THE NUTRITIONAL DEMANDS OF EGG PRODUCTION IN FEMALE ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

By

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SUMMARY

Egg production is a demanding process for female birds. A number of studies have shown that body condition declines during breeding and reserves of lipid and protein are depleted. The aims of this study were to measure the relative contributions of exogenous and endogenous nutrients to the formation of eggs by captive female Zebra Finches and to investigate the transfer of material from the body reserves to the developing eggs.

Analysis of eggs revealed that there was little change in egg size or composition between the eggs of a clutch. On average the eggs contain 58.3mg of lipid, 134.8mg of protein and 17.8mg of calcium. The amino acid composition of egg proteins was similar to that in domestic hens. The mean clutch size for the colony was 5.2 ± 0.9 eggs. The onset of ovarian development was detected at around Day -4 of the laying cycle (ovulation of first egg on Day 0). Similarly, the oviduct grows rapidly from Day -4 to Day -1, then it declines in weight as the clutch is laid. Taking the above information into account the investment of protein and lipid in the clutch was calculated. Demand for egg nutrients increases rapidly from Day -4. Peak protein demand occurs on Day 1 of the cycle and for lipid on Day 0, after this demand gradually falls as the eggs are laid.

The consumption and the nutritional value of seed was measured during the period of egg formation in an attempt to estimate the use of exogenous nutrients. There was no measurable increase in seed consumption by breeding pairs of Zebra Finches from day to day of the laying cycle. However, a marked increase in the consumption of cuttlefish bone was recorded. Comparing the composition of a clutch of five eggs and the food consumed at the time of their formation it was clear that the diet could not meet demand for egg protein or amino acids. There is a possibility that during egg formation there is an increase in digestive efficiency to liberate more nutrients from the diet. However, this would lead to relatively little protein becoming available. In terms of energy available the diet could go some way towards satisfying demands for lipid. Calcium for the eggshells could be supplied from the diet alone.

The body reserves of protein and lipid were investigated during the period of egg formation. The lean dry weight and lipid of the pectoral muscle, ovary, oviduct and total carcass was measured, together with the dry weight of the leg muscles, heart, liver, gut and gizzard. There was a decline in the lean dry weight of the pectoral muscles equivalent to 15% of the protein in a five-egg clutch. The total carcass lean dry weight declined also by an amount equal to 76.8% of the protein in a five-egg clutch. The timing of this decline closely matched the demand for egg protein. Other organs, except the heart, followed a similar pattern of decline across the laying period.

There is a decline in body lipid by 61% of the amount found in females at the start of the laying cycle. This amounts to much more than the lipid content of a clutch of five eggs. The bulk of this lipid is lost from lipid depots. Intramuscular lipid declines but the amount involved is relatively insignificant.

Ash weight of the carcass showed no significant change and the calcium content of the ash was the same in post and pre-breeding females.

In considering the budgeting of nutrients for the eggs;
Body reserves of protein decline by an amount equivalent to 74.6% of the total reproductive requirement (eggs plus oviduct). If there is an increase in digestive efficiency of the order seen in a previous study then up to 15.2% of protein could come from the diet. The remainder could be made available by a decrease in female activity that would free protein from metabolism for reproduction.

It is possible that the diet can make a significant contribution to lipid needed for the eggs. In addition, the body reserves of lipid fall by an amount much greater than that found in the eggs. This surplus of lipid indicates that as well providing for the eggs themselves the lipid reserves may act as an energetic buffer to offset the extra demands of egg production.

Calcium is likely to be obtained entirely from the diet. There was no evidence to suggest reserves were used.

The pectoral muscle was investigated in more detail. Direct measurement of the protein content of sarcoplasmic and myofibrillar fractions of muscle revealed a similar pattern of decline in both fractions to that seen in lean dry weight. Also, the total amount of measured protein lost by the pectoral muscle was close to the loss of lean dry weight, indicating that lean dry weight is a good indirect measure of protein in muscle. Gel filtration analysis of the sarcoplasmic fraction revealed three proteins, two of which were tentatively identified as myoglobin and haemoglobin. The remainder, of high molecular weight appeared to be responsible for the bulk of the decline in sarcoplasmic protein.

Isotope labelled methionine was used to provide evidence that protein from the body reserves is transferred directly to the developing eggs. There was a significant difference in the isotope content of the pectoral muscle and oviduct between breeding and non-breeding females suggesting a higher turn-over of protein in the breeding birds. Secondly, isotope was detected in the eggs. Protein demand is highest while the first eggs of the clutch are being formed. As the clutch progresses the demand for protein diminishes and this is reflected in the distribution of the isotope through the clutch. There was more isotope in the eggs at the beginning of the clutch than those laid last. Also, at the start of the clutch, protein from the reserves seems to be of most importance to the yolk and at the end of the clutch to the albumen.

DECLARATION

I declare that the work presented in this dissertation has been completed by myself unless otherwise acknowledged in the text.

David W Donnan

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CHAPTER 1

GENERAL INTRODUCTION

The production of a clutch of eggs by a female bird is a demanding process. In the past, many studies have concentrated on the energetic demands of reproduction (eg Ricklefs, 1974). The daily energy requirement for egg laying can range from 29% of basal metabolic rate in raptors to 135% in waterfowl. However, daily protein requirements can be even more pronounced, being in raptors 86% above maintenance requirement and up to 230% in waterfowl, gulls and terns (Robbins, 1981 and 1983). These demands, above the level of normal requirements, vary from species to species depending on the clutch size and relative size of the eggs, the rate at which they are laid, and the growth of the ovarian follicles and oviduct prior to laying (Astheimer, 1986, Robbins, 1983). There is also considerable variation in egg composition across species depending on the mode of development of the chick. The eggs of those with precocial young have an average yolk content of 37%, compared to 22% for altricial species (Robbins, 1983, Blem, 1990, Birkhead & Nettleship, 1984, Carey, 1983). The larger yolk of precocial species enables longer incubation and a relatively advanced chick at hatching.

The nutrients that are used for the production of eggs may be derived from food intake (exogenous) or they may be supplied from reserves that the female already possesses (endogenous). There is little known, however, about the relative importance and budgeting of these avenues in avian reproduction (Walsberg, 1983).

Table 1.1 records those species whose protein and lipid reserves have been investigated during the egg laying period. They show a spectrum from total reliance on reserves to no use of them at all. For example, the female Adelie Penguin does not eat for two to three weeks before and during laying thus all nutrients must be derived from reserves (Astheimer & Grau, 1985). Similarly, the Lesser Snow Goose relies entirely on endogenous sources for egg production. They produce a clutch without having to feed when they arrive at their Arctic breeding grounds (Ankney & MacInnes, 1978).

In other birds the reliance on reserves is not so obvious but in the Canada and Brant Goose reserves of protein from the body of females are lost that are approximately equivalent to the amount required by the clutch (Raveling, 1979, Ankney, 1984). In a granivorous passerine, the Red-billed Quelea, it is thought that protein for the eggs is supplied partly from depletion of reserves and partly from food intake (Jones & Ward, 1976).

In contrast with the above, female Mallard Ducks use some of their lipid reserves while breeding but show only a slight loss in body protein reserves and so most of the protein for the eggs comes from intensive foraging for protein rich food items (Krapu, 1981). The Wood Duck shows no use of protein reserves at all and gets all the protein it needs from the diet but, like the Mallard, their lipid reserves are important during egg production (Drobney, 1980).

Finally, an example of a bird that seems to require no endogenous nutrients to produce eggs is the Brown-headed Cowbird (Ankney & Scott, 1980). These birds can apparently obtain all the necessary nutrients from food eaten at the time of laying. It is worth noting that while there is no decline in the Cowbirds' body protein levels across the entire breeding period there is a small, but significant, decline while the eggs are being laid.

As mentioned above, the additional protein requirements of egg production are, in many cases, relatively higher than energy requirements. In a number of studies protein, rather than energy, is thought to be the limiting factor for ovigenisis (Jones & Ward, 1976, Raveling, 1979, Fogden and Fogden, 1979, Houston et al, 1983, Drobney & Fredrickson, 1985).

In most of the studies in Table 1.1 protein has not been measured directly. Instead, a protein index based on the lean dry weight of muscle, corrected for body size, has been used. Lipids are extracted by solvents and the remainder is taken as being representative of protein. In the light of recent work this assumption is not unreasonable. In a study of the House Sparrow the decline of lean dry weight of the flight muscle was highly correlated with a direct measure of protein in the muscle (Jones, M.M, 1991). Therefore, decline in muscle lean dry weight is likely to reflect loss of protein and not some other non-lipid material.

It is unlikely that the decline in protein reserves seen in so many studies in Table 1.1 is to meet extra energetic demands due to egg production. Protein is not generally viewed as an energy store and its breakdown is complex and inefficient. Unlike lipid and carbohydrate, toxic by-products result from the breakdown process (Blem, 1990). Protein yields little energy as a substrate, only 4.3 kcal/g in birds (Schmidt-Nielsen, 1979). Many of the birds in the table have large lipid reserves which would be more efficient to use for energy. However, it has been suggested that the use of protein reserves during reproduction is to supply protein directly to the developing eggs (Kendall et al, 1973, Schifferli, 1980, Jones & Ward, 1976).

There is some evidence to suggest that protein reserves may be utilised to meet a specific nutritional demand for amino acids. This has been suggested before (eg Kendall et al, 1973, Krementz, 1984). Eggs are known to contain some amino acids in unusually high concentrations particularly the sulphur amino acids, cysteine and methionine (Harvey, 1970). In a recent study of Lesser Black-backed Gulls supplementary feeding experiments involving laying birds were done. Those given additional protein in the form of fish did not show any difference in their eggs, compared to control birds. However, those given an equivalent protein mass in the form of cooked eggs produced significantly larger eggs. This suggests that the quality of protein available is important not just quantity (Bolton et al, 1992). Poultry are known to have increased egg production when fed diets supplemented by methionine, tryptophan and lysine (Fisher, 1976). Even when protein is not in short supply in a bird's diet there may therefore be a need for certain limiting amino acids and these might be supplied from protein reserves.

Lipid reserves may be used for transfer to developing eggs, as is undoubtedly the case with the Lesser Snow Goose and the Adelie Penguin (Ankney & MacInnes, 1978, Astheimer & Grau, 1985). In other cases the reason for lipid reserve depletion is not so clear cut. In some studies it has been observed that lipid reserves are used to provide energy that allows a change of feeding behaviour. In the Mallard Duck (Krapu, 1981) and the Wood Duck (Drobney, 1980) the female turns her attention to protein rich food items at the expense of the lipid reserves. Similarly, the passerine Grey-backed Camaroptera switches from energy rich seeds to protein rich food items and may fuel this by utilising lipid reserves to cover the

energy deficit (Fogden & Fogden, 1979). In the Red-billed Quelea, a switch of diet to include calcium rich items for shell production was observed. Again, this would reduce energy intake that could then be compensated for by the decline in lipid reserves (Jones & Ward, 1976). In those studies in Table 1.1 that did not use protein reserves all, except the Cowbird and the White-bellied Swiftlet, made use of lipid reserves during the breeding period.

This study used a captive colony of Zebra Finches to examine the nutritional demand of egg production, in particular the budgeting of protein, lipid (fat) and calcium. The study had the following aims;

To determine the extent and timing of the nutritional demand for egg production in female Zebra Finches by measuring the growth of the oviduct and ovarian follicles from the resting state plus the protein, lipid and calcium content of an average clutch of eggs. Amino acid composition of the eggs was also determined.

To measure food intake during the reproductive period and to compare this to the non-breeding state. In addition, to measure the nutritional value of the diet (ie exogenous nutrients) including amino acid composition. Such measurements of food intake can only be made adequately in the controlled conditions of a laboratory.

To measure any change in body reserves of protein, lipid and calcium (ie endogenous nutrients) by examining the carcasses of birds at various stages of the reproductive cycle compared to non-breeding birds using, not only traditional techniques of lipid extraction, but also direct measurement of protein in muscles.

From the above, to assess the relative importance of endogenous and exogenous nutrients in relation to the demand for these nutrients during egg formation by female Zebra Finches.

Finally, by the use of isotopes, to determine if there is direct transfer of material from the body reserves of laying female Zebra Finches to the developing eggs.

The Zebra Finch is a convenient bird to use for such a laboratory study where controlled conditions are necessary to measure such parameters as food intake. They will breed readily in captivity if favourable conditions are maintained. The use of body reserves during breeding has not been previously demonstrated in this species. However, it is a species where reserves might be expected to play a role. In the wild, the Zebra Finch lives in the semi-arid regions of Australia and even while breeding exists on a diet almost entirely of grass seed which is low in protein content (Zann & Straw, 1984, Morton & Davies, 1983). The Zebra Finch is unlikely to be able to meet reproductive protein demand from the diet alone.

The Zebra Finch colony was maintained as follows;
The temperature throughout was maintained at 23 ± 2°C and the lighting regime was 14:10 light/dark. The cages in which birds involved in experiments were kept and those breeding for stock were all identical, measuring 60x50x40cm arranged in pairs with a removable partition between them. Food, Haith's Foreign Finch Mix, and water were replenished daily. In addition, cuttlefish bone and grit were available in the cage and replaced when necessary. Non-breeding birds were kept in flight aviaries. The sexes were always kept separated unless being used for breeding or experiments. In addition to the normal seed diet the birds in the flight cages were given a food supplement to maintain them in good condition. This was Haith's Conditioning Food which has a higher protein content than the normal finch mix. Except during feeding trials (see Chapter 3) ICI Forest Bark was used as a litter on the bottom of the cages. This was replaced as necessary but not during experiments. All birds were fitted with numbered leg rings so that each individual's history could be recorded.

For breeding, cages were fitted with a nest box, approximately 12.5cm square. The lid of the nest box was hinged to allow examination of the contents. The bottom of the nest box was covered by a layer of wood shavings and nesting material (dried and fresh grass) provided in the cage. For the experiments that required birds to breed, a technique was developed to encourage the highest rate of successful pairings. Birds were selected from the flight cages and weighed. Those of less than 12g were rejected as experience indicated that these birds were unlikely to breed. Pairs of males and pairs of females were placed in adjacent cages separated by the partition. They were left for about a week and then the

partition was removed and the birds allowed to mix. The partition was then replaced so that a breeding pair occupied each cage. Copious nesting material was then provided in the cage and a little put into the nest box to encourage nest-building behaviour.

Table 1.1 Studies that show a decline in protein reserves of female birds associated with egg production

Species	References
Adelie Penguin	Astheimer and Grau, 1985
American Coot	Alisaukas and Ankney, 1985
Black Ducks	Reinecke, Stone & Owen, 1982
Brant Goose	Ankney, 1984
Canada Goose	Mainguy and Thomas, 1985
Canada Goose	Raveling, 1979
Canvasback	Barzen and Serie, 1990
Common Eider	Korschgen, 1977
Common Eider	Parker & Holm, 1990
Grey-backed Camaroptera	Fogden and Fogden, 1979
House Sparrow	Jones, 1991
House Sparrow	Krementz & Ankney, 1986
House Sparrow	Schifferli, 1976
Lesser Black-backed Gull	Houston, Jones and Sibly, 1983
Lesser Black-backed Gull	Bolton et al, 1993
Lesser Snow Goose	Ankney and MacInnes, 1978
Mallard Duck	Krapu, 1981
Pied Flycatcher	Ojanen, 1983
Red-billed Quelea	Jones and Ward, 1976
Sand Martin	Jones, G. 1987
Starling	Osborn and Ward, pers comm.

Studies that did not show a decline in protein reserves of female birds associated with egg production

Brown-headed Cowbird	Ankney and Scott, 1978
Northern Shoveler	Ankney and Afton, 1988
Ringneck Duck	Hohman, 1986
Ruddy Duck	Tome, 1984
White-bellied Swiftlet	Hails and Turner, 1985
Wood Duck	Drobney, 1980 and 1982

CHAPTER 2 - NUTRITIONAL INVESTMENT IN THE EGGS OF FEMALE ZEBRA FINCHES

2.1 INTRODUCTION

The purpose of this chapter is to examine the total and daily nutritional investment made by female Zebra Finches producing a clutch of eggs. The extent of the investment will be a function of egg mass, clutch size, nutrient content, the interval between successive eggs and the length of the rapid phase of ovarian follicle growth (Astheimer, 1986, Walsberg, 1983). I shall attempt to investigate each of these factors in relation to the female Zebra Finch.

It is useful, at this point, to consider the process of egg formation itself. The Zebra Finch like most other birds suppresses the development of the right ovary (Phillips et al, 1985). Only the left ovary develops and it is to be found at the anterior part of the body cavity, suspended from the dorsal body wall by a peritoneal fold. The ovary initially contains millions of oocytes, the majority of which degenerate. Only a small number of the original will mature and ovulate. Oocytes that do develop must first be incorporated into an ovarian follicle. This follicle is necessary to support the oocyte which will grow so large that its own cell membrane alone would rupture. In addition, this follicle extracts yolk material from the blood and transfers it to the oocyte. The yolk material is produced by the liver.

As the follicle approaches its maximum size and is ready to ovulate it secretes progesterone that promotes the final development of the oviduct and induces copulatory and nest-building behaviour. At ovulation the follicle ruptures and the oocyte passes into the body cavity from where it enters the oviduct. The post-ovulatory follicle regresses over a few days and is resorbed by the ovary. The oviduct is held in place by dorsal and ventral ligaments and during the period leading up to ovulation it has grown rapidly from its resting state. The oviduct only develops when the female is in breeding condition as its growth depends on the secretion of hormones by the ovary.

The oocyte enters the first part of the oviduct, the *infundibulum* where it is fertilised and the first of the layers of albumen are laid down around the yolky zygote. The zygote then passes to the longest portion of the oviduct, the *magnum*, where most of the albumen is deposited. Next, the egg enters the *isthmus* and membranes are laid down that give the egg its shape. The last part of the oviduct is the shell gland or *uterus*. At first water is passed into the albumen resulting in a doubling of its mass, then the outer membrane becomes progressively calcified forming the shell. Once this process is complete the bird can lay the egg (Lofts & Murton, 1973, Phillips et al, 1985).

In passerines, such as the Zebra Finch, the period of rapid follicular growth is likely to be of the order of 3-4 days (Calder, 1974). The entire egg white and shell is produced in the twenty four hour period following ovulation (Walsberg, 1983).

There is great variation in the composition of birds eggs among species and this has been well documented (eg Sotherland & Rahn, 1987). The percentage of yolk in an egg varies with precocity of the young (Ricklefs, 1977). The Zebra Finch is an altricial species and Sotherland & Rahn (1987) predicted yolk in such species approximated 20% of the total egg and its water content about 80%. Rahn et al. (1975) showed egg mass (M_e) as an allometric function of body mass (M_b);

$$M_e = 0.277 M_b^{0.77}$$

This would predict an egg of about 12.5% of the body weight of a Zebra Finch. There is, of course, substantial variation around these averages but it provides an estimate to work with.

Laying a clutch of eggs is a demanding process for a female bird, particularly for those of small body mass (Blem, 1990). While there has been much work on the energetics of egg production, relatively little attention has been payed to the nutrient aspects of reproduction in wild species. Robbins (1983) notes that while daily energy requirements for egg laying range from 29% of basal metabolic rate to 135%, the estimated daily protein requirement increases from 86% of the maintainance allowance to 230% for corresponding groups of birds. On this basis he questions the almost exclusive emphasis of many avian biologists on

understanding the energy parameters. Therefore, in this chapter I present data based on the analysis of the egg constituents of the clutch, and use this to consider the nutritional investment in egg production, in total and on a daily basis.

2.2 MATERIALS AND METHODS

2.2.1 Analysis of eggs

Pairs of Zebra Finches were introduced and allowed to breed. The nest-boxes were monitored daily between 0900 and 1000. Any eggs that had been laid were removed and replaced with a plaster dummy. The eggs were small and required delicate handling to prevent breakage. In most other studies, the eggs were boiled in order to harden the contents so that yolk and albumen may be easily separated (Birkhead & Nettleship, 1984, Schifferli, 1976). Initial trials using this technique with Zebra Finch eggs had a very high breakage rate while the samples were boiling, leading to a loss of albumen material from the egg. An alternative method, therefore, was developed that caused little breakage with the delicate finch eggs.

Each egg had a small hole made at the airspace and was then placed in a 100°C oven for one hour. The hole in the airspace allowed the expanding gas to escape thus preventing the egg from bursting. This process hardened the egg contents to facilitate the easy separation of the shell, yolk and albumen. An incision was made with a scalpel blade along the long axis of the egg and the shell could be removed in two halves. The albumen was then removed from around the yolk, taking care not to mix the two. The shell, yolk and albumen were placed on pre-weighed, marked aluminium containers and then placed in an oven at 70°C. The samples were weighed to 0.0001g at intervals until constant weight was obtained. This was called the Dry Weight.

The lipid content of the yolk and albumen was obtained by extraction with chloroform in a Soxhlet extractor. The dried yolk and albumen was wrapped in 15cm diameter filter paper and the ends stapled shut. The packets were placed into a 70°C oven until constant weight was obtained and then placed in the Soxhlet extractor with chloroform for 8 hours. After this extraction the packets were again dried at 70°C to constant weight. The lipid content of the sample was equivalent to the difference in the dry weights of the packets before and after extraction.

Therefore, for each egg, values for the Dry Weight of shell, yolk and albumen and also the Lipid Weight and Lean Dry Weight (LDW) of the yolk and albumen were obtained. Of the lean dry weight of eggs 95% is estimated to be protein and only 5% is carbohydrate (Sotherland & Rahn, 1987, Romanoff & Romanoff, 1949) and I have assumed these values apply to Zebra Finch eggs.

2.2.2 Amino acid analysis of eggs

Four eggs were obtained for amino acid analysis. The eggs were heated at 100°C for an hour to harden the contents. The yolk and the albumen were then separated and freeze dried to constant weight. The dried albumen was powdered using a mortar and pestle. Lipid was extracted from the yolk using chloroform solvent in a Soxhlet apperatus by the same technique as before. The lipid free yolk was then powdered using a mortar and pestle. Two eggs were analysed by Dr. I. D. Hamilton and Mr. J. Jardine at the Biochemistry Department, Glasgow University and two eggs were analysed by Dr. J. McNab at The Institute of Grassland and Animal Production, Roslin. The analyses were performed using the same technique and thus the results were pooled.

2.2.3 Calcium content of shell

The shells from three four-egg clutches were placed in pre-weighed and dried crucibles and ashed at 650°C in a muffle furnace for 21 hours. The crucibles were removed from the furnace and allowed to cool in a desiccator before weighing to 0.0001g on a Precisa A80-200 electronic balance. The ash was powdered and a sample of 0.0200g taken for analysis by atomic absorption spectrophotometry. 20mg of ash was dissolved in 4ml of 8N hydrochloric acid, to which 2ml of 2N nitric acid was added. Deionised water was used to wash the solution through filter paper into a 50ml measuring cylinder. The solution was then made up to 40ml with deionised water. This "stock" solution and was then stored in polythene bottles until analysis.

Standards were made up using BDH Spectrasol calcium nitrate solution to give a range from 0 to 5ppm. In order to bring the "stock" solutions within this range a further 1:50 dilution was required with deionised water to which lanthanum chloride was added to comprise 0.2% of the solution. The lanthanum is necessary to relieve the suppression of Ca

emission that is caused by certain non-dissociable salts, particularly phosphate. The lanthanum preferentially binds these leaving the Ca free (Wilson & Goulding, 1986). Therefore, the final dilution of 1:50 was made by taking 0.5ml of "stock", adding 0.05ml of La solution and making it up to 25ml with deionised water. This was done with two aliquots of the "stock" solution and each was measured in duplicate. All measurements were taken on a Phillips PU9200 Atomic Absorption Spectrophotometer.

2.2.4 Ovary and oviduct development

During the study, birds were taken at various stages of the laying cycle to allow analysis of the carcass. The ovary and oviducts of all of these birds were removed for anlaysis (see Dissection Procedure, Chapter 4).

When the ovary was in situ, the size of any developing follicles was measured to the nearest 0.05mm using calipers. The larger follicles can vary in shape considerably. Therefore, three different measurements were recorded for each and the mean taken as the diameter. Post-ovulatory follicles were noted if they were present. The ovary was then removed taking care not to include surrounding tissue (see Chapter 4).

Depending on the experiment that the bird was taken for, some of the ovaries were kept in 70% alcohol and some were frozen. The frozen ovaries were later oven dried at 70°C to constant weight and measured to 0.0001g. Neutral lipid was then extracted by chloroform in a Soxhlet extractor and the lean dry weight and lipid weight obtained.

The ovaries in alcohol were examined again under a x10 dissecting microscope to ensure that the presence or absence of post-ovulatory follicles had been correctly identified when the dissection was performed. Using the same technique as the frozen samples, a dry weight and lean dry weight was obtained. However, lipid values from these samples were not used as some lipid was lost from the follicles during storage in alcohol.

The oviducts were removed from the females and were treated in the same manner as the ovaries. A dry weight, lean dry weight and lipid weight were obtained as above. Whenever an oviduct contained an egg, the egg was removed before the oviduct was analysed.

2.3 RESULTS

2.3.1 Analysis of eggs

Unless otherwise stated, all means presented hereafter are given with standard deviation. 21 clutches of eggs were analysed giving a total of 105 eggs. Their mean clutch size was 5.0 ± 0.9, n = 21. The mean clutch size recorded for all females during the study from November 1987 until July 1990 was 5.2 ± 0.9, n = 120. The mean egg fresh weight was 1.098 ± 0.092g, n = 36, the mean length 15.4 ± 1.1mm, n = 36 and the mean breadth 11.0 ± 0.4mm, n = 36.

Table 2.1 presents the results for the analysis of the eggs. The albumen of Zebra Finch eggs contained little or no detectable lipid using the method employed. The lipid content of the yolk represents 53.3% of the yolk dry weight. Estimated mean carbohydrate and protein content is 4.2mg and 79.4mg in the albumen and 2.6mg and 48.6mg in the yolk (Sotherland and Rahn, 1987).

In some bird species there is a decline in egg size during the laying of the clutch, for example the coot (Alisaukas & Ankney, 1985). Analysis by Oneway Anova showed that there were no differences in composition between the eggs of a clutch, except for shell weight (Table 2.2). I also tested for within-clutch differences in four-egg and five-egg clutches separately, by repeated measures ANOVA, because if only the last eggs to be laid were lighter then this effect could be masked when considering different clutch sizes together. However, when analysed separately there were no significant differences in clutch composition, except for shell weight in four-egg clutches (Table 2.3). A Tukey multiple range test on the four-egg clutches indicated that the shell of Egg 4 was significantly lighter than the shell of the first egg laid. The same was not true of five-egg clutches where there was no significant difference between the components of any of the eggs.

2.3.2 Calcium content of shell

The mean dry weight of 12 shells chosen at random for this analysis was 52.5 ± 5.8mg, this being not significantly different from the 54.1 ± 8.6mg mean dry weight of all the eggshells

given in Table 2.1 ($t_{110} = 1.145$, $p > 0.05$). The results of this analysis are shown in Table 2.4. The mean value for calcium content of Zebra Finch eggs in this study was 17.8 ± 2.8mg of calcium.

With the mean clutch size for the study colony being 5.2 eggs, 89.0mg of calcium is required for the total clutch. Each egg is in the oviduct for one day only, and part of that time is required to deposit the albumen. Therefore, the calcium must be laid down in less than 24 hours.

2.3.3 Amino acid analysis of eggs

Table 2.5 shows the results of the amino acid analysis on four whole eggs with the amino acid composition of the domestic hen for comparison. These are presented as percentage of total amino acid content. While 11 of the amino acids are present in similar proportions differences can be seen in 7. The most marked differences are seen in alanine and isoleucine.

2.3.4 Ovarian follicle growth

Of all the females that were taken at the time of laying, 13 were found to be at the stage where the first yolk of a clutch had been ovulated (called here, Day 0). These birds were found to have an egg in the oviduct and only one post-ovulatory follicle. The mean diameter of the oviduct egg yolk and the remaining enlarging follicles on the ovary is shown in Figure 2.1. Zebra Finches lay one egg per day and so the measurements are indicative of daily growth increments, i.e. the largest follicle represented the size of a follicle on Day -1, the next largest Day -2 and so on. Developing follicles prior to Day -4 were difficult to distinguish from undeveloped follicles which had a mean diameter of 1.3 ± 0.1mm, n=237. I defined a follicle as enlarging if it had a diameter of at least 1.85mm and was yellow and not white.

This follicle growth curve allowed all pre-breeding females to be assigned to the correct period in their laying cycle by measuring the largest follicle and checking for the absence of post-ovulatory follicles. The stage in the laying cycle reached by birds that were laying, or had laid, was determined by; the number of eggs they had laid, the number and size of

the ovarian follicles and a count of post-ovulatory follicles to check whether an additional egg might have been laid but then had been broken and eaten.

The Mean follicle diameters were converted to volume ($4/3\ \pi r^3$). This assumes that the follicles were exactly spherical, which they were not, but the method gave a reasonable estimate. Figure 2.2a shows the increasing volume of a single follicle. Figure 2.2b shows the combined volume of ovarian follicles during the production of an average 5 egg clutch and illustrates the time scale of nutrient demand for yolk formation. Demand increases rapidly from Day -4 to Day 0 where it peaks, then declines less rapidly to Day 4 when the last follicle is ovulated.

2.3.5 Lipid and protein content of ovary

The ovaries that had been stored in alcohol could not be expected to give reliable measurements of lipid content and thus only frozen tissue was analysed.

Figure 2.3 shows the weight of lipid and the lean dry weight (protein) of the ovary across the laying cycle for females producing a five egg clutch. Up to Day 0 the ovaries were examined to determine the potential clutch size the birds would have gone on to lay. It was difficult in the early stage of growth to be certain of the exact clutch size that these birds would potentially lay. However, all the birds from Day 0 on were those that had laid five eggs or, due to the number of eggs laid and those left on the ovary, would lay five eggs. Unfortunately, there was no data for Day 2 and the value presented is an estimate calculated from the known average volume of follicles and the average contents of yolks of given volume (see below). Day 3 only had one bird and therefore error bars are not shown.

The mean lean dry weight of ovaries from birds that were not in breeding condition was 5.97 ± 3.6mg, $n = 11$. There was no detectable lipid in the ovaries at this stage. From Figure 2.3 it can be seen that lipid is not detected until Day -2. Thereafter it increases rapidly and then declines until the last yolk is ovulated on Day 4. Beyond this day there is no lipid present and the lean dry weight has returned to a similar value as in non- breeding birds.

As the tissue of the ovary itself does not increase in weight virtually all of the increase in both lipid and protein that occurs in the ovary during egg formation is due to deposition in the developing follicles. Also, from the dissections of post-laying females it was evident that surplus follicles were not enlarged and subsequently resorbed. An undeveloped ovary does not contain any measurable lipid and thus the change of a follicle's colour from white to yellow is a good indication that it is starting to develop.

2.3.6 Lipid and protein content of the oviduct

As with the ovary, only the frozen samples were used for this analysis. Figure 2.4 shows the mean oviduct lean dry weight through the egg laying period of birds laying a five-egg clutch. The data presented are for the 13 birds which contained an egg in their oviduct and those that laid, or were going to lay, a five-egg clutch. Data for Day 2 of the laying cycle was not available, Day 1 and 3 are based only on one bird each. The analysis did not reveal any detectable lipid in the oviduct at any time.

Again it can be seen that the increase in the oviduct's mass is significant and very rapid, resulting in a ten fold increase in just 3 days. The oviduct grows until Day -1 when it is ready to receive the first egg. After Day 0 the oviduct begins to regress. The consequence of this is that protein from the oviduct is available for other uses from this day on. This may be of some importance to egg formation. Assuming a linear decline in the tissue of the oviduct from Day 0 to Day 5 on which the last egg was laid, a minimum of 14.12mg of protein per day could be made available.

2.3.7 Nutritional investment in a five-egg clutch

From the above data it was possible to estimate the investment of lipid, protein and calcium on each day of the cycle for a five-egg clutch including the growth of the oviduct. The investment required for the clutch is less than the full cost as I am not considering factors such as the energetic cost of egg synthesis here. Rather I am illustrating the difference in nutrients required between each day and the next of the laying cycle.

Figure 2.5a shows the daily investment of protein required for a clutch of five eggs and the growth of the oviduct. The amount of protein needed for the yolks increases each day to

reach a peak on Day 0. This is the day on which most growing follicles are present on the ovary. Beyond this day the amount of yolk protein needed daily declines as each follicle reaches maximum size and is ovulated. The pattern for albumen protein investment is very different. The eggs are laid daily and only one egg is in the oviduct at any one time, thus a similar amount of albumen protein is required each day from Day 1 until the clutch is complete. The oviduct grows rapidly from its resting state to a size that is capable of accepting the first ovum. Most of the growth appears to occur between Days -2 and -1 and then growth stops. This leads to a slight discontinuity on Day 0 as no further growth of the oviduct occurs yet albumen protein is not required until after the first egg is ovulated. From this point on the oviduct is actually decreasing in protein content (see Figure 2.4) and this could be providing protein for the developing eggs. The significance of this will be discussed later in Chapter 4.

