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Daily Variations of Lipogenic Activities in Japanese Quail (Coturnix Coturnix Japonica).

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DAILY VARIATIONS OF LIPOGENIC ACTIVITIES
IN JAPANESE QUAIL
(Coturnix coturnix japonica)

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Zoology and Physiology

by
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Abstract

Studies were made in Japanese quail (*Coturnix c. japonica*) to determine if daily variations of plasma insulin and *de novo* lipogenesis exists and examine the roles of daily variations in lipogenic responsiveness to insulin and prolactin. A daily variation in plasma insulin levels exists in quail fed *ad libitum*, with photophase levels 4.6 times greater than scotophase levels. This variation was not driven exclusively by the feeding of the quail, because insulin levels had dropped by dark onset, yet all bird's crops contained food at this time.

There was a daily variation in the amount of $^{14}$C-acetate labelled lipids extracted from the liver and fat pads (FP). Liver $^{14}$C-acetate incorporation into lipids was lowest at 10:00 h and peaked from 18:00 to 22:00 h. A similar pattern of lipogenesis was observed in birds injected with bovine insulin. A difference exists between the trough and the peak of lipogenesis in insulin injected birds. FP $^{14}$C-labelled lipid content was constant throughout the 24-hour period, with a depression at 22:00 h.

Injections of bovine insulin depressed *in vivo* lipogenesis at all times-of-day (TOD). There was no change in sensitivity at different TOD, because bovine insulin injections depressed lipogenesis similarly at each TOD. Injections of chicken insulin did not alter lipogenesis as measured by $^{14}$C-labelled lipid in the liver and FPs.

Attempts to alter *in vivo* lipogenesis with the D2 type dopamine agonist, 2-bromo-α-ergocryptine, used previously to reduce fat stores, cholesterol and triglyceride levels in mammals, were unsuccessful in quail. Food and water intake,
body weight (BW), liver and FP weight, and $^{14}$C-acetate incorporation into liver and FP were unchanged. It is not known why quail react differently than mammals to this drug.

Injections of ovine prolactin or saline for 5 days produced BW changes in quail. Prolactin injected birds gained 5.4 g, non-injected birds gained 2.4 g, and saline injected birds lost 3.9 g of BW. BW changes were not due to differences in FP weights. TOD effects within each group were lacking, with all injection times producing similar results.
General Introduction

Although not normally associated with obesity research, studies using avian/poultry species are increasing. As the poultry industry has genetically selected animals that grow rapidly, they have also inadvertently selected animals that are proportionally and relatively fatter than unimproved varieties (Cartwright et al. 1986; 1988). This fatness presents the poultry industry with three “problems” (Mallard & Douaire, 1988). First, the increased fat deposition decreases feed efficiency (the weight of bird produced per unit of feed). Second, much of the fat produced has no commercial value, and is removed at the processing plant. The disposal of part of the bird not only reduces processing yield, but presents waste disposal problems. Third, excessively fat birds are viewed by the consumers as a less healthy food source. In summary, increasing fatness in birds has been recognized as a major problem by the poultry industry.

Three lines of research are attempting to address the problem: 1). Careful genetic selection for rapid yet lean growth. 2). Manipulation of dietary factors to curb fat production. 3). Hormonal/physiological alteration of fat production. Great advancements are expected with continued genetic selection and/or molecular genetics. However, progress with selection will be slow due to the length of inter-generational times, while molecular genetics progress has been slowed by a lack of suitable genetic probes to genes involved in lipogenesis and lipolysis (Mallard & Douaire, 1988; Leenstra, 1988). It has long been known that fatness in poultry can be influenced by manipulating dietary factors. Unfortunately, most of these
manipulations, such as lowering the intake energy to protein ratio or restrictive food regimes, also adversely affect growth rate. Hormonal or physiological alteration of fat production remains an area of active exploration. Bird species that undergo migrations are able to drastically alter their lipid metabolism, some more than doubling their body fat stores (Meier, 1977b). This is accomplished by an increase in feeding and an increased routing of excess energy into fat storage (Farner, 1960). De novo lipogenesis does not proceed at a constant rate, but in a rhythmic fashion with more lipogenesis occurring at certain times of day than others (Meier, 1977a). Understanding and being able to manipulate the physiological mechanism by which migratory birds alter their lipid metabolism could provide an effective method for limiting fat production in poultry species.

A review of the literature highlights several hormones which might play a major role in lipid control. Prolactin (PRL) has been known to influence body weight in birds for a number of years (Schooley et al., 1938, 1941), yet evidence to demonstrate its role in influencing fat stores was not obtained until the 1960's (Bates et al., 1962; Meier & Farner, 1964; Goodridge & Ball, 1967; Meier & Davis, 1967). Following these landmark studies have been a number of experiments indicating PRL is involved in lipogenic induction in birds (Meier & Farner 1964; Meier & Davis 1967; Goodridge & Ball 1967a; Chandola & Pavgi 1979; Дьяченко 1982; Sotowska-Brochocka et al. 1986; Chattopadhyay et al. 1991). Meier & Davis (1967) and Sotowska-Brochocka et al. (1986) indicated that there are times of day when exogenous PRL promotes lipid production, and other times of day when PRL is
ineffective in promoting lipogenesis. It is possible that some of the negative data reported in the literature are a result of the experimenters performing experiments at times during which PRL lacks lipogenic effects.

It is not clear how PRL influences lipogenic processes in birds. It has been proposed that prolactin injections resets a hypothalamic metabostat with circadian components in birds (Meier et al., 1981). Recent experiments in mammals by Meier and colleagues suggest that PRL also influences neural centers involved in the integration of physiological events (for discussion see Cincotta & Meier, 1989) and directly affects liver lipogenic machinery as well by altering the number of hepatic insulin receptors available (Cincotta & Meier, 1985a). Increased or decreased insulin-receptor interactions result in an alteration of lipogenesis (Cincotta & Meier, 1985b).

There is a great deal of conflicting experimental evidence concerning insulin's involvement in lipid regulation in birds. Supportive evidence includes the observation that genetically obese chickens have elevated insulin levels (Raheja et al., 1986) or increased insulin release after feeding or glucose challenge (Touchburn et al., 1981; Simon & Leclercq, 1982, 1983, 1985; Saadoun et al., 1988). Additionally, in vivo insulin injections stimulate fatty acid (FA) and triglyceride synthesis (Kompiang & Gibson, 1976; Gomez-Capilla et al., 1980; Vives et al., 1981), while in vitro insulin increases fatty acid synthetase and malic enzyme activity (enzymes involved in lipid synthesis) in hepatocytes.

As seen with PRL, there also exists a significant body of experimental evidence indicating a lack of insulin involvement in lipid metabolism or at least a significant
departure from what is known about insulin's involvement in lipogenesis in mammals. Goodridge (1964) found that while insulin injections lowered plasma glucose levels, they had no effect upon incorporation of labeled fat precursors (acetate) into liver or fat pad lipids. Simon et al., (1991) could not attribute differences in lipid metabolism in genetically fat chickens to differences in the number of, or activity of, hepatic insulin receptors. Burghelle-Mayeur et al., (1989) and Rosebrough & Steele (1987) were unable to increase FA synthesis or malic enzyme activity in vitro, while others (Grande, 1969; 1970; Goodridge, 1964; Heald et al., 1965; Lepkovsky et al., 1967; Langslow et al., 1970; Langslow & Hales, 1971; Nir and Levy, 1973) were unable to alter plasma free fatty acid (FFA) levels in birds given insulin injections.

As with PRL, there have been studies indicating circadian variations in lipogenic activities and responses of mammals to insulin (Cincotta & Meier, 1984) and specific times of day when most lipogenesis occurs (Cincotta & Meier, 1984; Martin et al., 1990). Experiments performed at different times of the day might yield drastically different results and fill the literature with contradictory reports.

Pancreatic polypeptide is yet another hormone that might play an important role in the regulation of lipogenesis. It has a number of "insulin-like" effects such as decreasing plasma levels of free fatty acids while increasing triglyceride levels and suppressing lipolysis (Kimmel et al., 1978; McCumbee & Hazelwood, 1978; Hazelwood, 1984).

Both triiodothyronine (T₃) and thyroxin (T₄) have been reported to play a role in the regulation of lipogenesis. T₃ appears to have both lipogenic and lipolytic properties. Plasma T₃ levels were found to be higher in broiler strains that had a
higher percentage body fat (Stewart & Washburn, 1983). Others report that T₃ alone (Rosebrough et al., 1992) or T₃ in concert with somatotropin (STH) (Cogburn et al., 1989) or somatostatin (SS) (Cogburn et al., 1990) reduce body fat content of broiler chickens. Timed T₄ injections have been shown to alter the fattening response of the pigeon to timed prolactin injections (John et al., 1972), while Saadoun et al. (1988) have found differences in T₄ levels in fed fat and lean chickens. It appears that both T₃ and T₄ play a permissive role in lipid control, acting synergistically with other hormones.

It has been suggested that lipogenic control in birds might be accomplished more by an alteration of lipolysis, than of lipogenesis (Calabotta et al., 1983), mainly through the actions of glucagon (glucagon/insulin balance -- Leclercq, 1984; Cieslak, 1984), SS (Strosser et al., 1983) and growth hormone (Scanes & Griminger, 1990). Glucagon is strongly catabolic and induces marked lipolysis and large increases in gluconeogenesis (Hazelwood, 1984). Somatostatin affects lipogenesis by inhibiting the release of pancreatic glucagon much more strongly than the other pancreatic hormones (Honey et al., 1981). Somatostatin also directly counteracts the lipolytic action glucagon on adipose cells (Strosser et al., 1981). The net result is an increase in the insulin/glucagon ratio, and a shift toward anabolic (lipid forming) metabolism. Growth hormone is usually thought of as lipolytic and anti-lipogenic (Harvey, et al., 1977; Campbell & Scanes, 1985; Campbell & Scanes, 1987; Campbell & Scanes, 1988), yet several studies indicate that GH is anti-lipolytic and/or lipogenic (Scanes et al., 1976; Campbell & Scanes, 1987; Cogburn et al., 1989; Scanes et al., 1990). The recent identification of lipolytic and non-lipolytic GH variants (Aramburo et al.,
1989) indicate that GH's array of seemingly conflicting biological activity is due to its heterogeneity. Because GH induces the release of IGF-1 in vivo, it is still unclear if GH's in vivo actions are direct or mediated through IGF-1 (Decuypere & Buyse, 1988).

Corticosterone, the main corticosteroid in birds, seems to have potent lipogenic effects in birds, with many studies demonstrating mild to large increases in fat pad weights after several days of injections (see Decuypere & Buyse, 1988 for review). Simkins & Smith (1976) were unable to demonstrate increases in liver weight or liver lipids after corticosterone injections in brown leghorn chickens, but they did not look at fat pad weights, and did not consider the possibility of simultaneous increases of transport of lipids from the liver. Time-of-day differences in corticosterones' lipid promoting ability have been reported (Buyse et al., 1987) but others have noted a lack of time-of-day effects (Bartov et al., 1980). Corticosterone has also been reported to entrain the rhythm of tissue (liver) sensitivity to prolactin (Meier & Martin, 1971; Simkins & Smith, 1976; Buyse et al., 1987), setting times-of-day when prolactin is effective in promoting lipogenesis and other times-of-day when it is not.

The catecholamines are also capable of inhibiting lipogenesis/stimulating lipolysis, but only at very high (pharmacological) concentrations (Langslow, 1972). This contrasts with mammals where epinephrine and norepinephrine are the major effectors of lipolysis. Indeed, β-agonists are reported to have little effect upon abdominal fat pad weight in broilers (see Peters, 1989 for review).

