SELECTION, STORAGE AND UTILISATION OF PROTEIN IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*)

By

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Thesis for the degree of Ph.D
University of Glasgow,
November, 1998.

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SUMMARY

The role of protein in the physiological ecology of the zebra finch (*Taeniopygia guttata*) was investigated using a colony of captive birds at Glasgow University. Trials based on dietary protein supplementation investigated self-selective ability, effect on egg production, endogenous protein storage, repercussions for protein synthesis in body tissues, immuno-competence trade-offs and the potential of bill colour as an indicator of physiological state.

A basic experimental diet was formulated for the investigation of self-selective ability in zebra finches. Nutrient supplements could be added to this without altering its visual / textural appearance, thus removing the non-nutritive cues to diet selection suggested to compromise previous studies. The outlined dietary formulation and delivery protocol facilitate the controlled nutritive provisioning of large numbers of birds with minimum disturbance.

The ability of zebra finches to self-select between basic and protein-supplemented diets to maximise egg production was investigated. When presented with a choice between diets ostensibly differing only in protein content, finches differentiated between the two, selecting 70% basic to 30% supplement. Though daily consumption did not differ significantly between dietary groups, birds receiving a choice between diets tended to maintain their body weight while producing the largest clutches and eggs, in comparison with birds maintained solely on either one of the diets. Having shown dietary discrimination to occur, "perceived taste", influenced by a specific nutrient appetite, is suggested as the basis for diet selection. Implications for lab and field studies are discussed.

Pectoral muscle is recognised as an important reserve of endogenous protein. Analysis of the sarcoplasm of the flight muscle of zebra finches supported previous observation of protein removal during egg production. However, SDS electrophoresis in combination with amino acid analysis of muscle protein fractions indicated this loss to be of a general nature and not confined to a specific protein. No evidence was found to support the previous findings of a high molecular weight storage protein specific to the amino acid requirements of egg production, and it is suggested that this observation may have arisen as an artefact of the experimental methodology. The theoretical benefits of specific storage proteins for free-living zebra finches are argued to be less advantageous than previously suggested.

The effect of dietary history and egg production on protein synthesis in female zebra finches was investigated using a "flooding dose" method. Fractional synthesis rate (FSR) was determined for liver, leg muscle and pectoral muscle in laying and non-laying birds. FSR of protein in the liver was elevated during egg production and by previous experience of a high protein dietary supplement. FSR of muscle types did not appear to differ significantly from one another, nor between experimental groups. Modification of liver FSR by previous dietary experience is suggested as a contributory mechanism for the promotional effects of protein supplementation on egg production.

The repercussions of dietary history and breeding status on immuno-competence were investigated. It was predicted that, were a nutrient allocation trade-off between egg and antibody production to occur, this might be alleviated by protein supplementation of the diet. Female zebra finches of differing dietary histories were immunised with sheep red blood cells (SRBCs) to present them with a potential resource allocation trade-off between egg and antibody production. Egg productivity did not differ between birds maintained, prior to breeding, on seed or protein supplemented diets. However, primary immune response was reduced in breeding birds maintained on a seed diet in comparison with both non-breeding birds and protein-supplemented breeding birds. Hence, females from a good nutritional background were able to maintain egg productivity, without reducing the strength of their immune response. When subjected to concurrent immunological challenge, rather than increasing short-term reproductive capacity, high quality nutrient reserves may promote the immuno-competence of the individual and consequently improve survivorship if exposed to a pathogenic challenge. It is suggested that the dietary protein supplementation observed in wild finches prior to breeding might potentially be regarded as much an immunological measure as a mechanism for increasing egg production.

The potential of bill colour as an indicator of physiological state in female zebra finches was investigated. Carotenoids are suggested to represent a possible link between pigmentation and physiological state. Female finches of different dietary histories were presented with a physiological "challenge" (egg and antibody production in combination with blood sampling), followed by a period of "recovery" (cessation of laying, immunisation and blood sampling in combination with maintenance on a protein supplemented diet). Computer analysis was used to obtain an objective measurement of bill colour over the course of the trial. Bill coloration did not differ significantly between birds maintained on different diets - indicating that bill colour per se might not be an indicator of nutritional plane. However, throughout the trial, physiological challenges provoked a reduction in bill yellowness and brightness, combined with an increase in redness - traits regarded as "unfavourable" in females. Physiological recovery encouraged a reversal of these trends. Deviation from original bill coloration was least, and hence most preferable, in birds previously experiencing a dietary protein supplement. The use of computer software packages in colour assessment, and the role of bill colour as an honest indicator of condition, are discussed.

DECLARATION

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

Matthew P. Cottam

15th November 1998

ACKNOWLEDGEMENTS

Foremost thanks to my supervisor Dr David Houston. A genuinely nice guy who's enthusiasm for the subject is real, and the best sort of motivation.

Thanks to the technical staff in IBLS. Dorothy Armstrong, John Laurie and Pat McLaughlin for assistance in the bird suite. June Freel for help with all things physiological. Liz Denton for trusting me with her Apple Mac. Kenny Ensor for re-installing the erased hard drive on Liz's Apple Mac.

The fact that this thesis involves a variety of scientific fields, means that it could not have been completed without the help and encouragement of a large number of people from outside the department.

Thanks to Dr Iain Hamilton and Alan Scott (Biochemistry), Prof Malcolm Kennedy (Infection and Immunity), Dr Neil Metcalfe, Dr Graham Ruxton (DEEB) and Dr James Currall for much needed stats help. Dr Peter Watt, Rob and Dr Ken Smith from University of Dundee for help with all aspects of the protein synthesis study. Dr Gerald Lobely and Dr David Brown at the Rowett Research Institute, Aberdeen, for advice and amino acid analysis of experimental diets and muscle fractions.

Thanks also for help, advice, (and friendship !) to Dr Scot Ramsay, Sarah Jarvis, Andrea Fidgett, Ben Ross, Stuart Bearhop, Dave (Caddis) Stevens, Dr Alan Taylor, Dr Iain Barber, Dr Steve Arnott, Mark Cooke, Dave Aird, Jim Jardin, Dr Dave Fraser, Dr Brendan Godley and Dr Annette Broderick (for a cool time in Cyprus), Alexandra Hay (and Tim), Dr Geoff Hilton, Paul Walton, Kenny Leung, Big James Carroll (for mints), Dr Ruedi Nager, Kirstine O'Connor, Dr Paul-Parslow Williams, Dr Lynn Sneddon, Amanda MacLean (once again... Amanda MacLean) and Ruth Greer (for instilling in me a balanced appreciation of art and vodka).

Thanks to my mum and dad for not pressurising me to get a proper job (yet).

Thanks especially to Mykela Heath (soon to be Cottam) for putting up with me during writing-up, and giving me so much to look forward to in the future. By the way, if there is some Tippex on this copy, just after "Heath", you can just assume that she didn't put up with me after all.

Contents

Chapter 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 *Small, but perfectly formed...*

Energetic limitation of reproduction in birds has been the subject of much investigation. However, the role of protein has received comparatively little attention (Selman and Houston 1996).

A captive colony of Australian zebra finches was used to investigate the role of protein in the physiological ecology of this small, granivorous grassfinch. The use of zebra finches in laboratory and field studies has a long history, much of which is covered in the recent book by Richard Zann (1996).

Zebra finches are grass seed specialists (Zann and Straw 1984). In the arid / semi-arid environment of the Australian outback, grass seed represents a comparatively stable and reliable food source (Morton and Davies 1983). However, the protein content and representation of specific amino acids in grain is impoverished in comparison with that of eggs (see Harvey 1970, Houston *et al.* 1995a, McCance and Widdowson 1978, 1980), and protein supplementation of the diet of breeding finches encourages the laying of more and larger eggs (Selman and Houston 1996, Williams 1996). As such, it would appear that egg production is limited by the availability of dietary protein and / or specific amino acids.

The additional nutrient demands of egg production are met by utilisation of endogenous and / or exogenous sources. In red-billed quelea, *Quelea quelea*, a finch which also feeds mostly on grass seeds, onset of breeding is linked to attainment of sufficient endogenous protein reserves (Jones and Ward 1976). However, in this species, the depletion of endogenous reserves is reduced by augmentation of the diet with insects, a protein rich food source (Jones and Ward 1976).

Though capable of capturing and eating insects, zebra finches are known to rarely do this (Zann 1996), leading to speculation that egg production may not be protein limited in the case of the zebra finch (Zann and Straw 1984). However, recent work indicates that the amino acid complement of unripened grain (which the finches are known to readily consume during the breeding period) closely matches that of egg (Allen and Hume 1997), and it would appear that the opportunistic timing of breeding to coincide with flushes of unripe grain (Zann *et al.* 1995) may represent a mechanism for the alleviation of the protein demands of egg production.

