Handling and field stress in a transplanted F1 ring-necked pheasant population as determined by corticosterone levels in plasma and adrenal glands

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Handling and field stress in a transplanted F₁ ring-necked pheasant population as determined by corticosterone levels in plasma and adrenal glands

by

Robert Charles Goetz, Jr.

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

Many state game organizations have been involved in stocking programs to establish a huntable population of ring-necked pheasant (Phasianus colchicus). A problem associated with the programs has been post-release mortality (Ginn, 1947; Kabat et al., 1956; Anderson, 1964). Mortality has been of such proportions in some instances that the birds released failed to establish a population. Many theories exist as to the reasons for the post-release mortality and release failure, but there has been limited evidence to support them.

A stocking operation was carried out in the fall of 1970 by the Iowa Conservation Commission using birds 3-4 months of age that were first generation progeny (F₁) of wild-trapped birds. These birds were reared at the Boone Station and moved to southeastern Iowa where they were released. The area of release was void of pheasants and closed to pheasant hunting.

In this study the birds were evaluated physiologically by measuring stress before and during the stocking operation. By sampling the birds before stocking, the condition of the birds prior to release could be examined to evaluate the effects of caging and crowding. Kabat et al. (1956) state that caging is a considerable stress, and according to other investigators (Siegel, 1959; 1960; Flickinger, 1961; Balding, 1967; Gross, 1972), penned birds undergo stress due to crowded conditions. These birds form a social hierarchy where the plasma corticosterone levels and adrenal size vary according to social position. Others
(Wolford and Ringer, 1962) do not agree with this concept but they appear to hold a minority opinion.

Stress of handling has been recognized by most researchers (Wolford and Ringer, 1962; Nagra et al., 1963b; Balding, 1967; Freeman, 1967; 1969; Harris, 1970; Perhach and Barry, 1970) as an important part of total stress. Handling has even been used as a deliberate source of stress (Freeman, 1969). Many efforts have been made to remove the effect of handling in studies concerning stress. The efforts ranged from putting the birds in small boxes, to confinement in large isolated pens, and use of anesthesia. The act of manipulating the birds to change their environment constitutes "stress of handling", as does restraining them for blood sampling. These attempts to remove stress of handling have been unsuccessful. In the present study, the stress of handling was removed from a second control group by killing the birds before sampling. These birds were unaware of human presence before their death.

Adrenal parameters were examined as a measure of the relative degree of stress during each stage of the stocking operation. Corticosterone was identified in the bird in 1957 (Phillips and Chester Jones, 1957), and later proven to be the principal corticoid in birds (Brown, 1961; deRoos, 1961a). Trace amounts of cortisol and cortisone were reported in certain species (Phillips and Chester Jones, 1957; Urist and Deutsch, 1960), but others (deRoos, 1961a; Nagra et al., 1960) have not been able to demonstrate corticoids other than corticosterone.

All groups in this study were compared with the second control group to obtain an evaluation of stress differences without the stress of
handling as an additive factor. Data from the second control group were contrasted with those from wild birds (Kirkpatrick, 1944; Anderson, 1972) to obtain an indication of the stress of caging and crowding.
LITERATURE REVIEW

Stress-Adrenal Relationship

According to the General Adaptation Syndrome Theory (Selye, 1946; 1973), an animal developed reactions that made it resistant to any stressful stimuli. The reactions were termed nonspecific or specific. That reaction causing the animal to resist a specific stress was a specific adaptation, while reactions to different stimuli were nonspecific adaptation. Selye termed the sum of these nonspecific responses to prolonged stress as General Adaptation Syndrome which can be divided into three series of reactions.

First was the "alarm reaction" which can further be divided into the "shock" and counter shock" phases. During the shock phase, one saw a hemoconcentration, depression of metabolic rate, lowering of body temperature, catabolism of tissue, and many other physiological responses. If one of these or all of these did not cause the death of the animal, it recovered by the responses termed counter shock. Here ACTH secretion reached high levels and as a result there was increased release of corticoids from the adrenal gland. These steroids enabled the animal to recover and develop a resistance to the stress.

Secondly, after the alarm response continued stress resulted in the development of additional resistance to stress ("adaptation stage" or "resistance"). The level of the cortical steroids was increased in the blood stream during this time. But the resistance to other stresses was not increased and may even be decreased.
Thirdly, the response could be maintained for great lengths of time. The resistance dropped and the animal died with symptoms similar to those animals in the shock stage. The animal had lost its ability to adapt or its "adaptation energy" as termed by Selye. The third stage was Selye's "exhaustion stage". Failure to adapt at any of these stages may result in death.

Adrenal Autonomy

Many investigators have reported various results in different species as to the relationship of the avian adrenal and the pituitary. There was controversy as to the extent, if any, to which adrenal corticoid secretion was controlled by pituitary hormones. The confusion began when investigators reported young and adult pigeons surviving hypophysectomy for one or more years (Schooley, 1939). The pigeon adrenal was found to be functional with only the anterior pituitary removed (Miller and Riddle, 1942). The authors noted a 20% drop in adrenal weight, but with stress applied, 4% formaldehyde solution injected daily, the adrenal weight increased sharply. Miller some 20 years later, repeated the experiment (Miller, 1961), and found the same results. The adrenal gland was denervated and the responses were the same as in Miller's original experiment.

It was first thought that the chicken was less resistant to hypophysectomy and therefore exhibited less autonomy but later, age was shown to influence the response. Hypophysectomized chickens were reported to live only 2 weeks (Mitchell, 1929) or according to Hill and Parkes
(1934), for no longer than 48 hours; chicks of less than 7 days of age lived only 5 days (Bates et al., 1940). Chickens in the age class of 40 to 50 days lived up to a year (Nalbandov and Card, 1943). It was reported that the operation caused an extensive degeneration of the adrenal, but some recovery was observed by Nalbandov and Card (1943). In a later study Ma and Nalbandov (1963) noted that the adrenal weight fell immediately following surgery, but the original weight was regained 13 days later and thereafter adrenal weights paralleled those in the control group.

Investigators using young chickens (Baum and Meyer, 1956; Brown, et al., 1958; Newcomer, 1959b) added support to the view that the avian adrenal possesses a high degree of autonomy since in their studies there was no significant decrease in adrenal weight, in the cholesterol content, in total protein, or excretion of uric acid. Brown (1960; 1961) supported the theory of autonomy because the levels of corticosterone in the peripheral blood remained unchanged after hypophysectomy. But Brown was unable to show corticosterone increases when the birds were stressed by cold and dehydration. In the chicken (Newcomer, 1959a), the $\Delta^4$-ketocorticoids remained constant after the operation. But in both the chicken and pheasant, Meyer (1962) reported a significant drop in corticosterone in the adrenal venous blood while the gland weight remained constant. In contrast, adenohypophysectomy was reported to cause a marked decrease in the adrenal weight of pheasants (Resko et al., 1964).
Urist and Deutsch (1960) added meaning to the controversy of adrenal weight and plasma corticosterone level by reporting that, in the rooster, increased levels of corticosterone were not accompanied by a significant increase in adrenal weight. In direct support of this thesis, Garren et al. (1961), showed that an increase in adrenal activity (decreased body weight and size of bursa of Fabricius) could occur without an increase in the weight of the adrenal gland. They also showed that with increased activity one may see an increase in adrenal weight but not necessarily in all instances. Brown et al. (1958) reached a similar conclusion in that adrenals of birds do not necessarily hypertrophy when stimulated to a highly secretory state. Certain investigators (Frankel et al., 1967c,d; Resko et al., 1964) were able to show solid evidence against adrenal autonomy with a series of in vitro experiments where hypophysectomy did cause a drop in corticosterone level in the plasma, and a decrease in corticosterone production by adrenal glands. Later Newcomer et al., (1972), comparing intact and hypophysectomized cockerels, reported a decrease in both plasma and adrenal tissue corticosterone levels, but it was not statistically significant. They suggested that the concentrations of free and bound corticosterone may throw some light on the autonomy picture.

The issue of autonomy gained its greatest support from the fact that a complete hypophysectomized animal would survive and respond to stressful agents. The plasma levels of corticosterone were the indicators of activity.
In an effort to disprove the theory of autonomy, where a bird without a pituitary can have adrenals functioning at a base level with no control from the central nervous system, directly or indirectly, pituitary autotransplants were implemented by Ma and Nalbandov (1963) with little success. Although the pituitary cells appeared revitalized, the adrenal weight, ascorbic acid content, and $^{32}$P-uptake of the adrenal glands corresponded to those of adenohypophysectomized cockerels. Resko and associates (1964) reported corticosterone levels in cockerels with autotransplants to be lower than in intact birds, and comparable to that of the adenohypophysectomized birds. But other investigators dealing with the domestic duck, quail, and pigeon (Boissin, 1967; Bayle et al., 1967) reported no significant difference in plasma corticosterone levels after autotransplants. Thus, there appeared to be a difference between species as to the degree of autonomy. The pigeon has appeared to be very contradictory in that the pigeon survives well with no pituitary, yet the adrenal can be significantly influenced by the relocated pituitary. Assenmacher and Bayle (1969), in work with the three species, duck, quail, and pigeon, showed a drop of corticosterone in the adrenal tissue, but not as low as that of hypophysectomized birds.