In summary, it is apparent that much remains to be elucidated about the control of lipogenesis in avian species. A great deal of conflicting evidence exists in the
literature. What can be said with some certainty is that the integrated control of lipid metabolism in avian species is not well understood.

References


Chapter 1

Daily Rhythm of Plasma Insulin in Japanese Quail (*Coturnix c. japonica*)
Fed *Ad Libitum*
Introduction

Circadian rhythms of plasma insulin concentration have been demonstrated in several mammalian species, including humans (review, Mejean, et al., 1988), dogs (Fischer, et al., 1985), Syrian hamsters (de Souza & Meier, 1987), rats (Bellinger et al., 1975) and mice (Pessacq, et al., 1976). Daily rhythms of insulin have not yet been reported in birds. Most of the avian work focuses on insulin responses to the animal's prandial state, especially starvation/refeeding experiments.

A few studies on daily variations of different indices of carbohydrate regulation have been reported in the chicken. Sollburger (1964) observed a circadian liver glycogen rhythm in chicks and Twiest & Smith (1970) reported a circadian variation of plasma glucose levels. Such metabolic rhythms might reflect rhythms of insulin and/or rhythms of other glucoregulatory hormones. The present study examined plasma insulin concentrations throughout the day in Japanese quail.

Materials and Methods

Thirty-six, sexually mature (five-week-old) Japanese Quail (Coturnix c. japonica) were obtained from the Poultry Science Dept., Louisiana State University. Birds were held on a 14L:10D photoregime in an environmentally controlled room, maintained at 22° C with food and water available ad libitum from hatch until five weeks of age. Birds were transferred to broiler batteries (six birds per level) in a Veterinary Science animal holding room, separated into six sample groups and placed on a 16L:8D photoregime (lights on at 06:00). After two weeks acclimation, blood samples were collected into non-heparinized syringes by cardiac puncture and then transferred to test tubes. Six different birds were sampled every four hours for 24
hours (02:00, 06:00, 10:00, 14:00, 18:00 and 22:00 h). Food trays for the group being sampled were removed immediately prior to sampling and care was taken to minimize disturbance to birds not being sampled. The samples at light onset (06:00 h) were taken before the birds began diurnal (photophase) feeding. Blood samples were placed on ice and allowed to clot before centrifugation. The serum was frozen and stored at -20° C until assayed.

Plasma levels of quail insulin were determined by radioimmunoassay, described by McMurtry, et al. (1983), using purified chicken insulin (Litron Laboratories, Rochester, NY) as the standard and for iodination (chloramine T method using 125I), guinea-pig antichicken insulin serum as the first antibody and antiguinea pig gamma globulin antiserum as the second antibody. Avian insulin appears to be highly conserved, with chicken, turkey and ostrich insulin having identical amino acid sequences and duck and goose insulin differing by three amino acid substitutions (Evans et al., 1988). The highly conserved nature of avian insulins sequenced to date coupled with inhibition curves obtained by serial dilution of Japanese quail plasma which were parallel to chicken insulin standard curves, suggested that the quail plasma insulin inhibits binding in the same manner as the standard chicken solutions. All samples were run in duplicate.

The data were analyzed using the SAS General Linear Models Procedure (SAS, 1985). Variations among treatment groups were examined by analysis of variance. Comparisons between time groups were made with the Waller-Duncan K-ratio T test.
Results

Concentrations of plasma insulin varied during the 24-h period (ANOVA, P < 0.003). Insulin levels were high from 06:00 (at light onset) until 18:00 h (four hours before light offset) (Figure 1.1). Mean insulin levels from 06:00 to 18:00 h (857 pg/ml) were more than four times as high as the mean values at 22:00 and 02:00 h (185 pg/ml). Insulin concentrations at 06:00, 10:00, 14:00 and 18:00 h did not significantly differ from each other as shown by Waller grouping. The 02:00 and the 22:00 h values did not differ from each other, but both differed significantly, by Waller grouping, from those of all other sampling times (P < 0.05).

Feed consumption in these birds appeared to be diurnal as evidenced by the observation of food in the crop sacs at all times of the day, except the 02:00 and 06:00 h time slots. It is noteworthy that crop sacs were especially full at light offset (22:00 h).

Discussion

Japanese quail fed ad libitum exhibited a pronounced daily variation of plasma insulin, with levels high from 06:00 to 18:00 h and low from 22:00 to 02:00 h. High levels during most of the photophase seem to support the conclusion of Raheja (1973) who suggested that plasma insulin variations in chickens are directly related to feeding. Indeed, insulin levels in birds are generally elevated after feeding (Anthony et al., 1990; Krestel-Rickert, et al., 1986; Simon & Leclercq, 1982; Simon & Rosselin, 1979). Furthermore, Japanese quail are diurnal feeders, consuming food throughout the photophase (with a marked increase in feeding just before dark onset) and halting consumption during the scotophase (Siopes T.D., pers. comm.). The
Fig 1.1: Plasma insulin concentrations over a 24-hour time period. The solid bars denote the dark period. Values marked with different superscripts are significantly different from each other (P < 0.05).
increase in feeding near the end of the photophase allows the quail to fill their crops
with food which is then digested during the next several hours of darkness. This
pattern is similar to patterns observed in chickens (Scanes, et al. 1987; Chandrabose,
1970). However, if feeding patterns were fully to account for the daily variations in
plasma insulin levels, then one would expect to observe elevated insulin levels at
10:00, 14:00, 18:00 and especially at 22:00 h, and see depressed levels at 02:00 and
06:00 h (plasma samples were drawn at light onset before diurnal feeding resumed).
This was not the case. Insulin levels were depressed at 22:00 h despite the fact that
the quail's crops were full of food and the birds were clearly in an absorptive state.
Conversely, plasma insulin levels were high at 06:00 h even though no food had been
consumed for 10 h beforehand. These "food deprived" birds (at 06:00 h) should be
metabolically equivalent to fasted birds, and as such might be expected to have low
plasma insulin levels (Anthony et al., 1990; Krestel-Rickert, et al., 1986; McMurtry,

Insulin rhythms that do not parallel feeding rhythms have been described in
other animals. Hulcher, et al. (1985) measured insulin levels in two strains of twice-
daily meal-fed monkeys (Cercopethicus aethiops). The vervet strain displayed a large
insulin peak following the 14:00 h feeding, but no such peak was associated with the
08:30 h meal. In fact, the lowest daily insulin level occurred 30 min after feeding in
the grivet strain monkeys. In ad libitum fed mice, Gagliardino & Hernández (1971)
and Roesler, et. al. (1985) independently demonstrated plasma insulin rhythms
wherein insulin levels rose, and subsequently peaked, during the period when the
mice consume relatively little feed (photophase), and were at their lowest levels
during the scotophase, when the mice fed most. A similar pattern of plasma insulin levels in rats was described by Jolin & Montes (1973). Our findings in a bird similarly indicate that factors in addition to feeding are responsible for the plasma insulin rhythm.

It is well known in mammals that pancreatic insulin release is not controlled solely by blood glucose levels. The rise in plasma insulin levels prior to any rise in plasma levels of absorbed nutrients, known as the preabsorptive insulin response (PIR) (Rohner-Jeanrenaund et al., 1983; Steffens & Strubbe, 1983), is just one example. Rich autonomic innervation of the endocrine pancreas (both parasympathetic and sympathetic) affect insulin release (Luiten et al., 1987). Neural regulation of pancreatic sensitivity to stimuli (e.g., glucose) via the vagus, and controlled by a hypothalamic pacemaker, allow for circadian regulation of insulin secretion (Strubbe et al., 1986; Marcilloux, J.-C. et al., 1985).

It has been demonstrated in rats that the circadian rhythm of feeding does not necessarily drive the IRI rhythm (Ikonomov, et al. 1985). Under conditions of continuous darkness (DD), the feeding rhythm of these rats persisted while the daily variations of IRI was lost. de Souza & Meier (1987) demonstrated plasma insulin rhythms in Syrian hamsters (Mesocricetus auratus) that shifted in phase seasonally with respect to the daily photoperiod, whereas feeding patterns did not. This finding suggests that, although the feeding and IRI rhythms may both be entrained by the light/dark cycle, the phase relationships are not fixed and one rhythm does not necessarily drive the other. Because there are also rhythms of metabolic responses to
insulin, a shift in phase of the insulin rhythm can change the net effect insulin has on liver lipid and/or carbohydrate metabolism (Cincotta & Meier, 1984).

The ability of an animal to alter its food intake or the way it metabolically responds to food intake is very important. Whereas many hormones work together, and are necessary for the proper regulation of feeding, body weight, and body composition, insulin is perhaps the most prominent. Insulin receptors in the brain, specifically in the ventromedial hypothalamic area (VMH) and the lateral hypothalamic area (LHA), are involved in the regulation of body weight and food intake (for review see Steffens, et al., 1990). Injections of exogenous insulin in mammals can induce increases in body weight (Lotter & Woods, 1977), increases in body fat stores in animals under food intake control (Torbay et al., 1985), and changes in feeding (Panksepp et al., 1975), although insulin injections have much greater lipogenic and hypoglycemic effects at one time of day than at other times (Cincotta & Meier, 1984).

The daily fluctuations in insulin levels observed could well be expressions of circadian neural control centers that regulate body weight, fat stores or feeding, in part, by modulating the phase relationship of the insulin rhythm and the rhythms of metabolic responses to insulin. The insulin rhythm may be an important mediator for neuroendocrine regulation of metabolism. Robust circadian variations in lipogenic and hypoglycemic responsiveness to insulin occur in hamsters in addition to circadian rhythms of plasma insulin concentration (Cincotta & Meier, 1984). Furthermore, the phase of the insulin rhythm varies so that the daily insulin peak coincides with the daily interval of lipogenic responsiveness in fat animals and not in lean animals.
These findings and those of the present study indicate clearly that the daily variations in plasma insulin concentration are not merely homeostatic reactions reflective of feeding.

References


Chapter 2

Daily *In Vivo* Variations in Lipogenesis and Lipogenic Responses to Avian and Mammalian Insulin Injections in Japanese Quail (*Coturnix c. japonica*)
Introduction

Insulin's role in the control of carbohydrate and lipid metabolism is well documented in mammals. Injections of exogenous insulin increase feeding (Panksepp et al., 1975), body weight (Lotter & Woods, 1977) and body fat stores (Cincotta & Meier, 1984), even in animals subjected to restricted food intake (Torbay et al., 1985). Insulin also stimulates triglyceride (TG) synthesis in the liver (Alberts et al., 1974; Assimacopoulos-Jeannet et al., 1977; Tiedgen & Seitz, 1980), decreases plasma free fatty acid (FFA) levels (Dole, 1956; Bierman et al., 1957) and promotes transport of lipids from the liver to adipose tissue for storage (Borensztajn et al., 1972; Topping & Mayes, 1972; Green & Newsholme, 1979). Insulin also increases the rate of synthesis and activity (Vydelingum et al., 1983; Speake et al., 1985) of adipose lipoprotein lipase (LPL), which hydrolyses triacylglycerols of plasma chylomicrons and very-low-density lipoproteins for uptake by adipose tissue (for review see Borensztajn, 1987). Insulin further influences lipogenesis through an antagonism of counter-regulatory (lipolytic) hormones, such as glucagon, growth hormone and the glucocorticoids (Brindley et al., 1989). This antagonism is achieved via insulin's ability to prevent/reverse the decrease in the activities of enzymes in the triacylglycerol synthesis pathway, brought about by lipolytic hormones (for review see Saggerson, 1989).