Endogenous protein reserves also play a part in egg production in the zebra finch. Endogenous stores acquired through protein supplementation of the diet prior to breeding have been shown to promote an increase in egg production for some time after cessation of the supplement (Selman and Houston 1996, Williams 1996), and the transfer of specific amino acids from the flight muscle to forming eggs has been previously demonstrated using labelled isotopes (Houston *et al.* 1995b).

1.1.2 *A history of investigation*

In recent years, Glasgow University Ornithology Group has performed a number of studies into the protein limitation of egg production (Bolton *et al.* 1992, 1993, Houston *et al.* 1995a, 1995b, 1995c, Ramsay and Houston 1997, 1998, Selman and Houston 1996). The current study arose as a continuation of two of these previous investigations.

"The role of protein reserves in egg formation" (Selman 1994), investigated the effect of dietary protein supplementation on egg productivity in zebra finches. Addition of boiled hens eggs to the diet of females prior to pairing promoted the laying of larger eggs and clutches (Selman and Houston 1996), despite the fact that during the period of egg formation, birds were maintained on a seed diet. This indicated that the birds were able to store nutrients endogenously during the pre-breeding period, effectively reducing subsequent dietary limitation of egg production. Supplementation with whole boiled egg represented an assortment of nutrients, however it was suggested that of these it was protein that was the most limiting factor. The first objective of this project was to refine the protocol of dietary supplementation, with the aim of determining whether egg production was limited by protein and / or specific amino acids.

"The nutritional demands of egg production in female zebra finches (*Taeniopygia guttata*)" (Donnan 1993), investigated the role of endogenous protein stores in egg production. It was suggested that the sarcoplasm of the pectoral muscles might represent a reservoir for the storage of protein, from which protein could be acquired (for egg production) without compromising the flight performance of the laying female. This study included a biochemical analysis of the muscular sarcoplasm (Houston *et al.* 1995c), indicating the presence of a high molecular weight protein, the concentration of which was greatly reduced over the course of the breeding cycle. The protein was suggested to constitute a "specific protein reserve" for egg production. The second objective of this project was to investigate this protein further, with particular reference to it's amino acid composition.

1.1.3 *Development of the current study*

The investigation of Selman and Houston (1996), was replicated using experimental diets enhanced with energy rich and protein rich supplements (2.3.1). This suggested that the promotion of egg production, arising from dietary supplementation with hens eggs, was due to protein rather than energy limitation in the seed diet, confirming the predictions of the previous study. Completion of the experimental work unfortunately coincided with the publication of a near identical, and more refined study (Williams 1996), and it was thought counter-productive to waste further time on this avenue of investigation.

Replication of the investigation of Houston *et al.* (1995c), indicated that the observation of the high molecular weight sarcoplasmic protein, suggested to represent a specific storage protein, may have arisen as an artefact of the experimental technique used (4.4.1).

From this unpromising start, the objectives of the project broadened, with the aim of investigating the role of protein in the physiological ecology of zebra finches, with special reference to egg production.

A palatable diet was developed (*Chapter 2*), to which nutrient supplements could be added without compromising its visual or textural appearance. Basic and supplemented diets were then used to investigate the ability of pairs of finches to self-select between a choice of diets, solely on the basis of protein content (*Chapter 3*). Having acquired protein from dietary sources, the ability of the birds to store protein endogenously was examined (*Chapter 4*), with the aim of discovering whether the sarcoplasm of the flight muscle might contain specific storage proteins for utilisation during egg production. Fractional protein synthesis rates of specific body tissues were determined (*Chapter 5*), in order to investigate the role of modification of protein turnover as a possible link between dietary protein supplementation and the promotion of increased egg production. Immunological repercussions of protein availability and egg production were studied (*Chapter 6*), and a possible protein allocation trade-off considered between short-term reproductive benefit and long-term survival of the laying female. Finally, having demonstrated a variety of potentially beneficial consequences of dietary protein acquisition, the beaks of female zebra finches were examined, using a computer package, to determine whether bill colour might represent a phenotypic indicator of internal nutritional plane or physiological state of the birds (*Chapter 7*). The investigation eventually encompassed aspects of behaviour, biochemistry, physiology, immunology and functional ecology, illustrating the multiplicity of roles of protein in the physiological ecology of the zebra finch.

1.1.4 *Standardisation of experimental groups*

The "character" attributed to zebra finches by Zann (1996) was evident in the extraordinary individuality of the birds used in these trials. The degree of variation between individuals, while impressive, represented a potential source of error for many of the studies, especially with sample sizes being fairly restricted. Females maintained under otherwise standard conditions differed markedly in weight, dietary preference, reaction to a standard immunological challenge, bill colour and number and size of eggs laid. It was not possible to control for all these variables. However, egg production being the factor common to all experiments, it was thought necessary, at least, to standardise all experimental birds for egg producing ability (i.e. total mass of a clutch produced under standard conditions).

Prior to initiation of experimental work, birds to be included subsequently in treatment groups were assigned to a standardisation trial.

Birds were kept in cages measuring 60cm x 50cm x 40cm, under a lighting regime of 8 hour dark to 16 hour light using timer controlled "daylight" UV lighting (*Lightbox*). All birds received a mixed seed diet, cuttlebone, water and oyster grit *ad libitum*. Ambient temperature was maintained at 20.5°C (+/-2).

Equal numbers of male and female finches were selected and maintained on a mixed seed diet for at least one month. During this time, sexes were separated by cage partitions which allowed visual and vocal communication between birds, but prevented physical contact. This was done in order to encourage the formation of a pair bond between neighbouring birds. After this period neighbouring birds were re-united, and their display behaviour noted. Males and females that readily associated with one another were transferred to a separate breeding cage and provided with hay lined nest boxes and fresh grass to encourage breeding.

Nest contents were monitored daily. Eggs were removed and measured on the morning of laying and replaced with replicas. Eggs were weighted (+/- 0.001g) and length and width measured (+/- 0.1mm). After two successive days without additional eggs being laid, the clutch was considered complete and the pairs were separated once more.

Pairs were ranked according to the total mass of the clutch of eggs produced. The ranked birds were sub-divided into stratified groups according to the following protocol:

	Rank (1st = largest total clutch mass)
Group.1	1st, 6th, 7th...
Group.2	2nd, 5th, 8th...
Group.3	3rd, 4th, 9th, 10th... etc...

As such, each group contained an equal number of birds, with a standardised range of egg producing ability.

1.1.5 *Overview of statistical techniques*

Basic statistical analyses were performed using PC software packages for Windows: Minitab Version.5 (MTB) and SPSS Version.7 (SPSS).

When data sets conformed to the constraints of normality and homogeneity of variance, analysis proceeded by comparing the means of the groups using analysis

of variance (ANOVA) (MTB). In all cases, a probability value (p) of less than 0.05 was regarded as indicating a significant difference between data sets. When significance was signified, Fisher's Pairwise Comparisons (MTB) were performed as a post-hoc test in order to identify data sets which differed significantly. Comparisons of specific individuals over time were performed using Paired T-Tests (SPSS) to remove variation arising from group variation within the data set.

When constraints of normality were violated, standard normalising transformations (e.g. square, log transformation) were attempted. When these failed to normalise data, non-parametric analysis was performed. Mann-Whitney U-test (MTB) was used to compared the median averages of non-normal data sets.

In several experiments clutch characteristics were repeatedly monitored over time. When analysing successive data sets, Repeated Measures ANOVA (SPSS) was first performed. Significant trends in data were taken as statistical justification for performing repeated ANOVA analyses of data sets from specific time periods, while minimising the probability of obtaining significant results by chance, through repetition of analyses (see Williams 1996).

Chapter 2

DEVELOPMENT OF AN EXPERIMENTAL DIET FOR THE STUDY OF FEEDING IN ZEBRA FINCHES (A SMALL GRANIVOROUS BIRD)

...it's not "mistakes", it's "method development"...

Paul Parslow-Williams

SUMMARY

A range of methods was investigated for the delivery of experimental diets to zebra finches. A basic experimental diet was formulated for the investigation of self-selective ability in zebra finches. Nutrient supplements could be added to this without altering its visual / textural appearance, thus removing the non-nutritive cues to diet selection suggested to compromise previous studies. The outlined dietary formulation and delivery protocol facilitate the controlled nutritive provisioning of large numbers of birds with minimum disturbance.

2.1 INTRODUCTION

2.1.1 *The basis for standardisation of experimental diets*

Following on from the work of Selman and Houston (1996), this thesis describes supplementary feeding trials which were performed on a captive group of zebra finches. The objective of the study as a whole was to investigate the effects of dietary nutrient supplementation on aspects of egg production and body condition in breeding females. In order to proceed with this study investigations were performed into various methods by which nutrient supplements could be delivered to the birds.