Miller (1961) reported data in opposition to adrenal autonomy. He put forth three postulations to explain the reactions of adenohypophysectomized birds to stress: a) there may be one or more sources of ACTH other than the pituitary; b) two stimuli cause adrenal enlargement; and c) stresses such as formaldehyde may activate a pathway through which
ACTH may exert its effect. Miller placed the first postulation as most unlikely, but Ma and Nalbandov (1963) supported this postulation. Many investigators agreed with the theory of extrahypophyseal ACTH or ACTH-like substances in the bird (Resko et al., 1964; Frankel et al., 1967b, c,d,e; Salem et al., 1970a,b).

The hypothalamus was suggested as the most likely area for the location of an extrahypophyseal regulatory mechanism. This suggestion was made because hormones of the posterior hypophysis did not stimulate corticosterone increases in plasma. Resko et al. (1964) used pitressin which failed, and Frankel et al. (1967d) used the avian posterior pituitary hormone, arginine vasotocin, with no stimulation of corticosterone production. Because investigators using total hypophysectomy had failed to achieve any insight, the hypothalamus was the next logical area for investigation. Frankel (1970) listed reasons that would justify research on the hypothalamus: a) the ventral tuberal nucleus was involved with ACTH in the intact bird; b) dexamethasone, a hypothalamic inhibitor, completely depressed adrenal function in the adenohypophysectomized bird; c) electrolytic lesion in the ventral tuberal nucleus seemed to enhance adrenal function.

Frankel et al. (1967d,e), working in vitro with hypothalamic and cerebral cortex tissue from adenohypophysectomized birds failed to demonstrate any ACTH-like activity. Later, studies were made by Salem et al. (1970a) where chicken hypothalami were assayed on hypophysectomized rats; the rats showed ACTH-like responses. Salem et al. (1970b) with more work demonstrated that the ACTH-like substance in the hypothalamus
was different than the ACTH of the pituitary by virtue of its resistance to boiling. Both mammalian ACTH standard and chicken pituitary lost their adrenal ascorbic acid depletion (AAAD) activity after 30-60 minutes exposure to boiling. The hypothalamic extracts retained activity after 60 minutes of boiling. Salem et al. (1970a,b) suggested that the ACTH-like substance may be in the hypothalamus and its true identity may be alpha melatonin stimulating hormone (α-MSH). Van Tienhoven (1969) suggested that the extrahypophyseal source of ACTH-like substance could be the pineal gland. The pineal gland produces 5-hydroxytryptamine, and in vitro acting on the adrenals of hypophysectomized rats, 5-hydroxytryptamine can stimulate corticosterone production. Additional research is necessary before the question of adrenal autonomy can be answered.

Avian ACTH

Brown et al. (1958), Newcomer (1959a), Newcomer and Connally (1960) working with chickens, and Flickinger (1959) working with California quail reported little or no change in the activity of the adrenal gland following treatment with mammalian ACTH. These investigators had a great deal to do with establishing the autonomy theory. Even a decrease of activity (lower corticosterone level) was observed after a massive dose of ACTH in birds by Phillips and Chester Jones (1957). Other workers (Bates et al., 1940; Jailer and Boas, 1950; Zarrow et al., 1962; Conner and Shaffner, 1954; Miller and Riddle, 1942; Miller, 1961; and Garren et al., 1961) reported results in direct opposition to those investigators who demonstrated a response of the adrenal gland to ACTH. Brown et al.
(1958) reported data which showed a relatively small increase in adrenal weight of intact young chickens and hypophysectomized chickens both treated with ACTH.

The early studies of the effect of ACTH on the adrenal gland activity indicated a range from a decrease to a very slight increase to a large significant increase. Phillips and Chester Jones (1957) reported a decrease characterized as adrenocortical exhaustion (Holmes et al., 1961; Phillips et al., 1961), due to the massive dose of ACTH. Nagra et al. (1960, 1963a), Urist and Deutsch (1960), Brown (1961), Breitenbach (1962), Resko et al. (1964), Frankel et al. (1967c), and Macchi et al. (1967) failed to substantiate the decrease of corticosterone even with massive doses of ACTH. More light was shed on the effect of ACTH by Siegel (1961, 1962a,b) when age and sex were reported to modify the response of young chickens. Siegel suggested factors to account for the conclusions reached by Bates et al. (1940) in his studies. Zarrow et al. (1962) pointed out a fault with some research data in that cortisone was used as the corticoid treatment and the adrenal activity failed to lessen. Zarrow stated that cortisone was shown not to be released from the avian adrenal, and that if ACTH did not elicit a response it was because of improper injections.

Siegel and Beane (1961) did a study to investigate the effectiveness of ACTH as a result of time. Garren et al. (1961) reported the influence of dosage and frequency of injection, and pointed out the importance of the vehicle for ACTH, since it was found that the zinc used
in mammals did not work in birds. The vehicle importance was reported by Zarrow et al. (1962). Beeswax-oil was shown to give better results than the saline suspension used by Garren (Zarrow et al., 1962). Zarrow and Garren drew similar conclusions that increased frequency of injection or, better still, the continuous release of ACTH promotes more activity and hypertrophy of the adrenal gland. Salem et al. (1970b) suggested the thesis that the avian pituitary gland contains relatively more ACTH than that of the rat, which was not understood in earlier work and was a source of error as to effective dosage.

DeRoos and deRoos (1964), established the existence of an avian ACTH which until that time was merely assumed to be present. The purified avian ACTH has yet to be available. The need for avian ACTH has been brought to light by Macchi et al. (1967), who worked with the gull, a species rather unresponsive to mammalian ACTH. The degree of insensitivity of other species to mammalian ACTH can only be guessed at. Avian ACTH can now be said to elicit a response similar to that seen in mammals, but the question of adrenal gland autonomy still is unanswered.

Adrenal Histology

The avian adrenal is structured not as the mammalian adrenal with a distinctive cortex and medulla, but with the two areas intertwined. The tissue which corresponds to the cortex is termed interrenal tissue, and that tissue comparable to the medulla is referred to as chromaffin. Work describing in much detail the anatomy and histology of the avian adrenal gland was done in the 30's, 40's, and 50's. Researchers worked with the
domestic chicken (Uotila, 1939), and the pigeon (Miller and Riddle, 1942). Hartman and associates described the adrenal glands of hundreds of wild bird species (Hartman, 1946; Hartman et al., 1947; Hartman and Brownell, 1949; Hartman and Albertin, 1951; Knouff and Hartman, 1951). Later Flickinger (1959) described the gland in quail while Balding (1967) described to a limited extent the pheasant adrenal.

In general, the avian adrenal gland is encapsulated by a thin capsule under which is found a vascular area. Nerves course through the subcapsular area. The interrenal tissue is organized in strands which project up to the capsule and then arc back to the center of the gland where they associate with other strands. When sectioned, these strands or cords appear as rounded structures of tightly arranged cells. The chromaffin tissue exists as a connecting network throughout the interrenal tissue and, in sections, appears as dark staining islets. DeRoos (1963) shows a distinct difference, according to species in the relative amounts of these two tissues.

Most investigators agree that the interrenal tissue exhibits no zonation except in the brown pelican. In the pelican three zones do exist and resemble the zones of the mammals as to position, but not to proportions (Knouff and Hartman, 1951). Yet, many investigators reported an atrophy of the central cortical region in the absence of the pituitary and a zonation of this region similar to that of the mammalian adrenal gland. Miller (1961) reported atrophy in pigeons, Assenmacher (1958) in ducks, and Frankel et al. (1967e) in cockerels. Bhattacharyya and Ghosh (1972) failed, as did most work of others, to demonstrate any
zonation of the interrenal tissue in quail, parakeet, and myna. Most investigators agree that with hypophysectomy there is a mid-region atrophy but little effect in the periphery; the exception is the work of Nalbandov and Card (1943) who showed an increase in the proportion of interrenal tissue in adenohypophysectomized cockerels. Frankel (1970) suggested this occurred due to a stress which led to the hypertrophy of the gland. The question of zonation is left to more investigation.