Although many of insulin's activities in mammals have also been demonstrated in birds (Hazelwood, 1984), the evidence for a regulatory role in lipid metabolism is equivocal. Several lines of evidence suggest that insulin is involved in lipogenic regulation. Genetically obese chickens have elevated insulin levels (Raheja et al.,

Depancreatized birds experience decreasing plasma triglyceride concentrations and the eventual atrophy of adipose tissue (geese, Sitbon, 1967; Karmann & Mialhe, 1976 and ducks, Sitbon et al., 1980). In vivo insulin injections stimulate the synthesis of fatty acids and triglycerides from glucose in liver, adipose tissue, skeletal muscle and heart muscle (Kompiang & Gibson, 1976; Gomez-Capilla et al., 1980; Vives et al., 1981). The addition of insulin to an incubation containing chick hepatocytes also increases glucose (Tarlow et al., 1977) and acetate incorporation into fatty acids (Capuzzi et al., 1971; Goodridge, 1973; Harvey et al., 1977) and triglycerides (Laurin & Cartwright, 1993). Griffin & Windsor (1993) found that freshly isolated hepatocytes incubated in the presence of porcine insulin were able to maintain elevated rates of lipogenesis for 48 hours or more. Insulin increases fatty acid synthetase activity in chick embryos (Joshi & Wakil, 1978) and newly hatched chicks (Joshi & Sidbury, 1976), as well as malic enzyme activity in chick (Laurin & Cartwright, 1993) and chick embryo (Goodridge et al., 1989) hepatocytes. Insulin also synergistically increases 3,3',5-triiodo-L-thyronine (T₃) stimulation of liver malic enzyme activity (Goodridge & Adelman, 1976), acetyl CoA carboxylase activity in liver slices of five-week-old chicks (Kompiang & Gibson, 1976) and LPL activity in adipose tissue of eight- and twelve-week-old chickens and turkeys (Borron et al., 1979). All of the previously listed enzymes are involved in lipogenesis or lipid storage. Insulin stimulates acetate incorporation into isolated chicken adipocytes when co-incubated with glucose (Montes et al., 1981) as well as increasing plasma
FFA uptake by the liver, in ducks, both in vivo and in vitro (Gross & Mialhe 1978, 1982, 1984).

Contrariwise, other studies indicate that dramatic differences exist between the way insulin influences lipid metabolism in birds and mammals. In vivo injections of mammalian insulin, which significantly lower plasma glucose levels, have no effect on the incorporation of labeled acetate into liver or fat pad of the house sparrow, *Passer domesticus* (Goodridge, 1964). Simon *et al.* (1991) showed that fattening in a genetically fat line of chickens could not be accounted for by differences in the number of, or kinase activity of, liver insulin receptors. Neither Rosebrough & Steele (1987) (3-4 week old chicks) nor Burghelle-Mayeur *et al.* (1989) (16 month old normal and dwarf laying hens) were able to modify in vitro lipogenesis in liver slices with insulin. Furthermore, insulin fails to stimulate fatty acid synthesis, and decreases malic enzyme activity in chick embryo or 1-day-old chick hepatocytes maintained in tissue culture (Goodridge *et al.*, 1974). These results indicate that insulin does not stimulate lipogenesis in birds. Additional studies indicate that insulin seems to have little or no anti-lipolytic role in birds (Langslow & Hales, 1971).

Insulin injections have been shown to have no significant effect on FFA levels (geese & owls; Grande, 1969; Grande, 1970) or to elevate (= thirty minutes post injection) circulating FFA levels in chickens (Goodridge, 1964; Heald *et al.*, 1965; Lepkovsky *et al.*, 1967; Langslow *et al.*, 1970; Langslow & Hales, 1971; Nir and Levy, 1973). In contrast, injection of insulin in mammals leads to a decrease in plasma FFA levels, primarily due to a decreased release of fatty acids from tissue stores (Bierman *et al.*, 1957). Heald *et al.* (1965) suggested that increases in FFA levels in birds following
insulin injection are due to an increase in glucagon release from the pancreas. It is the increased glucagon levels that then stimulate FFA release from storage tissues. Work by Veiga et al. (1983) also suggests that insulin itself does not increase FFA levels, rather the hypoglycemic conditions following insulin injection stimulate the release of adrenal glucocorticoids, which have a lipolytic effect upon adipose tissue.

It is not at all clear why there are so many conflicting results concerning a possible insulin role in avian lipogenesis. Perhaps this body of conflicting data is the result of circadian variations in lipogenic and hypoglycemic responses to insulin. Cincotta & Meier (1984) demonstrated in mammals that insulin injections have much greater lipogenic and hypoglycemic effects at one time of day than at other times. Additionally, most lipogenesis occurs during restricted daily intervals in birds (Meier, 1977), fish (Meier & Burns, 1976) and mammals (Cincotta & Meier, 1984; Martin et al., 1990). Experiments performed at a time of day when lipogenesis normally does not occur or when there is a lack of lipogenic responsiveness to insulin might falsely demonstrate a lack of insulin's involvement in lipogenesis. Another possibility is that mammalian insulin is not effective in some birds. There are 6-7 amino acid differences between bovine insulin and the avian insulins sequenced to date (Evans et al., 1988). Avian insulin appears to be highly conserved, with chicken, turkey and ostrich insulin having identical amino acid sequences and duck and goose insulin differing by three amino acid substitutions (Evans et al., 1988). For these reasons the effects of both mammalian and chicken insulin upon lipogenesis were tested in the Japanese quail at six different times of day.
Materials and Methods

Experiment 1 - Mammalian Insulin Injections: Eighty-four, nine week-old Japanese quail (Coturnix coturnix) were obtained from the Louisiana State University Poultry Science Department's random bred line. The birds were held on a daily 16L:8D photoregime (lights on at 06:00 h) and allowed ad libitum access to a standard quail breeder ration (2.75 Kcal ME/g feed, 17% protein).

Six groups of 14 quail each were injected (i.p.) with 1-[14 C]-sodium acetate (ICN Biomedicals, Inc., Irvine, CA, U.S.A.; 2 μCi in 0.1 ml 0.9% [w/v] saline; 55 mCi/mnmole acetate) at a rate of 2 μCi/100g B.W. at 02:00, 06:00, 10:00, 14:00, 18:00 or 22:00 h. Within each group, seven experimental animals were preinjected (i.p.; 30 min before) with mammalian insulin (MI; 5 i.u. bovine insulin; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in 1.0 ml 0.9% (w/v) saline at a rate of 0.1 ml/100g B.W. and seven controls were preinjected (i.p.) with 0.9% (w/v) saline at a rate of 0.1 ml/100g B.W. Insulin was preinjected to allow it to reach maximal hypoglycemic activity at the time of acetate injection. Insulin doses and period of preinjection were estimated from studies done with chickens (O’Hea & Leveille, 1969; Simon & Leclercq, 1985). Thirty minutes after acetate injection the birds were sacrificed by an overdose of halothane, an inhalation anesthesia. The liver and fat pads (leaf and mesenteric) were rapidly removed, blotted dry, weighed and then frozen until lipid extraction. Total lipid content was extracted by the method of Folch et al. (1957) without the use of the washing procedure, and analyzed for 14 C content in a liquid scintillation counter. A 0.002 M CaCl₂ solution was used in place of water.
to force phase separation. \(^{14}C\)-acetate incorporation was expressed as DPM per gram of tissue.

**Experiment 2 - Avian Insulin Injections:** Eighty-four, ten week-of-age Japanese quail (*Coturnix coturnix*) were obtained from L.S.U. Poultry Science Department's random bred line. Experimental conditions were as previously described, except that experimental birds were preinjected (i.p. 30 min before) with avian insulin (AI; 2 I.U. chicken insulin/kg B.W., lot #8706; Litron Laboratories, Rochester, New York, U.S.A.) in 1.0 ml 0.9% (w/v) saline at a rate of 0.1 ml/100g B.W.

Triglyceride and cholesterol content was extracted using matrix solid-phase dispersion (MSPD) (Barker & Long, 1990; Long et al., 1991). This method of extraction allows for rapid isolation of triglycerides and cholesterol while utilizing a minimum volume of volatile solvents. Preliminary studies on the feasibility of this methodology indicate that most of the recovered radiolabel in the acetate-incorporated liver (91%) was eluted in the hexane (52%), di-chloromethane (18%) and methanol (21%) fractions (Table 2.1). These fractions primarily contain the triglycerides (hexane), cholesterol/sterols (di-chloromethane) and inositol/non-phosphorylated sugars (Barker & Long, 1990). The non-phosphorylated sugars and inositols are polar in nature and primarily water soluble, and are not found in the chloroform fraction of a chloroform:methanol extraction (Folch et al., 1957). When quail liver tissue was "fortified" with radiolabel after removal from the animal, more than 96% of the recovered radiolabel was located in the ethyl acetate (80.5%),
Table 2.1: Results of MSPD extraction of $^{14}$C-acetate "spiked" and $^{14}$C-acetate "incorporated" liver lipids (DPM ± SEM) in Japanese quail. Hex = hexane; DCM = di-chloromethane; EtOAc = ethyl acetate; ACN = acetonitrile; MeOH = methanol; H$_2$O = water.

<table>
<thead>
<tr>
<th></th>
<th>Hex</th>
<th>DCM</th>
<th>EtOAc</th>
<th>ACN</th>
<th>MeOH</th>
<th>H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-Acetate &quot;Incorporation&quot;</td>
<td>3421±178</td>
<td>1207±120</td>
<td>269±26</td>
<td>155±18</td>
<td>1381±54</td>
<td>160±16</td>
</tr>
<tr>
<td>% Total Recovered</td>
<td>52</td>
<td>18</td>
<td>4</td>
<td>2</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>$^{14}$C-Acetate &quot;Spiked&quot;</td>
<td>466±24</td>
<td>938±41</td>
<td>33985±1400</td>
<td>2606±831</td>
<td>3124±1810</td>
<td>217±26</td>
</tr>
<tr>
<td>% Total Recovered</td>
<td>1</td>
<td>2</td>
<td>81</td>
<td>6</td>
<td>9.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
acetylnitrile (6.2%) and methanol (9.5%) fractions (Table 2.1). Consequentially, only the hexane and di-chloromethane fractions were eluted and used as indicators of de novo lipogenesis. For each sample, two grams of octadecylsilyl (C_{18}, 40 μm, 18% load, endcapped derivatized) silica packing material was placed into a glass mortar with 0.5g tissue. Samples were then gently blended (=2 min) into the C_{18} with a glass pestle until the mixture was homogeneous in appearance.

The resultant C_{18}/tissue matrix was placed in a 10 ml plastic syringe barrel plugged with a filter paper circle (Whatman No. 1). The column head was covered with two filter paper circles and the column contents were compressed to a final volume of 4.5 ml by a syringe plunger with the rubber end and pointed plastic portion removed. A modified pipet tip (100 μl) was placed on the column outlet to increase residence time of eluting solvents on the column. The resulting column was then eluted with 8 ml each of hexane (HEX), which elutes the triglyceride fraction, followed by di-chloro methane (DCM), which elutes the cholesterol fraction (Barker & Long, 1990). Flow through the column was gravity controlled in all cases. If initial flow through the column was hindered, positive pressure was applied to the column head with a pipet bulb to initiate gravity flow. When flow ceased, excess solvent was removed from the column with positive pressure as described above, before continuing with the next solvent. A one ml sample of each eluant was then aliquotted into a 20 ml scintillation vial, scintillation fluid added, and the samples counted for ^14C activity.