In previous studies dietary supplements have usually been presented as a discrete addition to a basic diet. In the case of Selman and Houston (1996), a basic diet of *Panicum* millet seed was supplemented with a wet mash of rusk and whole boiled hens eggs, while Williams (1996) used a basic diet of mixed seed (canary seed and white, yellow and red millet) supplemented with hens egg white.

In both these cases a range of selection criteria was presented, allowing birds the opportunity to select from the diets according to a range of nutritive/non-nutritive preferences, all of which may be prone to individual bias. This makes accurate estimation of the nutrient intake of experimental groups difficult. With reference to the methodology of Selman and Houston (1996), oral factors including feedback from mechanoreceptors in the bill and mouth have been implicated in the regulation of food intake in birds (*review* Gentle 1979). For example, a preference for specific consistencies of diet has been recorded in white-crowned sparrows, *Zonotrichia leucophrys gambelii*, which preferred a semi-synthetic diet in "seed" form above a powdered form, and the powdered form above a mash (Murphy and King 1982). Also, with reference to the methodology of Williams (1996), the presentation of a variety of seed-types potentially allows the birds to select specific seeds from the mixture. Determinants of seed selection are numerous and include handling time (Keating *et al.* 1992), colour, shape and size (*review* Gentle 1979). In the case of zebra finches, determinants of grass seed selection include size and net energy gain per unit handling time, both of which are subordinate to familiarity (see Zann 1996). As such, accurate estimation of the nutrient intake of a bird maintained on a mixed seed diet would be difficult, without counting seeds individually.

The initial aim of this investigation was to develop a standard methodology for delivering diets of differing nutrient quality to the finches. The formulated diet should be palatable, allow accurate determination of the amount of food eaten and enable easy production of feed sufficient to provision a large number of birds. Finally, to guard against nutrient-independent selective bias, diets of differing nutrient content should not be distinguishable on the basis of visual appearance or texture. The suitability of a variety of methods of food provisioning was investigated for nutrient supplementation of the diet of zebra finches, and these are described in this chapter.

2.2 METHODS

2.2.1 *Method.1 Intubation*

Following the method outlined by Houston *et al.* (1995b), attempts were made to feed the birds directly into the pharynx, via silicon tubing attached to a hypodermic syringe. However, as a result of the hydraulic resistance of the narrow-bore tubing, aqueous components of the diet tended to become separated from the solids. Murphy and King (1986) encountered similar problems using this method, and noted that "only a very dilute extract of unknown and variable composition was extruded". Use of a larger bore tube by Murphy and King (1986) overcame this problem, but resulted in the death of a number of birds through oesophageal perforation. Experiments with larger bore tubing were not attempted in the current study.

Additionally, this method was time consuming, and so not suitable for simultaneous provisioning of large numbers of birds. It also involved prolonged handling of individuals, which probably caused some stress. With reference to the objective of measuring egg production in the birds, repeated capture and handling of breeding individuals would probably have resulted in undesirable repercussions for breeding performance, outwith nutrient content of the diet.

2.2.2 *Method.2 Force-feeding*

Murphy and King (1986) detail a method for force-feeding granivorous birds by placement of semi-synthetic food pellets directly into the bird's pharynx using forceps. This caused the birds to swallow reflexively. In the current study, this methodology was repeated with some success, using individual de-husked seeds as food items.

However, provisioning of birds was a lengthy process, and the production of pre-formed pellets represented an additional and time consuming step to diet preparation. Additionally, the method of delivery of the supplement, though not as invasive as that of intubation (see above), still required capture and handling of the birds. For the reasons stated previously (2.2.1), this was regarded as undesirable for the current study.

2.2.3 *Method.3 Semi-synthetic and supplemented diets*

Semi-synthetic diets have been successfully used in the past to investigate nutrient intake in granivorous birds (Murphy and King 1982, 1991b). The composition of such diets, especially with reference to the correct balance of amino acids is critical.

Slight deviations from the optimum balance of certain amino acids can result in a range of disproportionate and adverse reactions, including toxic shock (*review* Harper *et al.* 1970). Rats have been observed to eat a protein free diet (incapable of sustaining growth) in preference to an amino acid imbalanced one (capable of supporting limited growth), (Leung *et al.* 1968, Sanahuja and Harper 1962). Amino acid imbalances are subject to "difficulty in generalising about effects" (*review* Harper *et al.* 1970), as "surplus" and "disproportionate" supplementation are as much a consequence of the nutritional status of the individual, as the composition of their diet.

Attempts were made to supplement a basic rusk diet with a limited range of specific amino acids (sulphur amino acids and lysine) previously implicated in the limitation of egg production in birds (e.g. Fisher *et al.* 1964). Efficient dietary supplementation can only occur if all essential amino acids are acquired simultaneously (Cannon *et al.* 1947, from Longenecker and Hause 1958), and purified supplemental amino acids might be expected to be absorbed more rapidly than those obtained via digestion of dietary protein. However, Longenecker and Hause (1958) showed that the amino acid complement of blood plasma effectively acts as a short term buffer to compensate for the temporary imbalance arising over the course of a supplemented meal, and hence suggested that the addition of specific amino acids to a basic diet represented a valid method of dietary supplementation. However, in the current study, adverse reactions were noted in a number of individuals maintained on diets supplemented with specific amino acids. This probably arose as a result of an imbalance to requirements, and attempts to supplement diets with specific amino acids were abandoned.

2.2.4 *Method.4 Provision of a separate protein supplement*

Previous feeding trials of Selman and Houston (1996) presented protein supplement (boiled hens eggs) in a mash form, separate from the seed diet. The main advantages of this method are the minimising of stress (daily exchange of dishes in the base of the cages being the only interruption to the birds) and the speed with which large number of birds could be provisioned. Additionally, the use of boiled hens eggs as a gross nutrient supplement might reasonably be assumed to represent a fairly balanced source of many of the nutrients required for egg production (outwith the requirements of shell formation).

It was decided to provision the birds in the current study on a similar basis. However, diets were modified to remove visual and textural differences between diets of differing nutrient content, and facilitate the homogenous distribution of nutrients throughout the supplement. Additionally, the gross nutrient supplement was refined by using boiled hens egg white as a (virtually) lipid free, protein rich supplement. Personal observations, and the study of Williams (1996), recorded that supplementation of the diet of zebra finches with this purer medium of egg protein promoted the laying of larger eggs and clutches.

2.3 RESULTS

2.3.1 *Formulation of experimental diets*

Experimental diets were based on whole seed (*Foreign finch mix, Haithes, Cleethorpes*) ground to a fine flour (using a coffee grinder). As such, these diets represented a nutrient complement similar to that of mixed seed with some added fibre (in the form of ground seed husks). To this flour an equal volume of dry, white wheat rusk (*Haithes white rusk*) was added. This mixture was hydrated to varying degrees and hand mixed to produce a bread-crumb consistency which the birds found palatable. To this basic mixture, nutrients (e.g. protein, lipid or specific amino acids) and water could be variably added so that the consistency of the supplemented mash matched that of the basic mash. The composition of basic and protein supplemented diets used later in this thesis are detailed in Tab.2.1, the concentration of protein being similar to that used by Selman and Houston (1996).

Diet	Millet Flour	White Rusk	Boiled Egg White	Water	Percentage Water Content
Basic	23g	23g	none	54g	57%
Protein	17g	17g	46g	20g	63%

Tab.2.1 Composition of basic and protein supplemented experimental diets. Ingredients are shown per 100g experimental diet.

The majority of the water contained in the protein supplemented diet was present in the form of boiled hens egg white. Eggs were boiled for 10 minutes and then allowed to cool. Whites were then separated from the shell and yolk and homogenised using a food blender.

To obtain a similar consistency to the diets, it was necessary to add slightly more water to the protein supplemented diet than the basic diet. A 30g portion of the protein diet would thus contain 11.2g of diet solids, while a similar wet weight of basic diet would contain 12.8g.

In the previous study of Williams (1996), an increase in the size and number of eggs laid was observed when zebra finches were given access to a dietary supplement of boiled hens egg white. Williams estimated the intake of the supplemented birds to approximate to 0.6g of egg protein per day per bird. With reference to the current study, when maintained wholly on the protein supplemented diet, mean

consumption was of the order of 5g per pair per day (Fig.3.2). Assuming both birds to eat roughly the same amount of food, this would approximate to an intake of 0.3g of egg protein per day. Amino acid composition of experimental diets was determined by the Rowett Research Institute (Aberdeen) and is detailed in Tab.2.3.