The interrenal cords are composed of tall columnar cells which in cross section form a circle with a sinus in the center. The nuclei of the cells are located in the end bordering the central sinus. The opposite end of each cell borders a blood sinus. The tissue is highly vascularized with sinusoids seen between the cords and between cords and the chromaffin tissue. Fujita (1961) examined the chicken adrenal under the electron-microscope, and reported a perisinusoidal space between the basement membrane of the endothelial cell and the interrenal cell. A parenchymal space was found between the chromaffin and interrenal cells, thus secretions from the interrenal tissue must pass through these spaces before entering the blood vessels.

Fujita (1961) described the cortical cells as having a higher relative percentage of cytoplasm with many vacuoles and mitochondria. He suggested that the lipids associated with the cortical hormones are produced in the vacuoles by endoplasmic reticulum with support from the mitochondria. Sheridan et al. (1963), described the interrenal cells of the pelican as evenly distributed throughout the tissue.

The importance of histology was seen in the early studies where
the adrenal weight failed to change with increased activity; even though cells did not hypertrophy or go through hyperplasia, yet the level of corticosterone was elevated.

Adrenal Rhythms

From studies in mammals, a rhythmicity was established in diurnal animals, man, rat, and mouse where corticosteroid production was maximal at mid-morning hours 0600-0900, and fell to minimal levels by midnight. This cycle was shown to be just reversed in nocturnal animals. A different cycle was first shown in birds by Boissin and Assenmacher (1968) when they reported a 12-hour cycle in the quail. Dusseau and Meier (1971) demonstrated a 24-hour cycle in the white-throated sparrow.

The circadian rhythm in the domestic duck was demonstrated by Chan and Phillips (1973a) and the high activity periods for the adrenal were 0600-0800 hours and 1600-2000 hours. These periods compared very closely to those of the quail, a wild bird, whose adrenal activity periods were 0500-0900 hours and 1700-1900 hours. From the small amount of data in the literature, two things seemed to stand out; a) the need for further research to establish the values for more species, and b) the need of caution for collecting data during the same period each day.

The other rhythm reported in birds, the circennian rhythm, has been investigated to greater lengths. Resko et al. (1964) reported substantial differences in corticosterone levels in the same age group sampled at different times of the year. Soule and Assenmacher (1966) also found a seasonal difference in the corticosterone levels of ducks.
Chan and Phillips (1973c) found corticosterone production *in vitro* to vary seasonally in the herring gull. Other investigators using adrenal weights found seasonal differences in adrenal weight in the herring gull (Hartman, 1946), the mallard (Hohn et al., 1965) and the pheasant (Anderson, 1972).

As Dusseau and Meier (1971) found that the white-throated sparrow differed from the 12-hour cycle, so did Macchi et al. (1967) find that the duck differed in the seasonal activity cycle. Other investigators reported winter as the maximal period of activity and spring the low, but Macchi et al. (1967) showed spring to be the high and winter the low periods of activity. It was postulated that the adrenal activity may follow the gonadal cycle in an inverse manner, but Kirkpatrick (1944), Hine and Fladas (1957), Greeley (1957), and Anderson (1972) reported that adrenal activity followed the gonadal cycle directly.

Two investigators using the same method, adrenal fractional cortical volume, and both using passerines came to contrasting conclusions. Fromme-Bouman (1962) reported that in European blackbirds the period of minimal activity was fall, and the maximal period was in spring. Lorenzen and Farner (1964) reported the high period to be in winter and the low to be in summer.

From the literature, it can be seen that there is very limited knowledge of the avian adrenal rhythms. More species need investigation because of variation between the reported 12-hour and 24-hour cycles. Also, nocturnal species must be included in the future work, and more work must be done on seasonal activity of the adrenal as related to gonad cycle.
Corticosterone-Stress of Handling

Investigators of stress have used many criteria for evaluating the activity of the adrenal gland. The earlier workers used adrenal weights until it was shown that the gland need not enlarge to achieve a high level of secretory activity (Urist and Deutsch, 1960; Garren et al., 1961). Depletion of ascorbic acid was used in many studies, but a distinct controversy exists concerning its reliability as a parameter (Frankel, 1970). The adrenal ascorbic acid has been reported to be depleted, not to be depleted, or depleted and replenished in various studies involving different species of birds. The usefulness of AAAD is questionable, although Salem et al. (1970b) used it and obtained significant results in contrast to Zarrow and Baldini (1952), who found no depletion of ascorbic acid in the quail.

Most researchers involved with adrenal activity have used corticosterone levels in plasma as an index. This parameter has been used since Phillips and Chester Jones (1957) identified corticosterone as the major steroid in the bird. Many have verified corticosterone as the major secretory steroid of the adrenal gland in birds; deRoos (1961a,b) in the chicken, pigeon, duck, western gull, brown towhee; Nagra et al. (1960) in the pheasant; Brown (1961) in the turkey; and Sandor et al. (1965) in the goose. Cortisone and cortisol have been reported as secretory products of the chicken adrenal gland by Urist and Deutsch (1960) and deRoos (1960), but Sandor (1969) states that it is unlikely that cortisol is present in any avian species. DeRoos (1960, 1961), in an in vitro study, demonstrated 11-dehydrocorticosterone in a measureable
amount in the gull. DeRoos suggested its presence in the chicken, duck, and pigeon. Chan and Phillips (1973a) working in vitro and in vivo with the gull identified deoxycorticosterone. Yet with all data considered, the general conclusion is that corticosterone is the major secretory corticoid of the avian adrenal gland. In accord with this, most investigators use the plasma corticosterone as a basic measure of activity.

Most investigators have suggested or referred to the ever-present stress of handling and its influence on data. In working with the wild blue-winged teal, Harris (1970) attempted to reduce handling stress by using a holding box with a black interior. Harris examined blood for non-protein nitrogen, free fatty acids, glucose, acetoacetate, free amino acids, and acidophilin which responded to corticosterone. Balding (1967) used a holding box with pheasants in an effort to estimate handling stress, and used plasma corticosterone levels for his index. The point missed by both studies was that in taking the blood samples the stress of handling was still present. Wolford and Ringer (1962), using laying hens, reported that handling, feed deprivation, ACTH, and cold exposure were the only factors used that altered the heterophil or lymphocyte percentages significantly. Thus handling stress was an important factor for consideration.

Freeman (1967) working with young chickens reported depletion of ascorbic acid within 10 minutes of handling, and believed that the response may occur within a minute. Freeman believed that in others' experiments the handling during the sampling had depleted the ascorbic
acid equally in controls and the ACTH-treated birds. Freeman stated that the important fact was that the ACTH-treated birds had replenished the acid in the gland within one hour after the treatment, thus the depletion was not demonstrated by many previous investigators. Freeman (1967, 1969) stated that the stress response to handling occurs much more rapidly in birds than in mammals.

While sampling wild birds, the struggling of the birds is much more pronounced than in domestic birds so they must be restrained more forcibly, increasing stress. Investigators have not compared the degree of stress in restraining domestic fowl versus wild fowl. But Perhach and Barry (1970) working with laboratory rats, studied different types of restraint and the animals' response to each. Plasma corticosterone levels were measured at three intervals (1, 4, and 24 hours) in rats restrained by complete body immobilization and by neck restraint which allowed struggling. The neck restraint condition produced higher levels in all intervals.
MATERIALS AND METHODS

Birds

The birds used in this study were F<sub>1</sub> offspring of wild birds which were captured in southwestern Iowa by the Iowa Conservation Commission. Adult wild birds were kept at the Boone Game Farm as a laying flock. The eggs were collected and incubated. The young birds were reared to approximately 4 months of age, and were then caught and transported to an area void of pheasants in southeastern Iowa. Here they were released into predetermined suitable areas. Approximately 3,000 birds were released and 10% of this population was used as a sample.

Experimental Design

Birds were reared in 150 ft x 50 ft pens which had built-in catch chutes into which the birds were driven. Early each morning they were banded and weighed and placed in shipping crates which were divided in the center. Ten birds were placed in each side. The crates were stacked onto trucks and transported to the release areas. Birds were in the crates for approximately 5 hours before the actual unloading and release. Birds were allowed to acclimate to their new habitat for over a month before any birds were collected from the field. Samples were taken using 10% of the birds at five chosen points of the operation. The five groups used were:

1. Banding-birds sacrificed at time of banding
2. Release-birds sacrificed at time of release
3. Return-birds brought back in crates and returned to smaller holding pens to be caught and sampled next day.