Figure 2.5b illustrates the daily investment of lipid, which is required by the yolk only. The pattern is, therefore, similar to that for protein with a steady increase in demand for lipid as the follicles grow followed by a steady decline as each is ovulated. The peak of demand is reached on Day 0.

As mentioned above the demand for calcium is a steady 17.8mg (Table 2.4) per day from Day 1 to Day 5.

TABLE 2.1

Dry weight (mg) of eggs and egg components including yolk lipid and yolk lean dry weight for each egg in a clutch (± s.d.).

Egg	Total	Shell	Albumen	Yolk	Yolk - lipid	Yolk - LDW	n
1	249.6 (28.0)	58.7 (6.0)	82.9(8.8)	107.7 (17.3)	56.8 (15.5)	50.9 (7.7)	21
2	247.4 (23.2)	55.9 (7.2)	83.9 (10.1)	107.7 (12.5)	54.2 (15.7)	53.5 (12.2)	21
3	250.0 (21.0)	53.6 (7.3)	84.8 (8.3)	111.6 (12.2)	59.4 (11.2)	52.1 (11.1)	21
4	246.8 (31.1)	50.4 (10.5)	85.5 (10.3)	110.9 (18.6)	61.8 (13.4)	49.2 (12.1)	21
5	246.1 (20.2)	53.1 (6.3)	83.3 (6.9)	109.1 (17.2)	58.1 (16.6)	51.0 (10.8)	14
6	241.6 (18.8)	52.1 (12.3)	80.0 (14.6)	109.5 (14.1)	61.6 (16.3)	47.9 (7.1)	5
Pop. mean	**247.3 (25.3)**	**54.1 (8.6)**	**83.6 (10.2)**	**109.4 (15.1)**	**58.3 (14.3)**	**51.2 (8.6)**	105

TABLE 2.2

Results of a Oneway ANOVA on the composition of eggs for 21 clutches.

Variable, Eggs 1-6	Value of $F_{5,97}$	Significance
Yolk Dry Weight	0.222	ns
Albumen Dry Weight	0.391	ns
Shell Dry Weight	2.520	*
Total Egg Dry Weight	0.135	ns
Yolk Lipid Weight	0.693	ns
Yolk Lean Dry Weight	0.465	ns

ns = Not Significant, $p>0.05$
* = Significant, $p<0.05$

TABLE 2.3

Result of repeated measures ANOVA on the composition of eggs in four- and five-egg clutches.

Four-egg Clutches, n=7;

Variable, Eggs 1-4	**Value of $F_{3,18}$**	**Significance of F**
Dry Weight of Yolk	0.812	ns
Dry Weight of Albumen	0.0.624	ns
Dry Weight of Shell	5.140	*
Yolk Lipid Weight	1.217	ns
Yolk Lean Dry Weight	0.504	ns

Five-egg Clutches, n=9;

Variable, Eggs 1-5	**Value of $F_{4,32}$**	**Significance of F**
Dry Weight of Yolk	0.930	ns
Dry Weight of Albumen	1.728	ns
Dry Weight of Shell	2.564	ns
Yolk Lipid Weight	2.457	ns
Yolk Lean Dry Weight	0.235	ns

ns = Not Significant, $p>0.05$
* = Significant, $p<0.05$

TABLE 2.4

Calcium content of Zebra Finch Egg Shells

Eggs	Dry Weight (mg)	Calcium in Shell (mg)
1	55.1	21.8
2	54.5	18.0
3	53.4	18.9
4	41.3	11.9
5	59.1	15.4
6	53.7	18.7
7	53.1	18.2
8	45.1	14.8
9	57.6	16.6
10	58.8	22.0
11	50.3	18.3
12	44.8	18.6
Mean (±s.d.)	**52.2 (5.8)**	**17.8 (2.8)**

TABLE 2.5

Amino acid content of Zebra Finch eggs compared to hen eggs.

(Individual amino acids shown as mean percentage of total amino acid ± s.d., n=4)

Amino Acid	Zebra Finch	Hen (whole egg)*
Alanine	7.6 (1.2)	3.1
Argenine	5.8 (1.1)	6.4
Aspartic Acid	11.1 (0.5)	7.8
Cystine	2.6 (0.1)	2.2
Glutamine	13.6 (2.4)	13.3
Glycine	2.6 (0.0)	4.6
Histidine	4.1 (0.1)	2.6
Isoleucine	2.9 (0.1)	6.6
Leucine	7.6 (1.2)	9.2
Lysine	6.1 (0.1)	6.8
Methionine	2.7 (0.1)	3.6
Phenylalanine	3.7 (1.1)	5.5
Proline	4.7 (0.2)	4.6
Serine	9.4 (2.3)	6.0
Threonine	5.3 (0.1)	5.5
Tryptophan	trace	1.4
Tyrosine	3.7 (0.0)	3.3
Valine	6.4 (0.2)	7.2

* Hen egg data from Harvey, 1970.

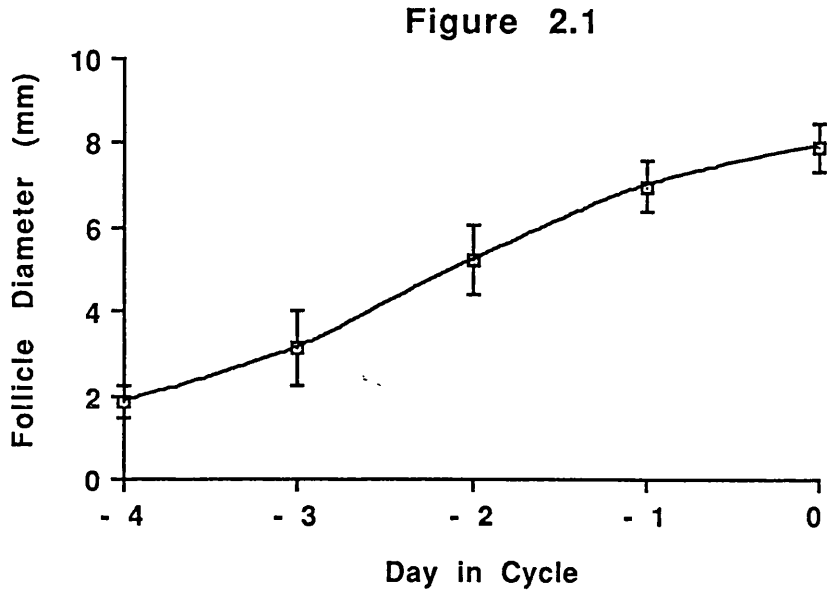


Figure 2.1

Diameter of ovarian follicles (mm) from Day -4 of the laying cycle to Day 0 (when the first egg of ovulated) of 13 females that had an oviduct egg and only one post-ovulatory follicle (Mean ± s.d.)

Figure 2.2a

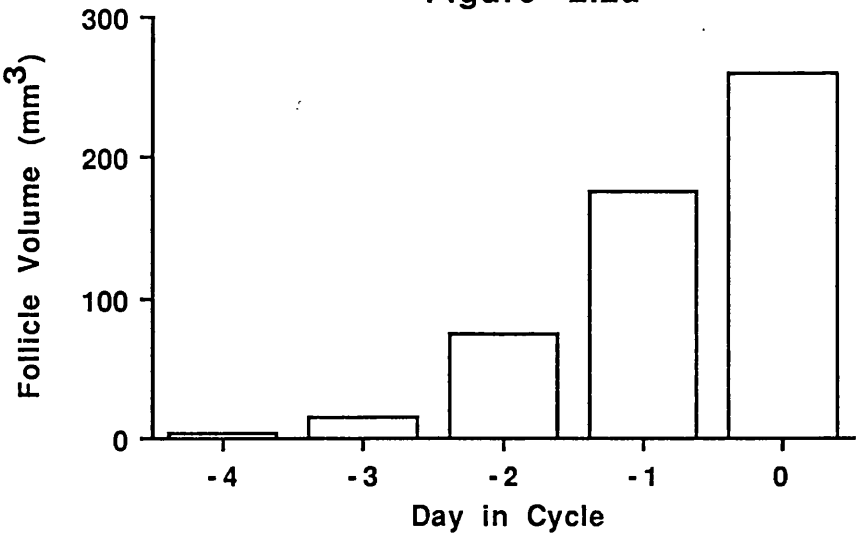


Figure 2.2b

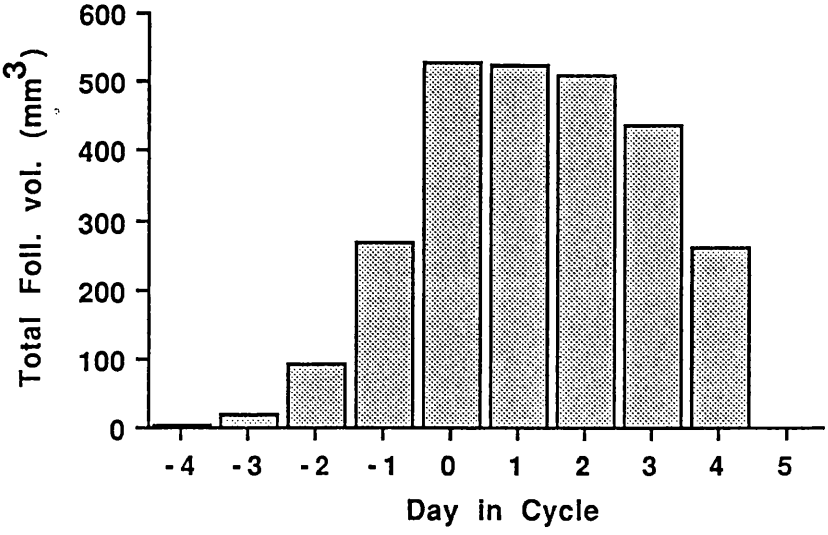


Figure 2.2

a) Volume (mm^3) of a single ovarian follicle from Day -4 to Day 0 calculated from the mean follicle diameters Figure 2.1

b) Total volume (mm^3) of ovarian follicles for the formation of a five-egg clutch

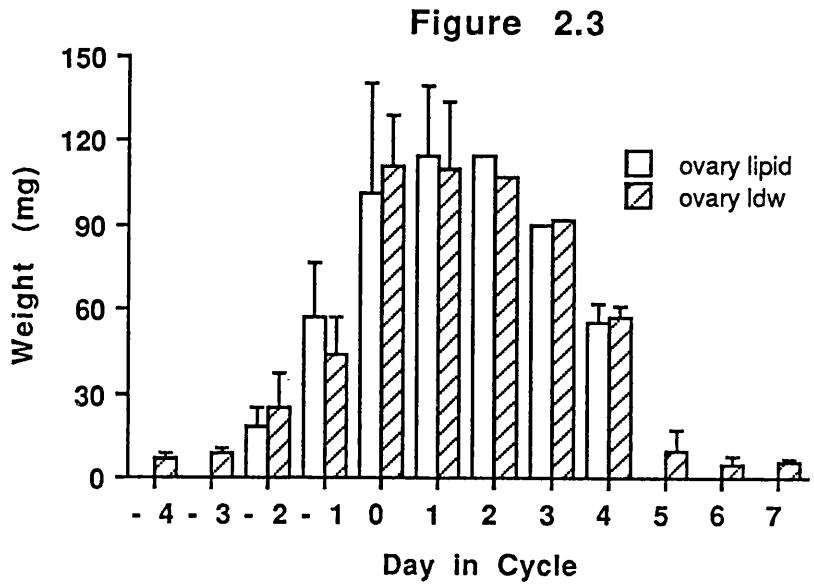


Figure 2.3

Lipid and Lean Dry Weight (protein) content (mg) of the ovary during the cycle of birds laying, or likely to lay, a five-egg clutch (mean ± s.d.)

Day	-4	-3	-2	-1	0	1	2	3	4	5	6	7
n	4	6	9	6	10	3	-	1	4	5	4	4

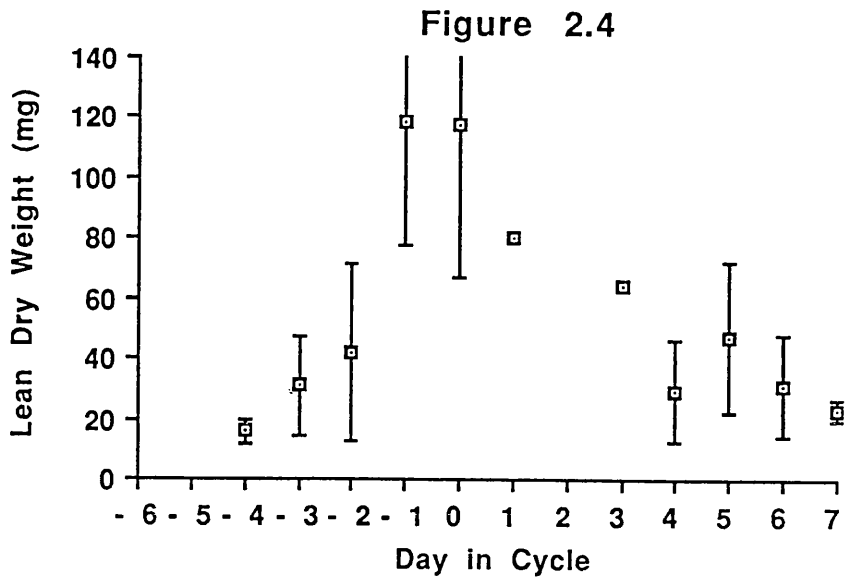
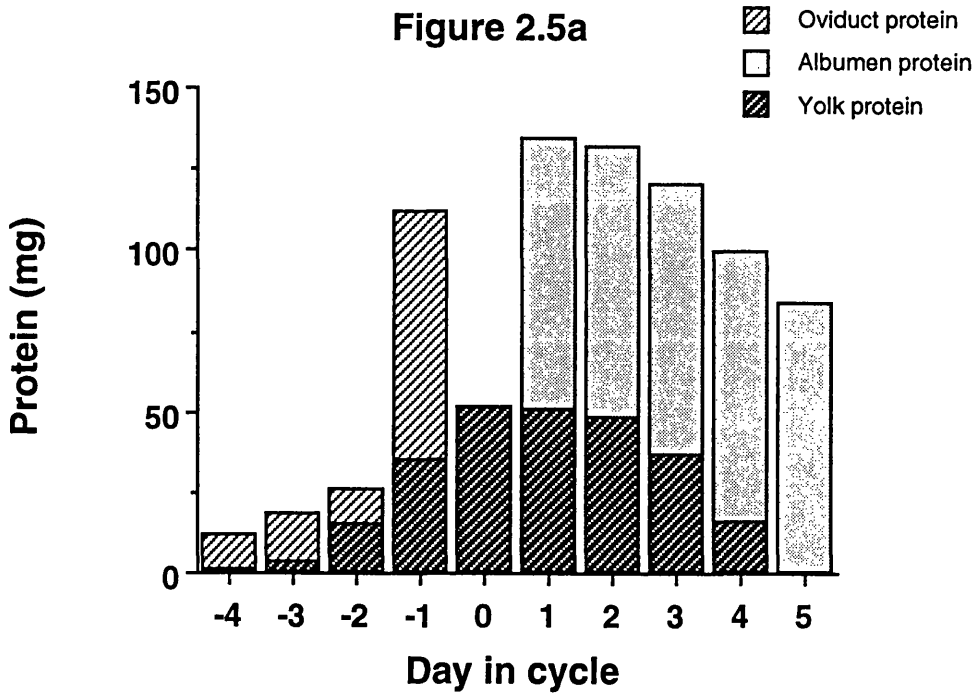


Figure 2.4

Lean Dry Weight (mg) of the oviduct during the laying cycle of birds laying, or likely to lay, a five-egg clutch (mean ± s.d.)

Day	-4	-3	-2	-1	0	1	2	3	4	5	6	7
n	4	6	9	6	10	3	-	1	4	5	4	4



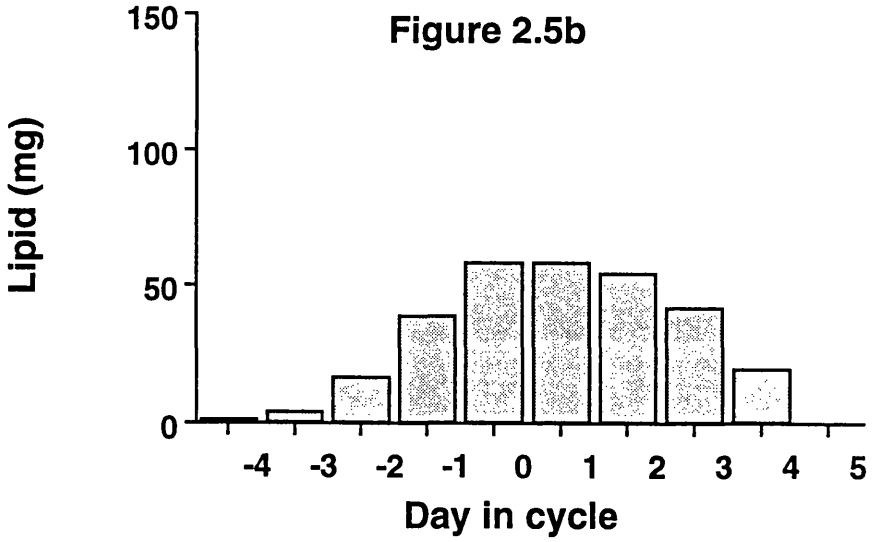


Figure 2.5

a) Quantity of protein (mg) required on each day of the laying cycle for the production of a five-egg clutch, including oviduct growth

b) Quantity of lipid (mg) required on each day of the laying cycle for the production of a five-egg clutch

2.4 DISCUSSION

The purpose of this chapter is to establish what investment the female Zebra Finch makes in her clutch. To do this the required parameters were investigated; clutch size, contents of the eggs, laying interval and the duration of the rapid growth phase of ovarian follicles (Walsberg, 1983). With these it is possible to determine the extent and the timing of the investment.

2.4.1 Analysis of eggs

The analysis of 105 eggs revealed that the eggs laid by female Zebra Finches in this study were not unusual for small passerines. Rahn et al (1975) estimated an egg of approximately 12.5% of body weight for altricial passerines. In fact the mean egg weight in this study was 1.098g which is 7.2% of the mean female body weight and the Zebra Finch egg is, therefore, representative in size for an altricial species. In addition, the predicted water content was 80% (Sotherland and Rahn, 1987) and the actual value was 77.5%. The mean egg weight of 1.098g is slightly heavier than those in the study of El-Wailly (1966) who recorded 0.98g as a mean egg weight. The clutch size in the same study was slightly less also, being 3.9 eggs compared to 5.0 eggs in this analysis. A possible reason for this difference is that the diet provided by El-Wailly (1966) was not seed but a powdered poultry pellet feed. Another possible cause may have been that the females in this study were considerably heavier, at 15.25 ± 0.19g (n = 110) compared to 12.8g (El-Wailly, 1966).

Analysis of the eggs by sequence of laying (Table 2.1) did not reveal any significant change in egg weight with sequence. This is not unusual, the Pied Flycatcher, which is of comparible size to a Zebra Finch, does not display any decline of egg size in its mean 6.3 egg clutch (Ojanen, 1983).

A decline in egg mass with laying sequence has been recorded for some species, e.g. Coot (Alisaukas and Ankney, 1985), Herring Gull (Parsons,1976). Krementz (1984) noted a trend towards decreasing egg size with sequence in the House Sparrow. In some other species of passerine egg mass tends to increase with laying sequence, for instance the Bengalese Finch (Coleman & Whittal, 1990) and the Tree Swallow (Wiggins, 1990), which

may be a strategy to counter differences in the size of nestlings following asynchronous hatching.

It was found that for Zebra Finches that laid a four egg clutch, the shell of the last egg was significantly lighter than the shell of the first egg (Table 2.3). This did not occur in larger clutches and suggests that in birds laying smaller than average clutches, the ability to provide calcium for the eggshell might be limited for the last eggs.

The lipid content of the eggs was 58.3mg, protein content was 128.1mg, carbohydrate content (estimated) 6.8mg and calcium content 17.8mg.

The carbohydrate content of eggs is generally ignored by authors due to its relative insignificance (2.7% of dry egg weight)(Alisaukas and Ankney, 1985, Houston et al, 1983, Krementz, 1984, Murphy, 1986, Schifferli, 1976). Of the material left after the extraction of lipid, Lean Dry Weight, 95% of this is estimated to be protein and only 5% carbohydrate (Sotherland & Rahn, 1987).

In the Zebra Finch egg lipid was confined to the yolk and none was measured in the albumen. Murphy (1986) and Schifferli (1976) found the same for the eggs of Eastern Kingbirds and House Sparrows respectively. The yolk was 56.6% of egg dry weight excluding the shell, and its lipid content was 30.2% of the dry weight of the egg excluding the shell. These values are typical of altricial species (Carey, 1983, Murphy, 1986).

2.4.2 Development of the Ovary

Investigation of the rate of follicular development revealed that the period of rapid growth in Zebra Finches lasts four days. No developing follicles were identifiable prior to Day -4 (Figure 2.1). The rapid growth phase has been cited for various species of passerines as between 2-3 and 5 days. For example the Pied Flycatcher is recorded at 4 days (Ojanen, 1983), House Sparrow, 4 days (Krementz, 1984, Schifferli, 1976). King (1973) points out that the maximum rate of investment by a female in her clutch is attained only when the clutch size is five eggs or more, the laying interval is daily and the yolk is deposited over

four days. The Zebra Finches in this study were fulfilling all of these criteria and therefore they would have reached the maximum rate of investment while producing their eggs.

I estimated that the ovary minus its developing follicles contained no lipid. The Pied Flycatcher is recorded as having very little lipid in the developing ovary, only 4.6% (Ojanen, 1983). However, this may have been an over-estimate because a petroleum ether-chloroform solvent mixture was used for lipid extraction. Dobush, et al.(1985) indicated that such a mixture will remove non-lipid material from tissue. This gives an artificially high measure of lipid content. Solvents used alone do not tend to do this. I assumed, therefore, that all of the lipid extracted from developing ovaries was from the follicles and not the tissue of the ovary itself.

2.4.3 Development of the oviduct

The oviduct increases in weight very rapidly (Figure 2.4) at the same time as the ovary. Lean dry weight undergoes a ten fold increase over just three days with the majority of growth during Day -2 to -1. Between Day -1 and 0 the weight does not change and the oviduct is ready to accept the first yolk. From this day on the oviduct loses weight. Although from Day 0 to Day 4 I have little data I think I am justified in assuming that the oviduct declines in a more or less constant manner (see below). This decrease in weight could potentially provide up to 14.12mg of protein per day, Day 0 to 5. I could detect no lipid in the oviduct and therefore I have assumed that the increase in lean dry weight is due to protein. Several species of bird display a similar pattern of oviduct growth during the pre-laying stage followed by a significant decline to the end of laying; House sparrow (Krementz, 1984, Schifferli, 1976), Brant (Ankney, 1984), Mallard Duck (Krapu, 1981), Canvasback Duck (Barzen and Serie, 1990) and the Pied Flycatcher (Ojanen, 1983).

It has been suggested that the oviduct may act as a storage organ for protein during reproduction (Krementz and Ankney, 1986). The possible consequence of this will be discussed later.

2.4.4 Nutritional investment in a five-egg clutch

Having investigated the criteria that are of importance to nutrient demand during egg production (egg contents, clutch size, laying interval, and the length of the rapid growth phase of follicular development) it was possible to construct a daily budget for protein, lipid (Figure 2.5a and b) and calcium. The results reveal that when laying a five-egg clutch the female experiences a rapid increase in demand for protein and lipid as the follicles on the ovary grow and the oviduct increases in size. This gives maximal demands for lipid on Day 0 and protein on Day 1. Thereafter the demands drop off each day as the eggs are laid. This nutrient budget is directly comparible with estimates of energy investment required for egg production in other passerines; House Sparrow (Krementz and Ankney, 1986), Pied Flycatcher (Ojanen, 1983).

This nutrient budget, in relation to food intake and nutrient reserve dynamics will be discussed more fully later.

CHAPTER 3 - LIPID, PROTEIN AND CALCIUM CONSUMPTION OF ZEBRA FINCHES DURING BREEDING

3.1 INTRODUCTION

Egg production is a major nutritional investment. In terms of energy the estimated peak daily cost of egg production ranges from 37-55% of basal metabolism (BM) in some passerine species to 160-216% of BM in ducks and the Brown Kiwi (Walsberg, 1983). The manner in which nutrients are acquired for ovogenesis is not fully understood for most wild birds. There are three possible routes; an increase in dietary intake, the use of internal stores or reducing allocations to other activities. For no species has the relative importance of all three of these avenues been determined (Walsberg, 1983).

There are extensive examples of each of these three potential routes to be found in previous studies of birds. An increase of 175% in the time spent foraging by the laying female compared to the male during the same period has been described for the Mallard Duck (Dwyer et al, 1979). In White-crowned Sparrows the difference was 11% (Hubbard, 1978). As mentioned above it is to be expected that an Anseriform would require a greater increase than a Passerine because of the difference in relative energetic costs of egg production between the two groups.

There are a number of studies that have investigated the role of energy/nutrient stores in ovogenesis. Laying female Wood Ducks lose body lipid equivalent to 88% of the energetic requirements of egg production (Drobney, 1980) and female Mallards loose up to 25% of their body weight in lipid during the pre-laying and laying period (Krapu, 1981). Body reserves of protein may be the limiting factor in egg production in Canada Geese as they are depleted by roughly the same amount as is required for the eggs (Raveling, 1979). A similar role for nutrient reserves in clutch formation is also suggested for the Lesser Snow Goose (Ankney & MacInnes, 1978).

Finally, reduction in the locomoter activity of the female at the time of egg formation has also been documented. The female Willow Flycatcher allocates 13% less energy to activity

during the laying period than does the male (Ettinger & King, 1980). This reduction appears to compensate for synthetic costs with the result that the female's daily expenditure averages 5% less during the laying period than does the male's.

The aim of this chapter is to investigate the daily intake of female Zebra Finches during egg production compared to non-breeding females. Having done this the nutritional content of the diet will be measured so that a budget of nutrient intake can be constructed for each day of the laying cycle. Budgeting of nutrients during egg production from food eaten (exogenous nutrients) will be discussed in relation to nutrients derived from body reserves (endogenous nutrients) in Chapter 4.

In the wild, Zebra Finches inhabit a wide variety of habitats throughout mainland Australia, from arid regions to farmland. The feeding ecology of wild populations has been studied by Serventy (1971), Davies (1977), Morton and Davies (1983) and Zann and Straw (1984). Zebra finches are predominantly granivorous and there is little evidence of insectivory, even while breeding (Zann and Straw, 1984). The species is considered to be an opportunistic breeder, responding to a combination of rainfall and temperature to initiate breeding (Davies, 1977).

The Zebra Finch has also been widely used in the laboratory, partly due to its ability to breed throughout the year. The energetics of egg laying and incubation by captive Zebra Finches has been studied by El-Wailly (1966) and the energetics of incubation by Vleck (1981).

3.2 MATERIALS AND METHODS

A series of feeding trials were used to measure the food intake of Zebra Finches during the egg producing period. Initially I compared separate pairs of males and females to see if there were any major differences in the food intake of the two sexes. I then compared pairs of mixed sex, some of which bred and some of which did not.

The experiments were carried out in cages modified to allow the collection of uneaten seed and the temperature was maintained at 23°C ± 2°C. Efficiency of food utilisation is maximal at 24.4°C for Zebra Finches (El-Wailly, 1966).

3.2.1 Comparison of food intake by males and females.

This initial trial was designed to establish if there was any difference between the food intake of males and females. This was necessary because when looking at the intake of breeding birds it would not be possible to keep the sexes separate.

The normal cages were modified slightly to allow the collection of all uneaten seed. When finches eat seed, they manipulate it in their bill to remove the husk, and in the process some seed is dropped. It was important that all spilt seed and husk was collected as well as the uneaten seed in the dish so that consumption was not overestimated. This was achieved by the fitting of a layer of Benchcoate to the base of the cage from which all uneaten seed could be swept. The cages also had a sheet of clear perspex attached to the open front of the cage to prevent seed and husk being lost by that route.

The birds were provided with a single seed diet (panicum millet, *Panicum miliacum*) instead of their usual mixed seed diet. This was done to allow easier interpretation of the results by preventing preferential selection of seeds from a mixture, which is known to occur (Diaz, 1990). The change of diet to a single seed was not thought to be of great significance to the birds. In studies of wild finches it was found that they tend to specialise on one variety of seed and that this accounts for most of the seed eaten (Morton and Davies, 1983, Zann and Straw, 1984). In addition, the seeds eaten in the wild are very similar to the millet supplied and in some cases are of the same genus, *Panicum*.

Three pairs of males and three pairs of females were used in this trial for 10 days. The birds were put into the cage and a known weight of seed provided daily. The seed, spilt seed and husk were weighed each day at the same time plus or minus one hour. The weight of the cuttlefish bone was also recorded daily.

3.2.2 Comparison of food intake by breeding and non-breeding pairs

The food consumption of breeding and non-breeding pairs was measured in a similar way, each pair being provided with a known weight of seed and cuttlefish bone each day. Because dried grass had to be supplied for nesting material, the spilt seed was collected by passing the contents of the cage base through a 5mm sieve to separate the seed from the nesting material. The weight of seed and cuttlefish bone consumed was measured each day. The nestbox was checked regularly for eggs. All eggs were immediately removed after laying and replaced with a plaster dummy. This was done because occasionally the birds will eat their eggs and this would have obviously influenced their food consumption. When eggs were eaten before the dummy was provided that trial was abandoned and the previous results ignored. The male and female were first kept in adjacent cages for seven days before being introduced. Food consumption was monitored from the day of introduction. Those pairs that produced a clutch were followed until seven days after the last egg was laid. Those that did not breed were followed for ten days and used as a control.

3.2.3 Nutritional value of seed and cuttlefish bone

Lipid, protein and calcium levels were measured in the panicum millet and lipid and protein only in the foreign finch mix. All analysis was done on dehusked seed because the finches remove the husk from the seed before ingesting it. This was done by lightly grinding the seed in a pestle and mortar and blowing off the husk.

3.2.3.1 Lipid content of seed

Lipid content of the seed was obtained by soxhlet extraction of the dried sample using chloroform, following the technique described for eggs in Chapter 2.

3.2.3.2 Protein content of seed

Dehusked panicum millet and mixed seed were dried at 50°C to constant weight and then powdered with a pestle and mortar. 1.0g aliquots were digested in 10.0ml of 0.3M sodium hydroxide (NaOH) at 37°C until the powder had completely dissolved. For each sample two 1.0ml aliquots of digest were taken and a dilution of 1:10 made with 0.3M NaOH.

The protein content of each sample was measured using a modification of the Lowry protein estimation (Lowry et al, 1957).

Reagents

Solution A - 2% $NaCO_3$ in 0.1M NaOH

Solution B - 1% $CuSO_4$

Solution C - 2% K.Na.tartrate

Solution D - 100ml of A + 1ml of B + 1ml of C (in that order).

Folin's reagent.

Technique

1 - 100 µl of sample added to 3ml of solution D, while mixing. Incubate at room temperature for 10 minutes.

2 - Add 0.3ml of half strength Folin's reagent while mixing. Incubate for 30minutes at room temperature.

3 - Read in a spectrophotometer at 750nm in a quartz cuvette.

Standards

Standards were made using bovine serum albumen (BSA). A stock solution of 2 mg/ml BSA was used to make standards of 0, 10, 20, 30 and 40 µg/100ml. Estimations of protein content in unknown samples were made by converting 750nm readings to µg protein /100µl using the equation derived from the standard curve (Figure 3.1).

$$\text{mg of protein / 100ml assay} = \frac{\text{Absorbancy at 750nm} - 0.002}{0.006}$$

3.2.3.3 Amino acid analysis of panicum millet

Amino acid analysis was carried out on the panicum millet only. Dehusked seed was freeze dried and powdered using a pestle and mortar. 1.00g samples of powdered seed were sent for analysis by Dr. I.D. Hamilton and Mr. J. Jardine of the Biochemistry Department, University of Glasgow and also by Dr. J. McNab at the Institute of Grassland and Animal Production, Roslin. The results obtained from both were very similar and so I have combined them.

3.2.3.4 Calcium content of panicum millet and cuttlefish bone

The technique used to determine calcium content was the same as that described in Chapter 2 for egg shells. The following slight differences were made in the preparation of the samples before the analysis.