When presented to the birds in separate bowls, the basic and protein supplemented diets were indistinguishable from one another visually and texturally. Hence any preference shown by the birds feeding on these diets could only have arisen from selection on the basis of taste and / or odour.

2.3.2 *Correction for water content of experimental diets*

The major disadvantage of this method of food presentation was that the mash dried out to some degree after being placed in the cage. This necessitated its replacement with fresh diet on each morning of the trial. Additionally, the weight of uneaten food in the bowl had to be standardised in order to obtain an accurate measure of the amount of food eaten, and to take into account water lost through evaporation. Small amounts of leftovers tended to dry out more than large amounts of leftovers.

To determine the evaporation profile of the experimental diets, 30g portions of diet were placed in cages and 1-4 birds allowed to feed from each over a period of 24 hours. At the end of this time, diets were removed and wet weight recorded, before being dried in an oven to constant weight, (70°C for 3 days). The relationship between wet weight of leftovers and dry weight approximated to a Weibull distribution (Fig.2.1).

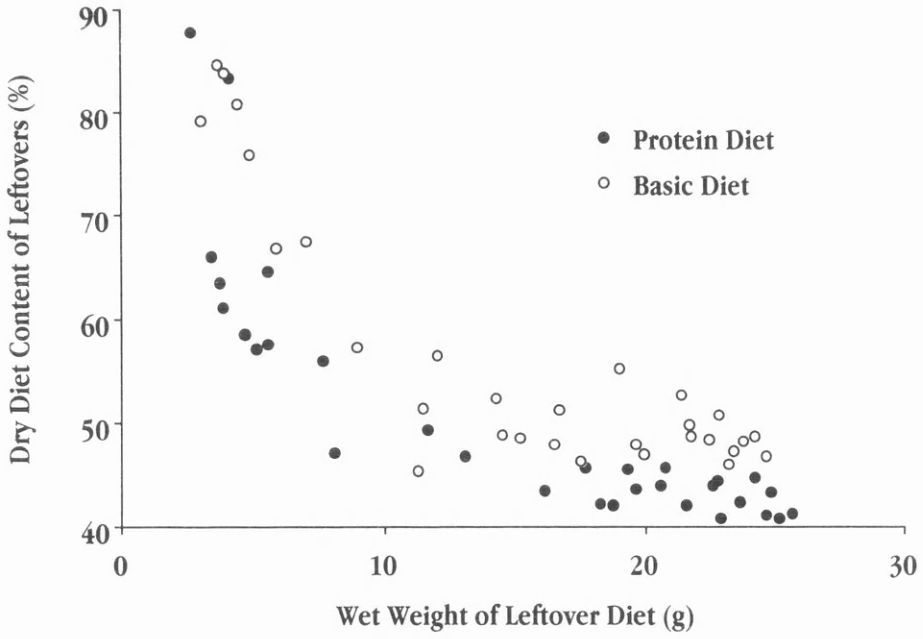


Fig.2.1 Relationship between wet weight of leftovers and percentage dry diet content of leftovers. Points represent individual observations.

The composition of leftover diets remained fairly constant (approx. 50% water) between 25g and 10g, the surface layer of the diet effectively insulating the diet below from drying out (Fig.2.1). Below 10g, insufficient diet was present for this to occur, and the leftovers tended to dry out more completely.

To obtain predictive parameters for the relationship between the weight of the diet leftover (after 24 hours in the cage) and its dry weight, non-linear regression analysis was carried out on the data (Fig.2.1), using the equation for a Weibull distribution as a model for the relationship (Tab.2.2). The equation of the fitted line was determined using the PC statistical package SPSS Ver.7.

Equation for Weibull Distribution		**Constant Values for Diets**	
$y = A \exp\left(-\left({}^{x}/_{B}\right)^{C}\right)$		**Basic**	**Protein**
y = dry weight of diet in (g) x = wet weight of leftover diet (g)	**A** **B** **C**	0.9738 ± 0.052 36.5917 ± 4.348 0.6008 ± 0.097	0.9517 ± 0.057 34.5253 ± 5.549 0.4633 ± 0.072
Goodness of fit	**Rsq**	0.861	0.843

Tab.2.2 Basic equation and constants for the prediction of dietary solids content from wet weight of dietary leftovers. Values are shown ± standard errors.

Predicted dry diet content of the protein supplemented diet tended to be slightly less than that of the basic diet over the range of leftover weights encountered in the trial (Fig.2.2). This difference reached a maximum in the region of 5-10g, and decreased slightly towards the extremes of the weight range. Hence equal wet weights of basic and protein diet contained different proportions of solids and water; the basic diet containing most solids and least water.

In the case of a diet choice experiment, if intake of dietary solids is the determinant of the amount of food eaten, this complication poses no problem for the calculation of the amount of solids eaten by the bird. However, if wet weight of food eaten determines satiation the slight difference in water content of the diets (Tab.2.1) should be taken into account. Previous studies have indicated distension of the crop by water (and even inflated balloons) to induce satiety in chickens (Richardson 1970, Gentle and Richardson 1972).

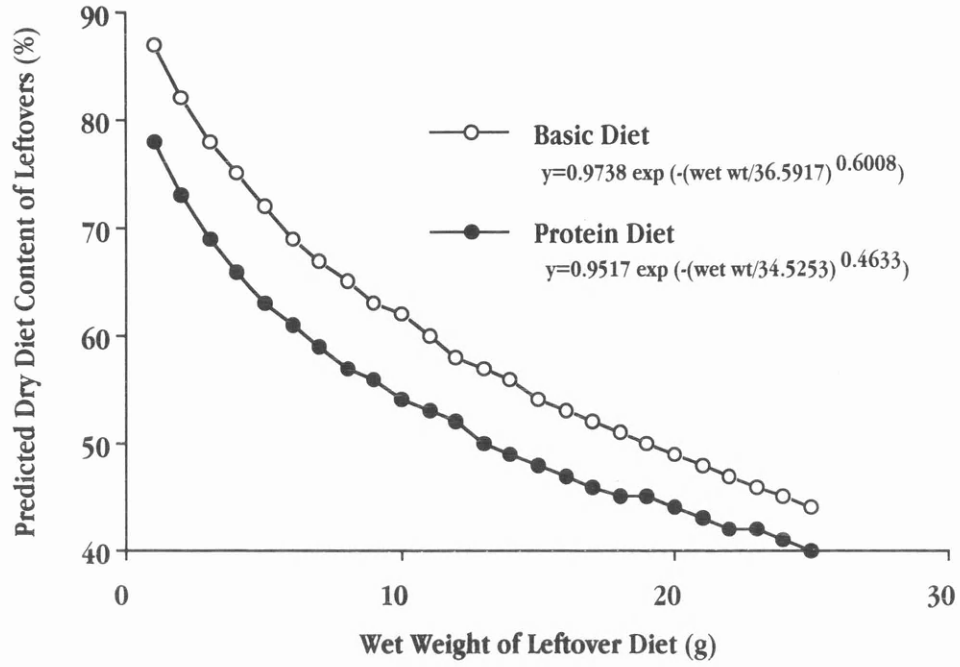


Fig.2.2 Relationship of wet weight of leftovers to predicted dry diet content, over the full range of leftover weights encountered in the dietary trial. See Tab.2.2 for calculation of predicted dry weight of diets. Points represent predicted weights correlating to specific wet weights (1g intervals).

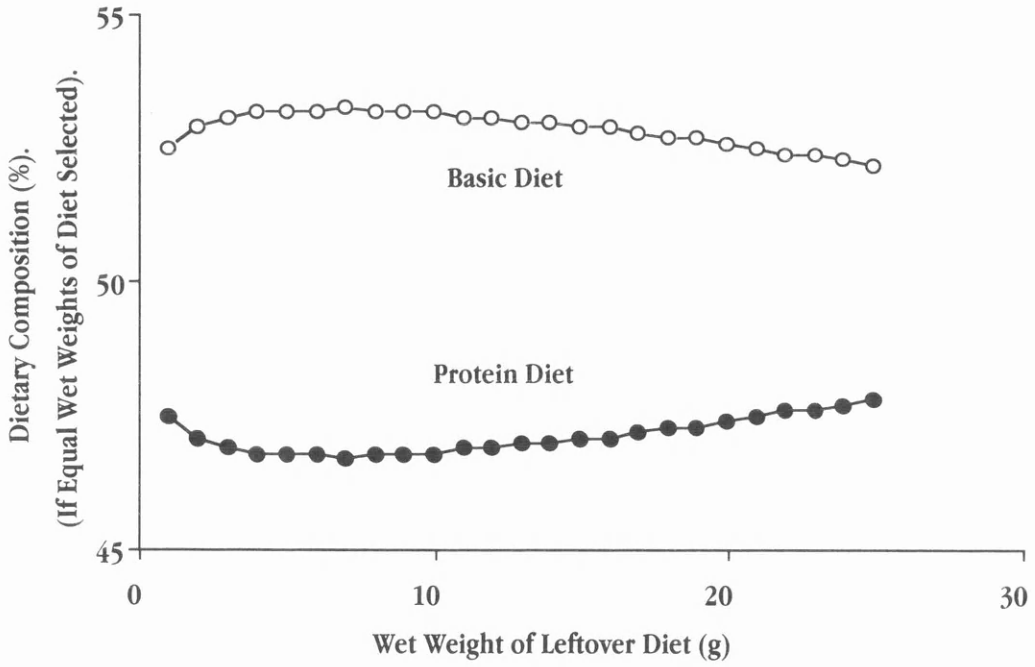


Fig.2.3 Comparative dry weight composition of a diet comprised of 50% basic and 50% protein diet by weight wet. Points represent predicted compositions correlating to specific wet weights (1g intervals).