4. Field-birds taken from the field in flight and on the ground with shotguns.

5. Control 1 - birds moved to smaller holding pens immediately after banding, and sampled to correspond to field birds.

6. Control 2 - birds shot in head while undisturbed.

Group 6 was added as a result of a supplemental study. The study was needed when it was found with preliminary assays that the original controls (Group 5) were not controls as to the stress of the handling and sampling. In order to get birds relatively free from the stresses of handling and sampling, 30 birds were maintained in a rearing pen and allowed to acclimate for 30 days until they reached the age of the previous groups. These birds were collected from a blind which was the brooder house in which they had been started as chicks. They were shot in the head while feeding at close range with birdshot from a .22 caliber rifle. The first five birds were collected one per day with at least 2 days between collections. The other birds were taken 4 per day or 5 per day until a total of 23 birds were collected. At least a 2-hour interval between collection of individuals was maintained with a 24-hour interval between group collections.

In each group, blood samples were taken and adrenal and thyroid glands were removed. The blood sample was split to obtain plasma to determine corticosterone levels, and to obtain serum to determine triiodothyronine-thyroxine (T₃-T₄) levels. The adrenals were separated and one was frozen.
for determining corticosterone and total protein content, and the other was placed either in Bouin's fixative or 10% formalin solution for histological work; right and left adrenals were alternated. Both the thyroids of each bird were also placed in one of the two fixing solutions for histological work. Body weight was recorded at the time of banding, sampling and at necropsy. At necropsy the livers were weighed and the digestive tracts were weighed. The gross appearance of fat was also noted.

Experimental Procedure

Two parameters were used to obtain an evaluation of the relative amount of stress at the five different points of the transplanting operation. The peripheral plasma corticosterone levels, and the adrenal gland corticosterone levels as related to the protein of the gland were used. The protein was used since it was impractical to carry a balance in the field to weigh the glands at the sampling point.

**Corticosterone levels in adrenal glands and plasma**

Sampling was done in the same manner for the four groups (banding, release, return, and control 1). The birds were restrained on their sides with neck outstretched so the feather tract could be moved aside to expose the jugular vein. A 5 ml syringe with a 21 ga needle was used to draw a 5 ml blood sample from the jugular. The vein was slightly distended by slight pressure of the thumb on the vein at the base of the neck (Appendix A). Two screwcap 2 dram vials were used to receive equal halves of the sample. One vial was heparinized in the drying oven the previous night and thus allowed for plasma collection needed for
corticosterone determinations. In the other vial the blood clotted, and the serum was collected for future T₃-T₄ determinations.

Sampling of the field group and the control 2 group differed from the other groups. The thoracic cavity of the shot bird was immediately opened to expose the cardiac area. A 22 ga needle was inserted into the inferior vena cava or heart and a 5 ml sample of blood removed (Appendix A). In most cases the heart was still beating and a sample was taken within 60 seconds. But when the heart was not beating, blood was removed from the heart plus large vessels such as the femoral, brachial, aorta, and common iliacs until a 5 ml sample was obtained or as maximal an amount as possible.

The adrenals were removed by incising the dorsal aorta just cranial to the adrenal glands (Appendix B). One tip of a small curved forceps was placed into the vessel where it courses between the paired glands. The glands were slowly removed by cutting the connective tissue and aorta posterior to the glands. The two glands were then separated and alternately frozen or placed in one of the two histological fixatives. This worked with no complications in the males, but with the females the ovary lies directly on and covers the ventral surface of the left gland. The first step was to slowly strip the quiescent ovary from the gland leaving no remnants of the tissue. From this point, the removal procedure was the same as for the males.

Frozen glands were thawed and then homogenized in 2.0 ml of 33% ethanol, and transferred to a 5.0 ml volumetric flask. The homogenate was then diluted to 5 ml with double-glass-distilled water and shaken
(Zarrow et al., 1964). The homogenate was transferred to screwcap culture tubes and centrifuged for 10 minutes at 1500 rpm. Then 0.5 ml aliquots of the supernatant were transferred to 15 ml glass stoppered centrifuge tubes for assay. Determination of the corticosterone of the glands (Appendix D) was made using fluorometric techniques (Van der Vies, 1961; Zarrow et al., 1964).

The plasma samples needed no preliminary treatment so 0.5 ml aliquots were transferred to 15 ml glass-stoppered centrifuge tubes. Again, fluorescence was used to determine corticosterone levels. Both the gland and plasma samples were evaluated in duplicate and compared to a standard curve of corticosterone to determine the corticosterone content.

**Total protein content of adrenal glands**

The adrenal gland homogenate used for the corticosterone determination was also used for the protein determination. A 0.2 ml aliquot was transferred to 10 ml of double distilled water and shaken. A 1.0 ml aliquot of this solution was transferred to a 50 ml round bottom test tube. The protein determination (Lowry et al., 1951) was carried out on the sample. All the samples were evaluated in duplicate and compared to a standard curve of casein for total content (Appendix E).
RESULTS

Data were analyzed by use of regression analysis. The statistics printed out were analysis of variance table, regression coefficients, and statistics of fit for variables. First, a regression was employed with unadjusted means, then the same analysis for variables was used with the means adjusted for age in order to determine effect of age on groups. Effects of sex, right or left adrenal used, and interactions of group-sex, group-side, and sex-side were determined for plasma corticosterone, adrenal corticosterone, adrenal protein, and the adrenal ratio of adrenal corticosterone to adrenal protein. Differences between groups as to variables were tested using Student's t test; data considered significant at the 0.05 level. The statistical mechanics were carried out with the aid of Dr. Jeffrey Berger, Animal Science Department, and the Statistical Laboratory, Iowa State University, Ames, Iowa.

Graphical and tabular summaries of the data are presented in this section, and the regression analysis is located in Appendix F. Results are reported with emphasis on group differences as compared to the group 6 (control 2) in reference to the four variables: plasma corticosterone, adrenal corticosterone, adrenal protein, and adrenal ratio.

Effect of Age

Age was the one largest possible extraneous factor because the ages of the birds ranged from 106 days to 172 days, with the younger birds concentrated mainly in groups 1 and 2. The age mean for group 1 was 115.4 days and the mean for group 2 was 114.9 days, and the means in
days for groups 3, 4, 5, and 6 were 150.6, 149.6, 149.6, and 121, respectively. Differences in body-weight means for each group were not statistically significant: group 1-887, group 2-883, group 3-894, group 4-875, group 5-895, and group 6-885 grams.

Age did not exert an effect as seen in Figures 1-6 and Tables 1-4 where the unadjusted means follow the adjusted means with no significant difference. The effect of age between groups in reference to each variable was not statistically significant as determined by the F test in the regression analysis with age as an effect (Appendix F).

Thus, the birds used in the experiment fell in an age range where maturity and growth had stabilized sufficiently that all birds could be considered essentially the same age and weight. The variables (plasma corticosterone, adrenal corticosterone, adrenal protein, and adrenal ratio) are free from the effect of age within and between groups.

**Effect of Sex**

With respect to three of the four variables, the effect of sex was significant. Males have a higher plasma corticosterone than females. The overall means were male-150.4 ug/100 ml blood and female-128 ug/100 ml blood with 128 males and 124 females sampled (Table 5). In all groups males had higher corticosterone levels than females (Table 5).

The plasma corticosterone level was significantly influenced by sex as the F test values had \( P < .0008 \) (adjusted) and \( P < .0006 \) (unadjusted); males had a mean of 150.4 ug/100 ml of blood and females had 128.9.
Figure 1. Concentration changes in plasma and adrenal corticosterone

Group 1 - Sampled at banding
Group 2 - Sampled after transport
Group 3 - Sampled after return to Boone
Group 4 - Sampled from field 30 days after release
Group 5 - Sampled from control pens to match field sample (Control 1)
Group 6 - Sampled from pens with no prior handling (Control 2)

Plasma corticosterone expressed per 100 mls blood
Adrenal corticosterone expressed per 5 mls homogenate or total gland
Points indicated by squares were means adjusted for age
Points indicated by dots were means unadjusted for age
Figure 1. CONCENTRATION CHANGES

**PLASMA**

**ADRENAL**

CORTICOSTERONE (µg)

GROUPS
Figure 2. Concentration changes in adrenal corticosterone, adrenal protein, and adrenal ratio
Groups as indicated in Figure 1
Means indicated as in Figure 1
Corticosterone/protein ratio used total corticosterone/total protein in gland
Figure 2. CONCENTRATION CHANGES

ADRENAL CORTICOSTERONE

ug

40

30

20

10

0

1 2 3 4 5 6

ADRENAL PROTEIN

mg

90

70

50

30

10

1 2 3 4 5 6

CORTICOSTERONE/PROTEIN

ug/mg

1.5

1.0

0.5

0.0

1 2 3 4 5 6

GROUPS
Figure 3. Plasma concentrations of corticosterone
Groups as indicated in Figure 1
Black bars represented age adjusted means
White bars represented unadjusted means ug/100 ml blood
Standard error represented on each bar
Figure 3. **PLASMA CONCENTRATIONS**