Calcium content was measured only for the panicum millet. Six samples of seed were dehusked and then dried at 70°C to constant weight. They were then placed in pre-weighed crucibles and ashed at 650°C for 6 hours in a muffle furnace. The crucibles were allowed to cool in a desiccator before weighing to 0.0001g. The ash was then powdered and samples of 0.0500g taken for analysis. Each sample was prepared as follows;

1- The 50mg of ash was dissolved in 10ml of 8N hydrochloric acid.
2- 5ml of nitric acid was added.
3- Deionised water was used to wash the solution through filter paper into a 100ml volumetric flask.
4- The solution was made up to 100ml with deionised water. This was called the "stock" solution and was stored in polythene bottles until analysis.

Standards were prepared as in Chapter 2 and the same analysis procedure followed. The final solution for analysis was 0.04ml of Lanthanum chloride made up to 20ml with the "stock" solution. Two aliquots of "stock" from each seed sample were prepared and read in duplicate on the spectrophotometer.

Samples of cuttlefish bone were also analysed for their calcium content. 10 pieces of cuttlefish bone were selected at random. The samples were dried at 70°C to constant weight and then placed in pre-weighed crucibles and ashed at 650°C for 21 hours in a muffle furnace. A 0.0500g sample of ash was taken from each and they were prepared exactly as described for the seed. However, before the final reading, the "stock" solution required a 1:50 dilution and the addition of lanthanum chloride. 0.50ml of the stock was taken with 0.05ml of lanthanum chloride and made up to 25ml with deionised water. This was done twice with aliquots from the "stock" solution and each was read in duplicate on the spectrophotometer.

3.3 RESULTS

3.3.1 Comparison of food intake by males and females

Three pairs of males and three pairs of females were monitored for 10 days. Table 3.1 shows the results. Male birds consumed about 7.6% more seed each day than females ($t=2.26$, d.f.=58, $p=0.03$).

I then compared the individual pairs by one way ANOVA and a Tukey multiple range test. This identified one pair of males that had a significantly higher mean seed consumption than the other pairs. The other two male pairs were not significantly different to the female pairs. This means that the significant result in the t-test is caused by only one of the male pairs.

A study performed by an honours student, Rachel Fairly (pers. comm.), using the same procedure showed no difference between the seed consumption of male and female pairs ($t_{94}=0.182$, n.s.).

Therefore, in most cases there is no significant difference in the seed intake of male and female birds.

3.3.2 Comparison of food intake by breeding and non-breeding pairs

Figure 3.2 shows the combined means for 11 pairs that were introduced but did not produce a clutch. Oneway ANOVA detected no difference in the daily means for the eleven pairs, $F_{9,84}=1.310$, $p=0.244$, not significant.

The mean seed consumption for this trial was 5.8 ± 1.2g per pair per day, n=94. Assuming that both sexes consumed equal quantities, each bird ate 2.9g/day when not breeding.

Figure 3.3 shows the mean daily seed consumption for 9 breeding pairs. As the different pairs did not begin to lay on the same day, the results were compiled from the day that the first egg was laid. Generally, the birds laid about a week after introduction, therefore I could only plot pre-laying data back to Day -6 for all the pairs. The daily seed consumption

of breeding pairs does not show any marked declines or increases. Oneway ANOVA showed there to be no significant changes in daily seed consumption ($F_{17,129} = 4.739$, $p=0.998$). The mean daily seed consumption for all the breeding pairs was 5.7 ± 1.2g, n=147 which was 2.85g/bird/day, assuming equal feeding.

The mean clutch size for these birds was 4 ± 0.5 eggs, n=9, compared to the colony average of 5.2 ± 0.9 eggs, n=120. This may have been due to the slightly different cage conditions that were required and also to the increased disturbance that was unavoidable as a result of daily monitoring seed consumption. In addition, the feeding trials were conducted on a single seed diet whereas all other birds were being fed the foreign finch mix which contained slightly a higher amount of protein (see Table 3.2)

3.3.3 Loss of weight from the cuttlefish bone

Cuttlefish bone was the only supply of calcium rich material to which the birds had access. The weight loss from the bone cannot be taken as the exact amount of material the bird had ingested, because some of it was spilt as fine dust and it was impossible to collect this lost material. Figure 3.4 shows the weight loss from the cuttlefish bone given to non-breeding pairs which was always less than 0.05g per day.

Figure 3.5 shows the mean daily weight loss from cuttlefish bone given to the breeding pairs. From Day -2 of the laying cycle there is a dramatic increase in weight loss that continues until Day 4 (from less than 0.05g on Day -5 to over 0.20g on Day 4). This represents a significant (Oneway ANOVA $F_{17,129}=5.028$, $p < 0.001$) increase and this period of ingestion coincides with the period when shells are being deposited onto eggs in the oviduct (see Chapter 2).

3.3.4 Nutritional Value of Seed and Cuttlefish Bone

3.3.4.1 Lipid and protein content of seed

Lipid and protein content was determined for both panicum millet and the foreign finch mixture, but calcium content and amino acid composition was measured only in the panicum millet. Table 3.2 shows the results for both of the seed types. Analysis of the

results by t-test revealed significant differences ($p < 0.001$) in protein, lipid, ash and water content. Carbohydrate was not measured directly but by inference from the other results. Panicum millet contains less lipid, protein and ash than the mixed seed, but has more carbohydrate.

3.3.4.2 Amino acid analysis of panicum millet

Table 3.3 shows the results obtained for panicum millet, presented as percentage of total amino acid content. They are presented with published results from two other sources for comparison and indicate that the amino acid composition of the millet used in the study was not unusual.

3.3.4.3 Calcium content of panicum millet and cuttlefish bone

The values for the calcium content of seed and cuttlefish bone have been presented in relation to the wet weight of the sample because this was how food consumption was measured in the feeding trials.

Panicum millet:

Six samples of panicum millet ash were analysed for calcium content and found to contain 0.103 ± 0.007mg calcium per 0.05g sample, and since ash represents 1.2% of wet dehusked seed weight, 1g of dehusked seed contains about **0.025g** of calcium.

Cuttlefish Bone:

Ten samples of cuttlefish bone were analysed. The result was;

0.05g of ash = 27.85 ± 1.96mg Ca

Calcium as a percentage of cuttlefish bone wet weight = **29.39 ± 2.04%**

TABLE 3.1

Seed consumption of pairs of male and female Zebra Finches.

Sex	Weight of seed consumed per pair per day	St. Dev.	n
Male	7.78g	0.87	30
Female	7.23g	1.01	30

TABLE 3.2

Contents of mixed seed and panicum millet (mg/gramme wet weight of dehusked seed).

Sample	Lipid	Protein	Water	Ash	C/hydrate*
Mixed Seed n = 10	46.0 (2.9)	158.0 (9.8)	103.0 (8.7)	46.0 (2.5)	647.0
Panicum millet n = 10	32.0 (4.0)	129.0 (6.7)	94.0 (6.2)	12.0 (1.5)	733.0
Value of t	8.485	21.481	6.216	77.273	-
Significance	***	***	***	***	-

*Carbohydrate derived by subtracting total of other values from 1000.

*** $p > 0.001$, d.f. = 18

TABLE 3.3

Amino Acid content of panicum millet, expressed as percentage of total amino acid (mean ± s.d.)

Amino Acids	This Study, n=4	Ref.1	Ref.2
Alanine	11.8 (1.5)	-	9.6
Argenine	3.0 (0.4)	2.9	3.1
Aspartic Acid	7.3 (2.6)	-	5.8
Cystine	1.6 (0.1)	1.7	1.3
Glutamine	16.4 (3.1)	-	21.6
Glysine	3.7 (0.6)	2.2	2.1
Histidine	2.2 (0.4)	1.7	1.8
Isoleucine	4.6 (0.7)	3.8	3.4
Leucine	10.4 (1.1)	9.7	10.7
Lysine	2.0 (0.2)	1.9	1.5
Methionine	2.7 (0.2)	2.2	2.5
Phenylalanine	5.2 (1.1)	4.6	4.5
Proline	10.2 (2.7)	-	5.0
Serine	8.0 (1.5)	5.4	6.7
Threonine	4.2 (1.0)	3.2	2.7
Tyrosine	2.2 (0.2)	2.0	1.9
Valine	4.7 (1.2)	4.7	4.1

1-Tables of Food Composition (Utah), Inernational Feedstuffs Institute (1984).

2- Harvey, 1970.

N.B. Tryptophan not present.

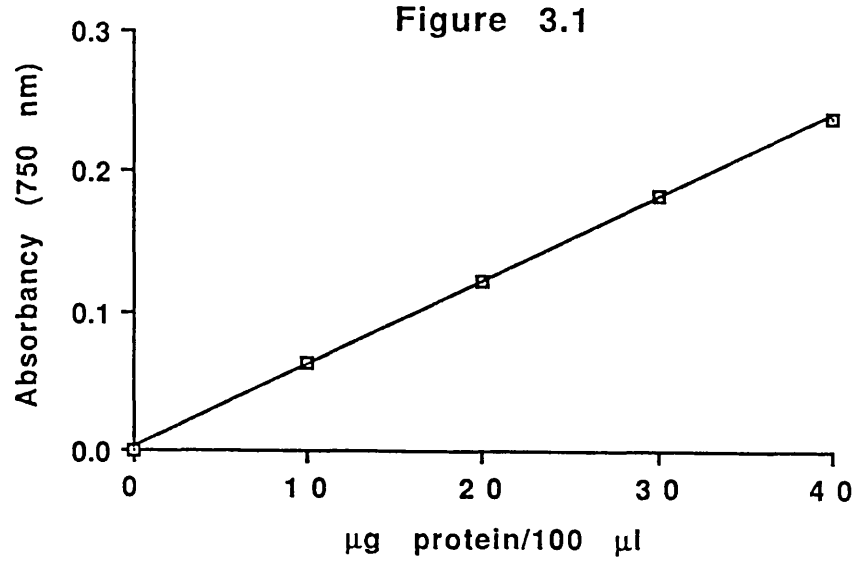


Figure 3.1

Standard curve for Lowry protein estimation obtained using Bovine serum Albumen

$y = 0.002 + 0.006x$, $R^2 = 1.000$

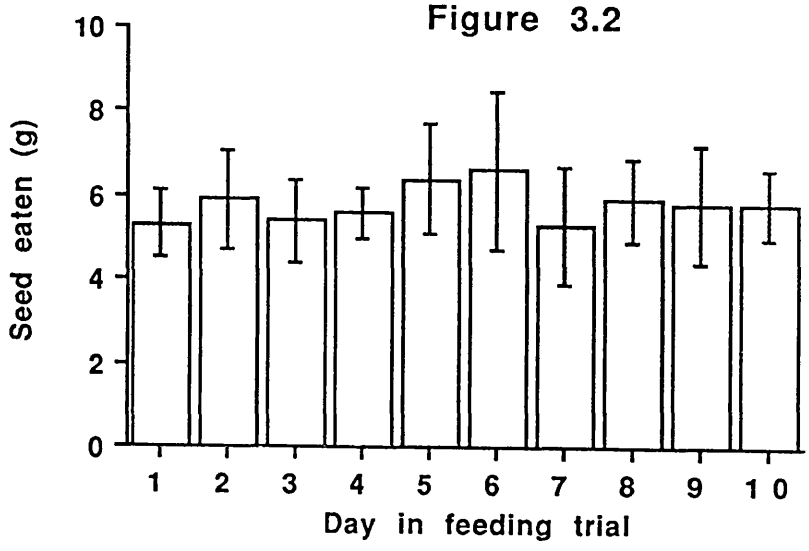


Figure 3.2

Daily seed consumption (g) of 11 non-breeding pairs of Zebra Finches during feeding trials (mean ± s.d.)

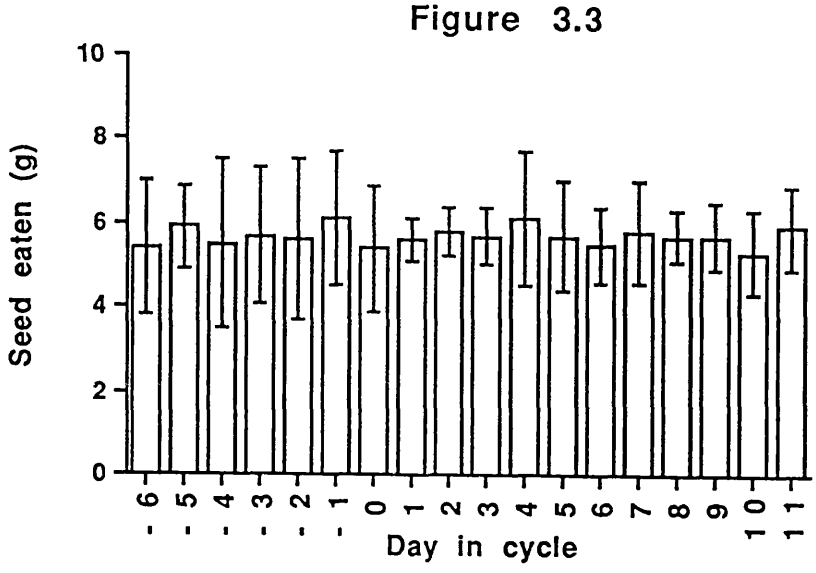


Figure 3.3

Daily seed consumption (g) of 9 pairs of breeding Zebra Finches during the laying cycle (mean ± s.d.)

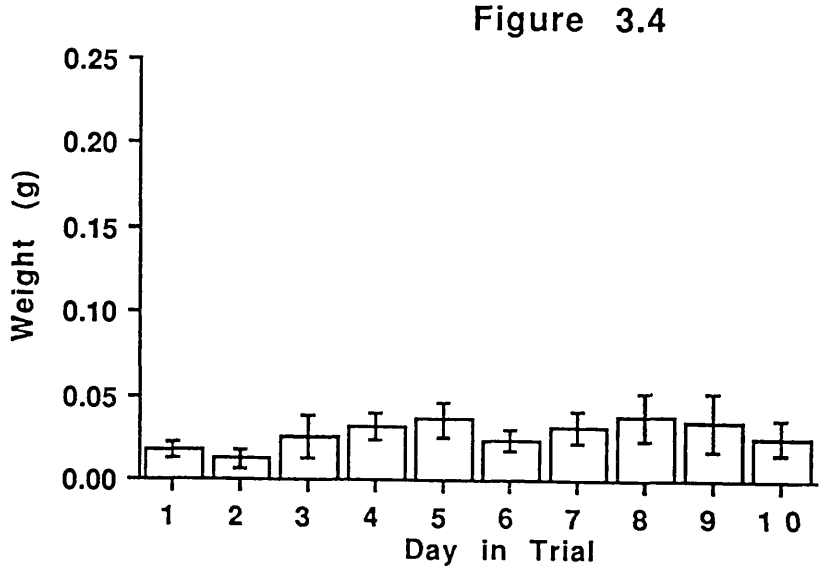


Figure 3.4

Daily loss of weight from cuttlefish bone (g) for 11 non-breeding pairs of Zebra Finches during feeding trials (mean ± s.d.)

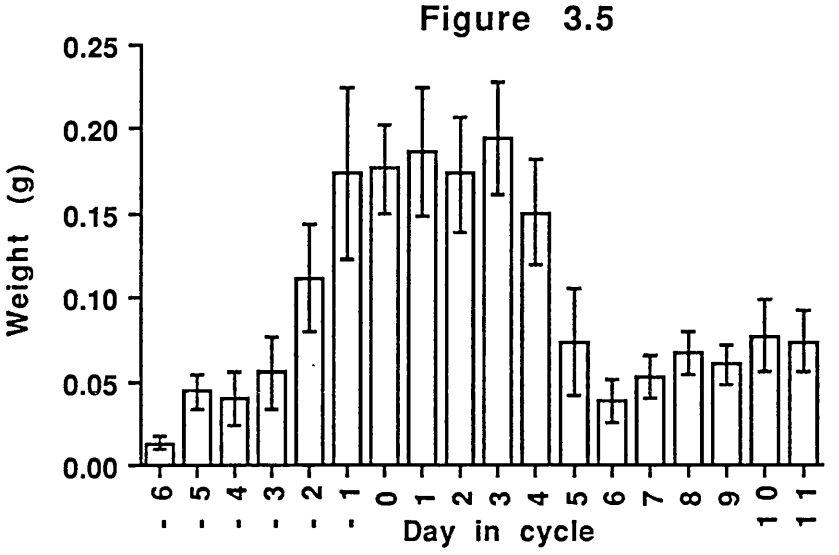


Figure 3.5

Daily loss of weight from cuttlefish bone (g) for 9 pairs of breeding Zebra Finches during the laying cycle (mean ± s.d.)

3.4 DISCUSSION

3.4.1 Food intake of males and females

The results of the feeding trial to compare the daily seed consumption of male and female Zebra Finches, showed no consistent significant difference between the sexes. El-Wailly (1966) also reported no difference in the consumption of food by the different sexes of Zebra Finch.

3.4.2 Food intake of breeding and non-breeding pairs

Comparing the consumption of breeding and non-breeding pairs did not reveal any significant increase or decrease during the period of egg formation (Figures 3.2 and 3.3). Mean non-breeding seed consumption was 5.8 ± 1.2g per day per pair, which is 2.9g per bird. For breeding pairs the value was 5.7 ± 1.2g day per pair, or 2.85g bird.

These values compare well with the 3.0g per day consumption recorded for captive Zebra Finches by Calder (1964). Wild Zebra Finches have an estimated seed consumption of 5.3g per day when non-breeding (Zann and Straw, 1984). Although this figure is probably an over-estimate it does reflect the difference in energy requirements of captive and free ranging birds.

Prior to migration the Yellow Wagtail doubles its body weight. Most of this increase is due to the deposition of fat but it is also partly due to hypertrophy of the flight muscles. During this build up of weight there is no evidence of hyperphagia and little of diet change (Fry et al, 1972). Therefore, the Zebra Finch is not exceptional in meeting a major nutritional demand without increasing food intake.

There are three possibilities that might explain why a female Zebra Finch can produce a clutch of eggs yet not increase her overall food intake;

1- The efficiency of digestion may be increased so that for a given quantity of food, more nutrients are extracted.

2- Activity may be reduced while the eggs are forming so that energy saved in this way may be diverted to the eggs.

3- The female may rely entirely on stored nutrient reserves to produce her clutch.

There is evidence to suggest that the first of these explanations may play some role. El-Wailly (1966) described an increase in food consumption during egg laying compared to a control period. In addition, the efficiency of food utilisation was significantly higher during the egg laying period than the control or for males and females that were separated. Control pairs were 77.8% efficient and nest-building/egglaying pairs 81.2% efficient (at 24°C). If the same were happening in my study then the finches could have liberated a small amount of additional nutrients for egg production without increasing seed consumption. The birds were receiving seed *ad libitum* and so there was no constraint on the amount of seed that could be eaten. Foraging time was not a factor as the birds did not need to forage as such and so would have been able to eat the same quantity of seed regardless of reproductive state.

The second possibility, that females decrease their activity while forming eggs has been discussed in previous studies. Fogden and Fogden (1979) failed to catch any female Camaropteras at the time when they would have had eggs at the early stages of development. They suggested that this may have been due to females remaining inactive in order to prevent damage to newly forming shells. However, Schifferli (1976) investigated this possibility in House Sparrows and found that eggs were not vulnerable to damage caused by activity even when the shells were very thin. Therefore, fear of damage to the eggs is unlikely to be the reason for reduced activity. The activity of breeding and non-breeding Zebra Finches was investigated by an undergraduate student, Michael Glover (pers. comm.), using the study colony. Electrically triggered perches recorded movements of the birds and provided an index of activity over set time periods. This technique found a significant difference in activity between breeding and control pairs. This difference was expected during the incubation period, from about Day 2 (Vleck, 1981). Interestingly, there was also substantially less activity recorded in breeding pairs during egg-laying; 4367 ± 173 perch movements compared to 10873 ± 703 perch movements of control pairs (t_{70}=

8.11, $p < 0.0001$). Therefore, a reduction of activity by the female, if that occurs, during the period when demands for egg production are high may be used to reduce overall energy expenditure and release nutrients for the eggs that would otherwise be used for routine metabolism.

The third possibility, that females do not rely on exogenous nutrients at all, but use endogenous, stored nutrients will be fully discussed in Chapter 4. The use of endogenous nutrients for egg production has been documented in a range of birds (see references in Chapter 1) to varying degrees, from providing for the entire clutch in the Lesser Snow Goose (Ankney and MacInnes, 1978) to not being used at all, as in the Cowbird (Ankney and Scott, 1980).

3.4.3 Nutritional value of seed and cuttlefish bone

The major components of single millet and Foreign Finch mix were measured and the results presented in Table 3.2. These results indicated that the Foreign Finch mix contained higher proportions of lipid and protein. This difference is due to the fact that the mixture is made up of seeds that have differing proportions of lipid and protein; some are protein rich, some lipid rich (Diaz, 1990). All seeds provided for the finches were, however, predominantly carbohydrate with only a small proportion of lipid and protein (3.2%, 12.9% and 4.5%, 15.8% for panicum millet and mixed seed diet respectively). A study of the small, granivorous passerine, the white-crowned Sparrow, indicated that a diet containing at least 8% protein would meet the maintenance requirements of many small birds (Murphy, 1993a). The requirement for egg production is presumably higher than this and in laying domestic fowl it is known that the diet should contain 17% protein (Nesheim et al, 1979), however it should be remembered that the hen is a non-determinate layer and has been selectively bred for as high an output of eggs as possible.

Of the three main nutrients being followed in this study; lipid, protein and calcium, what then is the contribution that exogenous supplies make to egg production in the Zebra Finch?

Lipid and Protein

The results of the feeding trial did not reveal any evidence of increased food intake associated with the production of eggs. Similarly, in a study of the Bengalese Finch, Coleman & Whittall (1990) reported that egg weight was not correlated with food intake, which did not increase during egg-laying. Therefore, only by reducing their activity or by increasing digestive efficiency, compared to non-breeding rates, could breeding Zebra Finches make nutrients available for egg production.

Using the figures for the change in digestive efficiency from non-breeding to breeding Zebra Finches published by El-Wailly (1966), I calculated the amount of nutrients that such an increase would liberate. This calculation is only intended to illustrate the order of magnitude of nutrients made available by enhanced digestive efficiency. I am also making the assumption that energy and protein absorption efficiencies are similar. At 24.4°C the digestive efficiency of non-breeding birds in El-Wailly's study was 77.8%. This increased to 81.2% in breeding birds. Brody (1945) gave the efficiency of the conversion of dietary nutrients to egg nutrients as 77%.

My feeding trial was conducted using a single millet, the contents of which were determined (see above). Table 3.4 shows the difference in availability that such an increase in digestive efficiency would make from the mean daily seed consumption of 2.85g. The amount of extra protein potentially released in this way (12.5mg) would only represent 9.3% of the protein content of a Zebra Finch egg (from Table 2.1). It is not as easy when considering lipid because lipids can be synthesised from carbohydrates. It is more convenient, therefore, to consider them both in terms of energy. In order to do this I have used the following figures for energetic value;

Lipid 9.0 to 9.5 kcal/g
Carbohydrate 4.0 to 4.5 kcal/g (Blem, 1990)

The range of values that this estimate would give is;

Lipid 0.003 x 9.0 to 0.003 x 9.5 = 0.027 to 0.028 kcal

Carbohydrate 0.071 x 4.0 to 0.071 x 4.5 = 0.284 to 0.320 kcal

In total this is a difference of between 0.311 and 0.348 kcal per day from the 2.85g of seed. In terms of energy, the lipid content of one egg is;

0.0583 x 9.0 to 0.0583 x 9.5 = 0.525 to 0.554 kcal

Therefore, the extra energy released by the increase of digestive efficiency would represent 59.2% to 63.0% of that required for lipid in an egg. These figures do not, however, take into account the conversion efficiency of changing dietry nutrients to egg nutrients which will not be 100%. Thus these figures are likely to be overestimates. Nvertheless, it appears that an increase of digestive efficiency at the time of egg production could potentially make a significant contribution to the lipid content of the eggs. The same is not true for protein. The low level of protein in the diet means that the increase of digestive efficiency would play a smaller role.

The values in Table 3.4 are likely to be underestimates of the amount of nutrients made available from the diet. El-Wailly (1966) fed his birds a powdered animal feed rather than seed and so efficiences for seed, which is the Zebra Finch's natural diet, may be higher. Shuman, et al. (1989) measured digestive efficiencies for four passerine granivores and for millet efficiency ranged from 84.8% to 92.1%. Calder (1964) estimated that the digestive efficiencies of non-breeding Zebra Finches was only 66%. Therefore, the potential difference between non-breeding and breeding efficiency may be even greater than that seen in El-Wailly's (1966) study.

Using the results of the amino acid analysis of both the eggs (Table 2.5) and the seed (Table 3.3) it is possible for a comparison to be made of demand for the eggs and supply in the diet. Table 3.5 shows the total amino acid requirements for a clutch of five eggs and the total amount of amino acids available from seed consumed over the seven days that protein demands are highest (Day -1 to Day 5, Chapter 2). This indicates that the amount of cystine needed for the eggs could not be met from the diet. This calculation, however, does not take account of either maintenance requirements or the utilisation efficiency of dietary amino

acids. In a study of essential amino acid requirements for maintenance in White-crowned Sparrows, Murphy (1993b) concluded that it would be advisable to assume utilisation efficiencies of no more than 75%. On this basis there would not be enough cystine or lysine in the diet of the Zebra Finches in this study to produce their eggs. In addition, it is also likely that arginine and histidine would be in short supply from the diet for egg formation.

Despite the potential for an increase in the digestive efficiency of the female while she is forming eggs, it is clear that this route alone is not sufficient to provide all the required nutrients. Due to the low protein content of the diet there is a deficiency that could not be overcome without exploiting other sources. In this study, the female finches are not producing a clutch of eggs from exogenous nutrients alone.

Calcium

The quantity of calcium that the female can obtain from seed is very small. Only 0.025mg of calcium per gramme of dehusked seed is available, and of this only 50 - 60% will be absorbed by the bird (MacLean, 1974). Only 0.04mg of calcium is available from the 2.85g of seed that is eaten daily. Each shell requires 17.8mg of calcium.

Calcium must therefore be obtained from another source. In the domestic fowl, medullary bone acts as a calcium store which is drawn upon during egg formation (Simkiss, 1961). The other source of calcium is from calcium rich food items. The deliberate selection of such items has been well documented for various species of bird at laying time. Lemming teeth and bones were found in the guts of laying female Sandpipers (*Calidris spp.*) but not in the gut of non-laying females (MacLean, 1974). Similarly Jones (1976) found calcareous grit, snail and egg shell in the guts of female *Quelea quelea* that were actively forming eggshells but not in non-breeding birds. Fragments of snail shell are found predominantly in the guts of laying House Sparrows but to a much lesser extent in those of non-breeding birds (Schifferli, 1976, Krementz, 1984). In all of these studies dietry intake of calcium was thought to be the major contributor of calcium to the shells. Wild Zebra Finches are known to include snail shell in their diet (Zann and Straw, 1984).

In this study, cuttlefish bone was the calcium rich food item available to the birds. Loss of weight from the cuttlefish bone was monitored daily as an index of consumption. The loss of weight cannot be interpreted as actual consumption but it does closely reflect the changes in attention that the finches paid to it.

Figures 3.4 and 3.5 illustrate the difference in consumption by non-breeding and breeding pairs. The non-breeding pairs maintained a more or less constant level of consumption, less than 0.05g per day. The breeding pairs, however, displayed a significant increase from about Day -3. This level of consumption remained high (about 400% of the non-breeding level) from Day -1 to Day 4, then dropping back to a lower level. The timing of this increase coincides very well with the requirement for calcium for eggshell deposition, which occurs daily from when the first egg is ovulated on Day 0. This pattern closely resembles that found in Sandpipers (MacLean, 1974) and *Quelea quelea* (Jones, 1976), where the peak of occurance of calcium rich food items was in females that were actively secreting shell material.

While the male must account for some of the consumption, perhaps half of the non-breeding level, I believe it was the female that accounted for the increase seen in breeding pairs. Only 1.9% of breeding male Sandpiper stomachs contained lemming teeth or bones compared to 38% of females (MacLean, 1974).

The mean calcium content of ten samples of cuttlefish bone was 29.39 ± 2.04%. If the females consumed only 0.15g of cuttlefish bone from Day -1 to Day 4, and only 50% of its calcium was available from digestion (MacLean, 1974), then 22mg of calcium would potentially have been available on each of these days. 17.8mg of calcium is required for each egg, therefore, ingested cuttlefish bone could easily meet this requirement.

In conclusion, it appears that while the female Zebra Finches in the feeding trial could probably meet the calcium requirment for egg production from ingested supplies, it is unlikely that protein requirements can be met by the diet alone. The diet may also make a significant contribution to lipid requirements.

TABLE 3.4

The quantity of protein, lipid and carbohydrate (mg) liberated from 2.85g of panicum millet by an increase of digestive efficiency from 77.8% to 81.2%.

Nutrient	From 2.85g seed	At 77.8%	At 81.2%	Difference
Protein	367.6	286.0	298.5	12.5
Lipid	91.2	71.0	74.0	3.0
Carbohydrate	2089.0	1625.0	1696.0	71.0

TABLE 3.5

Total amino acid requirements for a five-egg clutch, together with total consumption from the seed diet, to indicate which amino acids needed for egg formation may be poorly represented in the food.

Amino Acids	Seed Amino Acid (mg), 7 days	Egg Amino Acid (mg), 5 egg cl.	Seed Amino Acid / Egg Amino Acid
Alanine	0.303	0.051	5.9
Arginine	0.077	0.039	1.9
Aspartic Acid	0.188	0.075	2.5
Cystine	0.041	0.068	0.6
Glutamine	0.421	0.092	4.5
Glysine	0.059	0.018	5.7
Histidine	0.057	0.028	2.0
Isoleucine	0.188	0.019	6.21
Leucine	0.267	0.051	5.2
Lysine	0.051	0.041	1.2
Methionine	0.069	0.018	3.8
Phenylalanine	0.134	0.025	5.36
Proline	0.262	0.032	8.1
Serine	0.206	0.084	3.2
Threonine	0.108	0.036	3.0
Tyrosine	0.057	0.025	2.3
Valine	0.121	0.043	2.8

CHAPTER 4 - CHANGES IN BODY LIPID, PROTEIN AND CALCIUM CONTENT OF FEMALE ZEBRA FINCHES DURING BREEDING

4.1 INTRODUCTION

This chapter considers the changes which occur in body reserves of protein, lipid and calcium during the period of egg production in female Zebra Finches. Previous studies have shown considerable differences in the extent to which body reserves are used by different species of bird. In the case of protein condition the Introduction Table 1.1 listed studies from the Lesser Snow Goose (Ankney and MacInnes, 1978), which shows a substantial loss of protein reserves during egg formation, through to the White-bellied Swiftlet (Hails & Turner, 1985) which shows no loss of body protein.

In addition, those species that do utilise their reserves do not, in general, use them evenly. For instance the Northern Shoveler will deplete its fat reserves during egg production while not reducing the overall protein reserve because it gets adequate protein from its diet (Ankney & Afton, 1988). *Quelea* and *Camaroptera* females, however, deplete their protein reserves while their lipid deposits remain high (Jones & Ward, 1976, Fogden & Fogden, 1979).

The Zebra Finch is predominantly a granivore (Zann and Straw, 1984) and as such it has a diet that is relatively poor in protein and fat but rich in carbohydrate. The diet of wild Zebra Finches does not include a significant insect intake although the diet of breeding females has not been studied in detail (Zann and Straw, 1984, Morton and Davies, 1983). Other tropical granivores such as the Quelea (Jones and Ward, 1976) and the Camaroptera (Fogden and Fogden, 1979) are known to use reserves of both lipid and protein during the breeding period. The Zebra Finch is not dissimilar to the above two species in its life history and so is likely to have to use body reserves when under the stress of reproduction.

The previous chapters have dealt with, firstly, the nutritional investment in egg production and, secondly, the diet of the female during this period to estimate how much of the cost can be met by exogenous resources. This chapter aims to estimate the role that body

reserves play in meeting the cost of egg production and, in relation to food intake, produce a budget of how egg production demands are met by the female Zebra Finch.

4.2 MATERIALS AND METHODS

In order to investigate changes in body reserves of protein, lipid and calcium associated with egg formation, females were taken for carcass analysis. Pre-laying females were obtained by introducing a female to a male for two to three days. If courtship behaviour and nest building activity was shown the female would be taken one, two or three days after this started in order to get birds at various stages of the egg formation period. These birds were placed accurately in the laying cycle by examination of their reproductive tract (see Chapter 2). Other females were taken during the egg-laying period and post-laying females were obtained on the day the last egg of the clutch was laid. Post-laying females were taken specifically for carcass analysis and also from experiments where the eggs were required.