If birds were to eat an equal wet weight of each diet choice, by default, slightly more basic diet solids would be eaten than protein diet solids. This difference might be falsely interpreted as a selective preference for ingestion of the basic diet. Using the data in Fig.2.2, the dietary solids intake from a 50:50 wet mix of basic and protein diet choice was determined for the range of leftover weights encountered. This indicates that the reduced water content of the basic diet may account for a maximum of a 6% apparent elevation of solids intake above that of the protein diet (Fig.2.3).

2.3.3 *Amino acid composition of experimental diets*

Protein limitation of reproduction in birds may arise from gross protein restriction or limited availability of specific amino acids. The amino acid composition of ripe grain does not match that of zebra finch eggs. The essential amino acids lysine, threonine and methionine are highly concentrated in egg proteins in comparison with ripe seeds, and are hence potentially the most limiting for egg production (Allen and Hume1997).

The amino acid composition of the experimental diets was determined by acid hydrolysis performed at the Rowett Research Institute, Aberdeen (Tab 2.3). The total protein content of the protein supplemented diet was 24% by weight, double that of the basic seed diet (12%). Potentially limiting amino acids were additionally concentrated in the supplemented diet in comparison with the basic seed diet (lysine 480%, threonine 250%, methionine 350%).

However, it should be stressed that these data provide only an indication of the amount of protein / amino acids present in the experimental diets. These values should not be taken to give any indication of the availability of dietary constituents to the birds once ingestion has taken place. Gut characteristics, the physical form of the diet and chemical interactions between constituent amino acids and carbohydrates can all effect the bioavailability of dietary amino acids (for example Pond *et al.* 1995).

	Basic Diet (mg/g)	Protein Diet (mg/g)
Essential		
Arginine	6.60	16.18
Histidine	2.61	5.25
Lysine	2.84	13.57
Isoleucine	4.17	10.14
Leucine	11.05	20.56
Valine	5.11	12.50
Methionine	2.16	7.57
Cystine/2	2.12	5.03
Phenylalanine	5.79	12.64
Tyrosine	4.00	8.35
Threonine	4.01	10.02
Tryptophan	-	-
Non-essential		
Alanine	7.44	13.83
Aspartate*	5.79	21.72
Glutamate*	32.62	47.58
Glycine	3.32	6.95
Proline	9.92	12.80
Serine	6.24	14.60
Protein content of diet (mg/g)	115.8	239.29
% Protein Content of Diet	**12**	**24**

Tab.2.3 Amino acid composition of experimental diets. Amino acid analysis was carried out by the Rowett Research Institute, Aberdeen.

*** Note : Asparagine and glutamine are deaminated by acid hydrolysis to aspartate and glutamate.**

2.4 DISCUSSION

2.4.1 *Selection of methodology for diet supplementation*

A range of methodologies was investigated for the provisioning of dietary supplements to captive zebra finches. The invasive techniques of intubation (2.2.1) and force-feeding (2.2.2) were found to be time-consuming and caused unacceptable disturbance to the birds. The formulation of suitable semi-synthetic diets (2.2.3) using pure amino acids was complicated by the complexity of interactions of amino acid nutrition and the disproportionately adverse reactions of some individuals to slight imbalances in the composition of the diet.

A wet mash based on seed flour and rusk was selected as the most appropriate medium for delivery of the dietary supplement (2.2.4). Allowing birds to freely select experimental diets caused minimal disturbance. The use of boiled hens egg white as a protein supplement enabled large numbers of birds to be provisioned with elevated levels of protein without obvious pathological effects. Amino acid analysis of the diets, in combination with daily monitoring of consumption, provided a mechanism by which nutrient intake of the birds might be calculated.

The major disadvantage of this method of food provisioning was that calculation of the amount of diet ingested by the birds was complicated by evaporative water loss. Water loss varied as a function of the initial composition of the diet, time between serving and recovery, and the amount of diet remaining in the bowl (ambient temperature being maintained at 20.5°C). This necessitated the correction of the wet weight of leftovers prior to calculation of consumption (2.3.2). This calculation represented a potential source of error in the estimation of dietary intake.

The second disadvantage of this method was that, as the experimental diet was made freely available to the pairs, it was not possible to separate the intake rates of males and females. Hence, analysis of intake rates was restricted to consideration of pairs of birds, rather than individuals.

The dietary formulation facilitated the simple addition of nutrients to the basic diet, without effecting its visual or textural appearance. For example, the protein supplemented diet (Tab 2.1) was ostensibly indistinguishable from the basic diet, despite the former having twice the protein content of the latter.

In conclusion, the selected dietary formulation and provisioning protocol represent a flexible methodology for supplementation studies. Being similar in appearance, these diets would enable the investigation of dietary self-selective ability in the absence of visual or textural cues (*Chapter 3*). Additionally, the moist nature of the basic diet would enable the simple addition of food colorants for investigation of the visual determinants of diet choice.

Chapter 3

SELF-SELECTION OF A DIET CONDUCIVE TO THE PROMOTION OF EGG PRODUCTION BY ZEBRA FINCHES

"Stomachs, for example, vary in size, shape and contour... They also vary in operation... A Mayo Foundation study of about 5000 people who had no known stomach ailment showed that the gastric juices varied at least a thousand fold in pepsin content. The hydrochloric acid content varies similarly... Such differences are responsible for the fact that we do not tend to eat with equal frequency or in equal amounts, nor to choose the same foods..."

R.J. Williams (1978)
from Hughes (1993)

SUMMARY

The ability of zebra finches to self-select between basic and protein-supplemented diets to maximise egg production was investigated. When presented with a choice between diets ostensibly differing only in protein content, finches differentiated between the two, selecting 70% basic to 30% supplement. Though daily consumption did not differ significantly between dietary groups, birds receiving a choice between diets tended to maintain their body weight while producing the largest clutches and eggs, in comparison with birds maintained solely on either one of the diets. Having shown dietary discrimination to occur, "perceived taste", influenced by a specific nutrient appetite, is suggested as the basis for diet selection. Implications for lab and field studies are discussed.

3.1 INTRODUCTION

3.1.1 *Protein limitation of egg production*

Egg formation places nutritional demands on a bird in addition to those of routine metabolism (Robbins 1981). Dietary complementation with protein rich supplements has previously been demonstrated to promote an advancement of laying date and/or increase in egg/clutch size in a number of species of birds (Clamens and Isenmann 1989, Dhindsa and Boag 1989, Ewald and Rohwer 1982, Schoech 1996, Soler and Soler 1996, Svensson and Nilsson 1995) including the zebra finch (Selman and Houston 1996, Williams 1996). Additionally, egg production in wild birds feeding on a diet with a naturally high protein content has been shown, possibly, to be limited by quality of protein i.e. availability of specific amino acids (Bolton *et al.* 1992, 1993, Ramsay and Houston 1997, 1998).

3.1.2 *Strategies for protein supplementation*

The protein demands of egg production are met by utilisation of endogenous reserves and dietary intake. Onset of breeding in birds may be triggered by attainment of sufficient endogenous protein reserves (Jones and Ward 1976), or conducive environmental conditions - e.g. insect availability (Fogden and Fogden 1979), though in many species the relative importance of endogenous and exogenous nutrient sources is still being exacted (e.g. Bromley and Jarvis 1993).

However, the potential for utilisation of endogenous reserves is limited by the requirement for self-maintenance. During the period of egg formation, the deficit to internal reserves may be minimised by changes in the diet to increase the intake of limiting nutrients. For example, blue-winged teal, *Anas discors*, increase consumption of invertebrates (Swanson and Meyer 1977), while pink-footed geese, *Anser brachyrhynchus*, select specific species and age classes of grasses (Fox 1993) to maximise protein intake during the breeding season.