![Bar graph showing plasma concentrations for different groups.](image-url)
Figure 4. Total adrenal concentrations of corticosterone
Groups as indicated in Figure 1
Bars as indicated in Figure 3
ug/5 ml of homogenate
Figure 4. TOTAL ADRENAL CONCENTRATIONS

CORTICOSTERONE (ug/5ml)

GROUPS
Figure 5. Total adrenal content of protein
Groups as indicated in Figure 1
Bars as indicated in Figure 3
Figure 5. TOTAL ADRENAL CONTENT

![Graph showing total adrenal content across different groups. The y-axis represents protein (mg/gland) with values ranging from 0 to 100, and the x-axis represents groups 1 to 6. Each group has two bars, one for each condition, with error bars indicating variability.]
Figure 6. Adrenal concentrations of corticosterone/protein
Groups as indicated in Figure 1
Bars as indicated in Figure 3
Figure 6. ADRENAL CONCENTRATIONS

[Graph showing adrenal concentrations across different groups (1 to 6).]
<table>
<thead>
<tr>
<th>Group</th>
<th>Observations</th>
<th>Unadjusted Means</th>
<th>Significance**</th>
<th>Age Adjusted Means</th>
<th>Significance**</th>
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<tr>
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<tr>
<td>4 - Field</td>
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<tr>
<td>5 - Control 1</td>
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<tr>
<td>6 - Control 2</td>
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<td>124.17±10.74</td>
<td></td>
<td>127.57±10.74</td>
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</tr>
</tbody>
</table>

*Mean± Standard Error

**Comparison of groups 1-5 with group 6 with probability of .05 or less for significance. Probabilities less than .20 are also shown.
<table>
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<tr>
<th>Group</th>
<th>Observations</th>
<th>Unadjusted Means</th>
<th>Significance**</th>
<th>Age Adjusted Means</th>
<th>Significance**</th>
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*Mean ± Standard Error

**See table 1
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<th>Significance**</th>
<th>Age Adjusted Means</th>
<th>Significance**</th>
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*Mean ± Standard Error

**See table 1
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<th>Significance **</th>
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</table>

*Mean ± Standard Error

**See table 1
Table 5  Means Relative to Sex

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<tr>
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<tr>
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<td>26</td>
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<td>50.2</td>
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<td>Overall Means</td>
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<td>48.1</td>
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<tr>
<td>Overall Means</td>
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<td>133.9</td>
<td>150.4</td>
<td>44.3</td>
<td>56.8</td>
<td>0.92</td>
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</tbody>
</table>

* Units as used in Tables 1-4
The adrenal corticosterone was less in males than in females with means of 44.3 ug/5ml and 48.1 ug/5ml, respectively. These results were similar within all groups. The adrenal corticosterone level was definitely influenced by the sex of the individual sampled, as the F test values had $P<0.045$ (adjusted) and $P<0.0367$ (unadjusted).

The adrenal protein content was lower in males as contrasted to females; the means were 56.8 mg and 66.3 mg, respectively. This was true in the groups and among the groups. The adrenal protein content was affected by sex, as the F test values for means had $P<0.01$ (adjusted) and $P<0.013$ (unadjusted).

The adrenal ratio of corticosterone to protein was greater in males with a mean of 0.92 ug/mg as contrasted to the female mean of 0.88 ug/mg. This relationship was not consistent throughout all groups, for females in group 3 had a higher value, but the other groups followed the previously noted trend. The adrenal ratio was not significantly influenced by sex, as the F test values had $P<0.62$ (adjusted) and $P<0.70$ (unadjusted).

Use of the adrenal ratio eliminated most of the sex differences in the adrenal variables. This elimination was strengthened by the fact that in groups 1-5 the ratio of male to female was 1:1 except group 1, where there were 3 males in excess. Group 6 had a male to female ratio of 1:2.4. Consequently, regression analysis was used to remove any influence of the imbalance of sex ratio in groups 3 and 6. The group-interactions were not significant for any of the four variables ($P<0.05$).
Effect of Side

Effect of side refers to whether the right or left adrenal was used for evaluation (Table 6). No differences between sides were evident for any variable, but a definite trend was seen where the left adrenal had lower values than the right adrenal with the exception of group 4, the field group. In each group there was nearly equal representation of both the right and left side. Near significance was seen in the adrenal corticosterone values in both the adjusted and unadjusted means (P < .13).

Interaction between group and side in relationship to the three of the four variables was significant in all four cases. Plasma corticosterone levels cannot be affected by side.

Plasma and Adrenal Parameters

With the major effects and interactions examined, the four variables of groups 1-5 were compared with group 6 (control 2). The mean values are presented by Figures 3-6 and Tables 1-4. The means were tested by the t test. To be considered significant the P value had to be .05 or less, but higher values are presented in the tables for interest.

In evaluating plasma corticosterone levels, groups 4 and 5 were the only ones significantly different from the group 6 (Table 1). Group 4, which represents a chronic stress situation, and group 5, which represents an acute stress situation, both have similar mean values.

The adrenal corticosterone values were not significantly different from Group 6 (Table 2), but there seemed to be an inverse relationship to plasma levels (Figure 1).
Table 6 Means Relative to Adrenal Sampled

<table>
<thead>
<tr>
<th>Group</th>
<th>Side</th>
<th>Observations</th>
<th>Age</th>
<th>Corticosterone *</th>
<th>Corticosterone *</th>
<th>Protein *</th>
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<td>Plasma</td>
<td>Adrenal</td>
<td>Adrenal</td>
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<tr>
<td>R 18</td>
<td>L 18</td>
<td>151.3</td>
<td>---</td>
<td>40.9</td>
<td>59.8</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>R 28</td>
<td>L 7</td>
<td>148.2</td>
<td>---</td>
<td>47.1</td>
<td>63.2</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>L 7</td>
<td>R 10</td>
<td>121.0</td>
<td>---</td>
<td>43.3</td>
<td>37.9</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>R 10</td>
<td>L 7</td>
<td>121.0</td>
<td>---</td>
<td>47.2</td>
<td>44.9</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>Overall Means</td>
<td>L 122</td>
<td>132.3</td>
<td>---</td>
<td>44.2</td>
<td>59.9</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 130</td>
<td>133.8</td>
<td>---</td>
<td>48.0</td>
<td>63.0</td>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

* Units as used in Tables 1-4
The adrenal protein values for all groups significantly differ from group 6 except group 3, which was below the control 1 group, but not significantly so. The fourth group was high, which was due to an increase in adrenal size. The adrenal protein appeared inversely related to the adrenal corticosterone (Figure 2).

The inverse relationship of adrenal corticosterone to adrenal protein produced the adrenal ratio values. These values had greater differences between groups than adrenal corticosterone values. Thus, the suggested inverse relationship between adrenal corticosterone and plasma corticosterone was more pronounced by substituting adrenal ratio for adrenal corticosterone (Figures 1 and 2).
DISCUSSION

It has been demonstrated that corticosterone is the major circulating glucocorticoid of the bird (Phillips and Chester Jones, 1957; Nagra et al., 1960; Resko et al., 1964; Frankel et al., 1967a). This has been substantiated by in vitro work (deRoos, 1960, 1961; Sandor et al., 1963; Macchi and Brown, 1964). For the ring-necked pheasant, Nagra et al. (1960) and Balding (1967) established corticosterone as the major glucocorticoid. The validity of using corticosterone as an indicator of stress was examined by Brown (1961) in his work with turkeys.

Based on the above work, corticosterone levels in both plasma and adrenals were utilized as measurements of stress in a population of pheasants as they were relocated. The birds were sampled at six chosen points of the translocation to gain some insight into the relative levels of stress. The information was needed to shed light on the problem of high post-release mortality seen in massive releases, and the problem of failure of the release over the following years.

Before looking at the plasma and adrenal levels, major effects and interactions were examined.

Effect of Age

Because age within the six groups ranged from 106 to 172 days, age could have influenced the results. Age was known to have an effect on adrenal activity and the adrenal's response to ACTH. Siegel (1961, 1962a,b) investigated the effect of age in chickens on the ability of the adrenal to respond to ACTH. Freeman (1969) explored the same response
and examined the involution of the bursa of Fabricius as the birds matured. Both Siegel and Freeman recognized the limited response of younger fowl to ACTH and demonstrated age to be a critical factor.

The question that needed answering was: "Are the birds being sampled in a sexually immature state where their adrenal activity is in a state of flux?" The results were therefore compared with both age adjusted for and age not adjusted for; the age factor had no significant influence.