4.2.1 Dissection procedure

All birds were killed using either chloroform (CCl_4) or carbon dioxide (CO_2). Chloroform was used at the beginning of the study but was replaced by CO_2 because it is less hazardous to handle. Weight (to 0.01g) was recorded immediately, and the following external measurements taken with vernier calipers (to 0.05mm);

Wing Length - carpal joint to end of primary flight feathers.

Body Length - on horizontal surface with head extended, tip of bill to end of tail feathers.

Wing Span - with wings extended, tip to tip.

Leg - with foot folded back, from joint of tibiotarsus and tarsometatarsus to the end of the tarsometatarsus.

Head and Bill - tip of bill to back of skull.

Bill Length - tip of bill to nasofrontal hinge.

Bill Depth - base of lower mandible to upper mandible at nasofrontal hinge.

Skull Width - at its widest point.

Radius/Ulna Length - from joint with humerus to carpal joint.

At this point the majority of birds were frozen for analysis at a later date. The full dissection was carried out on the birds after thawing at room temperature for one to two hours. Firstly, the pectoral muscle blocks were removed (I am using the term pectoral muscle block to refer to the *pectoralis major* and *supracoracoideus*). In most birds the left pectoral muscle block was used for biochemical analysis and the right for lean dry weight determination (see below). Two internal skeletal measurements were then taken, the Sternum-Coracoid (from the abdominal tip of the sternum to the end of the coracoid) which is essentially the length of the pectoral muscle, plus the keel length (from the abdominal tip of the sternum to the end of the keel where it joins the furcula).

The rib cage was removed and the following organs taken for dry weight determination; the heart, liver, gizzard, gut (from gizzard to cloaca) and the leg muscles of the tibiotarsus were dissected out, taking care not to include any adjacent fat bodies. The omentum was also removed, this being the large fat body in the abdomen. The samples were dried at 70°C to dry weight (0.001g) and then returned to the carcass also. Ovary and oviduct were also removed and examined for breeding condition as described in Chapter 2.

4.2.2 Lean dry weight and lipid content of pectoral muscle

The right muscle block was dried to constant weight (0.0001g) at 70°C. Lipid was extracted using chloroform in a Soxhlet apparatus (see Chapter 2). Lean dry weight was recorded after redrying the samples and lipid content was taken as the dry weight minus the lean dry weight.

4.2.3 Lean dry weight and lipid content of carcass

The carcass was dried to constant weight (0.001g) at 70°C and together with the dried organs the lipid was extracted using chloroform in a soxhlet apparatus as above. Total lean

dry weight of the carcass was obtained by adding the lean dry weight of the right pectoral muscle, multiplied by two, to the carcass lean dry weight (reproductive tissue weights were not included).

4.2.4 Ash weight and calcium content of carcass

When the carcasses were lipid free an ash weight was obtained for each. The carcasses were placed in pre-dried and weighed crucibles and ashed in a muffle furnace at 650°C for 8 hours. The crucibles were removed from the furnace and allowed to cool in a dessicator before weighing to 0.0001g. An ash weight index that corrected for body size was calculated by dividing the ash weight by the sternum-coracoid length. This measure of size was used as it proved to be the one that explained most of the variation in body size (see Results).

Ash from five pre-breeding and five post-breeding birds was selected for calcium analysis. The carcass ash was powdered using a pestle and mortar and then treated in the same manner as the analysis of eggshell and cuttlefish bone previously described. A 1:10 dilution of the carcass ash "stock" solution was necessary to bring it onto the calibration curve. The final dilution for analysis included 0.5ml "stock" solution and 0.05ml lanthanum chloride made up to 25ml with deionised water.

4.3 RESULTS

4.3.1 Changes in pectoral muscle condition during egg formation

In order to investigate changes in body condition associated with the production of eggs it is necessary to obtain an index of body condition that corrects for differences in body size between individuals. Pectoral muscle lean dry mass is often corrected by some coefficient of linear body size (*eg* Houston et al, 1983, Hails and Turner, 1985). However, this may not be the best method (Freeman & Jackson,1990, Packard & Boardman, 1988). A more accurate correction is obtained from taking a sample of birds from the population and plotting the regression of lean dry weight on body size of the pectoral muscle (Bolton et al, 1991). The relative condition is derived from the deviation of the actual weight from that predicted by the regression equation on the basis of its body size, *ie* the size of its residual. This technique has been applied using the sternum-coracoid length as the best predictor of body size (Jones, M.M., 1983, Jones, G. 1987, Schifferli, 1976). The same measure also gives the best correlation with pectoral lean dry weight in Zebra Finches (r_{79}= 0.607, $p < 0.0001$). Indices based on one measure of body size have recently been criticised (Freeman and Jackson, 1990, Rising and Somers, 1989) and therefore a principal component analysis of four skeletal measures (sternum-coracoid, sternum, leg bone and radius/ulna) was used to produce a single "body size factor". However, this factor did not improve the correlation with pectoral muscle lean dry weight (r_{79}= 0.599, $p < 0.0001$) and so the sternum-coracoid measure was used.

The equation from the regression of pectoral muscle lean dry weight on sternum-coracoid ($y = 0.0228x - 0.2785$) was used to calculate estimated lean dry weights;

Estimated Lean Dry Weight = (0.0228 x Sternum-coracoid) - 0.2785

The observed value minus the estimated value (the residual) was calculated and the means for each day of the laying cycle shown in Figure 4.1. The results presented in this chapter are for female Zebra Finches that had or, from exmination of the ovary, were going to lay five eggs so that comparison could be made with the results presented in Chapter 2. (In this and subsequent figures, the regression equations were calculated using all data points.

However, for the sake of clarity, the figures are presented with the means for each day of the cycle only). The result shows that muscle lean dry weight declines significantly from the onset of egg production to the end of laying (r_{44}= -0.895, $p < 0.0001$). It appears that the majority of this decline in lean dry weight occurs from Day -3 to Day 1 as shown in Figure 4.1b (r_{27}= -0.992, $p < 0.0001$). It is perhaps not realistic to expect this decline in muscle weight to be linear given the pattern of demand seen in Figure 2.5a. However, the regression analysis gives an indication of the significance of the decline seen.

Such an index does not show the actual loss of muscle tissue that this decline represents, which is needed in order to work out the budgeting of nutrients at this time. To do this a Standardised Pectoral Muscle lean dry weight (SLDW) was calculated that corrected for body size but maintained the values in milligrams. This was done on the basis of how much the sternum-coracoid length of each bird differed from the population mean (28.07 ± 1.13mm, n = 110). This difference multiplied by the slope of the regression equation above (0.0228) gave a "body size" difference for each bird. This was subtracted from the observed pectoral muscle lean dry weight to give SLDW;

SLDW = Obs. LDW - (Obs. Sternum-coracoid - 28.07)0.0228

Figure 4.2 shows the mean of standardised pectoral muscle lean dry weight during the laying cycle. The decline observed is significant (r_{44} = -0.860, $p < 0.00001$). As in the pectoral muscle index, the majority of the decline is seen to occur from Day -3 to Day 1. Figure 4.2b illustrates this period (r_{27} = 0.852, $p < 0.0001$).

It was, therefore, possible to estimate the amount of lean dry weight that had been lost from the pectoral muscle blocks over the laying period by subtracting the mean on Day 1 from the mean on Day -3. The mean loss is **51mg**. Assuming that both muscle blocks are equal, then **102mg** of lean dry weight (protein) is lost from the pectoral muscles during the period of egg formation of a five egg clutch.

4.3.2 Lean dry weight of carcass

Lean dry weight (LDW) of the carcass was obtained by extracting lipid from the carcass. The total carcass lean dry weight (Total carcass LDW) was then calculated by adding the carcass LDW to the LDW of the pectoral muscles. Figure 4.3 illustrates the mean Standardised Total Carcass LDW across the laying cycle, the decline observed is significant ($r_{44} = 0.920$, $p < 0.0001$). This graph is for values that were corrected for body size. The "body size" factor extracted by principal components analysis proved to be the best measure of body size when looking at Total Carcass LDW ($r_{44} = 0.508$, $p < 0.0001$). A standardised carcass LDW was calculated in the same manner as for pectoral muscle using the "bodysize" factor instead of the sternum-coracoid length. Figure 4.3b shows the period Day -3 to Day 1 which, like the pectoral muscle, was when the majority of the decline occured ($r_{27} = 0.852$, $p < 0.0001$).

The mean standardised carcass LDW is 3.972mg on Day -3 and 3.454mg on Day 1, therefore, a loss of **518mg** has occured over this period. This loss is equivalent to 14.9% of the ash-free lean dry weight of the carcass on Day -3, which is 3.480mg (Mean Ash Weight = 0.492 ± 0.074g, n = 62). The loss of lean dry weight from the pectoral muscle blocks alone (102mg) makes up 19.7% of the total loss of body lean dry weight.

4.3.3 Dry weights of organs

Dry weights were recorded for liver, heart, gizzard, gut, left and right leg muscle and the omentum. Except for the omentum, which is a fat body, these organs had all adhering fat tissue removed and so I assumed that any decline in weight recorded from them would probably reflect a loss of protein. As such this data was used only to identify which of the major organs were experiencing declines similar to that seen in the pectoral muscle but could not be used to provide quantitative measures.

Leg muscle (Figure 4.4) and the gut (Figure 4.5) both displayed a significant decline in dry weight during the laying cycle ($r_{47} = -0.861$ and $r_{47} = -0.888$, respectively, $p < 0.0001$). Left leg only is shown as there was no significant difference in the dry weights of left and right leg muscles ($t_{73} = 0.150$, $p > 0.8$). Likewise, the liver (Figure 4.6) and the gizzard (Figure

4.7) displayed significant declines in dry weight (r_{47} =-0.647, r_{38} = -0.645 respectively, $p < 0.001$).

The heart (Figure 4.8) did not appear to lose any dry weight during the laying cycle r_{44} = -0.324, $p > 0.05$).

4.3.4 Ash weight and calcium content of carcass

The index of carcass ash weight did not change significantly over the laying cycle (Figure 4.9), $r_{26} = 0.297$, $p > 0.05$). The pre-laying mean calcium content (as percentage of ash weight) was 18.17% ± 1.04 (n=5) and the post-laying mean was 18.53% ± 0.55 (n=5). No significant difference in the calcium content was found ($t_8 = 0.227$, $p > 0.05$).

It appears that the female Zebra Finch does not draw on skeletal calcium during egg production.

4.3.5 Lipid contents of pectoral muscles and carcass

The lipid content of the whole carcass and the right pectoral muscle block was determined by extraction in a Soxhlet extractor. In addition, the dry weight of the omentum fat body was obtained as an indicator of the state of the lipid reserves while conducting the dissection. There is considerably more variation to be found in the lipid reserves than in the protein reserves of female Zebra Finches. No correlation of lipid content with body size could be detected; "body size" factor with total lipid content, r_{44} = 0.121, $p > 0.1$, and sternum-coracoid with total lipid content, $r_{44} = 0.119$, $p > 0.1$. Therefore, all data concerning total body lipid content is hereafter presented in absolute terms.

Figure 4.10 shows the mean total body lipid values (including the omentum dry weight) for females across the laying cycle. There is a significant drop ($r_{44} = -0.795$, $p < 0.0001$) in the quantity of lipid found in the birds over this period. The period from Day -3 to Day 1 of the cycle is when the majority of this drop in lipid content of the body occurs, Figure 4.10b (r_{27}= -0.931, $p < 0.0001$). In absolute terms the difference between Day -3 and Day 1 is **0.895g** of lipid.

An index of lipid contained in the right pectoral muscle was calculated that corrected for the different sizes of muscle;

Pectoral Lipid Index = Lipid in Pectoral Muscle , Pectoral Muscle LDW

This type of correction is commonly used (e.g. Perdeck, 1985). Figure 4.11 shows the lipid index over the laying cycle where a significant loss is evident, r_{44}= -0.587, $p < 0.01$). There is a very steep drop in intramuscular lipid (Figure 4.11b) during the period of Day -3 to Day 1 (r_{27}= -0.967, $p < 0.0001$) similar to that seen in the muscle lean dry weight. The amount of lipid in the muscles is rather small, being only about 6.9% of pectoral muscle dry weight.

There is a significant decrease in the dry weight of the omentum, Figure 4.12, (r_{44}= -0.778, $p < 0.0001$). These lipid depots are more important, in terms of quantity, than the intramusclular lipid. Once again, it is the period from Day -3 to Day 1 that experiences the bulk of this fall in lipid, Figure 4.12b (r_{27}= -0.940, $p < 0.0001$).

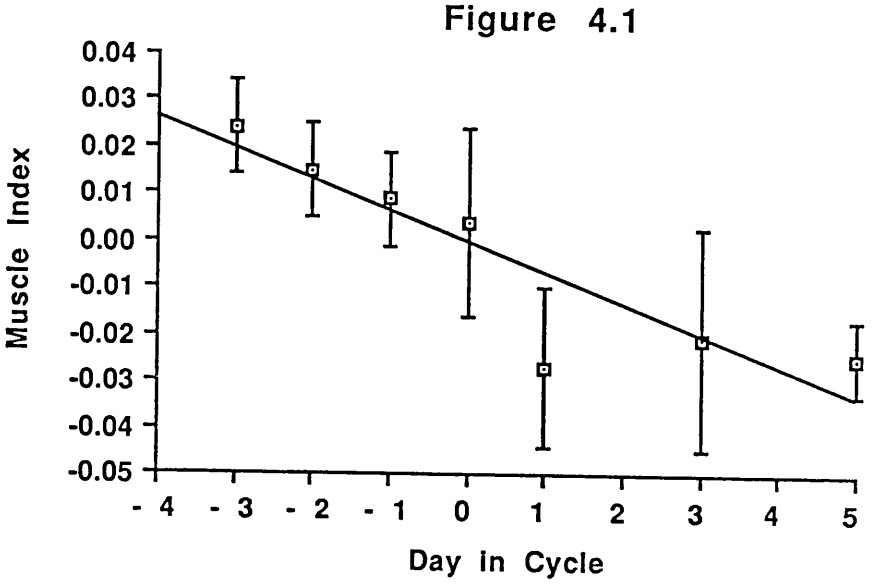


Figure 4.1

Pectoral Flight Muscle Index of female Zebra Finches during the laying cycle for a five-egg clutch (mean ± s.d.)

$y = -0.0002 - 0.0066x$, $r_{44} = -0.895$, $p<0.0001$

Day	-3	-2	-1	0	1	3	5
n	4	9	6	6	4	9	8

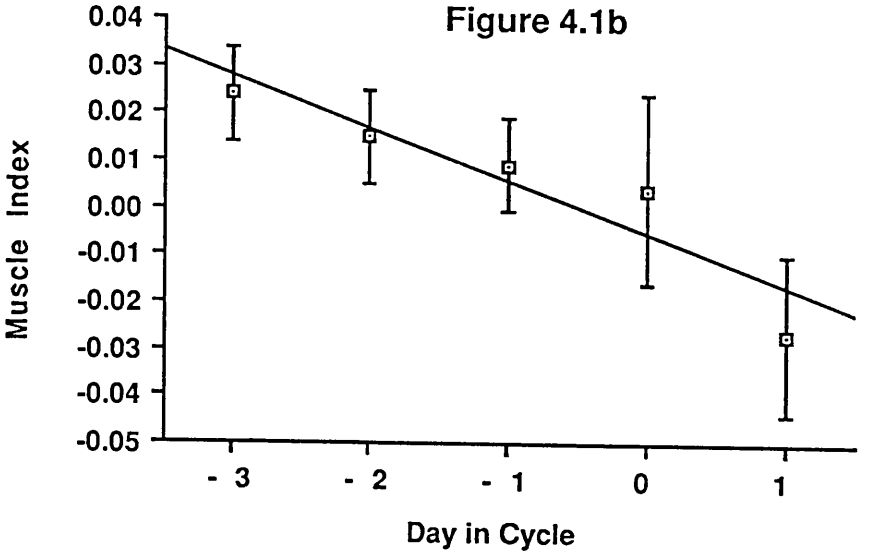


Figure 4.1b

Pectoral Flight Muscle Index of female Zebra Finches during the laying cycle from Day -3 to Day 1 only (mean ± s.d.)

$y = -0.039 - 0.011x$, $r_{27} = -0.922$, $p<0.0001$

Day	-3	-2	-1	0	1
n	4	9	6	6	4

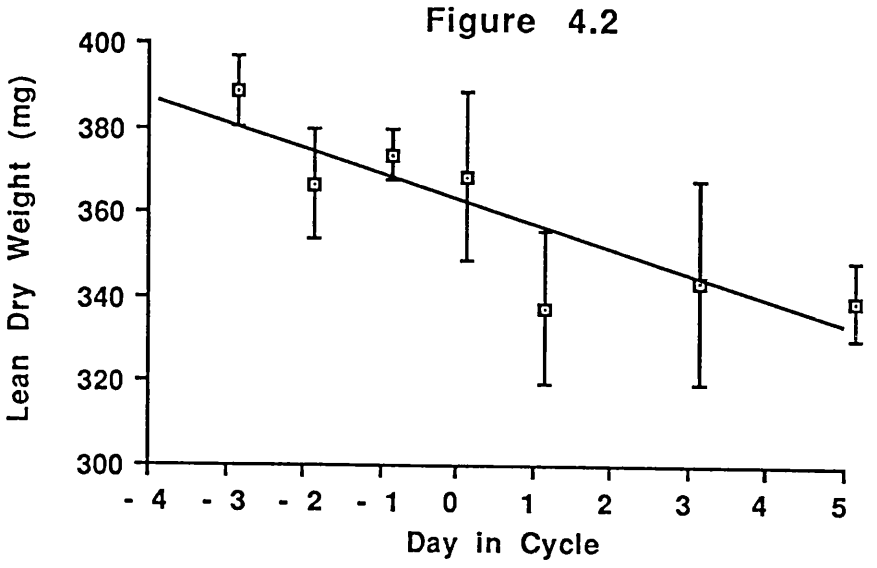


Figure 4.2

Standardised Pectoral Muscle Lean Dry Weight of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 359.71 - 5.982x$, $r_{44} = -0.860$, $p<0.0001$

Day	-3	-2	-1	0	1	3	5
n	4	9	6	6	4	9	8

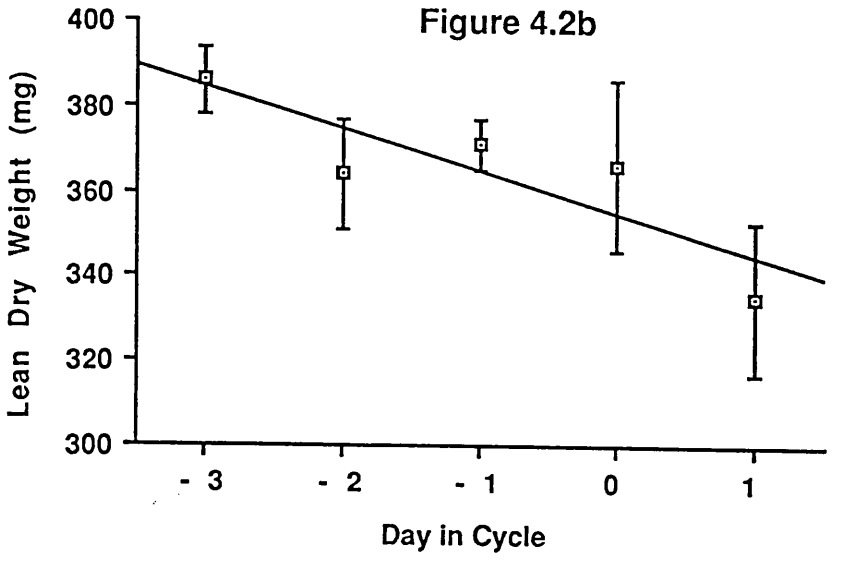


Figure 4.2b

Standardised Pectoral Muscle Lean Dry Weight of female Zebra Finches during the laying cycle from Day -3 to Day 1 only (mean ± s.d.)

$y = 394.40 - 10.00x$, $r_{27} = -0.852$, $p<0.0001$

Day	-3	-2	-1	0	1
n	4	9	6	6	4

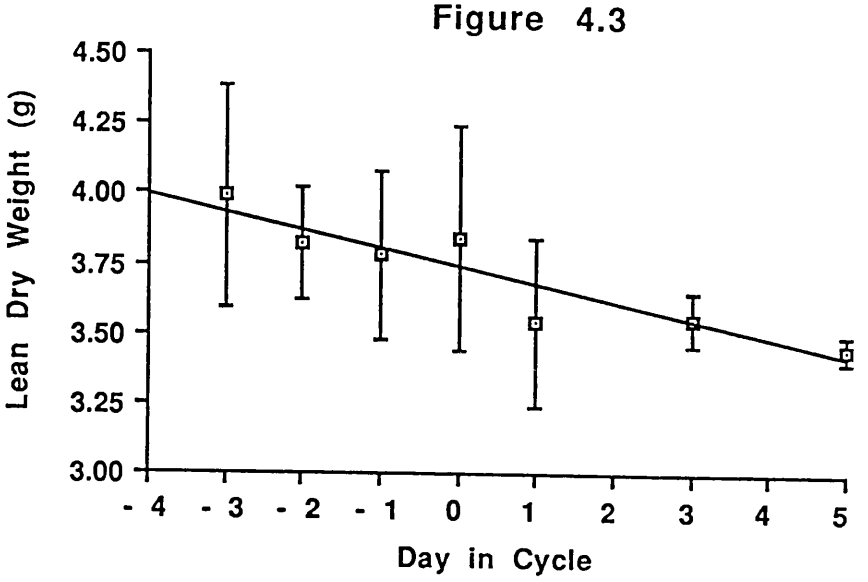


Figure 4.3

Standardised Total Carcass Lean Dry Weight of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 3.7390 - 0.064x$, $r_{44} = -0.920$, $p<0.0001$

Day	-3	-2	-1	0	1	3	5
n	4	9	6	6	4	9	8

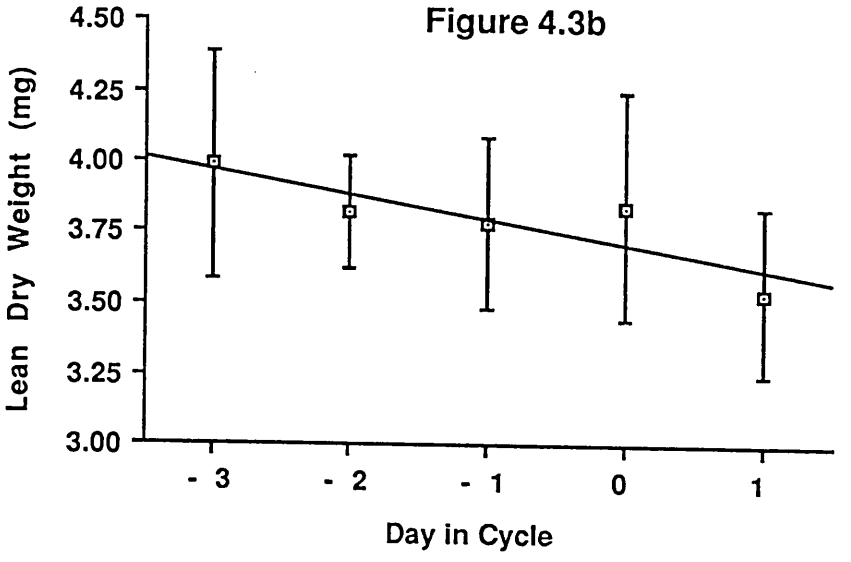


Figure 4.3b

Standardised Total Carcass Lean Dry Weight of female Zebra Finches during the laying cycle from Day -3 to Day 1 only (mean ± s.d.)

$y = 4.0569 - 0.087x$, $r_{27} = -0.851$, $p<0.0001$

Day	-3	-2	-1	0	1
n	4	9	6	6	4

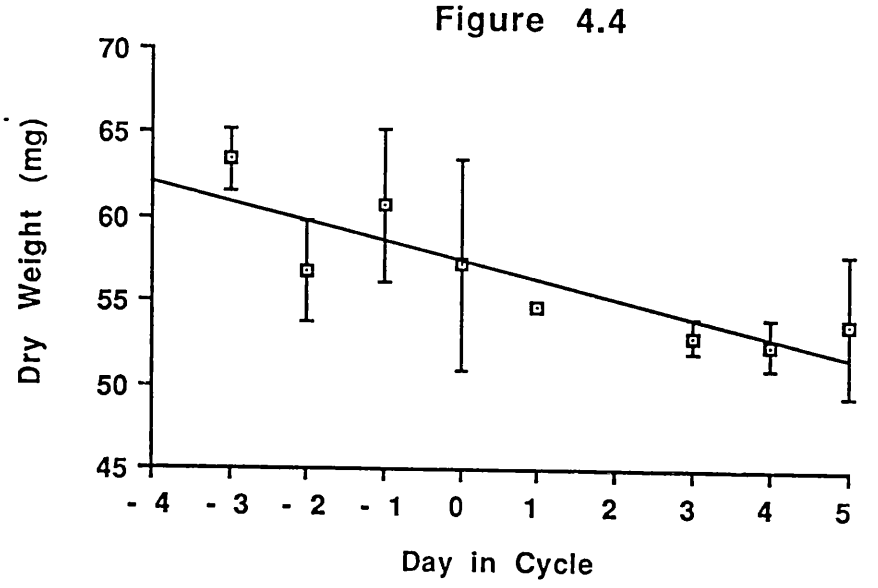


Figure 4.4

Dry Weight (mg) of the left leg muscle of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 57.508 - 1.152x$, $r_{47} = -0.861$, $p<0.0001$

Day	-3	-2	-1	0	1	3	4	5
n	4	9	6	6	4	9	3	8

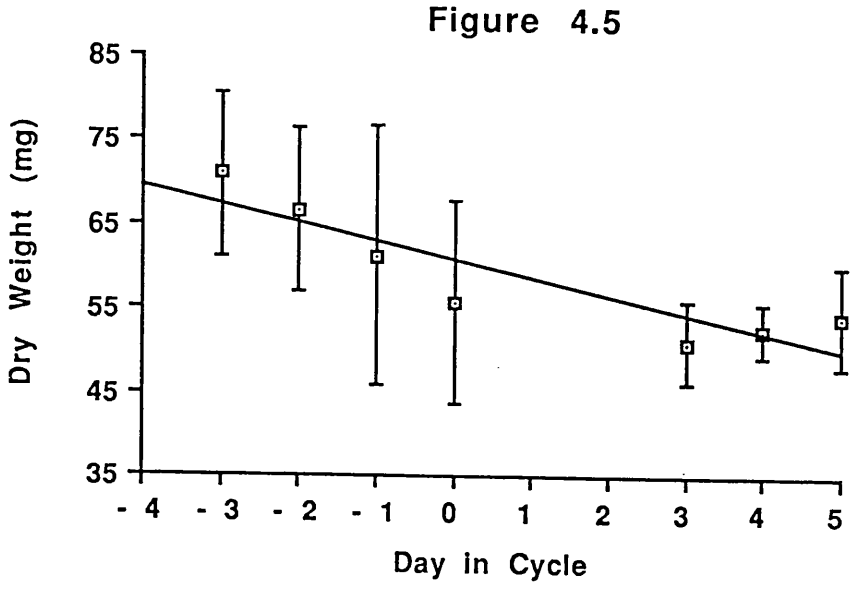


Figure 4.5

Dry Weight (mg) of the gut of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 60.687 - 2.152x$, $r_{47} = -0.888$, $p<0.0001$

Day	-3	-2	-1	0	1	3	4	5
n	4	9	6	6	4	9	3	8

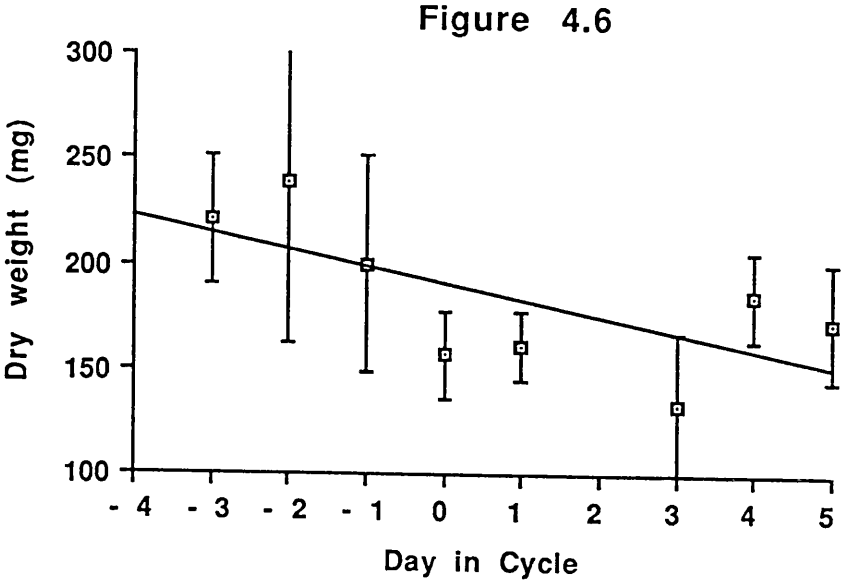


Figure 4.6

Dry Weight (mg) of the liver of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 190.48 - 7.836x$, $r_{47} = -0.674$, $p<0.001$

Day	-3	-2	-1	0	1	3	4	5
n	4	9	6	6	4	9	3	8

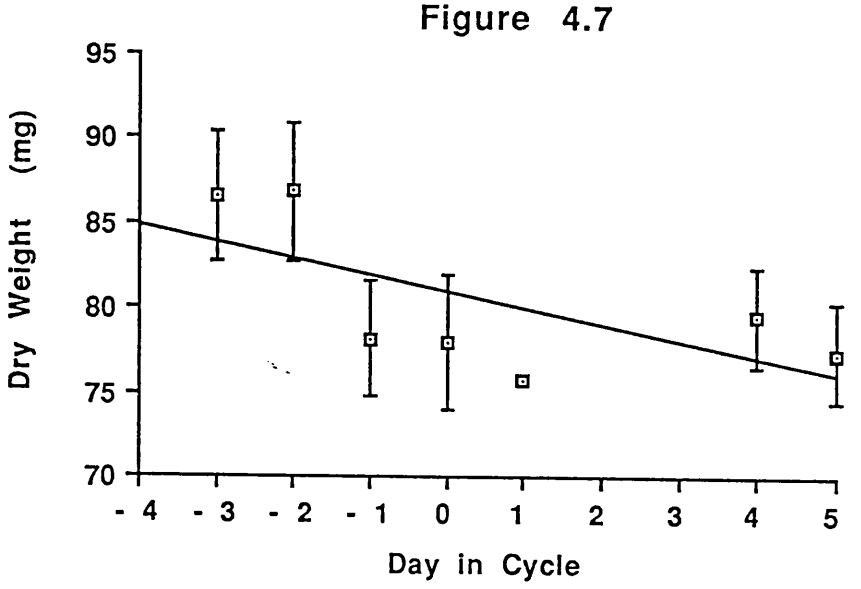


Figure 4.7

Dry Weight (mg) of the gizzard of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 80.869 - 0.971x$, $r_{38} = -0.645$, $p<0.001$

Day	-3	-2	-1	0	1	3	4	5
n	4	9	6	6	4	-	3	8

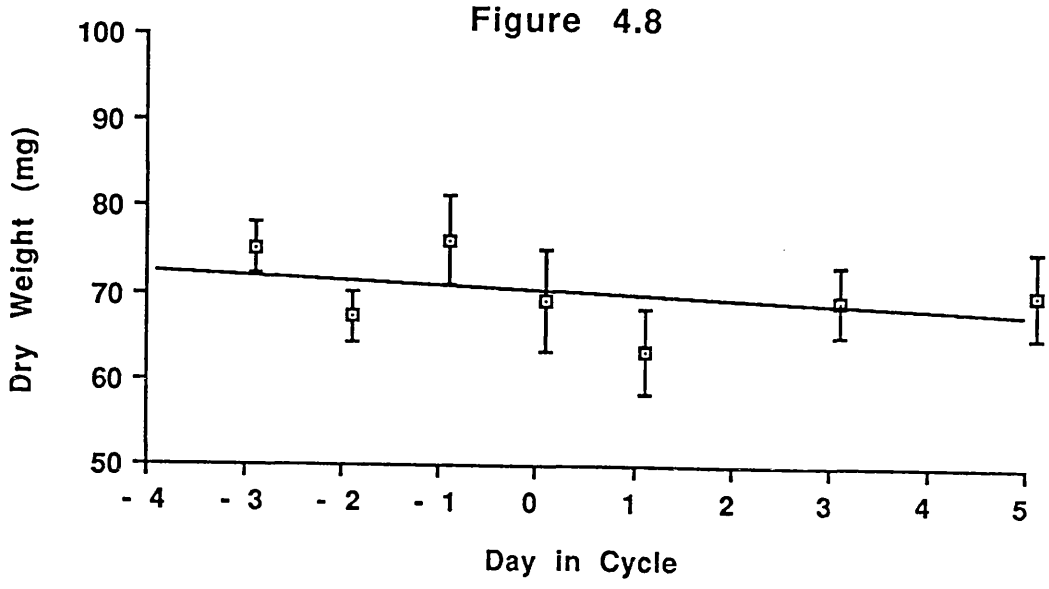


Figure 4.8

Dry Weight (mg) of the heart of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 69.075 - 0.515x$, $r_{44} = -0.324$, $p>0.05$