Though zebra finches have been occasionally observed to eat insects, such as ants, aphids and termites (see Zann 1996), and feed these to young nestlings (Davies 1977), less than one percent of birds sampled by Zann and Straw (1984) over a 15 month period, had insects in their crops and in these birds insects represented less than one percent of food items. Zebra finches feed almost exclusively on grass seeds (Zann and Straw 1984), predominantly *Echinochloa crus-galli*, though its representation in the diet varies, both seasonally and with location (Zann 1996).

Zann and Straw (1984) initially suggested that the exclusion of insects from the diet of wild zebra finches may indicate that egg production was not limited by protein availability. However, breeding in wild finches has been noted to be closely linked with the availability of unripened seed, which they readily consume (Morton and Davies 1983, Watson 1992, Zann 1996). Analysis of unripened grass seed by Allen

and Hume (1997), showed that the amino acid profile of the unripened grain not only closely matched that of eggs, but more closely resembled that of eggs than that of ripened seed. Thus, potential protein limitation of egg production may be ameliorated, in the case of the zebra finch, by the opportunistic timing of breeding to coincide with availability of unripe seed.

3.1.3 *Discrimination of dietary protein*

Protein supplementation of the diet of captive zebra finches has been shown to elicit the laying of larger eggs and clutches (Williams 1996), but without accurate monitoring of the amount of food eaten over the course of the trial, it is possible that the increase in egg production may have arisen as a result of more food being eaten when the birds were provided with the supplement: presentation of increased amounts of food has previously been shown to elicit increased consumption in domestic chickens, *Gallus gallus* (Ross *et al.* 1962).

Selman and Houston (1996) showed that birds receiving a protein supplement prior to pairing later produced more and larger eggs than those maintained on a seed only diet, despite the fact that during the period of egg formation and laying both groups were maintained on identical diets and ate similar quantities of food. This would indicate that there might be a strong selective pressure on the female to choose diets which enabled the development of high quality endogenous stores for utilisation in future breeding attempts. However, provisioning with visually/texturally distinct basic and supplemented diets represents an opportunity for selection on the basis of criteria outwith protein content (2.1). Hence, the study of Selman and Houston (1996) does not provide any indication of the ability of the birds to select between diets purely on the basis of protein content.

It should be remembered that the nutrient appetite of an individual is dynamic, and the degree of balance of a specific diet is as much a function of the metabolic state if the individual, as the nutrient composition of the forage. For example, self-selection of protein : carbohydrate balance by chickens has been demonstrated to decrease with ageing, as protein requirement for growth lessens (Forbes and Shariatmadari 1994). Heat stress has similarly been demonstrated to reduce protein selection in both rats (Musten *et al.* 1974), and chickens (Forbes and Shariatmadari 1994), possibly to satisfy an increased energy requirement for thermoregulation.

In the case of zebra finches, laboratory studies indicate that the factors which determine selection of grass seed include size and net energy gain per unit handling time, both of which are subordinate to familiarity (see Zann 1996). However, determinants of diet selection in captive and wild birds may be quite different. The nutrient status of wild birds may be expected to be different from that of captive birds, while other factors independent of the nutrient quality of the forage, e.g. predation risk, may heavily influence dietary complementation in the wild (Murphy 1994b).

An ability, innate or learned, to evaluate the relative nutritional value of a dietary option and moderate its intake appropriately, is necessary if a bird is to be able to respond effectively to a specific nutrient appetite. This being so, preferences for specific colours or familiar food items should relate to preconceived judgements of the nutrient content of that food item relative to nutrient requirement of the individual, rather than arbitrary preference.

If birds are not able to distinguish between diets outwith visual/textural indicators, then the relevance of the results of dietary supplementation trials to the mechanisms of nutrient provisioning encountered under more natural conditions is questionable.

3.1.4 *Self-selection of diet by birds*

Self-selective ability has been widely studied in rats. Among birds, the domestic chicken is most studied. In chickens, development of dietary selective competence arises from innate ability, modified by individual and social learning (*review* Hughes 1979). On hatching, chicks initially peck at small objects, both nutritious and otherwise, discriminative ability developing gradually (Hogan-Warburg and Hogan 1981), partly through young chicks reciprocating the food choices of the hen (Turner 1964).

Chickens are able to self-select adequate diets on the basis of gross protein content (Elkin *et al.* 1985, Forbes and Shariatmadari 1994, Kaufman *et al.* 1978), specific amino acids e.g. lysine (Newman and Sands 1983) and methionine, vitamins e.g. thiamine (Hughes and Wood-Gush 1971), and minerals, including calcium, zinc, and phosphorous (*review* Hughes 1979). Given a protein deficient diet, and a choice of specific amino acid supplements, chickens are able to self-select close to the National Research Council recommended intake (Summers *et al.* 1991), and maintain egg productivity (Emmans 1977). In fact, the ability of poultry to accurately self-select optimum diets has prompted the suggestion that time should be invested in aiding the discriminatory learning of poultry rather than the formulation of compound feeds (Forbes and Covasa 1995).

3.1.5 *The current study*

The following experiment was designed to investigate whether, given a choice between diets differing only in protein content, female zebra finches could self-select between the two, to maximise egg production.

A basic experimental mash based on cereals was formulated (2.3.1), to which a protein supplement (boiled hens egg white) could be added, while maintaining the visual/textural integrity of the diet. Egg white was used as a protein supplement in the assumption that it should represent a high quality source of many of the specific amino acids required for egg production. Egg white contains a similar complement

of amino acids to that of whole egg but lacks the complex of lipids, essential fatty acids and other nutrients present in the yolk (see McCance and Widdowson 1978, 1980).

Established pairs of zebra finches were divided into three standardised treatment groups (1.1.4) and provisioned, respectively, with a basic diet, a protein supplemented diet, and a choice between the two. Throughout the trial, dietary intake was monitored daily. Nest boxes were checked each morning and the size and weight of any newly laid eggs were measured.

Pairs were maintained on these diets prior to and during the formation of a primary clutch (removed on completion) and a replacement clutch of eggs (also removed on completion). Birds were encouraged to lay replacement clutches in the expectation that the requirement for dietary protein might be elevated during this period, were body reserves of protein depleted as a result of investment in the primary clutch.

After removal of the replacement clutch, all groups were provided with a choice between the two diets, and allowed to lay a third clutch. It was predicted that, under these conditions, were the protein diet an overly rich source of protein, birds previously maintained only on this diet might select more basic diet than those previously receiving a diet choice. Similarly, if the basic diet was protein impoverished, birds previously receiving the basic diet only, might be expected to select more protein when given a choice.

It was predicted that, when presented with a choice, the birds would be able to distinguish between basic and protein supplemented diets and moderate their intake to produce the largest eggs / clutches. The selection of protein was expected to: increase at the onset of laying, the first day of laying representing the maximum protein requirement of the female (Donnan 1993, Houston *et al.* 1995a); and also increase over the course of successive clutches, as endogenous reserves for egg production became progressively depleted.

3.2 METHOD

3.2.1 *Experimental protocol*

Birds were kept in cages measuring 60cm x 50cm x 40cm, under a lighting regime of 8 hour dark to 16 hour light using timer controlled "daylight" UV lighting (*Lightbox*). All birds received cuttlebone, water and oyster grit *ad libitum*. Ambient temperature was maintained at 20.5°C (+/-2).

Three groups of 15 standardised pairs of finches, 90 birds in total, were allocated to experimental feeding groups, (see 1.1.4 for standardisation procedure):

Group	***Diet*** *(see 2.3.1 for details)*
Basic	23% mixed seed flour, 23% white rusk, 54% water
Protein	17% mixed seed flour, 17% white rusk, 20% water, 46% egg white
Choice	both of the above diets in separate bowls

After the standardisation trial (1.1.4), all birds were maintained on a mixed seed diet for a period of two weeks prior to introduction of the experimental diets.

Experimental diets were accurately weighed out (+/- 0.1g) and each bird was provisioned with 15g of diet, (previous trials indicated 15g of diet to represent a daily excess for any individual bird). Each *Choice* bird was allotted 15g of basic and 15g of protein diet in separate dishes. Experimental diets were introduced to the cages on the first morning of the trial and replaced with fresh diet every 24 hours. Any uneaten diet was removed from the pot, weighed immediately and dry weight of leftovers was determined using the methodology outlined previously (2.3.2). Birds were maintained under these conditions for eight days, to allow them to become accustomed to the new diets.

During this time, due to limitations on space, females were kept in communal cages (each containing 7-8 females from the same experimental group), while males were transferred to individual cages and provided with nest boxes. This was done to allow them time to investigate the nest boxes before introduction of the females.

After eight days on the experimental diets, females were removed from the communal cages and reunited with their partners. Pairs were provided with hay lined nest boxes and fresh grass to encourage breeding. Protocol for delivery of experimental diets did not change, each cage being provisioned with 15g of fresh diet (15g of each diet for *Choice* birds) per bird each morning.