Sexual maturity and stabilized adrenal function can be correlated by the fact that sexual maturity was achieved under the direction of the endocrine system. No age effect was observed in the present study and these results were discussed by Sturkie (1965) where the ages are related to ages of birds in this study.

Sturkie (1965), in the chapters on male and female reproduction, stated that the female is reactive to gonadotrophins starting about day 35 and ending about day 120, which should represent sexual maturity. The male had secondary spermatocytes at about 70 days, and at about 116-140 days immature spermatids are found in the seminiferous tubules which represents sexual maturity in the male. The ages mentioned were all established in the chicken with the suggestion by Sturkie that wild birds mature somewhat faster.

Additional support was reported by Anderson (1972) who sampled ring-necked pheasants as juveniles during their growth period and in their first fall. He measured adrenal weights of adults at the same time of year in both hens and cocks and found juveniles of both sexes to be similar.
Age differences of the birds tested played no major part in the results noted in the parameters (plasma corticosterone, adrenal corticosterone, adrenal protein, and the adrenal ratio) as seen in the data and references cited.

Still another question in need of an answer was: "With growth, does the larger adrenal secrete more corticosterone?" Birds in the age ranges tested had essentially the same body weight with no significant differences. Therefore, general body growth cannot account for any adrenal size differences as demonstrated by adrenal protein.

Age had no significant effect on stress measures used in this study.

Effect of Sex

Sex was significantly related to plasma corticosterone level, adrenal corticosterone level, and adrenal protein in all groups. But the adrenal ratio was not significantly different due to sex, because the hens had larger protein values and higher adrenal corticosterone levels while the cocks had low protein values and low adrenal corticosterone levels. Thus, the ratio had the same proportions when comparing sexes.

In groups there was a significant difference between hen and cock values, but by using an approximate 1:1 sex ratio, the differences were averaged out in each group. Proof that the sex differences between groups were also removed can be seen in Appendix F where the sex-group interaction as tested by regression are shown.
Other investigators have suggested males to have larger adrenals than females and thus more total secretion of corticosterone. Siegel (1962a) suggested higher output from a larger adrenal after examining cholesterol content. However, the idea that a larger adrenal has more output was questionable, because it is commonly stated that with small or no increase in gland size there can be a large increase in gland activity (Garren et al., 1961). Therefore, adrenal size may have little influence on output.

The present results are in direct opposition to the theory that a larger adrenal has more output. By using protein rather than weight, errors such as moisture content and weighing errors were avoided. As a result the investigator showed that females had larger adrenals, yet had lower plasma corticosterone levels. Females had larger adrenals in the absolute sense, and in proportion to body weight also. Sturkie (1965) cites work of Sauer and Latimer wherein the females had 30 percent more cortical tissue than the males. In this study (Table 7), similar percentage values were as high as 72.1% and as low as 44.4% depending on the group.

The females appeared to respond more to stress (captivity) than males as seen when adrenal size was compared with data of wild birds (Anderson, 1972; Kirkpatrick, 1944). The female adrenals not only enlarged more than male adrenals, but became larger in size, reversing the male-female size relationship seen in the wild. By examining the adrenal ratio, one can see that the amount of corticosterone per unit of protein
Table 7 Adrenal Protein/Body Weight (Differences between Sexes)

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Body Weight</th>
<th>Adrenal Protein</th>
<th>Protein Weight %</th>
<th>% Adrenal Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - Banding</td>
<td>M</td>
<td>1022.1</td>
<td>54.5</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>745.4</td>
<td>59.5</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>2 - Transport</td>
<td>M</td>
<td>1016.4</td>
<td>56.5</td>
<td>5.6</td>
<td>42.9</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>809.4</td>
<td>52.5</td>
<td>6.5</td>
<td>44.4</td>
</tr>
<tr>
<td>3 - Return</td>
<td>M</td>
<td>1122.6</td>
<td>50.0</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>882.7</td>
<td>102.5</td>
<td>11.7</td>
<td>72.1</td>
</tr>
<tr>
<td>4 - Field</td>
<td>M</td>
<td>1246.9</td>
<td>85.0</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>784.1</td>
<td>64.5</td>
<td>8.2</td>
<td>54.7</td>
</tr>
<tr>
<td>5 - Control 1</td>
<td>M</td>
<td>1138.7</td>
<td>60.0</td>
<td>5.3</td>
<td></td>
</tr>
</tbody>
</table>

**Weight to nearest .1 gram
* Protein to nearest .1 milligram
was essentially the same in both sexes. Even though the female's gland had enlarged, the efficiency must be less than that of a male, for the female plasma levels were lower than male levels. Perhaps though, the level in the plasma was reduced due to a faster turnover of corticosterone leaving a lower net plasma level.

In this study only hens died with symptoms of "shock of handling" as described by Burger (1964). The hens are believed to have less resistance to stress as suggested by Conner and Shaffner (1954). These results were in direct opposition to the stress studies by Flakas (1950) where the hen was shown more resistant. Since only hens died, the investigator had doubts that rapid turnover occurred and therefore believed the hens were unable to increase plasma levels sufficiently to cope with highly stressful situations.

Although the effect of sex was removed in this study, sex is an important factor and adjustments must be made for its effect in future experiments.

Effect of Side

The influence on the data from choice of adrenal used for analysis was minimal as shown by the analysis (Appendix F). Although there was a side difference in the overall means, there was no consistency throughout groups where the right side was heavier than the left as stated by Siegel (1960), and Wolford and Ringer (1962). Flickinger (1959) in working with wild quail, found no side was consistently larger. No consistency was found in this study.
In the interaction of group-side, there was no significance. An equal number from each side was tested in each group to remove effect of side if present. The interaction of sex-side was also taken into account by testing an equal number of each sex per side in each group. As seen in Appendix F, there was no significant effect from the sex-side interaction. After looking at the data, even though there was no consistency throughout the groups, there was the impression that the right adrenal was heavier than the left. An equal number of sides per sex per group removed any chance of a side effect between groups.

Group Evaluations

Sampling of group 1 provided an evaluation of stress on birds as they were removed from rearing pens. Birds were driven into a funnel catch trap, removed by hand, banded, weighed, and placed in shipping crates. These birds were acutely stressed and at this point a loss of hens was witnessed, but there were no losses to cocks due to "handling shock". Hens appeared dazed and their wings tremored just before death. Plasma corticosterone levels of these individuals were well below the average of the other hens at this time, and the adrenal content was also below that of the others (Figure 1). It seems that these birds were unable to survive due to an inability to supply enough hormone. Birds in group 1 were acutely stressed as evidenced by an elevated plasma level and a low adrenal level (Figure 1).

Birds in group 2 provided an evaluation of stress after shipment (5-6 hours in the crates). At the release site, birds sampled had a drop
in plasma levels and a rise in adrenal content (Figure 1), but there was little change in adrenal protein. Because of the darkened shipping crates, the length of time, and movement of vehicles associated with shipping, the acutely stressed birds had time to achieve a degree of adaptation after the initial stress was met successfully (Selye, 1946, 1973). Also during this time, a number of hens died in a state of shock at the site or enroute; these deaths were easily differentiated from the accidental deaths due to the lack of physical damage. The birds unable to cope with the stress were, therefore, lost to the stocking operation and to this study. Less than one percent of the 2,690 birds handled were lost due to handling stress.

Group 3 birds were taken to the release site, but then returned to Boone and their pens. This group had a drop in plasma levels to the level of the control (Figure 1). The corticosterone/protein ratios of the adrenals of these birds, however, were high. The data suggested the condition where a bird has returned to a "normal" condition, but has an alarmed corticoid system readied for another possible acute stress. In other words, synthesis took place, but the hormone was not released (Figure 2), so the bird was still in a rebound or acclimating state.

Selye's description of a condition where the animal under a certain stress rallies and forms a resistance, but under a new stress must again raise its resistance was typified by group 4 (field group) birds. They had acclimated to the stress of penning and crowding, and survived the acute stress of handling, but now have a new chronic stress of survival in a new habitat. In response to this stress, the adrenal enlarged and
the levels of corticosterone in the plasma were very high as compared to the control 2 group (group 6). These birds were being pushed to the point of resistance exhaustion as the result of the numerous stresses. Since the high levels can only be maintained for a limited period, the birds must either have a decrease in stress or die.

The fifth group was birds replaced in pens after initial handling, weighing, and banding. These birds were killed by age and sex classes as close as possible to the birds sampled in the field. These birds were in the condition of readiness like those of group 3, yet the stress of handling for sampling was superimposed on the corticosterone parameters. A rise in the plasma levels was present, but not the increase in adrenal protein as seen in chronic field stress. Also the adrenal ratio was similar to the levels seen in groups 1 and 2, but not in the sixth group (Figures 1 and 2).