Day	-3	-2	-1	0	1	3	4	5
n	4	9	6	6	4	9	-	8

Figure 4.9

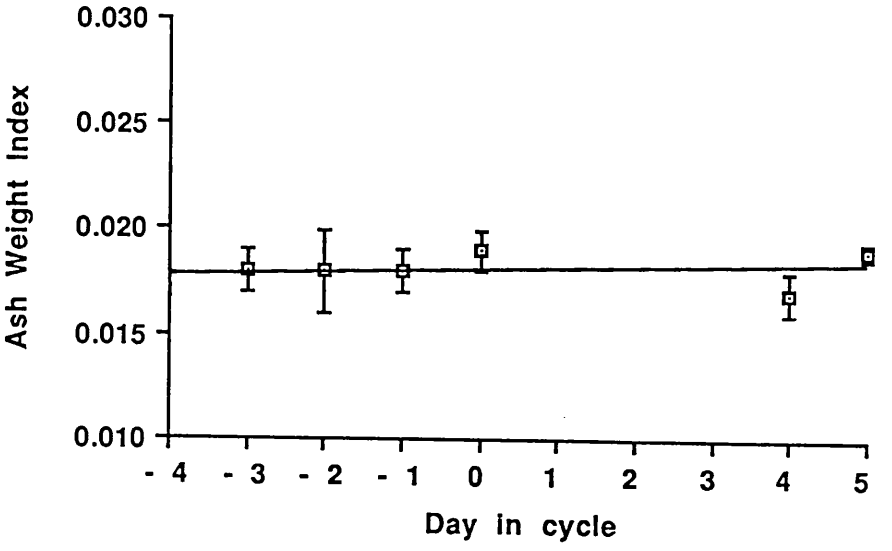


Figure 4.9

Ash Weight Index of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 0.018 - 0.00001x$, $r_{26} = 0.297$, $p>0.05$

Day	-3	-2	-1	0	4	5
n	4	4	4	5	3	8

Figure 4.10

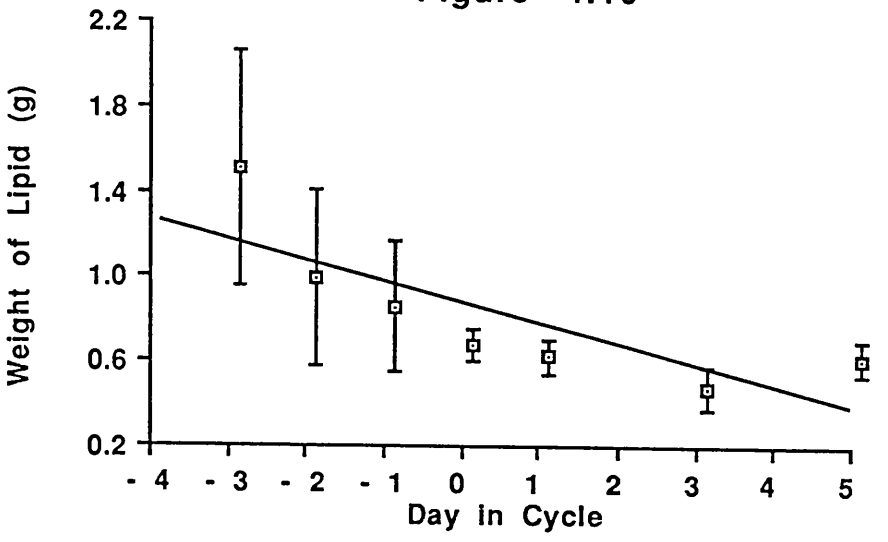


Figure 4.10

Total body lipid (g), including omentum dry weight, of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 0.813 - 0.098x$, $r_{44} = -0.795$, $p<0.0001$

Day	-3	-2	-1	0	1	3	5
n	4	9	6	6	4	9	8

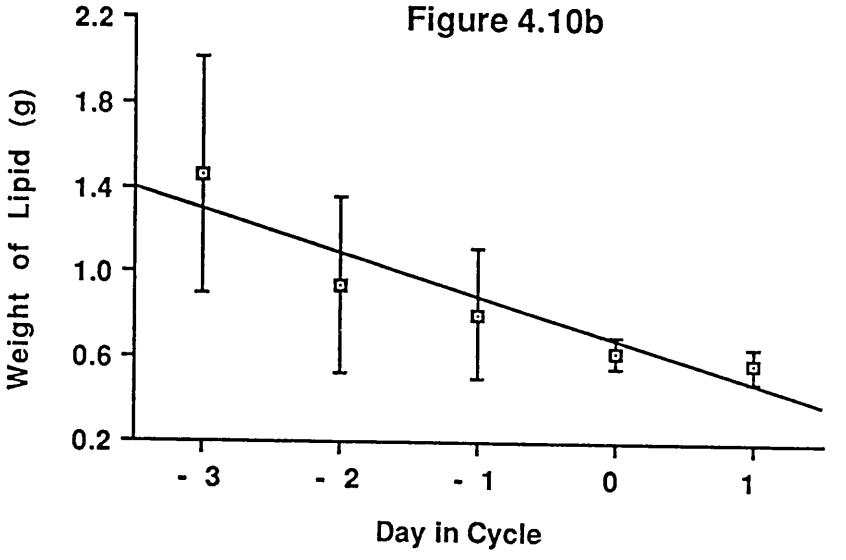


Figure 4.10b

Total body lipid (g), including omentum dry weight, of female Zebra Finches during the laying cycle from Day -3 to Day 1 only (mean ± s.d.)

$y = 1.505 - 0.208x$, $r_{27} = -0.931$, $p<0.0001$

Day	-3	-2	-1	0	1
n	4	9	6	6	4

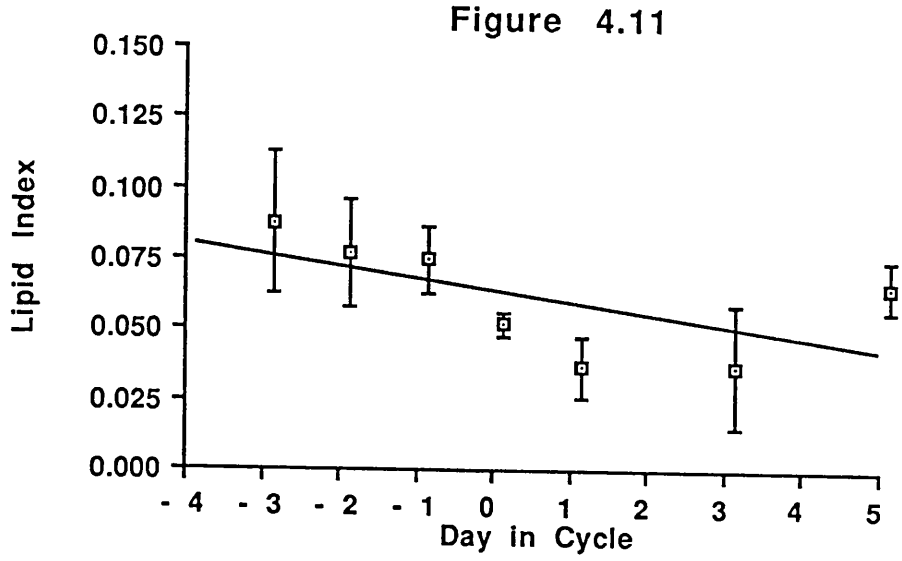


Figure 4.11

Pectoral Muscle Lipid Index of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 0.06 - 0.004x$, $r_{44} = -0.587$, $p<0.01$

Day	-3	-2	-1	0	1	3	5
n	4	9	6	6	4	9	8

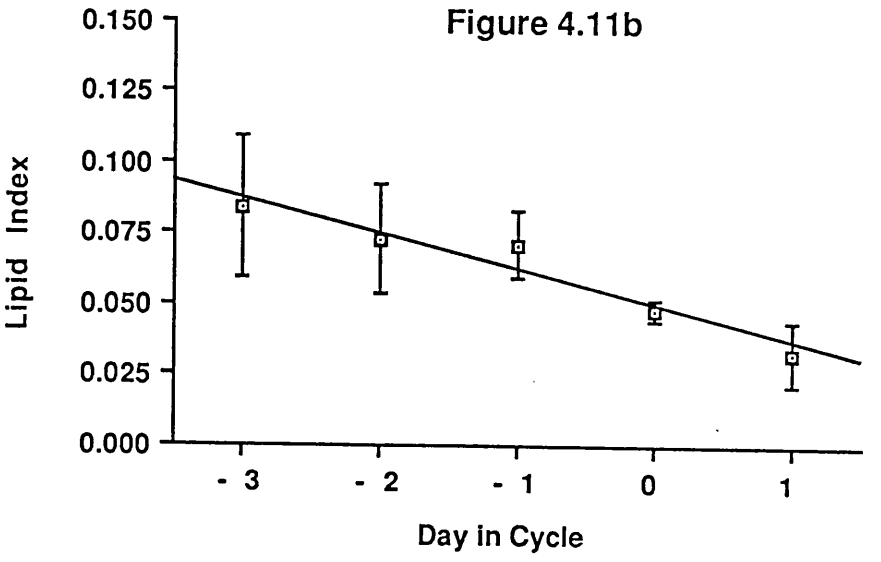


Figure 4.11b

Pectoral Muscle Lipid Index of female Zebra Finches during the laying cycle from Day -3 to Day 1 only (mean ± s.d.)

$y = 0.1 - 0.013x$, $r_{27} = -0.967$, $p<0.0001$

Day	-3	-2	-1	0	1
n	4	9	6	6	4

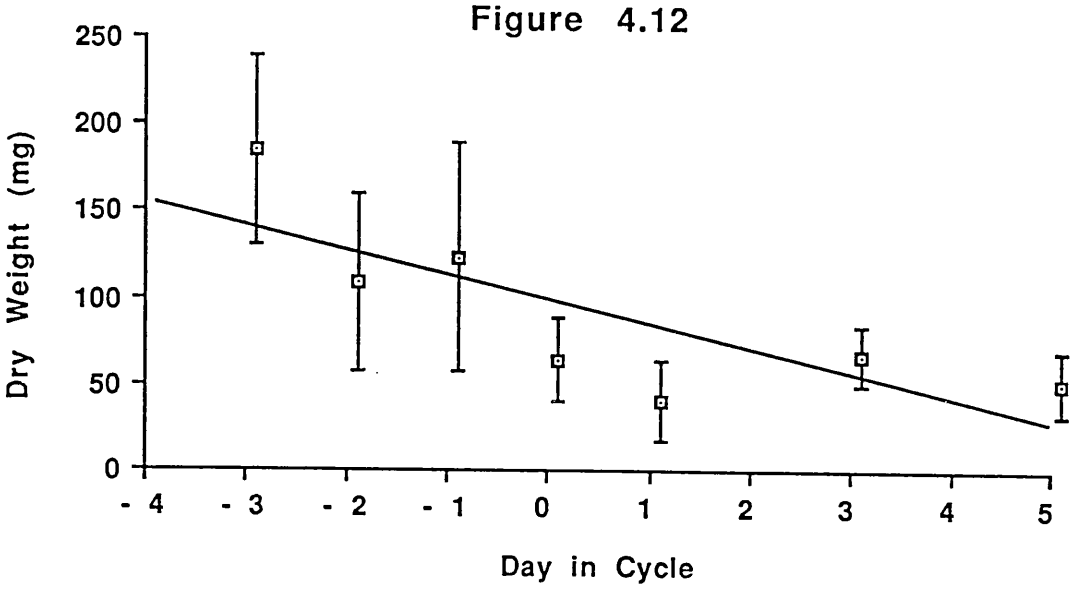


Figure 4.12

Dry Weight of the omentum (mg) of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 91.054 - 14.126x$, $r_{44} = -0.778$, $p<0.0001$

Day	-3	-2	-1	0	1	3	5
n	4	9	6	6	4	9	8

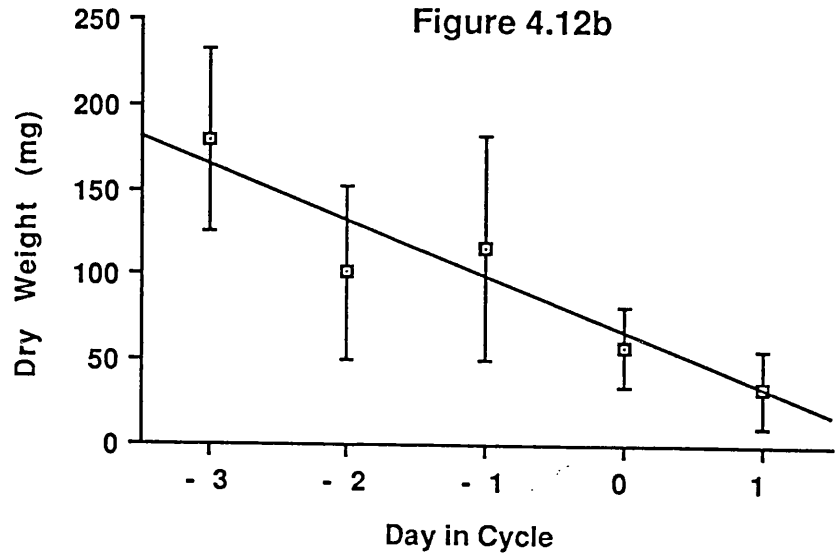


Figure 4.12b

Dry Weight of the omentum (mg) of female Zebra Finches during the laying cycle from Day -3 to Day 1 only (mean ± s.d.)

$y = 198.2 - 33.4x$, $r_{27} = -0.940$, $p<0.0001$

Day	-3	-2	-1	0	1
n	4	9	6	6	4

4.4 DISCUSSION

4.4.1 Changes in body reserves: Protein.

As in previous studies, the pectoral flight muscle (*pectoralis major* and *supracoracoideus)* was used as an index of changes in reserves of protein during the laying cycle. The results indicate that there is a significant decline in the protein condition of the female Zebra Finch under the stress of breeding (Figure 4.1 & 4.1b). In addition, an absolute value for the amount of protein that this decline represented was obtained (Figure 4.2 & 4.2b). This was a loss of 51mg from the right pectoral muscle block. Assuming equality with the left side, then a total of 102mg was lost from the pectoral muscles. This quantity represents 15% of the total protein content of a five-egg clutch (674mg, Chapter 2). The timing of this decline is significant also as from Day -3 to Day 1 it corresponds to the rapid increase in demand for egg nutrients occuring at this time (see Chapter 2). This pattern is similar to that seen in the Red-billed Quelea and the Grey-backed Camaroptera, both of which experience a decline in their flight muscle lean dry weight during the three days leading up to the laying of the first egg (Jones & Ward, 1976, Fogden & Fogden, 1979). In a study of the House Sparrow, the total protein content of the pectoral muscles was at maximum on Day -2 of the cycle and declined to Day 2 (Jones, M.M., 1991). This pattern is identical to that seen in the Zebra Finch but is on different days because the Sparrow lays a slightly smaller clutch. As in the Zebra Finch, this drop in the muscle protein content of the House Sparrow closely matches the pattern of demand for egg nutrients which increase rapidly from Day -3 to reach a peak on Day 0 (Krementz & Ankney, 1986).

The lean dry weight of the muscles declined by 13% of their weight at the commencement of the laying cycle. This value compares well with the 16% drop in flight muscle weight recorded in the Quelea (Jones & Ward, 1976). While appearing to be small this value represents almost half of the difference in muscle lean dry weight between Quelea in peak condition and those that had died of starvation.

In general, the *pectoralis major* and the *supracoracoideus* between them represent the largest single mass of muscle to be found on flying birds (George & Berger, 1966). The Zebra Finches in this study had a mean wet pectoral muscle mass of 3.012 ± 0.018g, n =

46, which is 19.8% of the whole body mean wet weight of 15.25 ± 0.19g, n = 110. The flight muscles represent a substantial part of any potential reserve of protein.

In addition to the pectoral muscles there was also a significant decline in the lean dry weight of the entire carcass (minus reproductive tissue), Figure 4.3, $r_{44} = -0.920$, $p < 0.0001$. The pattern of decline is similar to that seen in the pectoral muscles, occuring largely from Day -3 to Day 1 (Figure 4.3b). The total loss of protein, calculated from the difference between the mean on Day -3 and Day 1, was 518mg. Of this the 102mg from the flight muscle makes up 19.7%. The mean total loss of protein from the body over the laying period of 518mg, represents 76.8% of the protein content of a 5 egg clutch. The significant loss of dry weight from the leg muscle (Figure 4.4) provides evidence that the flight muscle is not the only muscle group undergoing a decline during the laying period.

Muscle is not a static tissue. In humans the protein turnover rate in skeletal muscle is 12% per day and it plays a vital role in maintaining blood amino acid homeostasis (Spargo et al.,1979). Millward (1970) reported that the skeletal muscles play the dominant role in the overall protein metabolism. In the breast muscle of domestic hens the rate of synthesis of new protein is as high as 20.4% per day (Hiramoto et al, 1989). Therefore it appears that the skeletal muscle system may have the potential to act as the major component of the body's protein reserves which may be drawn upon when the bird is under nutritional stress, as when producing eggs.

It was not just the muscles that were contributing to the loss of lean dry weight from the carcass. Evidence obtained from the dry weights of various organs suggested that other organs are important also. The gut (Figure 4.5), the liver (Figure 4.6) and the gizzard (Figure 4.7) all showed significant loss of dry weight across the laying cycle. Several other studies have implicated the role of organs other than muscle in potentially making protein available for egg production. The Black Duck (Reinecke et al, 1982), the Mallard Duck (Krapu, 1981) and the Lesser Snow Goose (Ankney & MacInnes, 1978) all have a significant loss of lean dry weight from the gizzard associated with egg production. The Brant Goose has a significant loss of lean dry weight from the gizzard and liver (Ankney, 1984). Some of the above birds do not feed while producing eggs and weight loss from the

gut is likely when it is not in use, however, the Zebra Finch continues to feed during the egg formation period. The heart (Figure 4.8) does not experience any loss of dry weight.

In addition, as shown in Chapter 2, the oviduct undergoes a decline in lean dry weight, potentially releasing material that may be used for egg production. In the female Zebra Finch this decline amounted to a total of about 70mg lean dry weight. Krementz & Ankney (1986) suggested that in the House Sparrow also, the oviduct acted as a storage organ for protein that is used in egg formation.

4.4.2 Changes in body reserves: Lipid.

Lipid (fat) reserves in the female Zebra Finch also decline under the stress of breeding (Figure 4.10, $r_{44} = -0.795$, $p < 0.0001$). As with protein reserves it can be seen that the bulk of this decline occurs from Day -3 to Day 1. The overall decline in absolute terms was 895mg. The total lipid requirement for a clutch of five eggs is 291.5mg (Chapter 2). Clearly, the loss of lipid from reserves can easily provide for the entire clutch but this suggests the lipid reserves may play a role in egg production beyond supplying the eggs themselves. In the studies of Quelea (Jones & Ward, 1976) and Camaroptera (Fogden & Fogden, 1979) it was suggested that the lipid reserve was used to fuel a change in the foraging strategy of the birds. This meant a switch from energy rich seeds to insects (Quelea) or to calcium rich food items (Camaroptera). The fat reserves could be drawn upon to make up for the deficit in energy intake.

Most of the stored lipid in birds is found in discrete sub-cutaneous depots (Blem, 1990) and the results indicate that these depots are utilised by the female when she is producing eggs. The dry weight of one of the fat bodies, the omentum, decreased significantly (Figure 4.12, $r_{44} = -0.778$, $p < 0.0001$). The lipid levels in the pectoral muscle, which constitute 6.9% of the muscle dry weight, are small by comparison but also decline significantly (Figure 4.11, $r_{44} = -0.587$, $p < 0.0001$). This level of lipid in the muscle is usual for passerines. The lipid content of pectoral muscle in the Sand Martin, for instance, is 7.9% (Jones, G., 1987). Intramuscular lipid was positively correlated with muscle lean dry weight in Eared Grebes when muscle condition was improving in preparation for migration (Gaunt et al, 1990).

In all of the lipid measures taken the timing of the decline was similar to that seen in the lean dry weight of pectoral muscle and the body as a whole, occuring between Day -3 and Day 1 (Figures 4.10b, 4.11b and 4.12b). This is the period when lipid is being laid down in the developing yolks. Demand for lipid increases rapidly from about Day -4 and is maximal on Day 0 (Figure 2.5b).

The overall decline of lipid from the carcass as a whole was 61% of the level recorded on Day -3. This represents a significant usage of lipid reserves. The final level of lipid reserves of 0.570 ± 0.080g is much lower than the mean level of lipid found in non-breeding females, 1.021 ± 0.186g, n = 24. The difference and more must be recovered between breeding attempts. Indeed, in females immediately prior to breeding the mean lipid content of the carcass was 1.5 ± 0.6g.

4.4.3 Change in body reserves: Calcium.

Calcium reserves are known to be drawn upon by many species of bird for shell production. The domestic fowl utilises medullary bone for this purpose (Simkiss, 1975) and to a lesser extent the House Sparrow does also (Krementz, 1983). Being a small bird, the potential for the use of the skeleton as a site for storing calcium in the Zebra Finch is reduced. The total calcium requirement for a five egg clutch is 89mg (Chapter 2). The mean ash weight of a Zebra Finch is 492 ± 9 mg of which only 18.3 ± 0.6% is calcium. This makes the total calcium reserve of a female Zebra Finch 90mg. Supplying calcium for a clutch from bone calcium stores would require a 100% increase in calcium which is highly unlikely. The results suggest that there is little evidence for storage and/or use of calcium reserves for reproductive purposes. The ash weight index did not change significantly during the cycle (Figure 4.9, $r_{26} = 0.297$, $p > 0.05$). Also, the calcium content of the ash of pre-laying females is no different to that in post-laying females ($t_8 = 0.227$, $p > 0.05$). Female Zebra Finches do not appear to draw upon their reserves of calcium when producing a clutch of eggs.

4.4.4 The budgeting of endogenous and exogenous nutrients during egg production

Chapter 2 considered the costs, in terms of protein, lipid and calcium of the production of a clutch of Zebra Finch eggs. In Chapter 3 the diet of the birds while forming eggs was

investigated. An estimate of the contribution that the diet made to the clutch was also calculated. In this Chapter the role of body reserves has been studied. By bringing these elements together it is possible to estimate a budget of exogenous and endogenous sources of material for egg production and their relative importance. For this budget I have used a five-egg clutch because the mean clutch size for the colony was 5.2 ± 0.9, n = 120, and the data in this chapter and Chapter 2 was based on females laying five-egg clutches.

4.4.4.1 Lipid

The lipid content of an egg is 58.3mg (Table 2.1). This is 291.5mg for a five-egg clutch. The energetic value of this is; **2.624 to 2.769 kcal.**

The total decline in lipid reserves resulted in a loss of 895mg of lipid. Using the values for energy content shown earlier, 9.0 to 9.5 kcal/g (Blem, 1990), the total energy that this could provide is; **8.055 to 8.502 kcal**.

In Chapter 3 I estimated that an increase of digestive efficiency, of the order described by El-Wailly (1966), could provide up to 63% of the lipid content of an egg.

It would appear, therefore, that the lipid requirements of the clutch can easily be met by the female finch, even from reserves alone. The excess of loss may be due to the female drawing upon the lipid reserves for energy to fuel normal metabolism and the demands of egg formation. In wild granivorous birds lipid reserves are thought to be critical during breeding. Jones and Ward (1976) reported dead female *Quelea* around breeding colonies after cold nights. These birds had very low lipid reserves. It was suggested that lipid reserves provided an important energetic buffer for the extra demands of egg formation and related behaviour. These birds had run their reserves so low that they had insufficient to survive an unusually cold night. In the artificial conditions of the experimental Zebra Finch colony where energy requirements for normal activity are much reduced, the levels of fat reserves are probably never reduced to the extent that the birds are in any danger. It is likely that the fat reserves of wild Zebra Finches are never as high as those seen in the captive birds.

4.4.4.2 Protein

The total protein content (lean dry weight of yolk and albumen) of a clutch of 5 eggs is **674mg**.

In Chapter 2 the mass of the oviduct was shown to decline from Day 1 to Day 5 of the laying cycle. This could potentially provide 70mg of protein (10.4% of egg protein). The other body reserves, as described above, could potentially provide 518 mg (76.8%). This is based on the decline of lean dry weight in muscles and other organs. Including the oviduct, the total protein that could potentially be available for egg formation and assuming a 100% conversion efficiency, is **588mg** (87.2% of egg requirement). I am aware of no studies which give the conversion efficiency of endogenous nutrients to egg nutrients. Astheimer & Grau (1985) used a figure of 75% for their study of Adelie Penguins. The conversion of exogenous nutrients into egg nutrients has been given as 77% (Brody, 1945). Krementz & Ankney (1985) assumed 100% efficiency and pointed out that their estimates were liberal and Alisaukas & Ankney (1985) also assumed 100% in the absence of any empirical alternative. It is reasonable, therefore, to assume that the efficiency of conversion of endogenous nutrients is at least 77% as the costs associated with protein accumulation and the conversion of digestible carbohydrates to lipid at the time of storage should not be incorporated in the costs at the time of laying.

The other cost of egg formation is the growth of the oviduct. The total protein (lean dry weight) needed for this was **113.8mg**. When taking this into consideration, the decline in body protein reserves could provide a maximum of **74.6%** of the total protein for eggs and oviduct.

Finally, the contribution that the diet makes to the budget. This is limited by the fact that seed is a relatively protein poor food. Based on El-Wailly's (1966) figures for increased digestive efficiency I estimated that an extra 12mg of protein could be liberated each day. If this was sustained for the ten days of the egg formation period a total of **120mg** (15.2% of egg plus oviduct) would potentially be available.

This gives a total of **708mg** which is **89.9%** of the protein required for a clutch of five eggs and the growth of the oviduct.

This leaves a deficit of just over 10% to be accounted for. There are some possible explanations for this. As mentioned earlier it is possible that the digestive efficiency of the birds may have been much higher than the estimate of El-Wailly (1966) and thus the protein available from the diet would be higher. In poultry it is known that when protein is at or below minimum levels the efficiency of absorption increases (Nesheim et al, 1979). Secondly, breeding pairs of Zebra Finches displayed a much lower level of activity compared to control pairs (see Chapter 3). This has been reported for other species, for example, the Camaroptera (Fogden & Fogden, 1979). It is possible that this leads to reduced energy and possibly protein metabolism that allows extra resources to be directed to egg production.

In the feeding trials the mean clutch size of pairs involved was 4.0 ± 0.5 eggs, whereas the colony mean was 5.2 ± 0.9 eggs. The total protein contained in 4 eggs plus the oviduct growth was 653mg. In this case the protein from the diet and body reserves would have been enough to meet the demand. A possible reason for the birds in the feeding trial laying a smaller clutch was that these birds were fed a single seed diet during the experiment compared to the mixed seed that the birds were usually fed. The single seed diet had only 69.6% of the protein available in the mixed seed.

It is apparent, however, that protein reserves play a crucial role in supplying protein for egg production in female Zebra Finches.

Calcium reserves play little or no role in supplying the developing egg shells. Therefore, all of the calcium that is required for the eggs is derived from exogenous sources. The substantial increase in weight loss from the cuttlefish bone and the timing of the increase support this conclusion.

In summary, therefore, it appears that female Zebra Finches are heavily reliant on endogenous reserves of protein for egg production. 74.6% of the total protein requirement

for eggs and oviduct could potentially be obtained from body reserves assuming 100% conversion efficiency. All of the lipid requirements could be met from reserves also. Lipid reserves may also be drawn upon to allow changes in feeding strategy or behaviour when the diet alone cannot provide sufficient. Finally, calcium for the eggs seems to be derived entirely from exogenous sources because the size of the Zebra Finch does not allow for a useful storage capacity of this nutrient.

CHAPTER 5 - CHANGES IN LIPID AND PROTEIN CONTENT OF THE PECTORAL FLIGHT MUSCLES OF FEMALE ZEBRA FINCHES DURING BREEDING

5.1 INTRODUCTION

The preceding chapter presented data indicating that body reserves are playing the major role in supplying protein for the developing eggs of female Zebra Finches. The aim of this chapter is to look more closely at the flight muscles to determine how much and from where in the muscle, protein is being lost.

In common with other studies concerning the changes in body condition associated with breeding (e.g. Alisaukas & Ankney, 1985, Ankney & MacInnes, 1978, Jones & Ward, 1976, Krementz & Ankney, 1986) a simple technique involving the extraction of lipid by a solvent and the calculation of the lean dry weight was used in the previous chapter. This technique generally assumes that lean dry weight is representative of protein content. In studies on the House Sparrow, Jones, M.M. (1979, 1980 and 1991), used a more sophisticated technique to directly measure the protein content of the flight muscle. As the sarcoplasm has been suggested as a possible site for the storage of protein in birds (Kendall et al, 1973) it was necessary to investigate if the decline in lean dry weight observed in the Zebra Finch was caused by a decline of the myofibrillar portion of the muscle or the sarcoplasm, or both. The technique described by Jones, M.M. (1980) allowed this distinction to be made.

In addition to this, work conducted on the Starling (Osborn & Ward, unpublished data) revealed a high molecular weight protein in the sarcoplasm which declined under the stress of breeding, while other detected proteins did not. Amino acid analysis of this protein suggested that the tyrosine, methionine and cysteine content declined faster than the decline in total protein content. It has been proposed that one possible reason for the use of body protein in the production of eggs could be to supply certain amino acids that might be limiting in the diet (Schifferli, 1976). There is some potential for this to be a factor with the Zebra Finch as they eat a diet that is relatively low in protein.

The purpose of this chapter was to obtain an accurate measure of the decline in protein from the pectoral flight muscles of female Zebra Finches while producing eggs. Secondly, it was necessary to identify to what extent each portion of the muscle, sarcoplasm or myofibrillar, was contributing to this observed decline. Thirdly, as there was some evidence to suggest the sarcoplasm as the site of a possible protein store, and indeed of a specific storage protein, the sarcoplasmic extract of the flight muscle was examined by the process of gel filtration.

5.2 MATERIALS AND METHODS

5.2.1 Collection of material for analysis.

Samples of pectoral muscle were obtained as recorded in Chapter 4. In most cases only the left pectoral muscle block was used. However, in some cases both muscles had to be used for biochemical analysis and therefore no lean dry weight of muscle was obtained and the carcass not used for body reserve estimation. Sample sizes are not, therefore, exactly the same as those in Chapter 4.

As before, pre-breeding females were those that had not laid any eggs but possessed developing follicles. These were allocated to the correct day in the laying cycle following the technique described in Chapter 2. All other birds used were those that had laid or would lay (confirmed by examination of the ovary) a five-egg clutch.

5.2.2 Analysis of pectoral muscle tissue

Once the muscle was removed from the bird, it was immediately weighed (to 0.001g). Using a scalpel it was chopped coarsely and 1.00g of the chopped muscle taken for the analysis. The remainder was refrozen and retained.

The 1.00g of muscle from the left pectoral muscle block was placed in a glass tube suspended in ice, with 4ml of 0.25M sucrose in 60mM tris, pH 7.5. The muscle was then homogenised using an Ultra-turax homogeniser for a total of 60 seconds. This was done in twelve 5 second bursts with 35 seconds cooling between each burst to prevent excessive heat build up which could denature proteins. The resulting homogenate was placed into two 15ml Corex tubes that had been pre-weighed to 0.0001g. The homogenate was centrifuged at 10,000rpm at 4^{o}C for 1 hour in an MSE 18 centrifuge (8 x 50ml head).

The supernatent was refered to as the **Water soluble extract** and was taken to represent the contents of the sarcoplasm. The volume of recovered supernatent was recorded (to 0.1ml) and 2ml of this extract was used for gel filtration (see below), the remainder for direct protein determination.

