amazons in the present study is similar to that reported by Dhabhar and McEwen (1995). Birds which were handled daily from 25 days of age through fledging may have interpreted the handling as a chronic stress, causing impairment in thymus function and cell-mediated immunity. In contrast, the nonhandled control group may have experienced only an acute handling stress at the time of testing. The greater DTH response of the nonhandled control group suggests that either (1) the chronic daily stress may have reduced the reaction to PHA-P in the handled birds, and/or (2) the acute stress in the nonhandled birds enhanced the reaction to PHA-P.

The nonhandled birds exhibited trends for higher H:L ratios. It is possible that H:L levels in nonhandled birds may have been higher had measurements been taken closer to the time of restraint. H:L ratios of Japanese quail that were significantly elevated 3 h after a restraint period had dissipated by 12 h after restraint, the time period that we chose to measure H:L ratios (Mills et al., 1993). Nonhandled birds also had significantly higher serum corticosterone levels. Also, NDV titers tended to be greater in handled birds and might have reached the 0.05 level of significance had experimental groups been larger. Corticosterone and H:L ratios both indicate elevated levels of perceived stress in the nonhandled control group. This supports the hypothesis that the acute stress and the hormonal response to acute stress enhanced the DTH response of the nonhandled control birds.

5. Conclusions

Neonatal handling of an altricial species, the Orange-winged Amazon, had significant effects on both the behavior and immune responses of fledged birds. Handled chicks were clearly tamer by all behavioral tests. The DTH response, however, was signifi-

cantly less in handled chicks (Student's *t*-test; $P \leq 0.002$), as were serum corticosterone levels (Wilcoxon; $P \leq 0.05$), while NDV antibody titers levels were higher ($P \leq 0.09$). H:L ratios were not significantly different.

The lower corticosterone levels of the handled birds suggest that postnatal handling permitted birds to be held at fledging by a method that was not interpreted as stressful. The lesser DTH response of handled birds suggests that either chronic neonatal handling was stressful or that the DTH response of nonhandled control birds was actually enhanced by the stress of being restrained in a towel. In either event, periodic neonatal handling produced both behavioral and immune differences to types of restraint that would typically be encountered in the course of normal captive husbandry of Amazon chicks.

Acknowledgements

We sincerely thank Prof. Sharon Hietala, California Veterinary Diagnostic Laboratories, University of California, Davis' for hemagglutination inhibition testing and analysis and Ms. Christina Craig-Veit, Dept. of Avian Sciences, University of California, Davis for invaluable editorial assistance.

References

- Ader, R., Grotta, L.J., 1973. Adrenocortical mediation of the effects of early life experiences. *Prog. Brain Res.* 39, 395–406.
- Aengus, W.L., Millam, J.R., in press. Taming parent-reared Orange-winged Amazon parrots by neonatal handling. *Zoo Biol.*
- Bolhuis, J.J., 1991. Mechanisms of avian imprinting: a review. *Biol. Rev. Cambridge Philos. Soc.* 66, 303–345.
- Brinkmann, V., Kristofic, C., 1995. Regulation by corticosteroids of the Th1 and Th2 cytokine production in human CD4+ effector T cells generated from CD45RO- and CD45RO+ subsets. *J. Immunol.* 155, 3322–3328.
- Campbell, T.W., Dien, F.J., 1984. Avian hematology: the basics. *Vet. Clin. North Am.* 14, 18–24.
- Dhabhar, F.S., McEwen, B.S., 1995. Enhancement of immune function by acute stress. *Soc. Neurosci. Abstr.* 21, 1396.
- Dhabhar, F.S., Miller, A.H., Stein, M., McEwen, B.S., Spencer, R.L., 1994. Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. *Brain Behav. Immun.* 8, 66–79.
- Gross, W.B., Siegel, P.B., 1979. Adaptation of chickens to their handler, and experimental results. *Avian Dis.* 23, 708–714.
- Gross, W.B., Siegel, P.B., 1982. Socialization as a factor in resistance to infection, feed efficiency, and response to antigen in chickens. *Am. J. Vet. Res.* 43, 2010–2012.
- Gross, W.B., Siegel, H.S., 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Dis.* 27, 972–979.
- Hanson, R.P., 1980. Isolation and identification of avian pathogens. *Am. Assoc. Avian Pathol.*, Arnold Printing, New York, NY.
- Hawkey, C.M., 1989. Color atlas of comparative veterinary hematology: normal and abnormal blood cells in mammals, birds and reptiles, 1st edn. Iowa State Univ. Press, Ames, IA.
- Hemsworth, P.H., Coleman, G.J., Barnett, J.L., Jones, R.B., 1994. Behavioural responses to humans and the productivity of commercial broiler chickens. *Appl. Anim. Behav. Sci.* 41, 101–114.