Group 6 was sampled without the stress of handling, but with the stress of crowding and penning. This group was used as the relative baseline to evaluate the other groups. Plasma levels were lower than in other groups, but these birds had a higher adrenal ratio. These birds were shot at a range of 10-12 feet while feeding or scratching and were totally unaware of the investigator. The blood sample was taken within 60 seconds so the stress of handling was removed. This left the stress of penning and crowding, which appears to be a rather large stress (Freeman, 1967; Flickinger, 1961). In terms of absolute values, Nagra et al. (1960) only obtained such high corticosterone values with 8 International Units of ACTH given intravenously to the pheasants. However, these values were
obtained with the use of non-avian ACTH, and with the birds under Nembutal anesthesia; these factors must be considered when interpreting the meaning of the results. For groups 1-4 the stresses of relocation were all superimposed on an already present stress of penning and crowding. Ideally, a seventh group of birds shot unaware in the wild would give a good relative index to stresses associated with pen-rearing, and would enable a better understanding of stress.
CONCLUSIONS

The adrenal stress response in the ring-necked pheasant as measured by plasma and adrenal corticosterone levels and adrenal protein was directly related to sex.

Data were not affected by the choice of whether right or left adrenal was used for evaluation. Cocks have a higher plasma level of corticosterone than the hens and this relationship is reversed for the adrenal corticosterone concentration. If there are increased concentrations in the plasma then there should be less corticosterone in the adrenal gland. The size of the adrenal gland was greater in hens than in cocks (Figure 2). The birds are under a considerable stress while being reared and the hens' adrenals have responded with an increase in size, but their efficiency still is not as great as that of cocks as reflected by the plasma and adrenal concentrations. Hens had less plasma and adrenal corticosterone; this would suggest they required less adrenal corticoid secretion to successfully meet the stress, but this was contradicted by the observation that only hens died in the acute stress situation. This was further supported by the enlargement of hen adrenals that suggested increased adrenal activity to meet stress. Thus, the investigator believed the hens were unable to respond with adequate synthesis and release of corticosterone.

Based on this study one could predict a large loss of hens after the stocking operation as the new stresses of habitat adaptation, winter survival, and breeding come to bear on the hens. Kirkpatrick (1944) and Anderson (1972) reported adrenal enlargement in hens associated with both
winter and breeding conditions. Hens in this study were already heavily stressed at release so they would have little reserve left for increased adrenal activity as they were stressed in winter and during reproduction and death would likely result.

May (1973) carried on independent field studies of the same population used in this study. He observed a hen to cock ratio of 1:1 following the first breeding season as contrasted to the starting ratio of 4:1. He suggested that the hatch, with a ratio of 1:1, would add proportionally more to the cock population. However, the author believes that hen mortality caused by stress accounted for the drop in hens between 1971 and 1972 that May noted. The hen mortality coupled with the hatch ratio of 1:1 created a high percentage of cocks in the sex ratio.

Many biologists believe, and based on this study the author supports, the theory that one must release a high number of hens to cocks in large numbers if success in pheasant stocking is to occur. If too small a number of hens is released the ability of a population to recover from stress losses is greatly endangered. Pheasant populations traditionally suffer annual losses of 60-75 percent, and under stress of stocking, losses may be even higher. If enough hens are not produced for replacement, no increase or growth of population will occur. In this study, the large number of birds released may have been sufficient to maintain a breeding stock throughout stresses of adaptation and reproduction.

Any effect of age should be of no consequence in success of releases after birds have reached the approximate age of 106 days or more, for the corticoid system has matured and stabilized by that time.
As to the future releases, the investigator suggests that the hens should be reared separately and a method or methods should be devised where the hens could have the stresses of pen-rearing and handling removed. The simplest method would involve drugs administered through drinking water or food. This has been attempted in Wisconsin (Flakas, 1952), where the aim was to remove stress from the cock which they found less resistant than the hen. Here estrogen was used, and the cocks became very docile, losing their "wildness". One would want a drug whose effects were short-lived once its use was discontinued. Once birds are crated and shipped, the effects of the drug should dissipate rapidly so the birds are released in an alert and wild state to avoid excess predation and loss to exposure. The drug would allow the hens to have that reserve margin for adrenal activity needed to respond to winter and breeding stresses. Stress of handling would be the only stress one could remove with the tranquilizing drugs, but perhaps treatment with androgens would increase the hens' resistance to stresses. The investigator believes work should be done to investigate this possibility.
SUMMARY

A. To better understand the high post-release mortality of ring-necked pheasants, stress levels were evaluated in these birds as they were transported and released in southeastern Iowa.

B. Adrenal corticoids were used as a measure of stress. Plasma and adrenal corticosterone levels, and adrenal protein content were measured.

C. Experimental birds were divided into five groups:
   1) birds sampled during banding and crating
   2. birds sampled after transport to the release site
   3. birds sampled after transport to the release site and returned to rearing quarters
   4. birds sampled 1 month after release
   5. birds retained at the rearing quarters, used as a control group

D. A sixth group of pheasants was maintained and sampled in such a way as to avoid the stress induced by the sampling procedure itself. This group served as a basis of comparison for the other five groups.

E. The influences of age, sex, and right adrenal versus left adrenal upon plasma and adrenal corticosterone levels, adrenal protein, and adrenal corticosterone:protein ratio were evaluated. The interactions of group-sex, group-side, and sex-side on the adrenal measures were also examined.

F. The hens had larger adrenals which was the opposite of that reported in studies of wild populations of pheasants. As indicated by the larger adrenals, hens responded more to stress than did the cocks.
G. During the release procedure, although the hens had larger adrenal glands than the cocks, the hens had lower plasma corticosterone levels, and in stress situations only the hens died from stress of handling.

H. Age, right versus left adrenal, group-sex, group-side, and sex-side had no significant effect upon the measures of adrenal activity.

I. Results suggested:

1. Hens were able to respond to the stress of penning and crowding, but some were unable to survive the additional stress of handling. Hens that did survive the handling had the additional stresses of adaptation to new habitat, winter survival, and reproduction superimposed on an adrenal system working near maximal limits.

2. Results predict a loss of hens perhaps even to the extent that the population might not maintain itself after the first breeding season. Thus, the need for release of a large number of birds with a high percentage of hens was suggested.

3. Particular attention should be directed to reduce stress of captivity and handling on the hen population. This would allow a higher reserve for stress of adaptation, winter survival, and reproduction to insure release success.


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I can never repay my wife, Karen, for the encouragement she gave me, nor for the many hours my investigation forced me to be away from her and the family. Without her love and understanding I would never have finished the education I desired, and I hope that in the future I will be able to show her my deep appreciation.
APPENDIX A: BLOOD SAMPLING TECHNIQUES

In live birds it was important to remove the blood within 60 seconds from the time the birds were caught with as little trauma to the bird as possible. After many trials using the radial artery and heart puncture, it was found much easier to use the jugular vein to draw blood.

The bird was grasped by the legs and at the base of the head by one person while the other person drew blood. In order to expose the jugular, the cervical feather tract was pushed to one side and the vein rolled up to set squarely on the lateral side of the neck that was facing upward. The person holding the bird held the feather tract off to the side while the sampler applied a slight pressure to the vein where it entered the body cavity at the base of the neck. This distended the vein sufficiently for the placement of a 22 ga needle, and a sample was slowly drawn.