The pellet in each of the corex tubes had 2ml of 0.3M sodium hydroxide added and the pellet resuspended using a glass rod shaped to fit the centirifuge tubes. This was then incubated at 37°C for an hour and then spun at 10,000rpm in an MSE 18 centrifuge (8 x 50ml head) for 30 minutes. The supernatent collected this time was refered to as the **Alkali soluble extract** and is taken to represent the myofibrillar portion of the muscle. The volume recovered was measured to 0.01ml. The tubes containing the pellet were dried at 100°C to constant weight at 0.0001g. This gave the dry weight of the **Insoluble material** of the muscle, which is likely to be mostly collagen (Jones, M.M., 1980).

The water and alkali soluble extracts were then stored frozen in eppendorf vials until analysis.

5.2.3 Analysis of the water soluble extract of pectoral muscle by gel filtration

When the water soluble extract was being prepared a 2ml sample was immediately taken for gel filtration analysis. The sample was kept chilled at all times and the analysis performed at a constant temperature of 4°C.

The gel filtration column used was filled with LKB Ultrogel ACA 34 and had a bed dimension of 87.5cm x 2.6cm. A 60mM tris buffer at pH 7.5 was used throughout, pumping at 30ml per hour. The eluted buffer was monitored at 280nm by an LKB UVCord spectrophotometer. A pen recorder provided a permanent record of each sample, recording absorption at 280nm against time. In this way a characteristic trace for each sample was obtained. Material could be collected in the eluted buffer by means of a fraction collector set to take 5ml fractions. These samples were then read at 280nm on an LKB Ultrospec spectrophotometer to identify those which were to be kept.

A gel filtration calibration kit supplied by **Pharmacia Fine Chemicals** was used for molecular weight determination of the fractions collected. The column used was calibrated using aldolase, catalase, ferritin and Blue Dextran 2000. Manufacturer's instructions were followed throughout to produce a calibration curve that allowed molecular weight to be calculated from the elution volume of the unknown peak. The procedure is as follows;

1 - A K_{av} value for each of the above proteins is calculated using the equation;

$K_{av} = (V_e - V_o) \div (V_t - V_o)$ Equation 5.1

V_e = elution volume for each protein

V_o = column void volume = elution volume of Blue Dextran 2000

V_t = total gel bed volume

2 - Using semi-logarithmic graph paper, the K_{av} value for each of the protein standards (on the linear scale) against the corresponding molecular weight (on the logarithmic scale). K_{av} of ferritin, catalase and aldolase was 0.15, 0.26 and 0.41 respectively.

3 - The regression equation can be calculated from the graph and used to convert elution volumes to molecular weight. The equation obtained was;

log Molecular Weight = $-1.676(K_{av}) + 5.860$ (Equation 5.2)

5.2.4 Protein content of water and alkali soluble extracts of pectoral muscle

The protein content of each of the extracts was calculated using the same technique used in Chapter 3 for protein content of seed. Standards were made using 2mg/ml bovine serum albumen (BSA) and a standard curve plotted as before, which allowed conversion of measured absorbancy at 750nm to μg of protein per 100μl.

Each sample was diluted 1:50 using 0.1M sodium hydroxide to bring the samples within the calibration range. 100μl aliquots of the diluted sample were assayed in duplicate throughout.

5.3 RESULTS

5.3.1 Protein content of the water soluble and alkali soluble extracts of pectoral muscle

The technique used to analyse the muscle produced two fractions of the pectoral muscle; the water soluble extract was representative of the proteins found in the sarcoplasm and the alkali soluble extract was representative of the proteins of the contractile elements of the muscle (Jones M. M., 1979). The protein content of each extract from 56 female Zebra Finches was measured. Figure 5.1 shows the mean values for sarcoplasm protein over the laying cycle, up to Day 5 when the last egg was laid (As in Chapter 4 all figures show means with standard deviation for each day of the laying cycle. The regression equations were calculated from all data points). The decline in protein content is significant, r_{54}= -0.865, $p < 0.0001$. In common with the results for pectoral muscle lean dry weight, the decline in protein content occurs mostly from Day -3 to Day 1 (Figure 5.1b), r_{44}= -0.976, $p < 0.0001$.

A very similar pattern was found for the myofibrillar protein (Figure 5.2), $r_{54} = -0.862$, $p < 0.0001$, over the whole cycle and from Day -3 to Day 1 (Figure 5.2b), r_{44}= -0.982, $p < 0.0001$.

The timing of both of the declines is similar and the combined mean values for protein in both of the extracts is shown in Figure 5.3, $r_{54} = -0.873$, $p < 0.0001$. Figure 5.3b shows the period Day -3 to day 1, r_{44}= -0.993, $p < 0.0001$. The dry pellet of residual material from each of the muscles was weighed to determine if any component of the muscle other than the protein was also declining. The mean dry weight of the pellets on each of the days across the laying cycle (Figure 5.4) does not decline at all, $r_{54} = -0.338$, $p > 0.05$. Therefore, it appears that it is only protein that is being lost from the flight muscles during the laying cycle and this implies that the decline in the measured lean dry weight (Chapter 4) is due to levels of protein.

The absolute values of protein that these declines represented were calculated so that the relative importance of the sarcoplasmic and myofibrillar extracts in supplying protein could be determined;

Water Soluble Extract

The following calculation was used to estimate the average quantity of protein that was lost from the water soluble extract of the flight muscles of the 56 birds used. The calculation was made using mean values and as such is intended only to provide a mean figure so that a comparison between sarcoplasmic and myofibrillar protein could be made.

Difference in mean, Day -3 to Day 5 = 25.4 - 11.4 = 14 μg/100μl
Mean recovery of extract = 2.5 ± 0.02 ml, n=56
Mean wet weight of right pectoral muscle = 1.500 ± 0.010g, n=110

0.25ml of the extract was diluted to 12.5ml and 100μl aliquots of this dilution were assayed. Therefore, the quantity of protein in 0.25ml of extract is;

(12.5/0.1) x 14.0 = 1750μl = 1.75mg

The mean recovery of extract was 2.5ml, therefore the total sarcoplasmic protein obtained from 1.00g of muscle was;

(2.5/0.25) x 1.75 = 17.5mg

The total pectoral muscle (both left and right side) therefore loses;

(1.500 x 2) x 17.5 = **52.5mg** of sarcoplasm protein.

Alkali Soluble Extract

Exactly the same calculation was used to estimate the amount of protein that the myofibrillar part of the muscle would have lost over the laying period.

Difference in Mean, Day -3 to Day 5 = 27.1 - 15.9 = 11.2 μg/100μl
Mean recovery of extract = 3.9 ± 0.01 ml, n=56.

Applying the above calculation to these figures gives the following;

Protein in 1.00g of muscle = 21.84mg

Protein in total flight muscle = (1.5 x 2) x 21.84 = **65.52mg**

The combined loss of sarcoplasm and myofibrillar protein from the flight muscles is **118.02mg**.

5.3.2 Gel filtration analysis of water soluble extract of pectoral muscle

The water soluble extract was passed through an AcA 34 gel that separated the contents on the basis of their molecular weight. The eluted buffer was monitored at 280nm with the spectrophotometer zeroed on the tris buffer used. This meant that a distinctive trace was produced for each sample on the pen plotter as it was eluted. Figure 5.5a and b show examples of typical traces obtained for the Zebra Finch water soluble extract. Figure 5.5a is from a female immediately prior to laying condition and Figure 5.5b is a post-laying female.

A number of peaks are defined and were labelled 1 to 3. The absorbancy at 280nm (A_{280}) of each peak was recorded for each of the birds analysed in this way (33). However, it was found that only the first of these peaks (Peak 1) declined during the laying cycle (r_{31} = -0.828, $p < 0.0001$), while Peak 2 and Peak 3 did not change significantly (r_{31}= 0.097, $p > 0.05$, r_{31}= 0.074, $p > 0.05$, respectively). Figure 5.6a-c shows the mean A_{280} of Peak 1, 2 and 3 during the laying cycle.

Figure 5.7 is the period of Day -3 to Day 1 of Peak 1 absorbancy at 280nm (r_{25}= -0.941, $p < 0.0001$). The major part of the decline in A_{280} came during this period in common with the measured protein content of this fraction of the pectoral muscle (Figure 5.1b).

The K_{av} values for the three peaks were determined so that molecular weight could be estimated using the equation (Equation 5.2) obtained from the calibration curve. Peak 1 lay very close to the exclusion limit of the column with a mean K_{av} of 0.05 ± 0.001, n=33. This meant that it must have a molecular weight of over 400,000 but an accurate estimate is not dependable when that close to the limit.

Peak 2 had a mean K_{av} of 0.65 ± 0.02, n=33. This gives a molecular weight of 59,000. Peak 2 also absorbed heavily when read at 418nm whereas the other peaks did not. It is likely that Peak 2 is avian haemoglobin, the human equivalent has a similar molecular weight of approximately 64,500 (Stryer, 1981).

Peak 3 had a mean K_{av} of 0.99 ± 0.02,n=33, giving a molecular weight of around 16,000. It is likely that this is myoglobin. Human myoglobin has a similar molecular weight of 17,900 (Stryer, 1981).

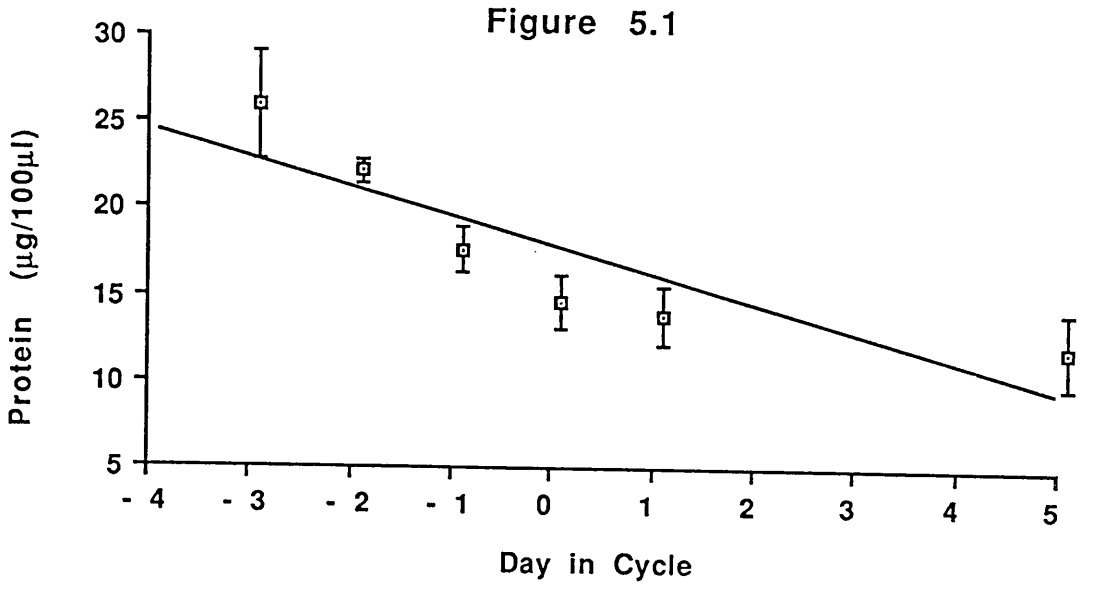


Figure 5.1

Protein content (µg/100µl of extract) of water soluble (sarcoplasmic) extract of the pectoral flight muscle of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 17.100 - 1.655x$, $r_{54} = -0.865$, $p<0.0001$

Day	-3	-2	-1	0	1	5
n	6	16	8	12	4	10

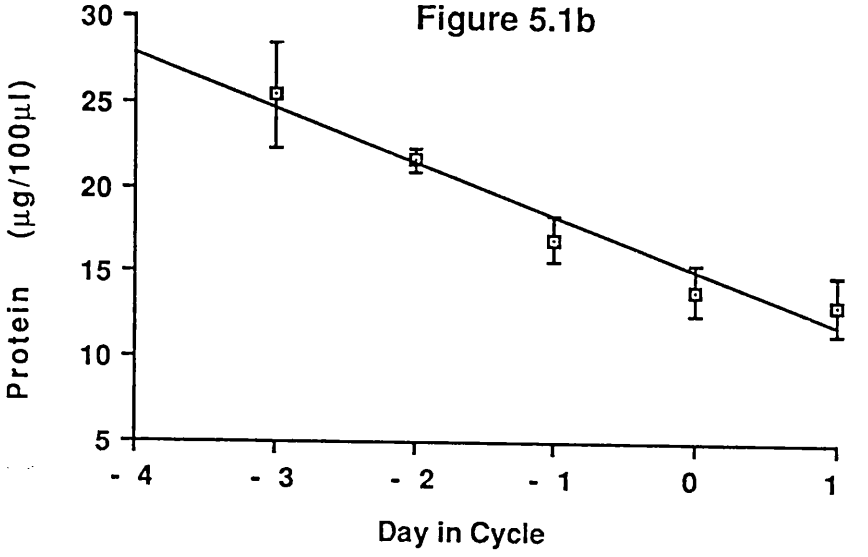


Figure 5.1b

Protein content (µg/100µl of extract) of water soluble (sarcoplasmic) extract of the pectoral flight muscle of female Zebra Finches during Day -3 to Day 1 of the laying cycle only (mean ± s.d.)

$y = 15.040 - 32.000x$, $r_{44} = -0.976$, $p<0.0001$

Day	-3	-2	-1	0	1
n	6	16	8	12	4

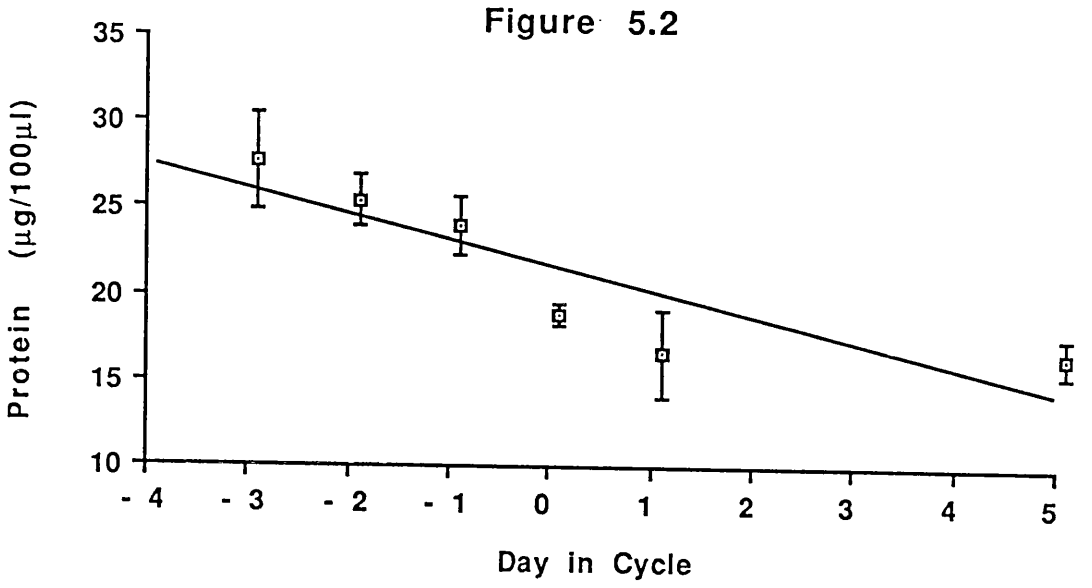


Figure 5.2

Protein content (µg/100µl of extract) of alkali soluble (myofibrillar) extract of the pectoral flight muscle of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 20.883 - 1.468x$, $r_{54} = -0.862$, $p<0.0001$

Day	-3	-2	-1	0	1	5
n	6	16	8	12	4	10

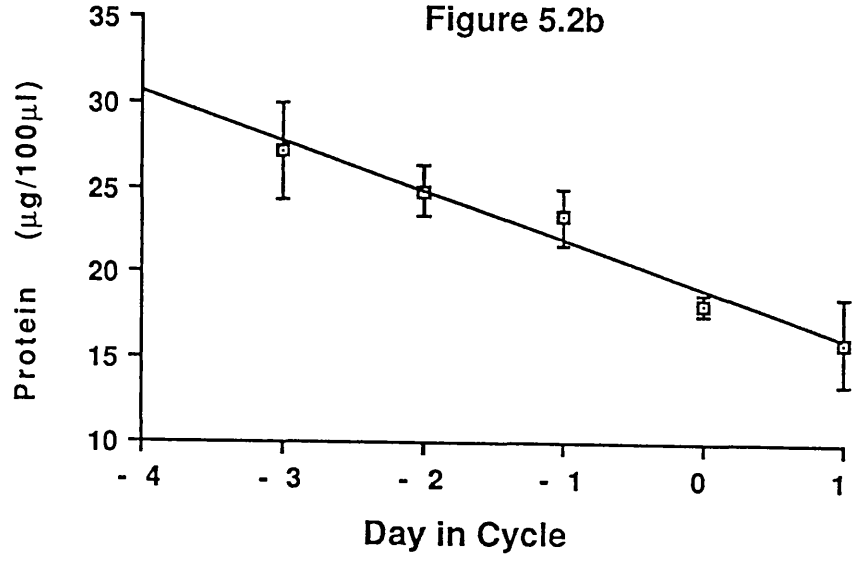


Figure 5.2b

Protein content (µg/100µl of extract) of alkali soluble (myofibrillar) extract of the pectoral flight muscle of female Zebra Finches during Day -3 to Day 1 of the laying cycle only (mean ± s.d.)

$y = 19.000 - 2.880x$, $r_{44} = -0.982$, $p<0.0001$

Day	-3	-2	-1	0	1
n	6	16	8	12	4

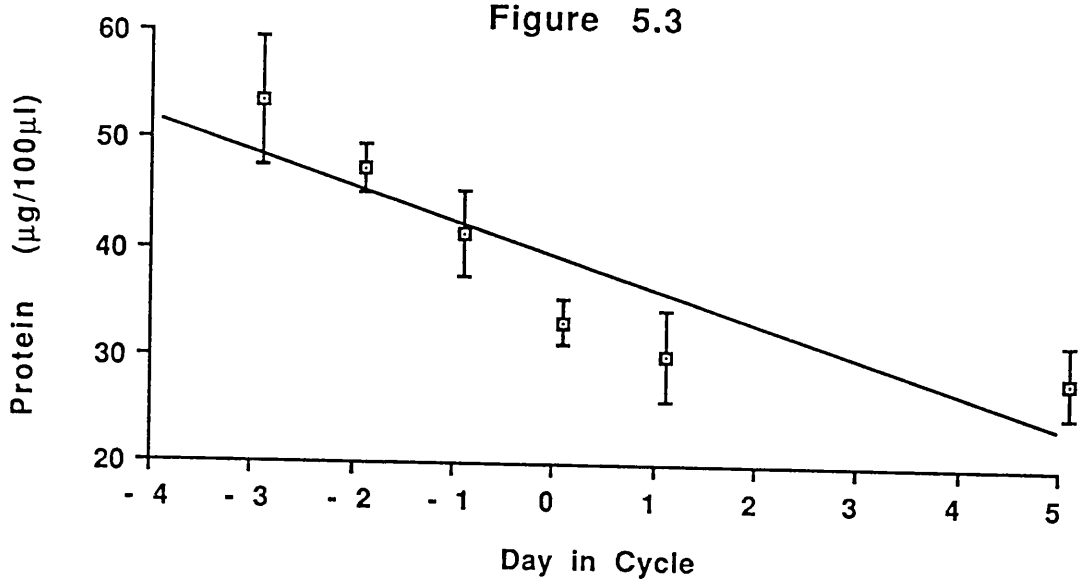


Figure 5.3

Total protein content (µg/100µml of extract) of the pectoral flight muscle extracts (water and alkali soluble) of female Zebra Finches during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 37.983 - 3.122x$, $r_{54} = -0.873$, $p<0.0001$

Day	-3	-2	-1	0	1	5
n	6	16	8	12	4	10

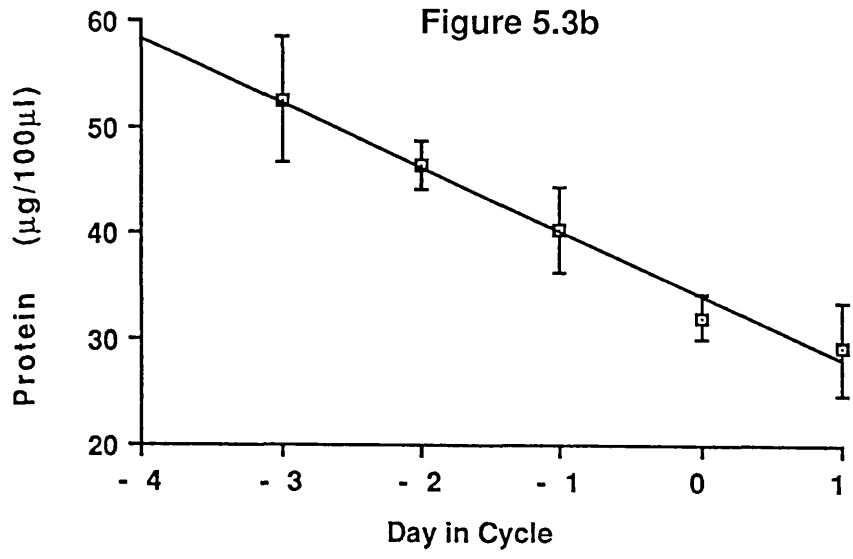


Figure 5.3b

Total protein content (µg/100µl of extract) of the pectoral flight muscle extracts (water and alkali soluble) of female Zebra Finches during Day -3 to Day 1 of the laying cycle only (mean ± s.d.)

$y = 34.040 - 6.080x$, $r_{44} = -0.993$, $p<0.0001$

Day	-3	-2	-1	0	1
n	6	16	8	12	4

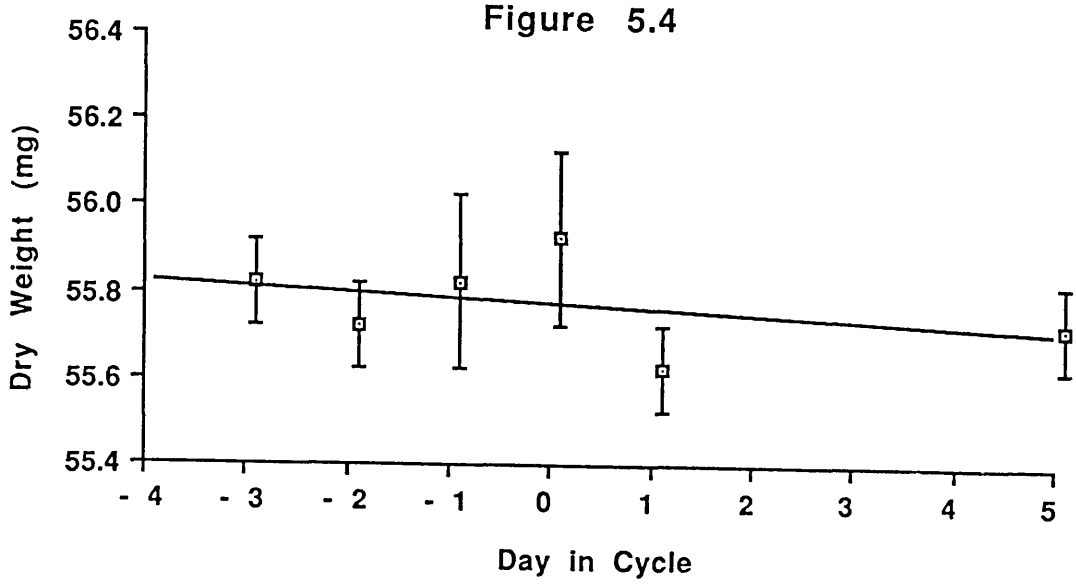


Figure 5.4

Dry weight (mg) of the residual pellet of pectoral flight muscle of female Zebra Finches, after extraction of protein, during the laying cycle of a five-egg clutch (mean ± s.d.)

$y = 55.750 - 0.012x$, $r_{54} = 0.338$, $p>0.05$

Day	-3	-2	-1	0	1	5
n	6	16	8	12	4	10

Figure 5.5a

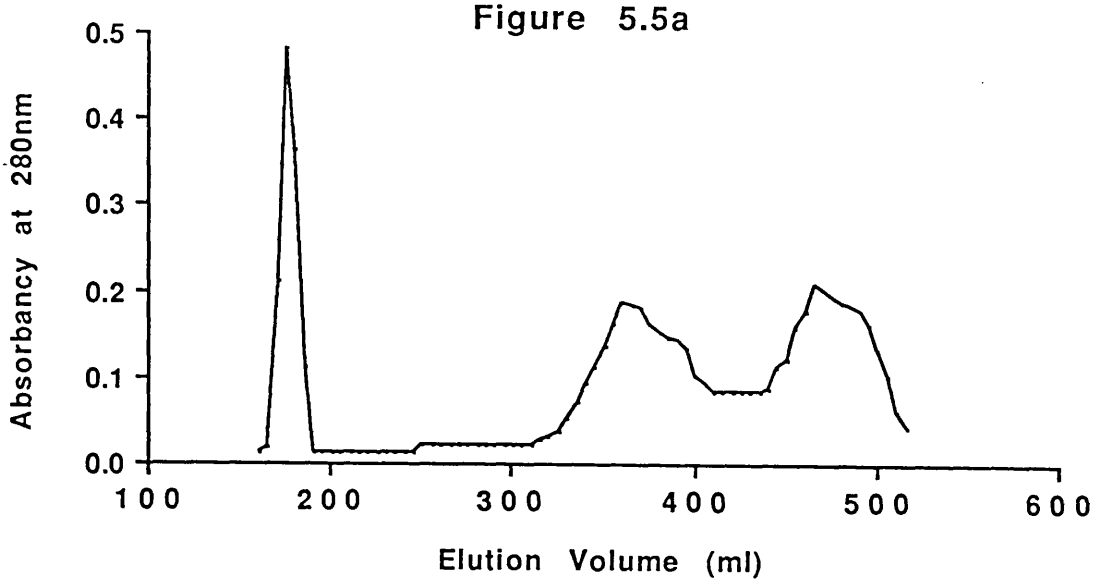


Figure 5.5b

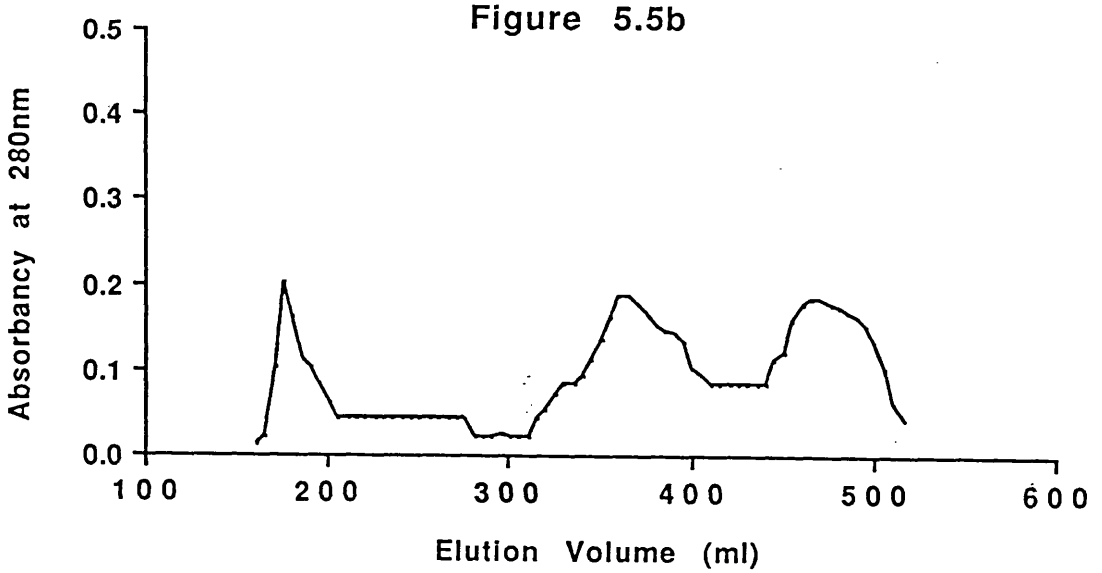


Figure 5.5

Typical gel filtration profile of the water soluble extract of female Zebra Finch pectoral flight muscle monitored at 280nm
a) Pre-laying female
b) Post-laying female

Figure 5.6

a) Absorbancy at 280nm of Peak 1 of water soluble (sarcoplasmic) extract of the pectoral muscle of female Zebra Finches on each day of the laying cycle of a five-egg clutch

$y = 0.293 - 0.023x$, $r_{31} = -0.823$, $p < 0.0001$

b) Absorbancy at 280nm of Peak 2 of water soluble (sarcoplasmic) extract of the pectoral muscle of female Zebra Finches on each day of the laying cycle of a five-egg clutch

$y = 0.181 - 0.002x$, $r_{31} = -0.097$, $p > 0.05$

c) Absorbancy at 280nm of Peak 3 of water soluble (sarcoplasmic) extract of the pectoral muscle of female Zebra Finches on each day of the laying cycle of a five-egg clutch

$y = 0.021 + 0.002x$, $r_{31} = 0.074$, $p > 0.05$

Day	-3	-2	-1	0	1	5
n	5	6	6	6	4	6

Figure 5.6a

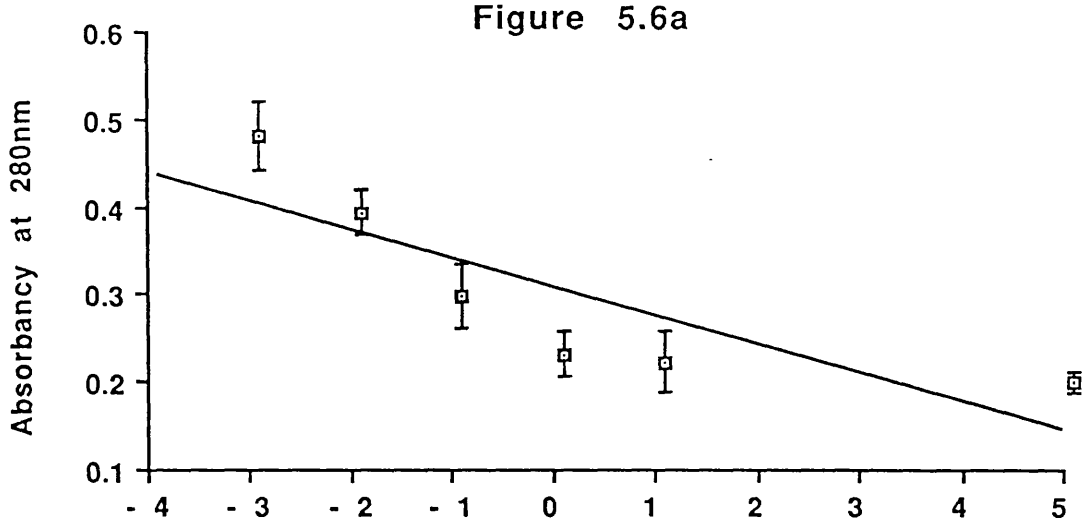


Figure 5.6b

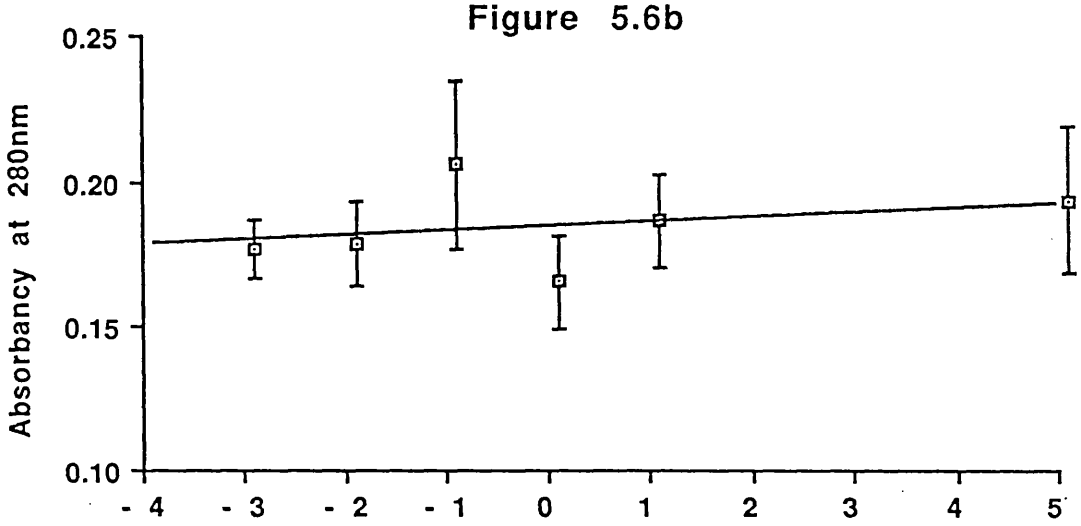
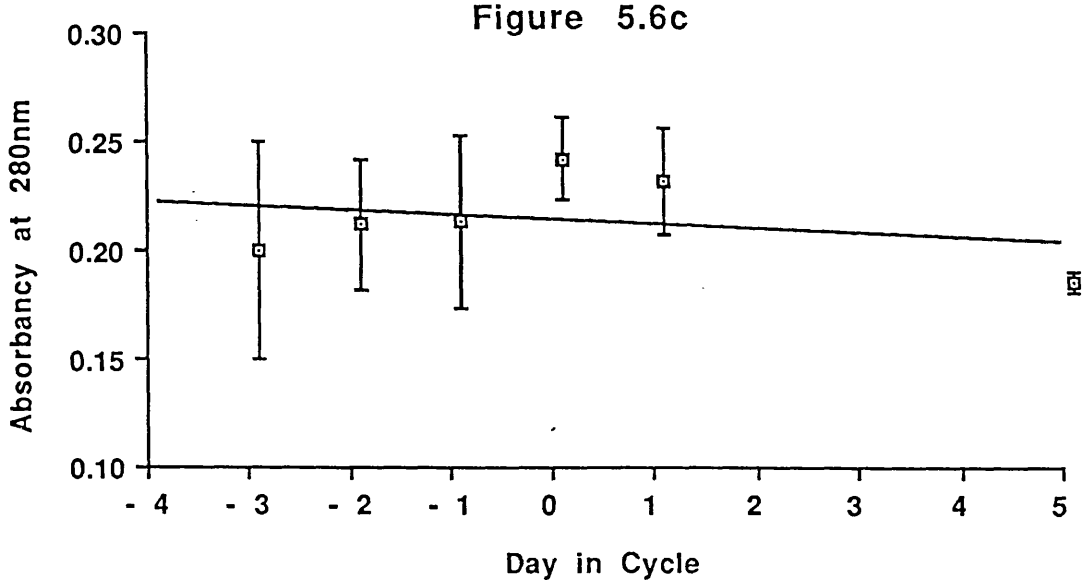


Figure 5.6c



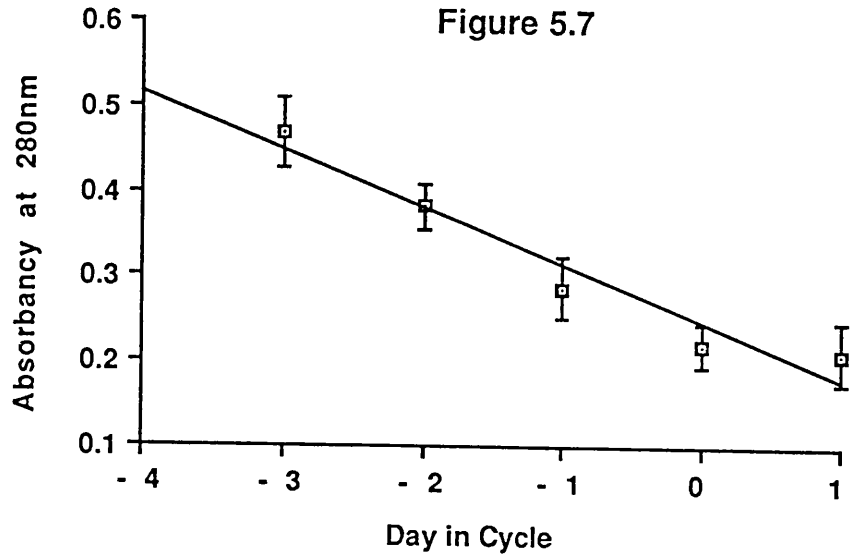


Figure 5.7

Absorbancy at 280nm of Peak 1 of water soluble (sarcoplasmic) extract of the pectoral muscle of female Zebra Finches from Day -3 to Day 1 of the laying cycle of a five-egg clutch

$y = 0.246 - 0.068x$, $r_{25} = -0.941$, $p < 0.0001$

Day	-3	-2	-1	0	1
n	5	6	6	6	4

5.4 DISCUSSION

5.4.1 Loss of protein from water soluble and alkali soluble extracts of pectoral muscle

The measured protein content of both of these fractions was seen to decline significantly across the laying cycle (Figure 5.1 & 5.2). However, it was the period from Day -3 to Day 1 of the cycle that saw the majority of this decline (Figure 5.1b & 5.2b). This timing closely matches the pattern that was seen in the measurements of lean dry weight of the pectoral muscles (Figure 4.1b & 4.2b). Figure 5.3 & 5.3b illustrates the same pattern for the combined values of the alkali and water soluble extracts.