- Hess, J.L., Denenberg, V.H., Zarrow, M.X., Pfeifer, W.D., 1969. Modification of the corticosterone response curve as a function of handling in infancy. *Physiol. Behav.* 4, 109–112.
- Jones, R.B., 1993. Reduction of the domestic chick's fear of humans by regular handling and related treatments. *Anim. Behav.* 46, 991–998.
- Jones, R.B., Waddington, D., 1993. Attenuation of the domestic chick's fear of human beings via regular handling: in search of a sensitive period. *Appl. Anim. Behav. Sci.* 36, 185–195.
- Keller, S.E., Weiss, J.M., Schleifer, S.J., Miller, N.E., Stein, M., 1981. Suppression of immunity by stress: effect of a graded series of stressors on lymphocyte stimulation in the rat. *Science* 213, 1397–1399.
- Levine, S., 1957. Infantile experience and resistance to physiological stress. *Science* 126, 405–406.
- Levine, S., 1962. Plasma-free corticosteroid response to electric shock in rats stimulated in infancy. *Science* 135, 795–796.
- Li, T., Harada, M., Tamada, K., Abe, K., Nomoto, K., 1997. Repeated restraint stress impairs the antitumor T cell response through its suppressive effect on Th1-type CD4+ T cells. *Anticancer Res.* 17, 4259–4268.
- Low, R., 1991. Hand-rearing parrots and other birds. Sterling Publishing, New York, NY.
- Lown, B.A., Dutka, M.E., 1987. Early handling enhances mitogen responses of splenic cells in adult C3H mice. *Brain Behav. Immun.* 1, 356–360.
- Meaney, M.J., Aitken, D.H., Bodnoff, S.R., Iny, L.J., Tatarewicz, J.E., Sapolsky, R.M., 1985. Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. *Behav. Neurosci.* 4, 765–770.
- Meaney, M.J., Aitken, D.H., Sharma, S., Viau, V., Sarrieau, A., 1989. Postnatal handling increases hippocampal glucocorticoid receptors and enhances adrenocortical negative-feedback efficacy in the rat. *Neuroendocrinology* 50, 597–604.
- Millam, J.R., Kenton, B., Jochim, L., Brownback, T., Brice, A.T., 1995. Breeding orange-winged amazon parrots in captivity. *Zoo Biol.* 14, 275–284.
- Mills, A.D., Jones, R.B., Williams, J.B., 1993. Responses to isolation in Japanese quail genetically selected for high or low sociality. *Physiol. Behav.* 53, 183–189.
- Myers, S.A., Millam, J.R., Roudybush, T.E., Grau, C.R., 1988. Reproductive success of hand-reared vs. parent-reared cockatiels (*Nymphicus hollandicus*). *Auk* 105, 536–542.
- Persinger, M.A., Falter, H., 1992. Infantile stimulation produces mild enhancement in a primary humoral response of adult albino rats. *Psychol. Rep.* 70, 976–978.
- Plotsky, P.M., Meaney, M.J., 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Mol. Brain Res.* 18, 195–200.
- Raymond, L.N., Reyes, E., Tokuda, S., Jones, B.C., 1986. Differential immune response in two handled inbred strains of mice. *Physiol. Behav.* 37, 295–297.
- Siegel, H.S., 1995. Stress, strains and resistance. *Br. Poult. Sci.* 36, 3–22.
- Solomon, G.F., Levine, S., Kraft, J.K., 1968. Early experience and immunity. *Nature* 220, 821–822.
- Steplewski, Z., Vogel, W.H., 1986. Total leukocytes, T cell subpopulation and natural killer cell activity in rats exposed to restraint stress. *Life Sci.* 38, 2419–2427.
- Tarcic, N., Ovardia, H., Weiss, D.W., Weidenfeld, J., 1998. Restraint stress-induced thymic involution and cell apoptosis are dependent on endogenous glucocorticoids. *J. Neuroimmunol.* 82, 40–46.
- Von Hoersten, S., Dimitrijevic, M., Markovic, B.M., Jankovic, B.D., 1993. Effect of early experience on behavior and immune response in the rat. *Physiol. Behav.* 54, 931–940.