Diets were presented in similar sized, clear glass dishes, which were labelled with the same colour of marker pen. Dishes were positioned on the floor of the cage. In the case of the choice diets, dishes were placed side by side and their positioning swapped each morning to guard against possible spatial bias in preference.

Nest contents were monitored daily. Eggs were removed and measured on the morning of laying and replaced with replicas. Eggs were weighed (+/- 0.001g) and their length and width measured (+/- 0.1mm). After three successive days without additional eggs being laid, the first clutch was considered complete and the replica eggs were removed. Fresh nesting material was provided to encourage the birds to lay a second clutch, under a continuation of the dietary regime.

On completion of the second clutch, the replica eggs were again removed. *Basic* and *Protein* groups were transferred to a choice between the two experimental diets, while *Choice* birds continued to receive a choice between the experimental diets. Fresh nesting material was provided to encouraged the birds to lay a third clutch of eggs.

3.3 RESULTS

3.3.1 *Dietary intake and egg production*

The dietary intake of the female zebra finch could not be easily separated from that of the male. It could have been assumed that the male was responsible for half of the food eaten (e.g. Williams 1996), consumption of male and female zebra finches having previously been shown to be similar (Donnan 1993, El-Wailly 1966), however, with the male not having a nutrient demand for egg production, this assumption may have become flawed as the breeding cycle progressed. It could also have been assumed that the dietary intake of the male was constant throughout the trial. However, feeding is a social activity in many species of birds (see Hughes 1993), and hence, feeding behaviour exhibited by the female may be reciprocated by the male. As such, it was decided not to attempt to isolate the consumption of males and females, but instead consider consumption rates of pairs of birds.

To qualify for inclusion in the statistical analysis of results, pairs were required to lay at least one egg within 13 days of introduction / removal of the previous clutch. Birds which did not lay were excluded from the analyses for that time period, and from subsequent periods. Birds previously receiving *Basic* only or *Protein* only diets were re-included in the analysis of *Clutch.3* if the presentation of a choice diet encouraged them to resume laying.

For the purposes of statistical analysis, the trial was divided into ten discrete periods. The first period of intake was:

Introduction
Intake on the first day of the trial.

Each of the three laying periods were sub-divided into three sections as follows:

Pre-Laying
Mean daily intake over the three days prior to the laying of the first egg of a clutch.

Laying
Mean daily intake from the day of laying of the first egg of a clutch, to the day of the last egg.

Post-Laying
Mean daily intake from the day after laying of the last egg, to the day of removal of the clutch (i.e. 3 days in total)

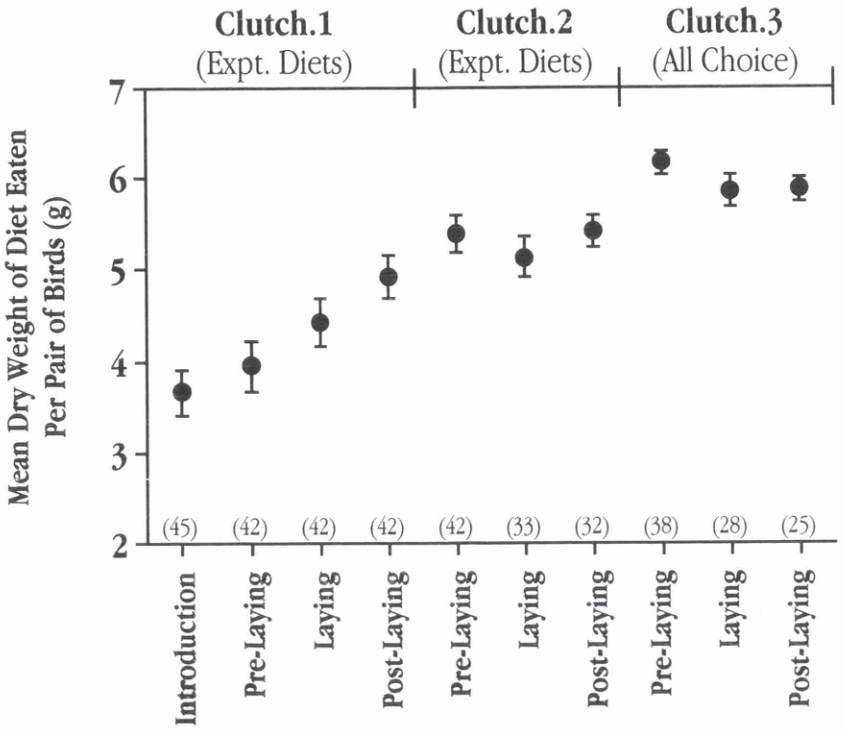


Fig.3.1 Daily mean dry weight of diet eaten by pairs of zebra finches over the course of three successive clutches. Dietary intakes from all experimental groups are combined. Bars indicate standard errors. (n) = number of pairs.

Mean dietary intake increased significantly over the course of the trial (ANOVA $F_{9,359} = 14.27$, $p = 0.000$), and over successive *Laying* periods (ANOVA $F_{2,100} = 9.07$, $p = 0.000$), (Fig.3.1), with mean intake significantly increasing during the *Laying* period of each successive clutch (Fisher's pairwise comparisons).

Repeated Paired samples T-Tests (Tab.3.1) indicated that intake generally increased significantly over each period of the trial in comparison with the previous period. Exceptions to this trend occurred during the *Laying* period of both second and third clutches, during which dietary intake fell in comparison with the previous periods (highlighted in Tab.3.1).

Laying Period	Comparison of intakes Paired samples T-test	
Introduction v Pre-Laying.1	n = 42, t = 2.05, p = 0.047	*Introduction < Pre-Laying.1*
Pre-Laying.1 v Laying.1	n = 41, t = 4.15, p = 0.000	*Pre-Laying.1 < Laying.1*
Laying.1 v Post-Laying.1	n = 42, t = 3.89, p = 0.000	*Laying.1 < Post-Laying.1*
Post-Laying.1 v Pre-Laying.2	n = 42, t = 3.39, p = 0.002	*Post-Laying.1 < Pre-Laying.2*
Pre-Laying.2 v Laying.2	n = 33, t = 1.62, **p = 0.115**	***No significant change***
Laying.2 v Post-Laying.2	n = 32, t = 2.14, p = 0.041	*Laying.2 v < Post-Laying.2*
Post-Laying.2 v Pre-Laying.3	n = 32, t = 4.70, p = 0.000	*Post-Laying.2 < Pre-Laying.3*
Pre-Laying.3 v Laying.3	n = 28, t = 2.29, **p = 0.030**	***Pre-Laying.3 > Laying.3***
Laying.3 v Post-Laying.3	n = 25, t = 0.75, p = 0.458	*No significant change*

Tab.3.1 Summary of statistical comparisons of dietary intake of pairs of finches over the course of the trial. Exceptions to the general trend of increasing dietary intake are highlighted.

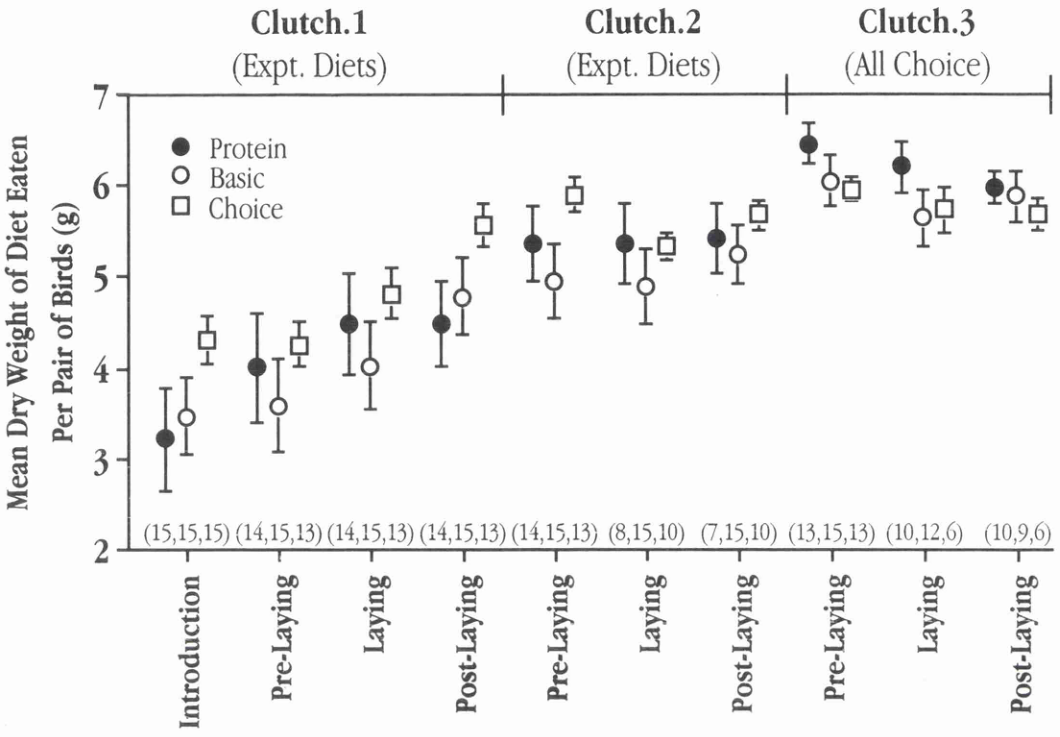


Fig.3.2 Daily mean dry weight of food eaten by pairs of zebra finches, divided into dietary groups. Bars indicate standard errors. (n) = number of pairs.