**Data Analysis:** Data were analyzed for normality using the SAS Univariate Procedure (SAS, 1985). Where necessary, data were ranked to provide approximate
normality and equality of variance and then analyzed by ANOVA (for unbalanced data) for significant treatment, time-of-day, and treatment by time-of-day effects using the GLM Procedure (SAS, 1985; p < 0.05). Additional analysis of each treatment group was performed, post-hoc, where indicated, using Tukey's studentized range test.

**Results**

**Experiment 1 - Mammalian Insulin Injections:** Data are presented as mean ± SEM for liver weights and fat pad weights (Table 2.2), [14C]-acetate incorporation by the liver (Fig. 2.1a), and [14C]-acetate-labeled lipid content of the fat pads (Fig. 2.1b).

**Daily variation of organ weights:** Analyses of daily variations in liver weights, as well as fat pad weights, indicate that there was a significant time-of-day variation of the weights of both organs (P=0.0001; Table 2.2). Heaviest liver weights (3.3g) were at 06:00 h while heaviest fat pad weights (5.5g) were recorded at 22:00 h. Lightest liver weights were at 18:00 h (2.2g) and lightest fat pad weights were recorded at 10:00 h (1.9g).

**Rhythm of lipogenesis/lipid incorporation:** There was a significant daily variation of *in vivo* [14C]-acetate incorporation into liver lipids (P=0.004) (Fig. 2.1a) and [14C]-acetate-labeled lipid content of the fat pads (P=0.0001; Fig. 2.1b) when expressed as disintegrations per minute (DPM) per gram of liver or fat pad tissue. Liver [14C]-acetate incorporation rates were lowest at 10:00 and highest at 22:00 h. [14C]-acetate-labeled lipid content of the fat pads were relatively constant throughout the day, with a single depression of labeled content at 22:00 h.
**Insulin injection effects:** Thirty minute preinjection of mammalian insulin had no significant effects on fat pad weight (Table 2.2). Significant overall treatment effects were observed on liver weight ($P=0.003$; Table 2.2), liver $[^{14}C]$-acetate incorporation rates (DPM/gram liver; $P=0.001$; Fig. 2.1a), $[^{14}C]$-acetate-labeled lipid content of the fat pads (DPM/gram fat pad; $P=0.0002$; Fig. 2.1b).

Liver weights of birds pre-injected with mammalian insulin (3.2 g) were significantly heavier (11.7%) than those of birds injected with saline (2.8 g; $P=0.003$; Table 2.2).

Liver $[^{14}C]$-acetate incorporation rates (DPM/gram liver), were significantly depressed by mammalian insulin injection (53.2% decrease; $P=0.001$; Fig. 2.1a) as were fat pad $[^{14}C]$-acetate-labeled lipid contents (40.9% decrease; $P=0.0002$; Fig. 2.1b). There was no significant treatment by time-of-day interaction in the depression of liver $[^{14}C]$-acetate incorporation rates or fat pad $[^{14}C]$-acetate-labeled lipid content, i.e., mammalian insulin depressed "incorporation" similarly at all times of day (Fig. 2.1).

**Experiment 2 - Avian Insulin Injections:** Data are presented as mean ± SEM for liver weight, fat pad weight, liver triglycerides and cholesterol, as well as $[^{14}C]$-acetate incorporation into liver triglycerides and cholesterol. Fat pad $[^{14}C]$-acetate incorporation was not measured in this experiment.

**Daily variation of organ weights:** Neither liver weights nor fat pad weights varied significantly over the 24-h period (Table 2.3).

**Rhythm of lipogenesis/lipid incorporation:** There were daily variations in the amount of triglyceride ($P=0.02$), cholesterol ($P=0.0001$) and lipid (triglycerides
Table 2.2: Mean wet weights (g±SEM) of livers and fat pads after bovine insulin or saline injection. Means for each organ without a common superscript differ significantly (P < 0.05).

<table>
<thead>
<tr>
<th>Time of Day (h)</th>
<th>02:00</th>
<th>06:00</th>
<th>10:00</th>
<th>14:00</th>
<th>18:00</th>
<th>22:00</th>
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<tbody>
<tr>
<td><strong>Liver Weights</strong></td>
<td></td>
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<tr>
<td>Saline Injected</td>
<td>2.7±0.3&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>3.3±0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.6±0.2&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>2.7±0.2&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>2.2±0.3&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.2±0.2&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin Injected</td>
<td>3.4±0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.0±0.1&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>3.4±0.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.6±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.4±0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Fat Pad Weights</strong></td>
<td></td>
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<tr>
<td>Saline Injected</td>
<td>3.3±0.8&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.5±0.4&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.9±0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.7±0.2&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.7±0.2&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>5.5±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin Injected</td>
<td>2.9±0.5&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.7±0.3&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.6±0.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.9±0.5&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>3.3±0.2&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>4.1±0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
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Fig. 2.1: Rhythm of $^{14}$C-acetate incorporation/content after bovine insulin injections. The solid bars denote the dark period.
+ cholesterol; \(P=0.005\)) extracted from the liver (Table 2.4). In general, liver triglyceride, cholesterol and lipid content was low at 10:00 h. Differences were noted between the lowest liver cholesterol content at 10:00 h and the increased levels seen at 18:00 and 22:00 h \((P=0.05)\).

There were daily variations \((P=0.0001)\) of \(^{14}\text{C}\)-acetate incorporation into liver triglycerides, liver cholesterol and liver lipids, expressed as disintegrations per minute per gram of liver tissue \((\text{DPM/g liver})\) (Fig. 2.2). \(^{14}\text{C}\)-acetate incorporation rates into liver triglycerides, liver cholesterol and total liver lipids were lowest at 10:00 and highest at 14:00 and/or 18:00. When the data were expressed as DPM/g extracted lipids the pattern is similar (Fig. 2.3), with time-of-day effects \((P=0.0001)\), a nadir of incorporation at 10:00 h and peak \(^{14}\text{C}\)-acetate incorporation rates at 14:00 and/or 18:00 h.

**Insulin injection effects:** Thirty minute preinjection of avian insulin increased liver cholesterol by 8.95\% (8.6mg) over controls (7.9mg; \(P=0.003\); Table 2.4). There were no significant effects on any of the other variables studied. Liver weights and fat pad weights (Table 2.3) and \(^{14}\text{C}\)-acetate incorporation rates into liver triglyceride, cholesterol or "total" lipid (Table 2.4) were not significantly affected by avian insulin injections.

**Discussion**

**Daily variation of organ weights:** There are few reports concerning daily variations in liver weights. When Raheja (1973) placed young (5-week-old) chickens on a three-hour on, three-hour off feeding schedule, and sampled birds one hour postprandially, no diurnal rhythm of liver weights was observed. Based on these
Table 2.3: Mean wet weights (g±SEM) of livers and fat pads after avian insulin or saline injection.

<table>
<thead>
<tr>
<th>Time of Day (h)</th>
<th>02:00</th>
<th>06:00</th>
<th>10:00</th>
<th>14:00</th>
<th>18:00</th>
<th>22:00</th>
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<tbody>
<tr>
<td><strong>Liver Weights</strong></td>
<td></td>
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<tr>
<td>Saline Injected</td>
<td>2.9±0.4</td>
<td>3.5±0.9</td>
<td>2.7±0.6</td>
<td>3.3±0.6</td>
<td>3.7±0.3</td>
<td>3.3±0.4</td>
</tr>
<tr>
<td>Insulin Injected</td>
<td>2.9±0.5</td>
<td>3.6±0.8</td>
<td>3.2±0.8</td>
<td>3.0±0.2</td>
<td>3.3±0.5</td>
<td>3.0±0.5</td>
</tr>
<tr>
<td><strong>Fat Pad Weights</strong></td>
<td></td>
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</tr>
<tr>
<td>Saline Injected</td>
<td>2.9±1.2</td>
<td>2.5±0.7</td>
<td>2.6±2.1</td>
<td>2.0±1.2</td>
<td>2.1±1.0</td>
<td>2.5±1.4</td>
</tr>
<tr>
<td>Insulin Injected</td>
<td>3.2±2.0</td>
<td>2.3±1.5</td>
<td>2.7±1.8</td>
<td>3.0±1.0</td>
<td>2.6±1.5</td>
<td>2.6±1.5</td>
</tr>
</tbody>
</table>
Table 2.4: Mean amounts (mg±SEM) of total lipids extracted from liver after avian insulin or saline injection. Means without a common superscript differ significantly from values within the same grouping (P<0.05).

<table>
<thead>
<tr>
<th>Time of Day (h)</th>
<th>02:00</th>
<th>06:00</th>
<th>10:00</th>
<th>14:00</th>
<th>18:00</th>
<th>22:00</th>
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<tbody>
<tr>
<td><strong>Extracted</strong></td>
<td></td>
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<tr>
<td>Saline Injected</td>
<td>9.0 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.1 ± 7.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.7 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.8 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.2 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin Injected</td>
<td>11.9 ± 3.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>16.9 ± 4.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 ± 6.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.8 ± 1.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.5 ± 1.6&lt;sup&gt;*&lt;/sup&gt;</td>
<td>11.3 ± 1.1&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td></td>
<td></td>
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<tr>
<td>Saline Injected</td>
<td>8.1 ± 0.4&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>7.2 ± 0.5&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>6.5 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.1 ± 0.4&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>9.1 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.5 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin Injected</td>
<td>8.6 ± 0.6&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>9.2 ± 0.5&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>7.3 ± 0.3&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>8.0 ± 0.4&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>9.9 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.8 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;</td>
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<tr>
<td><strong>Cholesterol</strong></td>
<td></td>
<td></td>
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<tr>
<td>Saline Injected</td>
<td>17.0 ± 1.5&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>24.3 ± 7.6&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>12.7 ± 0.6&lt;sup&gt;f&lt;/sup&gt;</td>
<td>22.8 ± 4.2&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>22.9 ± 2.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>21.6 ± 2.1&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin Injected</td>
<td>20.5 ± 3.9&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>26.1 ± 4.4</td>
<td>20.5 ± 6.4&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>18.8 ± 1.7&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>23.4 ± 2.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>20.2 ± 1.2&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Fig. 2.2: Liver lipogenesis after avian insulin injections. Solid bars denote the dark period.

A) Incorporation into triglycerides.
B) Incorporation into cholesterols.
C) Incorporation into triglycerides + cholesterol.
Fig. 2.3: Liver $^{14}$C-acetate incorporation after avian insulin injection. Solid bars denote the dark period.

A) Incorporation into triglyceride (DPM/g triglyceride)
B) Incorporation into cholesterol (DPM/g cholesterol)
C) Incorporation into triglyceride + cholesterol
   (DPM/g triglyceride + cholesterol)
results, and a study on the relationship of liver glycogen levels and feeding patterns by Sollberger (1964), Raheja concluded that liver weight was driven by the feeding pattern of the birds and not the light:dark cycle. When arriving at his conclusions, Raheja seemed to ignore the fact that, in order for them to eat, his experimental animals were exposed to a three-hour light period during their normal dark period (chickens normally do not feed during the scotophase). Because Raheja did not consider the effects that this skeleton photoregime might have had upon liver weights, it is difficult to compare the results of the studies.

A significant daily variation in liver weights was observed in both experiments. Because quail are known to eat continuously during the photophase (T.D. Siopes, pers. comm.), Raheja (1973) might have predicted that liver weights in quail would remain relatively high during the photophase, only to decline after dark onset. Our data indicate that this is not the case. There was no large decline of liver weight during the scotophase, a time when quail do not feed. Thus, liver weight variations do not directly reflect feeding patterns.