The residual pellet dry weight did not change during the laying cycle (Figure 5.4). The closeness of this pattern of decline with data for lean dry weight leads to the conclusion that protein is being lost from the flight muscle at this time and not some other material. In addition, the quantity of protein that was lost from the muscle measured directly by the described technique compared very closely with the estimate in Chapter 4 which was derived independently from lean dry weights. The difference between Day -3 and Day 5 was **118.02mg** for the measured protein loss from pectoral muscle presented here and the estimate based on lean dry weight data given earlier suggested a loss of **102mg**. The closeness of the estimates implies that the lean dry weight of muscle is a good indicator of protein content.

In her study of House Sparrows, Jones, M.M. (1979 & 1991), showed that they too lost protein from the pectoral flight muscle under the stress of breeding. However, this loss was attributed only to the myofibrillar fraction of the muscle and no significant decline was found in the sarcoplasmic fraction. The techniques used by this study and the House Sparrow study were very similar in most respects but sample sizes in the present study were considerably larger (56 compared to 28). It is possible that there was indeed a significant decline in sarcoplasmic protein from Day -2 to Day 3 of the Sparrow study which would coincide with the period of peak demand for protein in laying House Sparrows (Krementz, 1984, Schifferli, 1976). This could have been obscured by considering the perios from Day -4 rather than Day -3, as I did, as the peak protein condition in House Sparrows actually occurs on Day -3.

The loss of protein from the female Zebra Finch flight muscle amounts to 15% of the total protein requirements of a five-egg clutch plus the growth of the oviduct. In relation to the budget for egg protein requirements presented in Chapter 4, it would be possible to revise upwards the estimate of the contribution possible from the body reserves, from 74.5% to 76.6%.

5.4.2 Gel filtration of the water soluble extract of pectoral muscle

Kendall et al, (1973) proposed the sarcoplasm as a possible site for the storage of protein that was labile and available to be drawn upon when protein was in demand, such as when forming eggs. It was assumed that the decline seen in the myofibrillar protein content of the muscle reflected a loss from the contractile proteins, actin and myosin. The surrounding sarcoplasm may contain several proteins such as haemoglobin, myoglobin and various enzymes essential for the functioning of the muscle. The gel filtration technique allowed these proteins to be separated so that they could be measured individually. The results showed three proteins that occured in the sarcoplasmic fraction in high concentration Figure 5.5. Two of these were likely to have been haemoglobin (Peak 2) and myoglobin (Peak 3). Neither of these appeared to diminish significantly under the stress of laying (Figure 5.6b and c respectively). However, the first peak, Peak 1 (Figure 5.6a) did decrease significantly during the laying cycle and, once again, the greater part of the decline had occured by Day 1 (Figure 5.7). This material was of high molecular weight and similar characteristics to material that was isolated from the flight muscle of Starlings by gel filtration which also displayed a decline across the breeding period (Osborn & Ward, unpublished data).

It has been suggested that one possible reason for body protein being used in the development of eggs could be for the supply of amino acids that are normally limiting in the birds' diet (Schifferli, 1976). The data from the Starlings suggested that this may be occuring in them as the content of cysteine, methionine and tyrosine in the Peak 1 material declined faster than the overall decline in protein. Unfortunately, the technique used in this study of Zebra Finches did not allow the investigation of the amino acid content of Peak 1

material because not enough of it was recovered for the analysis to be successful. More work is therefore necessary to further characterise this material.

5.4.3 Conclusion

The results of the biochemical analysis of pectoral flight muscle suggest that the loss of lean dry weight during the laying cycle is due to a loss of protein. If this protein was available to the eggs then it could potentially supply up to 15% of the protein content of a five-egg clutch and the development of the oviduct.

Both the myofibrillar and sarcoplasmic fractions of the flight muscle lose protein. Further analysis of the sarcoplasmic fraction revealed one particular protein that declined while the others did not.

This trend is likely to be followed by the other muscles as it was found that leg muscle also declined in weight at the same time as pectoral muscles (Figure 4.4). The total decline of body lean dry weight will be due mainly to the skeletal muscles and as shown above this loss is probably protein.

What then was the fate of the protein being lost from the muscles? It is known that muscle is not a static tissue. The turnover of protein in human skeletal muscle has been measured at 12% per day (Spargo et al, 1979). The concept of protein being transferred from one muscle to another has also been discussed. In studies of geese during their moult it was suggested that the decline in the protein content of the flight muscles during the flightless period allowed hypertrophy of the leg muscles. These displayed a corresponding increase in weight (Ankney, 1984). The possibility exists, therefore, that protein from the skeletal muscles in the breeding female Zebra Finches was being transfered directly to developing eggs. The purpose of the following chapter was to demonstrate if such a process was occuring.

CHAPTER 6 - THE TRANSFER OF ISOTOPE LABELLED MATERIAL FROM BODY TISSUE TO THE DEVELOPING EGGS OF BREEDING FEMALE ZEBRA FINCHES

6.1 INTRODUCTION

In the preceding chapters it has been shown that when female Zebra Finches produce a clutch of eggs there is a decline in their lipid and protein condition. One possible explanation for this may be the direct transfer of nutrients from the body tissues to the developing eggs. This would be instead of, or in addition to, nutrients derived from the food intake.

This use of endogenous nutrients has been suggested before (eg Jones and Ward, 1976, Ankney and MacInnes, 1978, Jones, G., 1987, Jones, M.M.,1991). In Chapter 4 it was shown that in female Zebra Finches, body reserves of protein could play a major role in supplying the developing eggs providing up to 74.5% of the protein required for a five-egg clutch including that required for the growth of the oviduct.

There is not, however, any evidence that this direct transfer actually occurs. The purpose of this chapter was to investigate the use of radioisotopes to demonstrate the transference of proteins from the tissue of female Zebra Finches to the developing eggs.

The amino acid methionine containing the sulphur isotope ^{35}Sulphur (^{35}S) was used. This isotope is relatively safe to handle and has a 87.4 day half life which is long enough to make it suitable. The amino acid Cysteine also contains sulphur but was not considered suitable for this purpose because on oxidation the sulphur atom is lost (Stryer, 1981). This does not occur in methionine and, therefore, more of the isotope was likely to be incorporated into proteins in the bird. Also, cysteine can be synthesised from methionine, but not vice versa. As mentioned in Chapter 3, cysteine and methionine may be among the amino acids limiting in the diet of the Zebra Finch. It may, therefore, be expected to be involved in transferance from tissues to the eggs.

In this way it was hoped that labelled amino acid ingested by a female Zebra Finch some days before the start of breeding would be incorporated into proteins in its own tissue. Once breeding was initiated and the birds were on an unlabelled diet, any isotope that could subsequently be detected in eggs laid would provide evidence that direct transfer from tissues to the eggs does occur.

6.2 MATERIALS AND METHODS

Each bird was given a total 2 microcuries (μCi) dose comprising five daily doses of 0.4 μCi ^{35}Sulphur-methionine. The required amount of labelled amino acid was diluted in 0.05ml of distilled water and this was introduced directly into the birds' stomach via a narrow bore silicon tube fitted to a syringe. All birds were killed with CO_2 eleven days after the final dose.

6.2.1 Dissection and analysis of tissue

The dissection of the bird and the analysis of the tissue was carried out as swiftly as possible. The external measurements were recorded (see Chapter 4) then the left pectoral muscles were removed. The right pectoral muscles were retained frozen and the sternum and sternum-coracoid lengths were recorded. Other tissues for analysis were removed where appropriate (see below). The ovary was exposed and the diameter of enlarged follicles and the presence of post-ovulatory follicles in the ovary were recorded to allow the allocation of the bird to the correct day in the reproductive cycle. The ovary and oviduct were then removed for analysis.

The tissues were weighed in glass vials and distilled water added in the ratio of 1ml to 1g wet weight of tissue. The larger tissues, such as muscle, were homogenised by Ultra-turax for 10 seconds, whilst the smaller tissues, such as ovary, needed only to be chopped coarsely with scissors or a scalpel. **Amersham** NCS tissue solubiliser was then added, the volume being six times that of the tissue/water homogenate. The samples were incubated at 50°C until the solution became clear, which usually took about eight hours. The pH was reduced to pH6-7 with glacial acetic acid added dropwise. 1ml aliquots were then taken for analysis. For most samples the dilutions involved led to each aliquot containing 0.05g wet weight of tissue. Some samples, however, weighed less than 0.05g (eg the undeveloped ovaries). In such cases the technique was modified by dissolving the whole tissue in 1ml of tissue solubiliser (the minimum amount required to be effective). This meant that duplicates were not available. All results are corrected as for 0.05g wet weight of tissue.

Each 1ml aliquot was put into a plastic scintillation vial and 9ml of **Amersham** OCS scintillation fluid added. The vials were shaken and then kept in the dark overnight to allow the effects of chemiluminescence to abate. All samples were then counted on a **Phillips** PU4700 liquid scintillation counter for ten minutes.

Analysis of eggs differed slightly from the procedure for other tissues. In the first experiment the whole egg contents were analysed. Each egg was cracked into a glass vial and 5ml of tissue solubiliser was added in which the eggs readily dissolved at room temperature. The clear solution was neutralised to pH6-7 with glacial acetic acid and 1ml aliquots taken. 9ml of scintillation fluid was added and the samples were then counted as described above.

In the second experiment eggs were separated into yolk and albumen. A small hole was pierced in the air space at the top of the egg and then it was placed in an oven at 100°C for an hour. Once the contents had hardened the yolk was separated from the surrounding albumen. 3ml of tissue solubiliser was added to the samples and kept at room temperature until the solution was clear. Glacial acetic acid was used to neutralise to pH6-7 and then 1ml aliquots were taken for analysis as described above.

6.2.2 Conversion of counts per minute (CPM) to disintegrations per minute (DPM)

In order to compare the different samples it was necessary to convert the counts per minute (CPM) value, measured by the scintillation counter, to disintegrations per minute (DPM). A major disadvantage of scintillation counting is that of quenching, where chemical interference with the scintillant can result in a depression of the scintillation reading. Each sample may be affected to a different degree. This makes it necessary to determine the counting efficiency of the samples at various levels of quenching. Standardisation of my samples was achieved by the Channels Ratio technique which is less time consuming than other methods but is suited to even high levels of quenching. As the level of chemical quenching increases in the sample the effect on the scintillant is to alter the amplitude of the energy content of the light pulses, and if readings are taken at two energy levels the effect of increased quenching is to shift the spectrum towards the lower channel. The ratio of

counts over two channels can therefore be used to determine the level of quenching from the construction of a calibration curve.

A standard was prepared using ^{14}Carbon of a known activity, 1900 disintegrations per minute per ml (DPM/ml). 1ml of standard was added to 9ml of scintillation fluid, as used throughout the experiments. This was counted for 10 minutes in the scintillation counter and counts per minute (CPM) and the channels ratio (CR) values recorded. Using chloroform as a quenching agent I added 50 µl increments to the standard, giving values of CPM and CR for each from 0µl to 200ml and then 100µl increments from 100µl to 1200µ. By comparing the CPM recorded at each increment with the known activity of 1900 DPM, a percentage efficiency for each was calculated. Plotting the channels ratio against the percentage efficiency gave a quench curve, the regression equation of which could be used to convert CPM to DPM (Figure 6.1)

Regression equation; $y = 89.396 - 87.089x$, $R^2_{15} = 0.995$ Equation 6.1

x = channels ratio, y = % Efficiency = (CPM/DPM x 100)

This gives the equation;

DPM = CPM ÷ (0.894 - 0.871 CR) Equation 6.2

6.2.3 Comparison of breeding and non-breeding females

The intention of this experiment was firstly to determine whether there were differences in the amount of labelled material present in body tissues between females that had laid a clutch of eggs and a control group who had not. Secondly, did the eggs that were laid contain detectable levels of isotope?

Twenty-eight females were dosed with isotope as described above. Fourteen were introduced to males and allowed to breed. The nest boxes were inspected regularly and whenever an egg was found it was removed, marked with pencil and replaced with a plaster dummy. The remaining fourteen females were kept in similar conditions but were not

allowed to breed. All birds were taken on the eleventh day after the final dose. The birds were dissected as described and the pectoral muscle, liver, ovary and oviduct plus whole eggs were analysed.

6.2.4 Isotope uptake and decline in non-breeding females

The aims of this experiment were to determine how much of the total 2μCi dose of isotope was incorporated into the tissue by the day after the last dose of 0.4μCi and, secondly, to determine the rate of loss of labelled material from the tissue of non-breeding birds during the subsequent eleven days of the experiment.

Twenty-three females had isotope administered. Three were taken on the day after the last dose, three on the fourth day after and three on the eighth day. The results from the 14 non-breeding females in the first experiment were used for the eleventh day values.

The tissues analysed were pectoral muscle, leg muscle (as described in Chapter 4), ovary and oviduct. The leg muscle was analysed to allow a comparison of two different muscles to see if differences in muscle metabolic rates were reflected in the amount of labelled material lost or incorporated. Liver was not analysed as logistical constraints meant that four tissue types were the maximum that could be analysed in each experiment.

6.2.5 Distribution of isotope in the eggs

The aim of this experiment was to determine where in the egg labelled material was being deposited, the yolk or the albumen. Ten females were given the 2μCi dose and then introduced to a mate and allowed to produce a clutch of eggs. The nest boxes were monitored for eggs and those found were removed, marked with pencil and replaced with a plaster dummy. The yolk and albumen were separated and analysed separately.

6.3 RESULTS

For the sake of clarity the results are presented in an order slightly different from the order in which the experiments were actually done. First, I shall consider the uptake and decline of isotope in non-breeding females, secondly, the differences between non-breeding and breeding females. Finally, the results from analysis of the eggs are presented. All means are given with standard deviation.

6.3.1 Incorporation of isotope in the tissue

Three birds were taken on the day after the last dose of isotope was given. The total dose was 2μCi. By considering the mean level of isotope in the four tissues analysed it was possible to estimate roughly how much of the total dose had been incorporated. The isotope was contained in methionine and as such it was targeted at incorporation into proteins. I therefore used the mean wet body weight minus the main non-protein components (ash and fat) from the results in Chapter 4 for this estimation.

Calculation;

Mean DPM/0.05g for Pectoral muscle,
leg muscle, ovary and oviduct = 2612 ± 531

Mean (n=109) lean/ash free body weight of female = 13.4 ± 1.4g

DPM/g = 20 x 2612 = 52240,

Therefore, DPM/female = 13.4 x 52240 = 700016 DPM (divide by 60)
= 11667 DPSecond

1 DPsecond = 1 Bequerel (Bq), therefore = 11.667 kBq per bird

1μCi = 37kBq, therefore, 11.667/37 = 0.32 μCi

Percentage of total dose incorporated in bird = 0.32/2 x 100 = **16%**

Therefore, an estimated 16% of the isotope administered became incorporated in tissue one day after the final dose. This amount of incorporation gave easily measurable levels of labelled material in the tissue. Control samples of tissues from non-dosed birds gave a mean background reading of 22.4 ± 2.9 DPM/0.05g wet tissue (n=16).

6.3.2 Decline of isotope in tissue with time

All four tissues analysed displayed a decline in the level of isotope present over the 11 day period after the isotope had been administered. All figures show only the mean and standard deviation for each day but regression statistics were calculated from all data points. Figure 6.2 shows a significant decline (r_{21} = - 0.986, $p < 0.0001$) in isotope level in pectoral muscle. The amount lost was 62.1% of the total present immediately after dosing.

Activity in the leg muscle one day after, four days after and eight days after the final dose is shown in Figure 6.3 (r_7 = -0.940, $p < 0.05$). Whereas pectoral muscle had lost 1031 DPM/0.05g (34.9%) by the eighth day, leg muscle had lost significantly less, 514 DPM/0.05g (18.2%), t_4 = 2.875, $p < 0.05$. The amount of isotope in the two muscle types was, initially, similar (t_4 = 0.792, $p > 0.05$). The implication of this result is that labelled material was being lost from the pectoral muscle more rapidly than from the leg muscle.

The oviduct (Figure 6.4) contained much less labelled material immediately after dosing than the muscles (1836 ± 155 DPM/0.05g)(t_4 = 7.844, $p < 0.001$). The proportion of labelled material lost over the 11 days (61.0%) was similar to that lost from the pectoral muscles (62.1%), r_{21}= -0.993, $p < 0.0001$.

The ovary (Figure 6.5) contained similar levels of labelled material to the muscles at the start of the experiment (t_4 = 0.142, $p > 0.05$) but the percentage lost up to the eleventh day was much higher, at 82.5% (r_{21}= -0.938, $p < 0.0001$), than for any of the other tissues.

The loss of labelled material from tissue would be expected because of two factors. Firstly, the isotope decays radioactively and, secondly, normal protein turnover in tissues would lead to some loss. In order to estimate the relative importance of isotope decay I used the

^{35}S decay tables (Amersham, 1990) to calculate the expected loss of activity by this means during the experiment. Using values for pectoral muscle as an example;

Total loss of activity = 2956 - 1120 = 1836 DPM/0.05g

Ten days of radioactive decay would result in activity of;
*0.924 x 2956 = 2731 DPM/0.05g
(* From Amersham decay tables for ^{35}sulphur)

Therefore the loss due to decay is;
2956 - 2731 = 225 DPM/0.05g

This value is only 12.2% of the total loss. Therefore, the majority of the loss of labelled material from tissue is probably due to factors other than isotope decay.

6.3.3 Comparison of non-breeding and breeding females

Table 6.1 presents isotope levels in the four tissues analysed; pectoral muscle, liver, oviduct and ovary. There was a significant difference between the two groups in pectoral muscle and oviduct but not in ovary or liver. There was, therefore, a greater loss of ^{35}S methionine from muscle and oviduct tissue of breeding birds than from the tissues of non-breeders.

6.3.4 Occurrence of isotope in the eggs

In the first experiment whole egg contents were analysed. Fourteen females were introduced to males and six pairs produced a five-egg clutch and eight pairs laid six-egg clutches. As the amount of isotope in the female was declining daily (see above) it was necessary to correct for this when considering the eggs in their laying sequence. The first egg laid had a larger quantity of isotope available to it than the next egg and so on through the clutch. Taking into account the four tissues in which decline was measured, I corrected for a decline in availability of isotope of 5.6% per day (the mean decline of the four tissues over ten days was 56%). It was also assumed that there was no decline in egg size through the clutch, as was the case in the clutches analysed in Chapter 2.

The results showed that there were measurable quantities of isotope present in all the eggs. In addition, there was a significant decline in the level of isotope in subsequent eggs as the clutch advanced (in both clutch sizes); five egg (Figure 6.6) $r_{28} = -0.991$, $p < 0.0001$ and six egg (Figure 6.7) $r_{46} = -0.971$, $p < 0.0001$.

In the second series of eggs isotope levels were analysed in the yolk and the albumen. Of the ten pairs introduced only six produced a clutch and all of these were five-egg clutches. As with the eggs above there was an overall decline in isotope content from the beginning of the clutch to the end. Figure 6.8 shows the percentage of isotope for each egg found in the yolk and the albumen. This shows that while the proportion of isotope found in the yolk declines through the clutch ($r_{28} = -0.861$, $p < 0.0001$), the albumen, however, has an increasing proportion as the clutch advances ($r_{28} = 0.861$, $p < 0.0001$).

Therefore, material containing the isotope (*ie* that which is sourced from the body reserves and not from the diet) is more important in the yolk at the beginning of the clutch and in the albumen at the end of the clutch.

TABLE 6.1

The amount of ^{35}S methionine found in tissue samples from breeding females (n=14) and from those that had not laid (n=14) and the result of an unpaired t-test on them. Both groups sampled 11 days after the administration of the last isotope dose.

(DPM/0.05g, mean ± standard deviation)

	Pectoral Muscle	**Oviduct**	**Ovary**	**Liver**
Laying Females	869 (138)	339 (114)	367 (87)	631 (248)
Non-laying Females	1129 (235)	715 (470)	496 (224)	556 (184)
Significance	**	*	**ns**	**ns**
Control, n=1	25	20	20	25

**** $p<0.01$ * $p<0.05$ ns $p>0.05$**

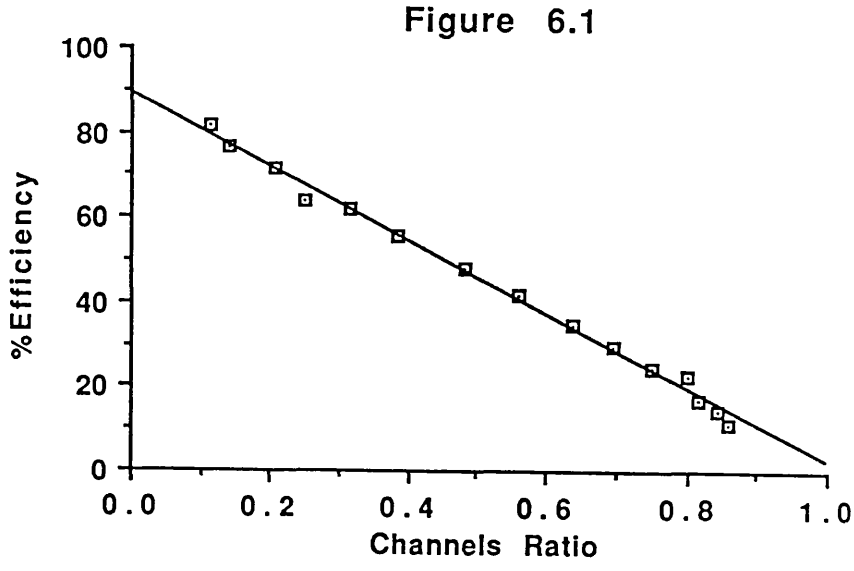


Figure 6.1

Calibration curve for the effect of quenching on Amersham OCS scintillation fluid using increasing volumes of chloroform as a quenching agent

$y = 89.396 - 87.089x$, $r_{13} = 0.997$

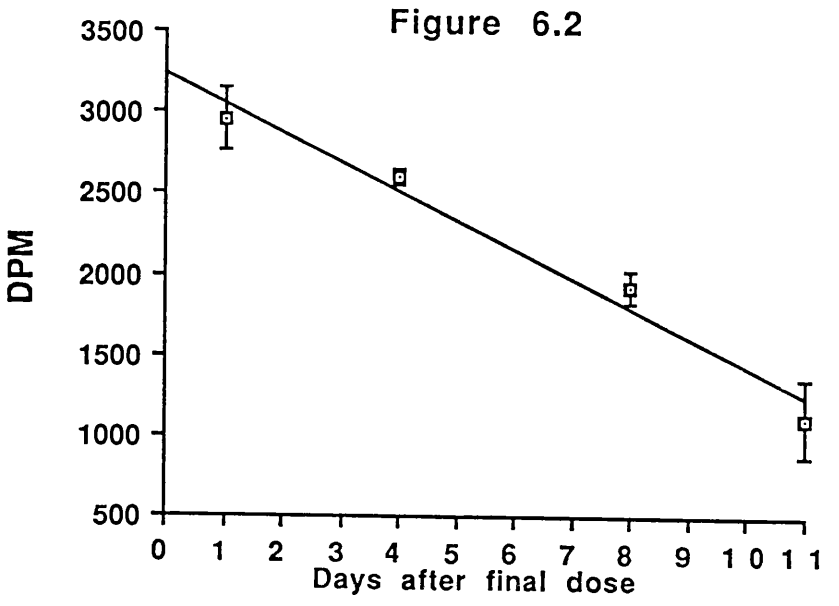


Figure 6.2

Activity of isotope (disintegrations per minute) in pectoral flight muscle of female Zebra Finches during the eleven day experimental period (mean ± s.d.)

$y = 3241.4 - 181.7x$, $r_{21} = -0.986$, $p < 0.001$

Day	1	4	8	11
n	3	3	3	14

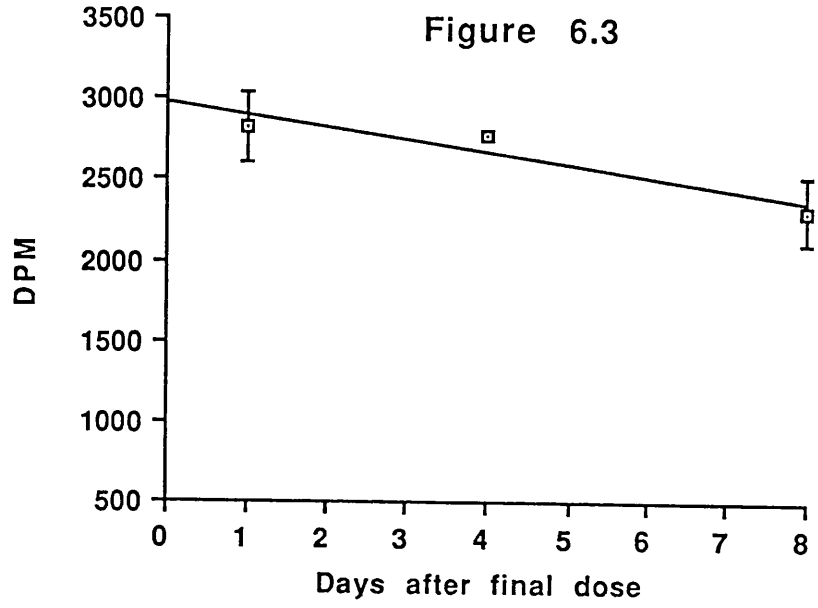


Figure 6.3

Activity of isotope (disintegrations per minute) in leg muscle of female Zebra Finches during the experimental period up to eight days after last dose of isotope (mean ± s.d.)

$y = 2962.4 - 75.703x$, $r_7 = -0.940$, $p < 0.05$

Day	1	4	8
n	3	3	3

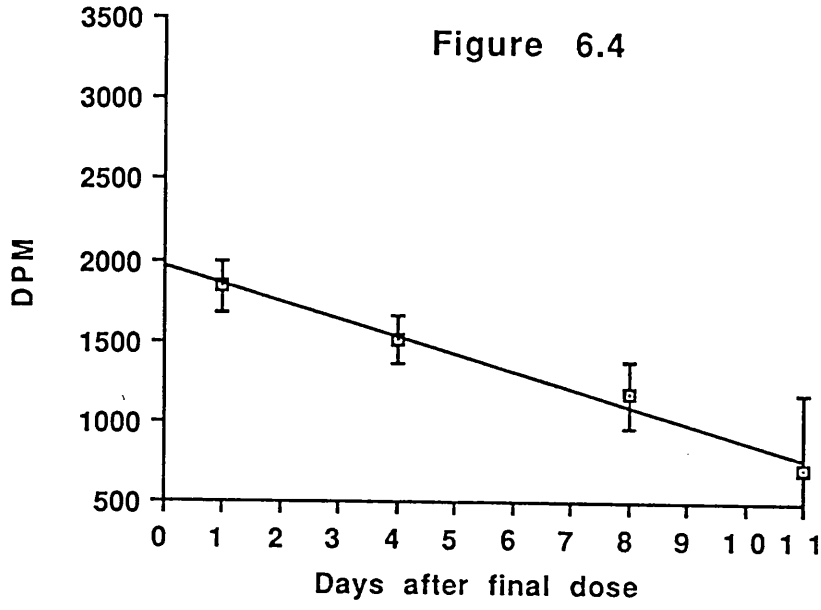


Figure 6.4

Activity of isotope (disintegrations per minute) in the liver of female Zebra Finches during the eleven day experimental period (mean ± s.d.)