In attempting to sample recently killed birds, the best method was found to be opening of the thoracic cavity. With the heart still beating, a sample was drawn from the post vena cava with a 22 ga needle and 5 cc syringe. When the heart had stopped, the sample was drawn from the heart's chambers and major vessels such as the aorta, femoral artery, brachial artery, and the common iliacs. Collections in the field were the most difficult and required quick recovery once the bird was shot. In the second control group shot with a rifle, a little different situation existed. The bird was shot only 6 to 12 feet away, but it was found that by using hollow-point cartridges, a large blood pressure drop
prevented obtaining much of a sample. By using birdshot cartridges, the
bird was killed but with very limited blood loss, and the heart remained
beating. The critical part of the procedure was the lifting of the breast.
This was done by slitting the skin at the point of the keel and pulling the
skin loose anteriorly to the neck leaving the breast free of skin and
feathers. Next the breast was lifted with incisions along both sides
until the ribs were reached. Only a few ribs were cut so as not to cut
any sizeable vessels. By lifting the breast, the heart and post vena cava
could be reached with the syringe. The above procedure was accomplished
by one person by grasping the thrashing bird and placing it on its
back. Its wings were outstretched and pinned beneath the person's knees
and its legs were dislocated from the body to allow better access to
the thoracic cavity. Using these techniques samples were obtained in
60 seconds or less from the time of the shot.
APPENDIX B: ADRENAL GLAND REMOVAL

Adrenals were easily removed once the keel was raised and the blood sampling done. More ribs were cut until the breast could be pushed anteriorly so that it did not interfere with access to the abdominal area. The bird was picked up, turned over, and the back broken by pushing down on the dorsal surface of the lumbar region. When the bird was again turned over the adrenals were prominent just posterior to the vertebral separation. The aorta was then severed and the point of one blade of a small curved forceps was carefully pushed through the vessel where it coursed between the glands. By grasping and raising the aorta, the adrenals were lifted and the connective tissue holding them could be cleared away. Finally the aorta just below the adrenals was severed to complete removal. The glands were then easily separated from the aorta.
APPENDIX C: CHEMICALS FOR CORTICOSTERONE AND PROTEIN DETERMINATIONS

1. Methylene dichloride purification
   a. Allow dichloromethane to stand over 1/10 its volume of concentrated H₂SO₄ for three days, with occasional shaking.
   b. It was then washed three times with 1/10 its volume of 2N NaOH, and then washed three times with 1/10 its volume of distilled water.
   c. Dry overnight over anhydrous sodium sulfate.
   d. It was filtered and distilled through a Dufton column (the fraction distilling between 40 and 41 degrees centigrade was collected).

2. Ethanol (absolute alcohol) purification
   a. Reflux for two hours with 5g/liter of 2,4-dinitrophenylhydrazine and 10 ml/liter of concentrated HCl.
   b. Then twice distill through a Dufton column and collect the fraction distilling at 78 degrees centigrade.

3. Fluorescence reagent: Consisted of a 3:1 ratio of concentrated sulfuric acid (3) to ethanol (1). Mix the solution cautiously by running the acid down the sides of the container. Acid should be added to the alcohol, and the container should be immersed in ice water (Important).

4. Corticosterone standards
a. 260 mg of corticosterone (Nutritional Biochemical Corporation) was dissolved into 5 ml of ethanol.

b. The ethanol solution was transferred to a one liter volumetric flask and diluted to one liter with distilled, deionized water. This produced a stock solution of 260 ug/ml.

c. The standard curve was formed from three dilutions of the stock solution.

(1) 1.25 ml of the stock solution was pipetted into a 100 ml volumetric flask and brought to volume with the distilled, deionized water (concentration 3.25 ug/ml).

(2) 50 ml of this solution was transferred into a 100 ml volumetric flask and brought to volume with distilled water (concentration 1.625 ug/ml).

(3) 50 ml of this solution was transferred into a 100 ml volumetric flask and brought to volume with distilled water (concentration 0.8125 ug/ml).

(4) 50 ml of this solution was transferred into a 100 ml volumetric flask and brought to volume with distilled water (concentration 0.40625 ug/ml).

(5) 50 ml of this solution was transferred into a 100 ml volumetric flask and brought to volume with distilled water (concentration 0.20313 ug/ml).

(6) 50 ml of this solution was transferred into a 100 ml volumetric flask and brought to volume with distilled water (concentration 0.10157 ug/ml).
(7) 50 ml of this solution was transferred into a 100 ml volumetric flask and brought to volume with distilled water (concentration 0.05077 ug/ml).

d. After step 4 the remaining 50 ml portions were stored for future dilutions to prepare fresh standards. The concentrations at the end of steps 5, 6, and 7 were the final dilutions used for the standard curve determination.

5. Reagent A (2% Na₂CO₃ in 0.1 N NaOH): 4 grams of NaOH were dissolved in about 100 ml of distilled water in a 1 liter volumetric flask and 20 grams of Na₂CO₃ were added. The volumetric flask was brought to volume with constant stirring.

6. Reagent B (0.5% CuSO₄·5H₂O in 1% sodium tartrate): 10 grams of sodium tartrate were dissolved in 500 ml of water to give a 2% solution. This was stored in its own container. CuSO₄·5H₂O in the amount of 5 grams was dissolved in 500 ml of distilled water to give a 1% solution. This was stored in its own container.

7. Reagent C (alkaline copper solution): Had to be mixed in order to prevent cloudiness from occurring. 1 ml of the 2% sodium tartrate solution was added followed by 1 ml of 1% CuSO₄·5H₂O which diluted them respectively to 1% and 0.5%. 100 ml of Reagent A was then added. The solution was mixed daily before the assay.

8. Folin-Ciocalteu phenol reagent (Fisher Scientific Company): The reagent was received as 2N and was diluted 1:1 with distilled water to obtain 1N Folin's reagent.
9. Protein standards
   a. 250 mg of casein (Nutritional Biochemical Company) was dissolved in approximately 50 ml of 0.1N NaOH.
   b. Double distilled water was added to bring the solution to 500 ml in a volumetric flask to produce a 0.5 mg/ml protein stock solution.
   c. Three volumes of this stock solution were used to determine a standard curve.
      (1) 0.5 ml was pipetted into 0.5 ml distilled water giving a concentration of 0.25 mg/ml.
      (2) 0.3 ml was pipetted into 0.7 ml distilled water giving a concentration of 0.15 mg/ml.
      (3) 0.1 ml was pipetted into 0.9 ml distilled water giving a concentration of 0.05 mg/ml.
APPENDIX D: DETERMINATION OF PLASMA AND ADRENAL CORTICOSTERONE IN THE PHEASANT USING FLUORESCENCE (Van der Vies, 1961; Zarrow et al., 1964)

1. Dichloromethane - extract 0.5 ml of sample with 5 ml of CHCl₃.
   a. Shake for 60 seconds and let stand for 30 seconds.
   b. Shake again for 30 seconds.
   c. Centrifuge for 1 minute (2,000 rpm).
   d. Remove top layer by aspiration.

2. Sodium hydroxide - wash the extract with 0.20 ml 0.1N NaOH.
   a. Add NaOH and shake for 60 seconds.
   b. Centrifuge for 1 minute (2,000 rpm).
   c. Remove top layer by aspiration.

3. Sodium sulfate - dry the extract.
   a. Add enough Na₂SO₄ to cover the bottom of the tube.
   b. Let stand for 5 to 10 minutes.
   c. At the end of this time transfer a 2.0 ml aliquot of the sample to another clean centrifuge tube.

4. Fluorescent reagent
   a. Mix the 2.0 ml of the extract with 5 ml of fluorescent reagent.
   b. Shake for 30 seconds.
   c. Centrifuge for 2 minutes (1,000 rpm).
   d. Remove top layer by aspiration.

5. One hour after shaking, read the fluorescence of the acid layer.
   a. Equip a Turner Model 111 with a high sensitivity kit and a No. 110-835 lamp.
b. Use primary filters Nos. 110-827 (3) and 110-831 (48) to provide exciting light at 470 millimicrons and cut off all light above 575 millimicrons.

c. Use secondary filters Nos. 110-822 (58) and 110-826 (2A-15) to pass light at 540 mu.

d. For the fluorimeter setting, to control the amount of light passing through the filters, use 3 for the plasma readings and 10 for the adrenal gland readings.

6. Run three internal standards and one blank with each set of samples and their duplicates.
APPENDIX E: PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT

(Lowry et al., 1951)

1. Mix Solution C in order fresh each day.

2. Place 0.2 ml of sample in 10 ml of double distilled water and mix on vortex mixer.

3. Take 1 ml of this solution and add it to 5 ml of Solution C and let set 10 minutes or longer after thorough mixing on the vortex.

4. Add standards directly to the 5 ml of Solution C skipping the water step for sample preparation.

5. Add 0.5 ml of the diluted Folin's reagent while mixing on the vortex mixer. Allow to develop for 30 minutes from time of shaking.

6. Run three internal standards and one blank with each set of samples and their duplicates.

7. Read on a Beckman spectrometer 70 set at 750 millimicrons.
APPENDIX F: REGRESSION ANALYSIS OF VARIABLES USING MEANS

ADJUSTED FOR AGE AND MEANS UNADJUSTED FOR AGE
<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees Freedom</th>
<th>Partial Sum Squares</th>
<th>F Value</th>
<th>Probability of a Greater F Value</th>
</tr>
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Table 9  Regression Analysis with Age Adjusted Means (Adrenal Corticosterone)

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Table 10  Regression Analysis with Age Adjusted Means (Adrenal Protein)

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Table 11  Regression Analysis with Age Adjusted Means (Adrenal Ratio)

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Table 12  Regression Analysis with Unadjusted Means (Plasma Corticosterone)

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### Table 13  Regression Analysis with Unadjusted Means (Adrenal Corticosterone)

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