Fat pad weight variation, like liver weight variation, does not seem to be driven by feeding patterns. Factors other than feeding (e.g., lipolytic activities) apparently have important roles in determining fat pad weight variation.

Daily variations in acetate incorporation: It has been reported that carbohydrate feeding increases liver lipogenic activity, as indicated by plasma triglyceride levels in chicks (Muiruri et al., 1975). This supposition is supported by the finding in chickens that fasting for two or more hours can diminish lipogenesis by as much as a factor of eight (Leveille et al., 1975 as adapted from Yeh &
Leveille, 1970, 1971). However, our results do not support such a generalization for adult quail. Since quail feed almost continually during the light period and not at all during the dark period (T.D. Siopes, pers. comm), one would expect liver lipogenesis to be high from light onset until shortly after lights off. The pattern of lipogenesis we observed, which was relatively constant throughout the 24-hour period with a depression at 10:00 h, suggests that lipogenesis is not driven exclusively by feeding. Variations in lipolysis and gluconeogenesis, for example, could be important determinants.

**Insulin injection effects:** Birds injected with mammalian insulin had significantly heavier liver weights than saline injected birds even when expressed as a ratio of liver weight vs. body weight. This weight difference did not seem to be the result of increased lipid production in the liver, for lipogenesis, as measured by 

\[ ^{14}C \text{-acetate incorporation,} \]

was depressed by mammalian insulin by almost 50%.

Duerden & Gibbons (1990) found that, in addition to stimulating synthesis, insulin enhances the storage of hepatocellular triacylglycerol in the cytosolic pool of cultured rat hepatocytes while depressing the secretion of very-low-density lipoprotein triacylglycerol (VLDL-TG) from those same cells. There is additional evidence in mammals that insulin, especially with long term exposure, can decrease triglyceride and apolipoprotein B secretion from the liver (Durrington et al., 1982; Patsch et al., 1983; Pullinger & Gibbons, 1985; Mangiapanne & Brindley, 1986; Sparks et al., 1986; Gibbons & Pullinger, 1987). If mammalian insulin injections in quail have the same liver lipid storage promoting effect, but lack lipogenic stimulatory effects, then the liver weight gain we observed might be due to an increase in triglyceride storage brought about by a decrease in triglyceride export.
The depression of acetate incorporation into liver and fat pad lipids after injections of mammalian insulin was an unexpected result. A number of studies of avian species had indicated that exogenous insulin might stimulate lipogenesis as well as lipid transport to storage depots (Capuzzi et al., 1971; Goodridge, 1973; Goodridge & Adelman, 1976; Harvey et al., 1977; Gomez-Capilla et al., 1980; Vives et al., 1981; Goodridge et al., 1989). One might argue that the lipogenic depression by mammalian insulin was due to differences in the in vivo potencies of the two insulins. In terms of the production of hypoglycemic effects, a 10-fold greater dose of bovine insulin was necessary to reduce plasma glucose levels as much as native insulin in the chicken (Hazelwood et al., 1968; Hazelwood & Barksdale, 1970). This is due, in part, to a higher binding affinity of avian insulin than mammalian insulin (Simon et al. 1977; Simon, 1979). Injections of "lower potency" mammalian insulin, which then competes with native insulin for binding sites, might act as a "partial antagonist", providing a mechanism for the depression of in vivo lipogenesis. The fact that lipogenic rates were not decreased by avian (chicken) insulin injections seems to support this argument. Even so, we were unable to elevate lipogenic rates in quail above control levels with avian (chicken) insulin injections at any of six times of day.

Mammalian insulin injections had no significant effects on fat pad weights. This was not surprising, in light of insulin's effects upon quail de novo hepatic lipogenesis. In mammals, an insulin injection would be expected to decrease adipose tissue lipolysis while simultaneously elevating de novo lipogenesis and lipid transport
to storage depots (see Williamson, 1989 for review). The results of these two effects would be to increase fat pad lipid storage, and weight. Mammalian insulin, however, lacks an anti-lipolytic effect in birds (Langslow & Hales, 1971). The absence of an anti-lipolytic effect coupled with a significant decrease in de novo liver lipogenesis should lead to significant depression of [14C]-acetate incorporation in the fat pads, which was observed, and the possible reduction of fat pad weights.

Insulin's role in avian lipogenesis may be strictly "permissive". If the dissociation constant (Kd) for chicken hepatocytes lies in the range of 0.3 nM (Cramb & Langslow, 1984), and peripheral blood concentrations are in the neighborhood of 3 \times 10^{-11} M to 1.6 \times 10^{-10} M (McMurtry et al., 1983), then portal levels of insulin may be high enough so that a large portion of the hepatic insulin receptors are continually occupied (Cramb & Langslow, 1984). Increases in the plasma concentrations of insulin (i.e. insulin injections) would have little or no additional effects upon hepatic lipogenesis or could trigger receptor down-regulation (Gavin et al., 1974). This scenario would also account for the seemingly contradictory information from in vitro hepatic studies, where insulin is necessary to maintain or stimulate lipogenesis (Alberts et al., 1974; Capuzzi et al., 1974; Tarlow et al., 1977; Griffin & Windsor, 1993). Additionally, if one compares the rhythm of plasma insulin in Japanese quail (Tedford & Meier, 1993a) to the rhythm of lipogenesis in quail (Fig. 2.1) it does not appear that increased plasma insulin levels bring about increases in liver lipogenesis.

Other investigators have claimed that insulin is lacking any hepatic short-term intracellular effect (reviewed in Cramb & Langslow, 1984). In isolated hepatocytes from 4- to 8-week-old birds, insulin had no effects on intracellular cAMP or cGMP
concentrations, glycogenolysis, glycogen synthesis, gluconeogenesis or lipogenesis (Anderson & Langslow, 1975; Dickson et al., 1978; Cramb et al., 1982, as reviewed in Cramb & Langslow, 1984). While there is evidence for embryonic liver sensitivity to insulin (Joshi & Aranda, 1979a, b) it may be that the hepatic insulin metabolic effector is lost or deactivated after hatching.

Insulin is considered an anabolic hormone in birds, with few whole body sensitivity differences in glucose control between birds and mammals, (as demonstrated by euglycemia clamp technique) (Chou & Scanes, 1988). It exerts its biological effects at the skeletal muscle fiber, adipocyte, and hepatocyte(?), where it encourages peripheral utilization of glucose, glycogenesis and glycogen deposition, antigluconeogenic and associated effects (Hazelwood, 1984). However, in this study insulin appears not to play a primary role in the control of lipogenesis, as quail liver and adipose tissue lack the lipogenic response to insulin seen in mammals. This lack of lipogenic responsiveness occurs throughout the day, unlike mammals, which have been shown to be unresponsive only at certain times of the day (Cincotta & Meier, 1984). Accordingly, insulin may have a secondary role but is probably not a rate-limiting factor for stimulation of lipogenesis.

At present, it is unclear which hormone(s) are responsible for the control of lipogenesis in birds. Prolactin has been shown to influence (stimulate and/or inhibit) the fat stores of a number of species of vertebrates (for review see Meier, 1969), including several species of birds (Goodridge & Ball, 1967; Meier & Davis, 1967; D'yachenko, 1974/75; Chandola & Pavgi, 1979; Д'яченко, 1982; Sotowska-Brochocka, et al., 1986; Chattopadhyay et al., 1991). However, other
studies, using Gallinaceous birds, have failed to demonstrate prolactin's lipid-affecting properties (Nagra et al., 1963; Simpkins & Smith, 1976; Wheeland et al., 1976; Bartov et al., 1980; Tedford & Meier, 1993b), indicating that prolactin's effects might be species specific.

Avian pancreatic polypeptide (APP) is known to have many "insulin-like" effects in fed birds, such as depressing plasma free fatty acids, glycerol, and alanine levels while increasing the levels of plasma triglycerides (for review see Hazelwood, 1984). APP is also antilipolytic (both in vivo and in vitro), favoring lipid accumulation in the nonfasting state (Hazelwood & Langslow, 1978; Kimmel et al., 1978; McCumbee & Hazelwood, 1978). Fasted birds injected with APP experience a depression of plasma free fatty acids and glycerols (Hazelwood, 1984). Except for its lack of effect on plasma glucose levels, APP's actions in birds is much like insulin's effects in mammals (Hazelwood et al., 1973).

Both triiodothyronine (T3) and thyroxin (T4) are known to respond to changes in the nutritional status of birds (for review see Eales, 1988). T3 and T4 also have been reported to play a role in the regulation of lipogenesis. T3 appears to have both lipogenic and lipolytic properties. It acts synergistically with insulin to induce lipogenic enzymes in chicken embryo hepatocytes (Goodridge & Adelman, 1976; Tarlow et al., 1977; Joshi & Aranda, 1979b). Plasma T3 levels were found to be higher in broiler strains which exhibited a higher percentage body fat (Stewart & Washburn, 1983). Others report that T3 (alone) (Rosebrough et al., 1992) or T3 in concert with somatotropin (STH) (Cogburn et al., 1989) or somatostatin (SS) (Cogburn et al., 1990) reduce body fat content of broiler chickens, decreasing the
plasma insulin-to-glucagon molar ratio \((T_3 & T_3 + STH)\) and decreasing SS's ability to inhibit lipolysis (Gapusan & Oscar, 1993). John et al., (1972) reported that timed T₄ injections could alter the fattening response of the pigeon to timed prolactin injections. Saadoun et al. (1988) have also found differences in T₄ levels in fed fat and lean chickens. It is not known if T₃/T₄ play a more direct role in lipid control at this time.

It has also been suggested that lipogenic control is accomplished by the regulation of lipolysis, through the actions of glucagon (Calabotta et al., 1983). If insulin's lipid promoting effects are held at maximum rates by the saturation of hepatic insulin receptors (Cramb & Langslow, 1984), then lipid control could be regulated by adjustments in the rates of lipolysis through glucagon. Freeman & Manning (1977, 1978) have demonstrated a circadian rhythm of lipolytic responsiveness to glucagon.

Still others have suggested that lipid control is accomplished through a regulation of the glucagon/insulin balance (Cieslak, 1984; Leclercq, 1984). Following meal ingestion, or during glucose loading, insulin levels increase. SS release during meal feeding seems to disproportionately inhibit glucagon release, with the net results being an increase in the insulin/glucagon (I/G) ratio. The increased insulin levels promote peripheral uptake of glucose and amino acids, while decreased glucagon levels favor a reduced level of lipolysis and reduced inhibition of lipogenesis (overall lipid promoting). As blood levels of glucose and amino acids return to normal, insulin levels decrease and glucagon levels rise (decreased I/G ratio) reducing lipid promotion at the appropriate time (for review see Hazelwood, 1984).
addition to the synergistic control of lipogenesis by SS (Honey et al., 1981; Strosser et al., 1983), there is mounting evidence that GH (Scanes & Griminger, 1990 for review) and T₃ (see previous section) are also involved in lipogenic control. It is apparent that there remain several viable hormonal candidates for control of lipogenesis. What remains to be discovered is which hormone, or combination of hormones, is responsible for the integration of lipid metabolism in birds.