$y = 1959.0 - 108.3x$, $r_{21} = -0.993$, $p < 0.0001$

Day	1	4	8	11
n	3	3	3	14

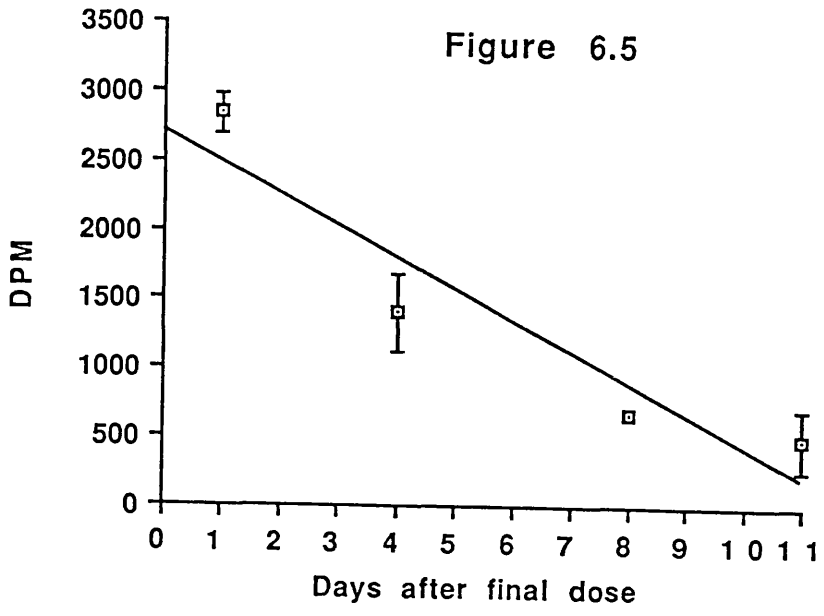


Figure 6.5

Activity of isotope (disintegrations per minute) in the ovary of female Zebra Finches during the eleven day experimental period (mean ± s.d.)

$y = 2712.5 - 226.3x$, $r_{21} = -0.938$, $p < 0.0001$

Day	1	4	8	11
n	3	3	3	14

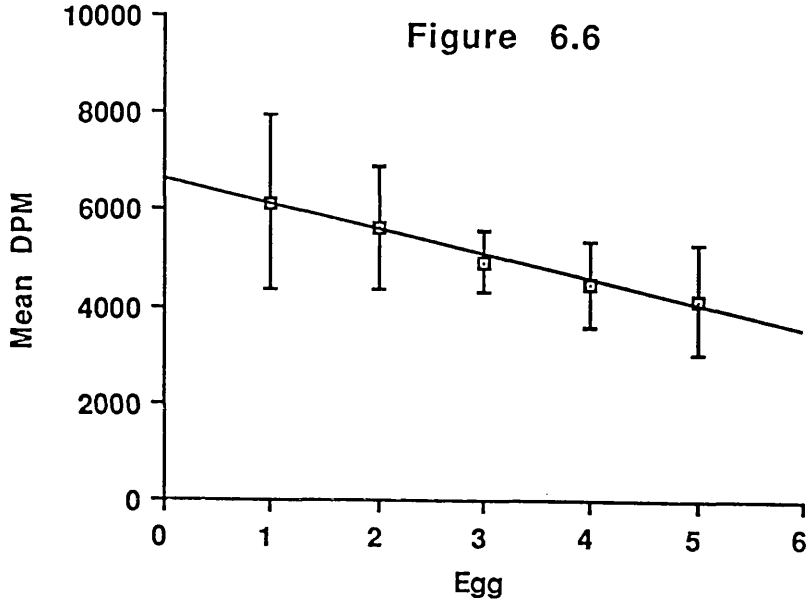


Figure 6.6

Activity of isotope (disintegrations per minute) in the eggs of five-egg clutches (n=6), corrected for declining isotope availability (mean ± s.d.)

$y = 6616.7 - 514.7x$, $r_{28} = -0.991$, $p < 0.0001$

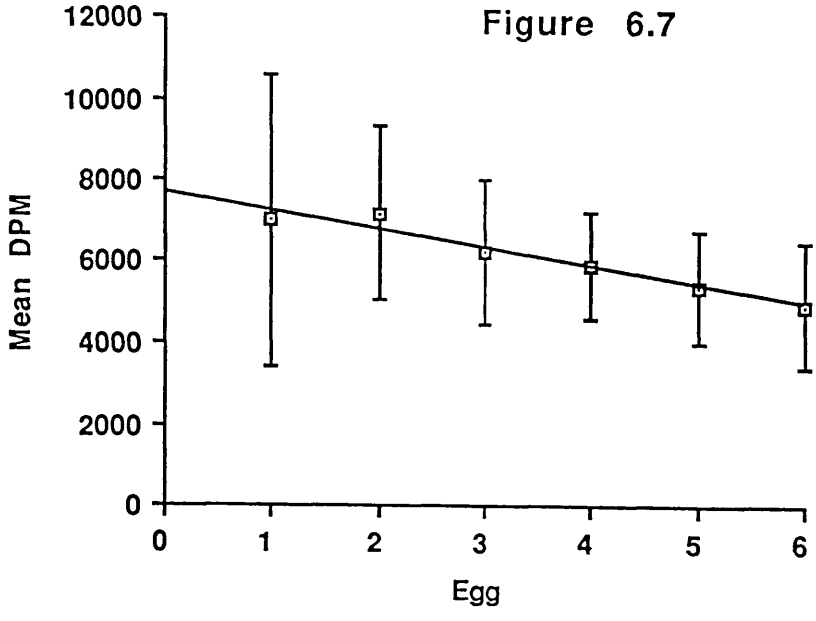


Figure 6.7

Activity of isotope (disintegrations per minute) in the eggs of six-egg clutches (n=8), corrected for declining isotope availability (mean ± s.d.)

$y = 7700.8 - 457.2x$, $r_{46} = -0.971$, $p < 0.0001$

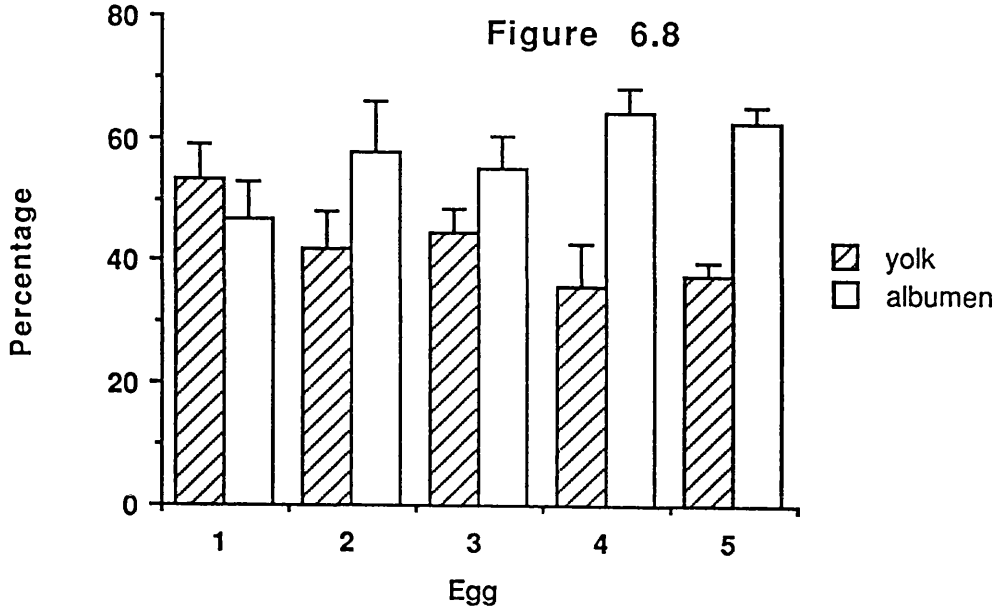


Figure 6.8

Percentage of isotope present in the yolk and albumen of each egg of 6 five-egg clutches, corrected for isotope availability (mean ± s.d.)

Yolk: $y = 59.980 - 3.760x$, $r_{28} = -0.861$, $p < 0.0001$

Albumen: $y = 59.980 + 3.760x$, $r_{28} = 0.861$, $p < 0.0001$

6.4 DISCUSSION

6.4.1 Incorporation of isotope in the tissues

The results indicated that up to 16% of the isotope given to the birds became incorporated in the tissues one day after the last dose was administered. There was some degree of variation in the levels of isotope measured in different birds and there are a number of factors that may have led to this. The rate of uptake may have been affected by the physiological status of the bird at the time. For example, a bird in the process of digesting food may have absorbed more isotope than a bird that was not. In addition, on several occasions it was noted that the bird would regurgitate some of the dose and thus some individuals will not have ingested the full quantity of isotope on each occasion that it was given. However, the technique did result in levels of isotope incorporation that were easily monitored in all tissues analysed.

6.4.2 Decline of isotope in the tissues with time

The decline of isotope from the tissues could have been caused by two factors. Firstly, by radioactive decay of the isotope or, secondly, routine metabolism that would lead to excretion of isotope from the body. In the pectoral muscle I calculated that decay could only have accounted for 12.2% of the lossduring the experimental period and was, therefore, of minor importance in comparison to metabolic turnover.

On the day after the last dose was given, the isotope levels in all four of the tissues analysed were similar. This could have been because during the dosing period the excess level of methionine may have led to a saturation point being reached. Once the dosing ceased the tissues would begin to lose isotope, presumably at a rate depending on their level of metabolic activity. The two types of muscle measured, leg and pectoral, appeared to display different rates of loss. By the eighth day after the final dose, the pectoral muscle had lost 35% compared to the leg muscle's 18.2% (Figure 6.2 and 6.3). In House Sparrows it is known that the oxidative activity of the flight muscles is higher than that found in leg muscle (Jones, M.M., 1979) and there are related differences in ultrastructure. In Eared Grebes there are differences in the mitochondria of the two muscles. The pectoral muscle mitochondria contain features not seen in those of the leg muscle, which suggest high

metabolic rates (Gaunt et al, 1990). Therefore, it is not unexpected that there is this difference in the rate of loss of isotope between these muscles in the Zebra Finch. The oviduct displayed a similar rate of loss to the pectoral muscle but the ovary lost the highest percentage of isotope (82.5%, Figure 6.5). The potential for error during analysis was highest with the ovary because in most cases they weighed less than 0.05g wet and thus could not be prepared in duplicate as the other tissues were. Also, the readings were corrected to 0.05g per sample in order to allow comparison with the other tissues. This would have compounded any error in the analysis. In any case, the loss of isotope from the ovary would only be a fraction of that from the pectoral muscle, for instance, due to the difference in their mass.

6.4.3 Differences between breeding and non-breeding females.

Table 6.1 presents the comparison of four tissues from females taken after laying a clutch of eggs compared to females that had not laid. Those birds that did lay had significantly less isotope present in their pectoral muscle and oviduct than the non-breeders but there was no difference in the ovaries or the liver.

In Chapter 4 it was shown that female Zebra Finches displayed a loss of lean dry weight from their pectoral muscles during the formation of eggs. In Chapter 5 I argued that this was due to loss of protein. A number of studies have shown similar findings and have suggested that this protein is transfered to the developing eggs (eg Fogden & Fogden, 1979, Jones,G. 1987, Jones, M.M., 1991). The results of this experiment follow that hypothesis because it would be expected that the egg-laying females would show a decline in protein levels, and therefore isotope, in comparison to birds that were not laying a clutch of eggs.

A similar result would be anticipated for the oviduct because it too loses protein during the laying cycle (Chapter 2). In Zebra Finches the lean dry weight (protein) of the oviduct increases rapidly prior to laying and then decreases from Day 1 in the cycle and can supply protein to the developing eggs thereby acting as a short-term storage organ. Krementz & Ankney (1986) suggested this for the House Sparrow and other studies have also reported a loss in the lean dry weight of the oviduct (Schifferli, 1976 and Barzen & Serie, 1990).

In contrast the ovary tissue of the female Zebra Finch was not observed to change in mass during the laying cycle, with growth being restricted to changes in the follicles (Chapter 2). There was no significant difference in the isotope content of this tissue between the two sets of birds in this experiment thus supporting the previous finding.

There was no difference in the isotope content of the liver of breeding and non-breeding birds (Table 6.1). A difference similar to that seen in the muscle and oviduct might have been anticipated given the loss of dry weight from the liver (Figure 4.6) during the laying cycle. The loss of dry weight from the liver was not as high as that seen in other tissues, and as the liver is involved in yolk production much of the material lost from the liver would be lipid. It is possible that any loss of protein from the liver was not enough to show up using this technique.

6.4.4 Occurence and distribution of isotope in eggs

Eggs were found to contain easily measurable quantities of isotope. The eggs were formed only after the isotope had ceased to be given to the female. Therefore, any isotope in the eggs must have come from that incorporated in the proteins of the bird rather than from the food that was eaten while the eggs were being formed.

This result provides further evidence for the hypothesis that the protein lost from the female Zebra Finch's tissue during the laying cycle (Chapter 4 and 5) is actively transferred to the developing eggs.

The second finding from the eggs was that there was a decline in the amount of isotope found in the eggs as the clutch advanced (Figure 6.7). This suggests that proteins sourced from the body of the finch are more important to the first egg than to the last. The findings of Chapter 2 showed that maximal nutrient demand for egg production was on the day that the first egg was laid. At this time the majority of the yolks for the clutch are forming rapidly, albumen is being laid down and shells being formed. By the end of the clutch the demands have diminished and there is less necessity to use body protein as the nutrients from food intake could cover a larger proportion of the required amount.

Eggs were also analysed for the isotope in the yolk and albumen separately. Figure 6.8 shows the percentage of isotope found in the yolk and the albumen of each egg in a five egg clutch. The yolk has only 61.2% (Table 2.1) of the protein that the albumen has. Despite this there is little difference at the start of the clutch in the percentage of isotope present in the two. By the last egg there is a significantly higher percentage in the albumen. Therefore, protein coming from the body must be more important to the yolk at the start of the clutch than at the end. This may be because the most demanding time for supplying protein to the yolks is near the start of the clutch when there are a number of yolks being deposited at the same time. As the clutch advances there are less yolks on the ovary and thus the demand for yolk protein falls. As the percentage of isotope in the yolk falls then that in the albumen rises correspondingly. Therefore, the last egg of the clutch contains less isotope in total than the first but the majority of it is found in the albumen rather than the yolk.

6.4.5 Conclusions

The conclusions to be drawn from this study are that:

1 - Direct transfer of protein from the body reserves of female Zebra Finches to developing eggs is indicated by the presence of isotope in the eggs.

2 - The results of the isotope experiments closely reflect the findings of the previous chapters. In breeding females, tissues that showed a decline in protein content during the laying cycle, the pectoral muscles and the oviduct, also had much less isotope in those tissues at the end of the experiment compared to non-breeders.

3 - There are differences between muscle groups in the contribution that they make to total isotope decline. The pectoral muscle showed a significantly faster rate of decline of isotope compared to leg muscle in breeding females.

4 - The distribution of isotope in the clutch indicates that endogenously derived protein is more important at the beginning of the clutch than at the end. Also, this material becomes less important for yolk production as the clutch advances, but correspondingly more important to the albumen. This reflects the pattern of demand for protein (outlined in

Chapter 2) which is maximal on Day 1, the day that the first egg is laid. Similarly, yolk protein demand is maximal on this day and thus endogenous protein is likely to be of most importance at this time.

CHAPTER 7

GENERAL DISCUSSION

The production of a clutch of eggs is a demanding process for a female Zebra Finch. The nutrients that are required for the eggs must be found from one, or a combination, of three routes. They may come from a) the diet or b) from the female's reserves or c) from changes in activity that free nutrients otherwise used in metabolism for egg production (Walsberg, 1983). This study was concerned largely with the role of the two former mechanisms.

It was found that the diet alone was unable to meet all of the requirements for the production of a five-egg clutch (Chapter 3). This was true for protein in particular. There was no evidence of hyperphagy in females in the process of forming eggs. One possible way by which extra nutrients from the diet could be obtained, therefore, was by an increase in digestive efficiency, as observed in breeding female Zebra Finches by El-Wailly (1966). Using El-Wailly's figures I calculated that if such an increase occurred it would result in the liberation of relatively little protein for egg production (Table 3.4). The situation for lipid was better, as the seeds are largely carbohydrate, but there was still a deficit. Calcium requirements, however, were probably met largely by dietary intake (see below).
This study was conducted entirely within a laboratory environment which was necessary to allow reliable measurement of food intake. During the feeding trials there was no opportunity for the birds to switch their diet from seeds to other, protein rich, food items. This sort of behaviour has been recorded in other, wild passerines. In the period leading up to laying the Grey-backed Camaroptera changes from its predominantly graniverous diet to start feeding on insects (Fogden & Fogden, 1979). However, studies of wild Zebra Finches have indicated that even when breeding their diet remains almost entirely of seeds (Zann & Straw, 1984, Morton & Davies, 1983). The Zebra Finches in this study had food available *ad libitum* and thus the requirement for foraging was minimal. It is, therefore, possible that Zebra Finches in the wild can obtain additional dietary nutrients during egg formation by increasing their foraging effort.

But in this study it would appear that the bulk of protein for egg production is derived endogenously. Chapters 4 and 5 showed that there was a significant decline in the protein reserves of breeding female Zebra Finches to the extent that up to 74% of the protein in a five-egg clutch could be supplied from this source. It was significant also that the timing of this decline, which occurred largely from Day -3 to Day 1 of the laying cycle, matched closely the period of rapid follicle growth and the peak of egg protein demand, which was on Day 1.

The concept of protein reserves in one part of the body being built up and then depleted again is not unusual. In studies of rats, for instance, protein reserves are estimated to make up more than 20% of total body protein and these reserves can be reversibly depleted and repleted (Allison & Wannemacher, 1965). Of all the tissues that may be involved it is the skeletal muscle that forms the largest part of the protein reserves. Skeletal muscle is highly mobile, with a protein turnover rate two to three times that seen in the liver, and it plays a crucial role in overall protein metabolism (Millward, 1970, Spargo et al, 1979).

Skeletal muscle, and in particular the pectoral flight muscles, are a highly important element of the protein reserves of the female Zebra Finch. The pectoral flight muscles undergo atrophy at the time of egg formation (Figures 4.1, 4.1b, 5.3, 5.3b). The measured loss of protein from these muscles alone could provide up to 15% of the protein of a five-egg clutch. The whole skeletal system is probably utilised for protein but the pectoral flight muscles are most important by virtue of the fact that they are by far the largest muscle group and also the most metabolically active (Jones, 1979, George & Berger, 1966). A study of Eared Grebes revealed differences between the structure of flight muscle and leg muscle. These differences were in relation to the mitochondria and suggested adaption of the flight muscle to rapid hypertrophy and atrophy (Gaunt et al, 1990). In the female Zebra Finch other organs, including the gut (Figure 4.5), the liver (Figure 4.6) and gizzard (Figure 4.7), contribute to the decline of total body protein during breeding. But the heart (Figure 4.8) showed no such decline in weight. Other studies have produced similar results for changes in organs associated with egg-laying. For example in a study of Northern Shovelers, Ankney and Afton (1988) measured no significant difference between pre-laying and laying females' heart weight. Korschgen (1977) and Ankney & MacInnes (1978)

reported a significant decline in the size of the gizzards of Eider and Lesser Snow Geese during breeding.

In previous studies that detected a decline in protein condition associated with egg production (Table 1.1) it was generally assumed that this protein could become available for egg formation. Therefore, in Chapter 6 an attempt was made using isotope labelled amino acids to demonstrate the transfer of material from the body of the female Zebra Finch to the developing eggs. The results of these experiments provide evidence that this is, in fact, what is happening. At the end of the experimental period, the pectoral muscle of breeding female Zebra Finches contained significantly less isotope than those Finches that did not breed (Table 6.1). In addition, isotope was present in the eggs and the distribution of the isotope within the eggs (and through the clutch) was consistent with previous findings for the timing of protein demand during egg formation *ie* most isotope was present in the eggs when protein demand was at its highest and protein from the body reserves would be most needed.

Having established that endogenous protein is of importance to breeding Zebra Finches the flight muscle was investigated in more detail. Kendall et al (1973) looked at the structure of Quelea flight muscle before and after laying. On the basis that sarcoplasm reduced in size while contractile elements did not they suggested that the sarcoplasm may be the location for a store of labile protein to be drawn upon under stress. In this study (Chapter 5) biochemical techniques were employed to measure both sarcoplasmic and contractile protein. It was found that both elements of muscle were involved in the decline of overall muscle protein (Figures 5.1 and 5.2). This direct measure of protein lost from the pectoral muscle during the breeding period was almost equal to the decline of lean dry weight of the muscle (Chapter 4). This backs up the assumption in previous studies that lean dry weight is a good, indirect measure of protein content. Jones, M.M. (1991 & 1979) found that only the contractile element of House Sparrow pectoral muscle declined in relation to breeding. The sarcoplasm proteins declined but this was not significant. Eared Grebes undergo a pectoral muscle atrophy of up to 50% and there is no evidence of change in overall ultrastructure, rather all of the muscle fibres become smaller (Gaunt et al, 1990).

It was possible to analyze the composition of the sarcoplasmic element further by gel filtration. This separated the individual proteins and it was found that one high molecular weight protein displayed a significant decline whereas two others remained relatively consistent (Figure 5.6). This result was similar to the findings of unpublished work on the sarcoplasmic fraction of the pectoral muscles of breeding Starlings (Osborn & Ward, pers comm.). Therefore, there is some evidence within the sarcoplasm that a protein of high molecular weight is lost in preference to others, but the status of the various alkali soluble protein is not known. This high molecular weight protein in the sarcoplasm is worthy of further investigation and characterisation.

Why do birds use stored protein during the production of their eggs and what benefits might accrue from such a strategy? Firstly, there is a range in the level of dependency on the use of stored protein. Some birds, by virtue of their life history, require to form all of their eggs from reserves. The Lesser Snow Goose (Ankney & MacInnes, 1978) and the Adelie Penguin (Astheimer & Grau, 1985) both have to do this as they are unable to feed immediately prior to breeding. At the opposite end of the spectrum are birds that do not utilise reserves of protein at all. The White-bellied Swiftlet appears to rely entirely on its diet to supply protein for eggs (Hails & Turner, 1985). In between these ends of the spectrum are those birds that use endogenous reserves of protein in conjunction with protein from the diet. For example, the American Coot loses protein equivalent to 80% of that required by the clutch (Alisauskas & Ankney, 1985) and the Red-billed Quelea obtains protein partly from its diet and partly from reserves (Jones & Ward, 1976). This use of endogenous protein may simply balance that available from the diet but there is some evidence that it may be important in supplying specific nutrients, most probably amino acids.

In the Lesser Black-backed Gull it has been shown that there is a positive correlation between the number of eggs laid and the level of flight muscle protein in females at the start of breeding (Bolton et al, 1993 and Houston et al, 1983). Further studies have demonstrated that this gull's clutch size can be depressed by protein, but not energy, limitation. Secondly, in an experiment using supplementary food, those breeding birds that received additional egg protein in the form of cooked egg showed an increase in the size of eggs laid. However,

those that were fed supplementary general protein (fish) did not produce larger eggs (Bolton, et al 1992). In general, eggs contain relatively high levels of certain amino acids, including the sulphur containing amino acids, cystine and methionine (Harvey, 1970). The egg of the Zebra Finch is not dissimilar to that of the domestic hen (Table 2.5) and studies of hens have shown that their egg production can be enhanced by increased methionine, lysine or tryptophan intake (Fisher, 1976). In Table 3.5 I compared the total amount of each amino acid needed for a five-egg clutch with the amount available from the total quantity of seed eaten each day over seven days. This indicated that cystine would be in deficit.
In addition, when taking into consideration the likely utilisation efficiencies of dietry amino acids (no more than 75%, Murphy 1993b), it is likely that both cystine and lysine requirements could not be met and that argenine and histidine would also be in very short supply.

The Zebra Finch exists on a diet that is protein poor. As mentioned previously, the Zebra Finch is predominantly graniverous even, as far as is known, under the stress of breeding (Zann & Straw, 1984). Other granivores will change their diet during egg formation to include more protein-rich items when necessary, for example the Grey-backed Camaroptera (Fogden & Fogden, 1979) and the House sparrow (Krementz, 1984). One possible reason why the Zebra Finch is so firmly graniverous is that in arid conditions small birds more readily attain favourable states of water balance on a seed diet than larger birds (MacMillen, 1990).

Even those birds that do not have a typically protein deficient diet may benefit from the use of protein reserves in egg production. Developing and utilising protein reserves may give the ability to lay larger eggs or perhaps larger and/or earlier clutches than the food supply at the time of egg formation would allow. Murphy (1986) found correlations between the body condition of female Eastern Kingbirds and their egg composition (especially in relation to lipid). It has been demonstrated that egg size has implications for chick size, growth and probability of fledging (Schifferli, 1973, Nisbet, 1978, Bolton, 1991). In addition, Furness (1983) showed that Great Skua chicks hatched from large eggs will remain larger during subsequent development. In Herring Gulls (Parsons, 1970), Common and Roseate Terns (Nisbet, 1978) chicks hatching from large eggs show a higher survival

rate than those that hatch from small eggs, although parental quality can be a factor in the survival of chicks regardless of egg size (Bolton, 1991). However, in the Lesser Black-backed Gull, Bolton et al (1993) found that there was no relationship between body condition of females and egg size but body condition was important for determination of clutch size. It has previously been suggested that female body condition acts as a proximate factor to trigger the onset of the rapid follicular development phase of egg production and also to determine the number of follicles that will be ovulated (Jones & Ward, 1976, Fogden & Fogden,1979, Houston et al, 1983 and Jones, M.M., 1991). Drent & Daan, 1980, put forward hypotheses to explain the possible relationships that may exist between food supply, female body condition and clutch size. They offered two models by which the monitoring of body reserve accumulation might be used for the timing of breeding and determination of clutch size. Firstly, there is the "Capital" model where clutch size and the timing of breeding is determined by the date on which a particular threshold is reached. Secondly, there is the "Income" model where it is the rate of accumulation, measured against a number of fixed thresholds, that is important.

Recent experimental field studies have provided evidence of the importance of body condition in relation to breeding. Bolton et al (1993) thought that the results of their study of Lesser Black-backed Gulls supported Drent & Daan's "income" model. The provision of supplementary protein to the breeding females led to an increase in clutch size but had no effect on laying date. In addition, there was no significant correlation between clutch size and the timing of laying among the supplementary fed birds. They suggested that their results were evidence of a causal link between body condition and clutch size, thus providing a possible mechanism by which clutch size can be adjusted to the available food supply in a given year or area or to individual foraging performance. Similarly, studies of Tengmalm's Owl (Korpimaki, 1987 &1989 and Korpimaki & Hakkarainen, 1991) concluded that clutch size is predominantly determined in this species by food supply during the period leading up to breeding and mediated by female body condition.

There are other occasions in the life history of birds, in addition to breeding, where there may be benefits from the accumulation of body reserves and the subsequent use of endogenous protein. In migratory birds lipid is the major energy substrate (Blem, 1990).

However, hypertrophy of flight muscle is also recorded. For example, the Grey Catbird undergoes hypertrophy of its flight muscle by up to 35%, with all the major components of the muscle contributing to the increase (Marsh, 1984). This augmentation of the pectoralis mass could make a significant contribution to flight performance however it may also be of significance once the bird reaches its migratory destination. In a study of migratory shorebirds Davidson & Evans (1986) found that the degree of increase in muscle mass prior to birds departing for their breeding grounds was unnecessary if considered from the standpoint of the power requirements of flight alone. In addition, they found no correlation between muscle size and the lipid content of fat individuals, which does not support the theory of a response by the muscles to the need for more power to carry the increased load of lipid. Their conclusion was that muscle hypertrophy in pre-migratory shorebirds was a method for storing protein for use primarily to enhance the chances of survival at the migratory destination in the face of unpredictable food availability. They estimated that this store could provide between one fifth and one half of the protein needed to form a clutch of eggs.

Moult is another part of the life cycle of birds where there is a requirement for protein. Most species obtain the energy and protein necessary for the production of new feathers from their diet (Blem, 1990). In a similar fashion to the use of endogenous protein in reproduction there are a range of examples of the use of endogenous protein for the moult. Some birds such as the Macaroni and Rockhopper Penguin are not able to feed during their moult and thus obtain all the necessary nutrients endogenously (Williams et al, 1977). It is thought that Redhead duck may use pectoral muscle protein in the formation of new feathers but cannot fully meet demand from this source (Bailey, 1985). At the other end of the spectrum to the penguins mentioned above is the Brant Goose which actually experiences an increase in the mass of muscle protein during the moulting period (Ankney, 1984).

The second major component of eggs considered in this study was lipid. Lipid reserves in the female Zebra Finch also underwent a significant decline during egg production (Figure 4.10). The extent of this decline was larger than the amount of lipid that is found in a five-egg clutch. Unlike protein, the diet may also be able to make a significant contribution to

the lipid content of eggs as lipids can be readily formed from the carbohydrate content of seeds. In other studies it has been suggested that during the period of egg formation the diet may be changed to include protein rich food items and/or calcium rich items for shells. The lipid reserves are utilised partly to fuel this change in feeding strategy (Krementz & Ankney, 1986, Schifferli, 1980, Jones & Ward, 1976, Fogden & Fogden, 1979). It is most likely that reserves of lipid act as an energetic buffer helping reduce the effect of lowered energy intake as a result of changes in behaviour or feeding strategy necessary for egg production. The Zebra Finches in this study displayed a marked change in feeding behaviour during breeding also. In Chapter 2 it was shown that weight loss from cuttlefish bone in the cages increased by as much as 400%. The implication of this is that Zebra Finches obtain all calcium for their eggs exogenously. In the wild this may result in a change of foraging strategy similar to that seen in House Sparrows (Krementz, 1984) and Quelea (Jones & Ward,1976).

The use of lipid reserves may also be of importance by helping to spread the energetic cost of egg production over a longer period. For instance, in the female American Coot, the use of reserve lipid and protein effectively reduced the required energy intake on Day 0 from 35% to 15% above that needed concurrently for daily energy expenditure (Alisauskas & Ankney,1985).

Finally, the third nutrient to be examined during this study was calcium. As mentioned above, the occurrence of calcium rich food items in the diet of laying birds is often noted (eg Krementz, 1984, Schifferli, 1976 and MacLean, 1974). Medullary bone can act as a store of calcium that will be drawn upon when the shell of the egg is being deposited. This can happen in even relatively small birds such as the House Sparrow where both stored and dietary calcium are used (Krementz, 1984). However, in this study there was no evidence that stored calcium was important (Figure 4.9) whereas there was a concerted effort to obtain calcium from the diet, from calcium-rich cuttlefish bone (Figure 3.5).

The final conclusions of this study are;

Female Zebra Finches have the ability to draw upon their reserves of protein for the production of eggs, largely because their diet is deficient in protein.

Reserves of protein may act as a reservoir of amino acids to supplement those that may be limiting in the diet.

Skeletal muscles, and in particular the pectoral flight muscles, are perhaps the most important element of the protein store but other organs such as the gut and the liver also play a role.

Both sarcoplasmic and myofibrillar proteins are involved in the decline of muscle protein during egg formation. A single, high molecular weight protein accounts for most of the decline of sarcoplasm protein.

Isotope experiments provide evidence that proteins are transferred directly to the developing eggs.

Lipid reserves also undergo a decline. The quantity of lipid lost from reserves greatly exceeds that found in the eggs. It is likely that reserve lipid is important not only for egg formation but to meet other energetic demands associated with egg formation.

Calcium requirements appear to be met from the diet alone. There was no evidence of the use of stored calcium.

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Scientific names of birds mentioned in the text

Adelie penguin	*Pygoscelis adeliae*
American coot	*Fulica americana*
Bengalese finch	*Lonchura striata var. domestica*
Black duck	*Anas rubripes*
Brant goose	*Branta bernicla*
Brown-headed cowbird	*Molothrus ater*
Brown kiwi	*Apteryx australis*
Cananda goose	*Branta canadensis*
Canvasback	*Aythya valisineria*
Common eider	*Somateria mollissima*
Common tern	*Sterna hirundo*
Eared grebe	*Podiceps nigricollis*
Eastern kingbird	*Tyrannus tyrannus*
Great skua	*Catharacta skua*
Grey-backed camaroptera	*Camaroptera brevicaudata*
Grey Catbird	*Dumetella carolinensis*
Herring gull	*Larus argentatus*
House sparrow	*Passer domesticus*
Lesser black-backed gull	*Larus fuscus*
Lesser snow goose	*Anser caerulescens*
Macaroni penguin	*Eudyptes chrysolophus*
Mallard duck	*Anas platyrhynchos*
Northern shoveler	*Anas clypeata*

Pied flycatcher	*Ficedula hypoleuca*
Red-billed quelea	*Quelea quelea*
Redhead Duck	*Aythya americana*
Ring-necked duck	*Aythya collaris*
Rockhopper penguin	*Eudyptes chrysocome*
Roseate tern	*Sterna dougali*
Ruddy duck	*Oxyura jamaicensis*
Sand martin	*Riparia riparia*
Starling	*Sturnus vulgaris*
Tengmalm's Owl	*Aegolius funereus*
Tree swallow	*Tachycineta bicolor*
White-bellied swiftlet	*Collacalia esculenta*
White-crowned Sparrow	*Zonotrichia leucophrys*
Wood duck	*Aix sponsa*
Yellow wagtail	*Motacilla flava*
Zebra finch	*Taeniopygia guttata*