References


Chapter 3

Effects of Bromocriptine Treatment on Lipogenesis and Fat Stores in Japanese Quail (*Coturnix c. japonica*)
Introduction

Numerous studies of physiological mechanisms controlling lipid synthesis and storage in birds have been performed. Considerable evidences suggest that prolactin (PRL) plays a major role in the modulation (stimulation and/or inhibition) of lipogenic activity in avian (Meier & Farner, 1964; Goodridge & Ball, 1967a,b; Meier & Davis, 1967; Stetson & Erickson, 1971; Д’yachenko, 74/75; Chandola & Pavgi, 1979; Чаттепадхайй et al., 1991) and other vertebrate species (Meier, 1969; Meier et al., 1969; Sotowska-Brochocka & Jaklewicz, 1984), and that its effects are time dependent (Meier & Davis, 1967; Mehrle & Fleming, 1970; Sotowska-Brochocka et al., 1986).

Recent work with golden hamsters (Mesocricetus auratus) by Cincotta & Meier (1985a,b) has shown that PRL, administered at the proper times, increases or decreases hepatic lipogenesis by altering hepatic sensitivity to insulin’s lipogenic effects. These investigators also reported a significant reduction in hepatic lipogenic activity and concomitant reduction in body fat stores (33-49%) with bromocriptine (Cincotta & Meier, 1987; Cincotta et al., 1989; Cincotta et al., 1993).

Bromocriptine, a dopamine (DA) agonist, may be expected to influence body fat stores in mammals by inhibiting prolactin secretion (Cincotta & Meier, 1987) and/or by altering circadian neuroendocrine rhythms (Cincotta et al., 1989).

The purpose of this study was to determine if lipid synthesis and storage could be altered in an avian species using bromocriptine alone or in combination with neurotransmitter agonists/antagonists, as has been shown in mammals (for review see Cincotta et al., 1993). Although some evidence suggests that a DA agonist (i.e.,
bromocriptine) may alter avian lipid metabolism (Wheeland et al., 1976), the findings are equivocal and the mechanism by which this might be accomplished is unclear. A possible dopaminergic role in the regulation of PRL secretion in birds is debatable (Scanes et al., 1976; Scanes & Harvey, 1981; Chadwick & Hall, 1983; El Halawani et al., 1984; Hall et al., 1986) and PRL's role in the regulation of lipid production in this (MacGregor, 1975; Wheeland et al., 1976; Tedford & Meier, 1993) and other Gallinaceous species (Nagra et al., 1963; Simkins & Smith, 1976; Bartov et al., 1980) is largely negative.

Three experiments were performed using young (5 week old) male Japanese quail (Coturnix coturnix japonica) from the Louisiana State University Poultry Science Department's random bred line. The experiments involved either:

1. Bromocriptine injection intramuscularly (IM) into the pectoralis major muscle.
2. Food treatment with bromocriptine.
3. Food treatment with bromocriptine and p-chlorophenylalanine (PCPA) and fed to the birds. PCPA was used to block the synthesis of 5-hydroxytryptophan (5-HTP) and serotonin (5-HT) (El Halawani et al. 1978; Guémené & Etches, 1989), which may be involved in stimulation of PRL release in birds (see El Halawani et al., 1984 and Hall et al., 1986 for reviews).

**Materials and Methods**

**Bromocriptine injections:** Twenty quail were individually caged, maintained at 23°C, provided with a 16 h daily photoperiod (16L:8D; lights on at 06:00 h) and given full access to food and water.
The birds were divided into two groups. Ten birds received bromocriptine (2-bromo-α-ergocryptine methane sulfonate, Sigma Chemical Co., St. Louis, Mo., U.S.A.) injections and 10 birds received sham injections (peanut oil only). Bromocriptine was dissolved in peanut oil and injected intramuscularly into the breast muscle mass (0.25 mg bromocriptine in 0.1 ml peanut oil) twice daily, at 06:00 and 18:00 h (0.5 mg bromocriptine/bird/day -- Wheeland et al., 1976), sufficient to provide DA agonist action for an entire day in mammals (Cincotta & Meier, 1987). Treatment continued through day 10. Food and water consumption were monitored for the duration of the experiment.

On the eleventh day of treatment, at 14:00 h, the birds were weighed and then injected intraperitoneally with 8.7 μCi [14C]-acetate per 100 g body weight. This time (14:00 h) was chosen because both liver and fat pad [14C]-acetate incorporation are near high levels or are beginning to rise at this time (unpublished results). Thirty minutes after acetate injection the birds were sacrificed by an overdose of the inhalation anesthetic, halothane (2-bromo-2-chloro-1,1,1-trifluoroethane). Livers, furcular and abdominal (leaf and mesenteric) fat pads were rapidly removed and placed in cold phosphate buffered saline until weighed. All samples were frozen until lipid extraction was performed.

Bromocriptine feeding: Twenty quail were divided into two groups of ten, individually caged, and maintained at 23°C on a 16 h daily photoperiod (16L:8D - lights on at 06:00 h). The birds were given full access to water and feed. Ten birds were provided feed containing 0.09 mg bromocriptine per gram food (= 0.5 mg
absorbed/bird/day at a consumption rate of 130 g food/Kg body weight assuming a 28% G.I. tract absorbance). Ten control birds were fed standard quail breeder feed.

On the eleventh day of treatment, at 14:00 h, the birds were weighed and then sacrificed by halothane overdose. Livers and abdominal fat pads were removed as rapidly as possible and placed in cold phosphate buffered saline until weighed. All samples were frozen until lipid extraction was performed.

**Bromocriptine/PCPA feeding:** Twenty quail were divided into two groups of ten, individually caged, maintained at 23°C, given full access to water and feed, and provided with a 16-h daily photoperiod (16L:8D - lights on at 06:00 h). Ten birds were fed standard quail breeder feed containing 0.09 mg bromocriptine and 0.8 mg PCPA (p-chlorophenylalanine, Sigma Chemical Co., St. Louis, Mo., U.S.A.) per gram of food (Guémené & Etches, 1989) (=100 mg/Kg body weight PCPA, at a consumption rate of 130 g food/Kg B.W.). Ten control birds received untreated feed.

On the eleventh day of treatment, at 14:00 h, the birds were weighed and then sacrificed by halothane overdose. Livers and abdominal fat pads were removed as rapidly as possible and placed in cold phosphate buffered saline until weighed. All samples were frozen until lipid extraction was performed.

**In vivo lipogenesis:** Livers and fat pads were homogenized separately, total lipid content was extracted by the method of Folch *et al.* (1957) (without use of the washing procedure), and analyzed for 14C content in a liquid scintillation counter. A 0.002 M CaCl₂ solution was used in place of water to force phase separation. [14C]-acetate incorporation into liver lipid and fat pad lipid was expressed as DPM per gram
of extracted lipid. A quench correction curve was constructed, and all sample counts were corrected for quenching.

Data analysis: Data are presented as treatment means ± SEM. Because the data were not normally distributed, differences between groups were evaluated nonparametrically, using the NPAR1WAY (SAS, 1985) procedure, with the probability of a Type I error being 0.05.

Results

Bromocriptine injection: Twice daily bromocriptine injections had no significant effect upon any of the parameters measured (Table 3.1). Body weight change, fat pad weight, incorporation of [14C]-acetate into liver or fat pad lipids, food consumption and water consumption of bromocriptine animals did not differ significantly from control values.

Bromocriptine feeding: Bromocriptine treated food (Table 3.2) had no significant effect on change in body weight, liver weight or fat pad weight.

Bromocriptine/PCPA feeding: Bromocriptine/PCPA treated food (Table 3.3) had no significant effect on change in body weight, liver weight or fat pad weight.

Discussion

Circadian neuroendocrine rhythms are important in the control of fat stores, and PRL, thought to be one of the circadian hormonal expressions of this mechanism, seems to have a central role (Meier & Davis, 1967; Meier & Russo, 1985). Experimental evidence indicates that plasma PRL "surges" (either injections or naturally occurring circadian peaks) are involved in lipogenic control in several
Table 3.1: Effects of bromocriptine injections upon change in B.W., liver and fat pad weight, liver and fat pad lipid weight, liver and fat pad $^{14}$C-acetate incorporation into lipid, food and water consumption (mean ± SEM) in Japanese quail.

<table>
<thead>
<tr>
<th></th>
<th>Bromocriptine Treatment</th>
<th>Control Treatment</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in Body Weight (g)</td>
<td>14.1 ± 3.7</td>
<td>13.6 ± 3.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>4.61 ± 0.29</td>
<td>4.14 ± 0.30</td>
<td>N.S.</td>
</tr>
<tr>
<td>Extracted Liver Lipids (g)</td>
<td>0.163 ± 0.025</td>
<td>0.192 ± 0.037</td>
<td>N.S.</td>
</tr>
<tr>
<td>Liver $^{14}$C-acetate incorporation (DPM/g-lipid)</td>
<td>5145 ± 1356</td>
<td>5019 ± 1178</td>
<td>N.S.</td>
</tr>
<tr>
<td>Total Fat Pad Weight (g)</td>
<td>3.69 ± 0.88</td>
<td>2.33 ± 0.39</td>
<td>N.S.</td>
</tr>
<tr>
<td>Extracted Fat Pad Lipids (g)</td>
<td>2.07 ± 0.45</td>
<td>1.57 ± 0.33</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fat Pad $^{14}$C-acetate Incorporation (DPM/g-lipid)</td>
<td>453 ± 116</td>
<td>420 ± 96.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Food Consumption (g/g-BW/day)</td>
<td>0.129 ± 0.006</td>
<td>0.131 ± 0.005</td>
<td>N.S.</td>
</tr>
<tr>
<td>Water Consumption (ml/g-BW/day)</td>
<td>0.257 ± 0.022</td>
<td>0.273 ± 0.018</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

$^1$NPAIR1WAY test (P < 0.05)
Table 3.2: Effects of oral bromocriptine on body weight, liver weight and fat pad weight of Japanese quail (Mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Bromocriptine Treatment</th>
<th>Control Treatment</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in Body Weight (g)</td>
<td>0.32±0.62</td>
<td>4.00±1.80</td>
<td>N.S.</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>4.02±0.18</td>
<td>3.69±0.15</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fat Pad Weight (g)</td>
<td>5.74±0.83</td>
<td>4.38±0.69</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

1NPAIR1WAY test (P <0.05)
Table 3.3: Effects of oral bromocriptine and p-chlorophenylalanine (PCPA) on change in body weight, liver weight and fat pad weight in Japanese quail (Mean ± SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Fat Pad Weight (g)</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromocriptine + PCPA Treatment</td>
<td>5.12±2.86</td>
<td>3.94±0.15</td>
<td>3.79±0.67</td>
<td>N.S.</td>
</tr>
<tr>
<td>Control Treatment</td>
<td>0.46±4.12</td>
<td>3.89±0.17</td>
<td>3.64±0.68</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

1NPATR1WAY test (P < 0.05)
avian species (Meier & Farner, 1964; Meier & Davis, 1967; Goodridge & Ball, 1967b; Chandola & Pavgi, 1979; Дъяченко, 1982; Sotowska-Brochocka et al., 1986). Cincotta & Meier (1987, 1989) demonstrated that lipogenesis could be inhibited by removing these stimulatory PRL "surges" in mammals with the DA agonist, bromocriptine. Bromocriptine (CB-154) has also been reported to decrease liver \[^{14}\text{C}]\text{glucose incorporation into liver lipids of Japanese quail (Wheeland et al., 1976). Wheeland et al. (1976) concluded that bromocriptine inhibits lipogenesis in quail, based solely on the observed reduction in liver \[^{14}\text{C}]\text{glucose incorporation into liver lipids. This data in itself could be misleading, as a change in the transport of newly formed lipids from the liver to extra-hepatic storage areas could be interpreted as a change in liver lipogenesis when, in fact, no such alteration has occurred (Meier, 1977; Legrand et al., 1987). The fact that hepatic enzymes involved in lipogenesis were unaffected by bromocriptine in the quail (Wheeland et al., 1976) argues against the assessment that bromocriptine reduces lipogenesis in this species. Our studies with quail do not support any effect of bromocriptine on lipogenesis and/or fattening.}

There is convincing evidence that regulation of PRL secretion in birds differs from that in mammals. In mammals, PRL secretion is under tonic inhibitory control by DA. Thus, supplementation of DA or a DA agonist (such as bromocriptine) inhibits PRL secretion (for reviews see Ben-Jonathan, 1985; Ben-Jonathan et al., 1989). In birds, however, it is unlikely that DA exerts nearly as potent a PRL-inhibiting effect as it does in mammals. Although bromocriptine was reported to lower PRL in chickens (Harvey et al., 1982), most of the evidence is equivocal or negative (Scanes & Harvey, 1981; Hall & Chadwick, 1983; El Halawani et al.,
Recent evidence supports the idea that PRL secretion in birds is mainly
controlled through a stimulatory component (Scanes & Harvey, 1981; El Halawani et
al., 1984; Hall, 1984; Hall et al., 1986). El Halawani et al. (1991) postulated that
DA effects upon PRL secretion, when observed, are indirect, with DA masking or
reducing the effects of PRL-releasing factor (PRF). Indeed, it has been suggested
that DA's effects are not observed unless PRL secretion has been previously
stimulated (for reviews see Hall et al., 1986 and Macnamee & Sharp, 1989).
Accordingly, bromocriptine may not have reduced PRL secretion in our studies with
quail.

While several neuropeptides have been considered as possible PRL releasing
factors (see Sharp et al., 1989 for review), 5-HT, working in series with vasoactive
intestinal polypeptide (VIP) has been identified as a major stimulatory component of
PRL release (El Halawani et al., 1984; Hargis & Burke, 1984; Sharp et al., 1984;
Fehr et al., 1985; El Halawani et al., 1988; Proudman & Opel, 1988; Sharp et al.,
1989; Macnamee & Sharp, 1989). PCPA, a 5-HT synthesis inhibitor, has been
shown to inhibit PRL secretion in turkeys (El Halawani et al., 1980; El Halawani et
al., 1983; Guémené & Etches, 1989). Accordingly, PCPA was used in combination
with bromocriptine in an attempt to remove a possible stimulator of PRL secretion
that might compensate for a possible moderate inhibitory action of bromocriptine.
The lack of a lipogenic or fattening effect of this combined treatment suggests that
PRL itself may not have a primary role in regulating lipid metabolism in Japanese
quail. The failure of PRL to affect in vivo lipogenesis in other Gallinaceous species
(Nagra et al., 1963; Simpkins & Smith, 1976; Bartov et al., 1980) as well as in
Japanese quail (MacGregor, 1975; Wheeland et al., 1976; Tedford & Meier, 1993) indicates the possibility of species differences in the lipogenic responses of birds to PRL.

In summary, lipogenesis in Japanese quail fails to respond significantly to bromocriptine treatments, unlike mammals. Bromocriptine may not alter neural circadian pacemaker activity and/or alter PRL levels in this avian species. Alternatively, PRL may have a lesser role in the control of lipogenesis in Gallinaceous birds, unlike in many other avian species and vertebrates generally.

References


Chapter 4

Effects of Timed Injections of Prolactin on Body Weight and Body Fat Stores in Japanese Quail (*Coturnix c. japonica*)
Introduction

The known physiological roles of prolactin (PRL) in vertebrates are so numerous (= 85; see Rillema, 1980 for review) and of such a diverse nature, that one author suggested that "versatilin" would be a more appropriate name (Nicoll, 1974). Six broad categories of PRL action have been outlined to date: Osmoregulatory, growth/developmental, metabolic, reproductive, ectodermal (integumental) and synergistic with or antagonistic to steroid hormone effects (Nicoll, et al. 1980).

One of prolactin's metabolic actions are its effects upon lipid metabolism (for review see Meier, 1977). Daily injections of PRL have been shown to alter the fat stores of representative species of every vertebrate class (Meier, 1975). This is accomplished either through PRL's ability to reset circadian neuroendocrine oscillations via feedback mechanisms (Meier et al., 1981) or through alteration of the liver's lipogenic response to insulin (Cincotta & Meier, 1985b). In mammals, PRL is involved in the alteration of hepatocyte membrane microviscosity, which in turn affects the number of hepatic insulin receptors available for binding (Cincotta & Meier, 1985a). These changes in the hepatic insulin receptor profile allow for modification of the liver's lipogenic responsiveness to available plasma insulin and are thought to play a role in the circadian rhythm of lipogenesis. Bromocriptine, an inhibitor of prolactin secretion in mammals (Mizokawa et al., 1993), sharply reduced lipogenesis (Cincotta & Meier, 1985a) and hepatic insulin receptor numbers in hamsters (Cincotta & Meier, 1985b). PRL replacement after bromocriptine-induced PRL blockade fully restored lipogenesis and hepatic insulin receptor numbers to that found in obese animals (Cincotta & Meier, 1987). Continued bromocriptine studies
have shown that body fat stores can be reduced in several mammalian species (Cincotta & Meier, 1987, 1989; Cincotta et al., 1991), including humans (Meier et al., 1992).

PRL's involvement in lipid metabolism is not as clearly established in birds as it is in mammals. Studies where the data are unclear or negative (Nagra et al., 1963; MacGregor, 1975; Simkins & Smith, 1976; Wheeland et al., 1976; Bartov et al., 1980) question PRL's involvement in lipogenesis in some birds. However, several other studies in birds indicate that PRL might have a lipid affecting influence similar to that in mammals (Meier & Farner 1964; Meier & Davis 1967; Goodridge & Ball 1967a; Chandola & Pavgi 1979; Дъдженко, 1982; Sotowska-Brochocka et al. 1986; Chattopadhyay et al. 1991). Additionally, Meier & Davis (1967) and Sotowska-Brochocka, et al. (1986) have demonstrated that there are times of the day during which PRL is ineffective in promoting lipogenesis. It is possible that some of the negative data reported in the literature are a result of the experimenters choosing an injection time during which PRL lacks lipogenic effects. This conflicting assembly of experimental results led to the present experiment, an attempt to clarify PRL's role in lipid metabolism in Japanese quail.

**Materials and Methods**

Sixty-three, male, seven-week-of-age Japanese quail (*Coturnix coturnix japonica*) were purchased from Knight's Quail Kuntry in Hammond, Louisiana. The birds were transferred to an environmentally controlled room and maintained at 22 °C on an 16L:8D photoperiod (lights on at 06:00 h). The animals were divided into nine weight matched groups of seven birds each (mean initial body weight = 186.0 g).
Four groups of birds (PRL group — twenty-eight birds in total) were given intraperitoneal injections (ip) of ovine PRL (NIDDK-oPRL-19; obtained from the National Hormone and Pituitary Program, Baltimore, MD) at either 0 (06:00 h), 6 (12:00 h), 12 (18:00 h) or 18 (00:00 h) hours after light onset (HALO), while four groups of birds (C group — twenty-eight birds in total) received sterile saline injections (ip), at either 0, 6, 12, or 18 HALO. Non-injected controls (NIC — seven birds in total) received no injections at all. Injections began when the birds were thirteen weeks-of-age and lasted for five days. As premigratory accumulation of fat can occur rapidly in migratory birds (5-10 days) (Famer, 1960), a short injection period was chosen to avoid "stress-related" effects brought about by repeated injections at the same time-of-day (MacGregor, 1975). Each bird receiving injections was injected with either 200 µg ovine PRL in 0.2mL of sterile saline vehicle (experimentals) or 0.2 mL of sterile saline. At the end of the five day injection period, the quail were weighed and then sacrificed by an overdose of halothane, an inhalation anesthesia. The fat pads (furcular, abdominal and mesenteric) were rapidly removed, blotted dry and weighed.

**Data Analysis:** Data were analyzed for normality using the SAS UNIVARIATE Procedure (SAS, 1985). Where necessary, data were ranked to provide approximate normality and equality of variance and then analyzed by ANOVA (for unbalanced data) for significant treatment, time-of-day (TOD), and treatment by TOD effects using the GLM Procedure (SAS, 1985: P < 0.05). Non-injected birds were excluded from all TOD analyses. Additional analysis of each
treatment group was performed, post-hoc, where indicated, using Tukey's studentized range test.

Results

Body Weight: Birds injected for five days with oPRL experienced an average body weight increase of 5.4 g. Saline injected control birds lost an average of 3.9 g in body weight while NI control birds gained 2.4 g (Fig. 4.1). The changes in BWs experienced by the three groups were significantly different from each other (P<0.05). In addition to significant treatment effects, there was a significant TOD effect (without regard to injection treatment) upon change in body weight (P=0.03), with the birds injected at 0, 6, & 18 HALO gaining weight (0.9, 1.7 & 2.9 g respectively) while the birds injected at 12 HALO lost an average of 0.3 g (Fig. 4.2). Significant differences in the average change of body weight existed between the 12 and 18 HALO birds. There were no significant TOD by treatment effect interactions, as none of the body weights at any of the four time slots differed from any other, within each treatment group (Fig. 4.3).

Fat Pad Weight: Fat pad weights (FPWs) were heaviest in the NI control birds (mean wt. = 8.5 g), followed by oPRL injected birds (mean wt. = 6.7 g) and S injected birds (mean wt. = 6.3 g) (Figure 4.4). Fat pad weights of NI control birds were significantly different from S injected birds (P<0.05). There were no significant differences in fat pad weights due to TOD or TOD by treatment effect interactions (Fig. 4.5).
Fig 4.1: Body weight changes in Japanese quail after five days of prolactin (PRL INJ), saline (SAL INJ) or no injections (NIC) (means ± SEM). Dissimilar lettered treatment groups are significantly different from each other (ANOVA P < 0.05).
Fig 4.2: Changes in body weight of Japanese quail as a function of injection times without regard to treatment. Results are plotted as means ± SEM. Dissimilar lettered treatment groups are significantly different from each other (ANOVA P < 0.05).
Fig 4.3: Body weight changes in Japanese quail as a function of injection time of prolactin (PRL INJ), saline (SAL INJ) or no injections (NIC) (Means ± SEM). Body weight changes within each treatment group did not vary from one another.
Fig 4.4: Fat pad weights after five days of prolactin (PRL INJ), saline (SAL INJ) or no injections (NIC). Results are plotted as means ± SEM. Dissimilar lettered treatment groups are significantly different from each other (ANOVA $P < 0.05$).
Fig 4.5: Fat pad weights of Japanese quail after five days of prolactin (PRL INJ), saline (SAL INJ) or no injections (NIC). Results are plotted as means ± SEM.
Discussion

As in numerous other experiments (Riddle, 1963; Meier & Farner, 1964; Renzoni, 1970; Chandola & Thapliyal, 1972; Silverin, 1980), PRL injections produced weight changes, and these changes vary with TOD of injection (Meier & Farner, 1964). Unlike Meier & Davis' (1967) and John et al. (1972) findings in white-throated sparrow (Zonotrichia albicollis), weight changes do not appear to be due to increases or decreases in FPWs, as oPRL injected birds and S injected birds FPWs were not significantly different from each other (Fig. 4.4). At the end of the experiment, NI control birds had the heaviest FPWs (8.5 g), yet did not experience the largest body weight increase. Even though PRL injections have been shown to have potent antigonadal effects in many birds, not all PRL injection experiments have been able to produce antigonadal effects (see Meier & Dusseau, 1968 for review). The differences in body weights of our quail could not be attributed to changes in the reproductive system of the animals, as testis size (and weight?), appeared to be the same in all three groups (all "maximally stimulated" - none regressed). While it is possible that the weight changes are due to increases in total body water (Loretz & Bern, 1982; Gerardo-Gettens et al., 1989) we believe it to be more likely that the weight changes are due to increases in feeding (Schooley et al., 1941; Miller & Riddle, 1943; Nagra et al., 1963; Meier & Farner, 1964; Buntin & Tesch, 1985; Denbow, 1985; Buntin & Figge, 1988; Gerardo-Gettens et al., 1989; Byatt et al., 1993), coupled with increased protein formation and organ weight increases (Breneman, 1942; Gerardo-Gettens et al., 1989; Byatt et al., 1993), as hyperphagia is not always coupled with increases in fat stores (Robinzon et al., 1978;
Gerardo-Gettens et al., 1989). Increased protein formation might be due to PRL-induced increases in growth hormone (GH) and/or somatomedin (IGF-1) levels. Studies by Emata (1990) with fish and Wilson & Meier (pers. com.) with hamsters, indicate that these PRL-induced releases are also time-of-day dependent.

While it has been demonstrated that PRL injections can affect lipogenesis and lipid stores in numerous species, including fish (Lee & Meier, 1967; Mehrle & Fleming, 1970; Joseph & Meier, 1971; de Vlaming & Sage, 1972), amphibians (Meier, 1969), reptiles (Meier, 1969; Trobec, 1974), many birds (see next paragraph), and mammals (Nicoll, 1973; Joseph & Meier, 1974; Meier, 1975), including man (Creemers et al., 1991), PRL's effects upon birds is inconsistent. It is possible that a mammalian PRL preparation is not effective in quail, whereas the native hormone would be. It also might be unrealistic to expect to observe effects upon the fat pads of a bird as large as a quail after only 5 days of PRL injections. However, Burns & Meier (1971) demonstrated a PRL-induced cropsac response in the pigeon, a comparably sized bird, with only 4 days of injections, while Дяченко (1982), in Fringilla coelebs - body weight 21-25 g, and Sotowska-Brochocka (1986), in 6-week-old chickens, produced increases in fat pad weight in response to PRL injections in 5 days, indicating that dramatic changes in metabolic machinery are possible in a short period of time.

Several investigators have demonstrated that PRL "surges" (either injections or naturally occurring circadian peaks) are involved in lipogenic control in several avian species (Meier & Davis, 1967; Goodridge & Ball, 1967b; D'yachenko, 1974/75; Chandola & Pavgi, 1979; Дяченко, 1982; Sotowska-Brochocka et al., 1986), while
others were unable to produce changes in body fat stores using PRL injections alone (Nagra et al., 1963; MacGregor, 1975; Simpkins & Smith, 1976; Wheeland et al., 1976; Bartov et al., 1980). All three species which failed to respond to PRL injections were Galliform birds, and included Japanese quail. The lack of a response to PRL injections in some Gallinaceous birds suggests that PRL's ability to reset circadian neuroendocrine oscillations via feedback mechanisms and/or directly alter lipogenic processes may vary by species. Given the widespread and potent effect of prolactin on the lipid metabolism of numerous vertebrate species, noninvolvement of PRL in some Galliform birds seems incongruous. The present results, however, do not support a TOD difference in prolactin effects on lipid metabolism in quail.

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Summary

Despite conflicting data, both insulin and prolactin are thought to influence lipid production in birds. Plasma insulin levels exhibit a robust daily variation, with peak levels more than 4.6 times greater than the nadir. This rhythm does not seem to be driven by the pattern of feeding in the quail, as levels drop during early scotophase, while the birds are still in a post-prandial state, and begin to rise before light onset and before feeding occurs. A rhythm not directly tied to feeding might indicate that the insulin rhythm was influencing a metabolic process other than blood glucose removal, such as lipogenesis, as we see in mammals. There is also a significant daily variation in \textit{in vivo} lipogenesis in \textit{Coturnix}, as measured by $^{14}$C-acetate incorporation into liver and fat pad lipids. Peak liver lipogenesis occurs between 18:00 and 22:00 h, with lowest incorporation at 10:00 h, while fat pad label incorporation is relatively constant throughout the day, with a significant drop at 22:00 h.

\textit{In vivo} insulin injections (bovine and avian) did not promote increased lipogenesis, as measured by $^{14}$C-acetate incorporation into liver and fat pad lipids. Equally surprising was the lack of any time-of-day effects. A variation in lipid stimulating effects was expected, with insulin lacking lipid promoting results at some times-of-day. A complete lack of lipid stimulating capability was unexpected. It appears that some other hormone is responsible for stimulating lipogenesis in Japanese quail, or that lipogenesis is under neural stimulation.
Prolactin was also expected to have lipid promoting ability at certain times-of-day. Meier (1975) has demonstrated prolactin's involvement in lipid production in a representative of every vertebrate class. Bromocriptine, a dopamine agonist thought to influence body fat stores by altering prolactin secretion or dopaminergic circadian neuroendocrine oscillators involved in prolactin control, was found to be ineffective in altering body weight, body fat stores, or food and water consumption. This lack of effect differs from bromocriptine's effects in mammals.

Bromocriptine's failure to alter lipogenesis in *Coturnix* questions prolactin's involvement in avian lipogenesis. Timed prolactin injections were able to induce significant changes in body weight which differed from saline injected and non-injected controls. These changes in body weight were time-of-day dependent as well. However, differences in body weight appear not to be related to changes in fat pad weights. As with insulin, the lack of prolactin's involvement with lipogenesis in *Coturnix* was unexpected.

To date, it is not clear which hormone or hormones exert dominant control over lipogenesis in *Coturnix* quail. Other possibilities include pancreatic polypeptide and triiodothyronine/thyroxin and several "catabolic" hormones, which might "control" lipid production by influencing the rate of lipolysis.

References

Appendix: Letter of Permission

27 May, 1993

Professor Gerald A. Kerkt
Executive Editor, Comparative Biochemistry & Physiology
Department of Physiology & Biochemistry
The Medical School, Boldrewood
Southampton University
Southampton SO9 3TU
UK

Dear Sir,

I am currently preparing my Ph.D. work for publication, and should be finished sometime next year (summer/fall). I am planning to include a study previously published in Comparative Biochemistry & Physiology (Vol. 104A, No. 1, pp. 143-145, 1993) entitled "Daily rhythm of plasma insulin in japanese quail (Coturnix c. japonica) fed ad libitum" in my dissertation. In accordance with Louisiana State University regulations, I am requesting written permission to reprint this previously published material in my dissertation.

If there is any additional information I need to supply, please feel free to contact me at (504) 346-3254 during the day, or (504) 766-6022 in the evenings.

Thank you.

Bruce L. Tedford

Bruce L. Tedford
Dear Mr. Tedford,

Thank you for your letter of 27th May.

Yes, you can certainly have permission to reprint your paper (published in CBP 1993, 104A pp 143-145) in your dissertation.

With best wishes for your Ph.D.

Yours sincerely

Gerald Kerkut

Gerald Kerkut
INTRODUCTION

Circadian rhythms of plasma insulin concentration have been demonstrated in several mammalian species, including humans (for review see Mejean et al., 1988), dogs (Fischer et al., 1985), Syrian hamsters (de Souza and Meier, 1987), rats (Bellinger et al., 1975) and mice (Pessacq et al., 1976). Avian species have received little attention, and daily rhythms of insulin have not yet been reported in birds. Most of the avian work focuses on insulin responses to the animal's prandial state, especially starvation/refeeding experiments.

A few studies on daily variations of several indices of carbohydrate regulation have been reported in the chicken. Sollburger (1964) observed a circadian liver glycogen rhythm in chicks and Twiest and Smith (1970) reported a circadian variation of plasma glucose levels. Such metabolic rhythms might reflect rhythms of insulin and/or rhythms of other glucoregulatory hormones. The present study examined plasma insulin concentrations throughout a 1-day period in Japanese quail.

MATERIALS AND METHODS

Thirty-six, sexually mature (5-week-old) Japanese quail (Coturnix c. japonica) were purchased from the Poultry Science Dept., Louisiana State University. Birds were held on continuous light (L: L) in an environmentally controlled room, maintained at 23°C with food and water available ad lib. from hatch until 5 days of age, at which time they were transferred to a 16L:8D photoregime (lights on at 6 a.m.) After 2 weeks acclimation, blood samples were collected into non-heparinized syringes by cardiac puncture and then transferred to test tubes. Six different birds were sampled every 4 hr for 24 hr (2 a.m., 6 a.m., 10 a.m., 2 p.m., 6 p.m., and 10 p.m.—6 birds in total). Care was taken to minimize disturbance to birds not being sampled. The samples at light onset (6 a.m.) were taken before the birds began diurnal (photophase) feeding. Blood samples were placed on ice and allowed to clot before centrifugation. The serum was frozen and stored at −20°C until assayed.

Plasma levels of insulin were determined by radioimmunoassay, described by McMurray et al. (1983), using purified chicken insulin (Litron Laboratories, Rochester, NY) as the standard and for iodination and guinea-pig anti-chicken insulin serum as the first antibody. Inhibition curves obtained by serial dilution of Japanese quail plasma were found to be parallel to chicken insulin standard curves, suggesting that the quail plasma insulin inhibits binding in the same manner as the standard solutions. All samples were run in duplicate.

The data were analyzed using the SAS General Linear Models Procedure (SAS, 1985). Variations among treatment groups were examined by analysis of variance. Comparisons between groups were made with the Waller-Duncan A-ratio t-test.

RESULTS

Concentrations of plasma insulin varied during the 24 hr period (ANOVA, P < 0.003). Insulin levels were high from 6 a.m. (at light onset) until 6 p.m. (4 hr before light offset) (Fig. 1). Mean insulin levels from 6 a.m. to 6 p.m. (mean = 857 pg/ml) were more than four times as high as the mean values at 10 p.m. and 2 a.m. (mean = 185 pg/ml). Insulin concentrations at 6 a.m., 10 a.m., 2 a.m., and 6 p.m. did not significantly differ from each other as shown by Waller grouping. The 2 a.m. and the 10 p.m. values did not differ from each other, but both differed significantly, by Waller grouping, from those of all other sampling times (P < 0.05).

Feed consumption in these birds was diurnal as evidenced by the observation of full crop sacs at all times of the day, except the 2 a.m. and 6 p.m. time slots. It is noteworthy that crop sacs were especially full at light offset (10 p.m.).
Vita

Bruce L. Tedford was born in Little Rock, Arkansas on September 9, 1954. He graduated with a B.S. in biology in July 1976 from the University of Arkansas at Little Rock. He continued his education at the University of California at Los Angeles, where he received his M.A. in Zoology in December, 1980. He then taught high school science and math at the American Cooperative School, La Paz, Bolivia, before entering Louisiana State University in the fall of 1983 to begin work on his Ph.D. in physiology. Bruce has also worked as a research associate at Louisiana State University's Veterinary School (since 1987) while finishing his Ph.D. studies.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Bruce L. Tedford

Major Field: Physiology

Title of Dissertation: Daily Variations of Lipogenic Activities in Japanese Quail (Coturnix coturnix japonica)

Approved:

Albert H. Mees
Major Professor and Chairman

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Dean of the Graduate School

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Date of Examination: October 19, 1995