Subdividing intake data between dietary groups (Fig.3.2), produced a similar trend to that in Fig.3.1, with intake increasing over the course of the trial (with the exception of the *Laying* periods of the second and third clutches).

There was no significant difference in dietary intake between experimental groups at any point in the trial. Therefore, any differences in egg production between dietary groups cannot be attributed to different amounts of food being eaten.

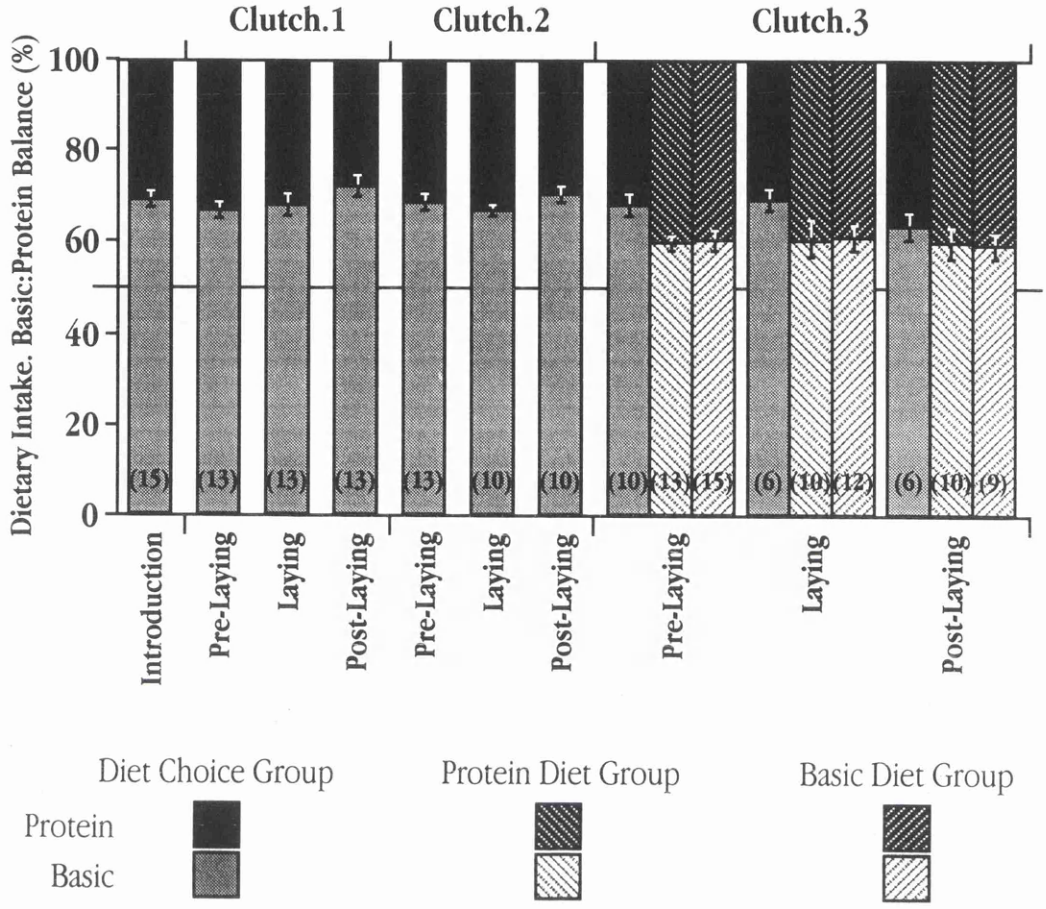


Fig.3.3 Ratio of Basic diet : Protein diet intake of birds presented with a choice of diets. Dietary intake over first and second clutches are shown for *Choice* group birds. Intake over third clutches are shown for all experimental groups. Bars indicate standard errors. (n) = number of pairs.

When presented with a choice between basic and protein supplemented diets, birds displayed a clear selective tendency (Fig.3.3). In the *Choice* group, diet intake was maintained fairly constantly at approx. 70% basic to 30% protein throughout the course of the trial. This balance did not vary significantly over the course of the trial (ANOVA $F_{9,99} = 0.98$, $p = 0.458$).

When *Protein* and *Basic* birds were introduced to a choice of diets, a similar tendency was observed. Despite having no previous experience of a choice between diets, intake in these groups was maintained at approx. 60% basic and 40% protein. There was no significant difference in intake between dietary groups during *Clutch.3, Pre-laying* (ANOVA $F_{2,35} = 3.06$, $p = 0.06$), *Laying* (ANOVA $F_{2,29} = 1.60$, $p = 0.219$) or *Post-laying* (ANOVA $F_{2,22} = 0.43$, $p = 0.655$).

The standard binomial confidence interval for dietary balance of *Choice* birds was calculated as follows:

$$\text{Confidence Interval} = Z \times \sqrt{(B \times P) / n} = +/- 0.09$$

Z = 1.95 (for 95% C.I)
B = Mean proportion of basic diet selected (= 0.32)
P = Mean proportion of high protein diet selected (= 0.68)
n = Sample size (= 109 mean diet selection events)
(15 pairs of birds x 10 time periods,
41 missing data values due to non-laying)

Mean Intake of Protein Diet = 32% (+/- 9%)
Mean Intake of Basic Diet = 68% (+/- 9%)

The 95% confidence intervals for the selection of each diet are exclusive of 50% mark, indicating that the balance of intake was outside that which could be attributed to chance. These confidence intervals are also exclusive of the additional maximum discrepancy of 47%:53% which may have arisen due to the slight difference in water content of the two diets (2.3.2). As such, the selection of diet is regarded as being outwith that which could be attributed to chance ($p < 0.05$).

It could be argued that the sample size of 109 is not statistically valid, as it includes repeated measurement of the diet choice of the same pairs of birds. These may be regarded as non-independent observations. However, as differential intake is the point of interest, this potential relatedness between observations is inconsequential. Indeed, it is not only expected, but may be regarded as re-enforcement of the validity of the test. i.e. differential selective ability between pairs of birds is more a confirmation of the occurrence of selection, than an indicator of random feeding.

Zebra finches have been shown to self-select a mean balance of 32% high protein diet : 68% basic diet. This ratio was combined with data on the amino acid composition of the diets (Tab.2.3.3) to allow calculation of the mean amino acid intake of this diet choice ratio (Tab.3.2).

	Basic Choice (mg/g)	Protein Choice (mg/g)	Combined Choice (mg/g)
Essential			
Arginine	4.49	5.18	**9.67**
Histidine	1.77	1.68	**3.45**
Lysine	1.93	4.34	**6.27**
Isoleucine	2.84	3.24	**6.08**
Leucine	7.51	6.58	**14.09**
Valine	3.47	4.00	**7.47**
Methionine	1.47	2.42	**3.89**
Cystine/2	1.44	1.61	**3.05**
Phenylalanine	3.94	4.04	**7.98**
Tyrosine	2.72	2.67	**5.39**
Threonine	2.73	3.21	**5.93**
Tryptophan	-	-	-
Non-essential			
Alanine	5.06	4.43	**9.48**
Aspartate*	3.94	6.95	**10.89**
Glutamate*	22.18	15.23	**37.41**
Glycine	2.26	2.22	**4.48**
Proline	6.75	4.10	**10.84**
Serine	4.24	4.67	**8.92**
Protein content of diet (mg/g)	78.74	76.57	**155.32**
% Contribution to Dietary Protein Intake	50.70	49.30	**100**

Tab.3.2 Mean amino acid intake of birds self-selecting between basic and high protein diets. (Assuming selection of 32% high protein diet : 68% basic diet. This selection ratio is based on 15 pairs of birds sampled over 10 time periods).

*** Note : Asparagine and glutamine are deaminated by acid hydrolysis to aspartate and glutamate.**

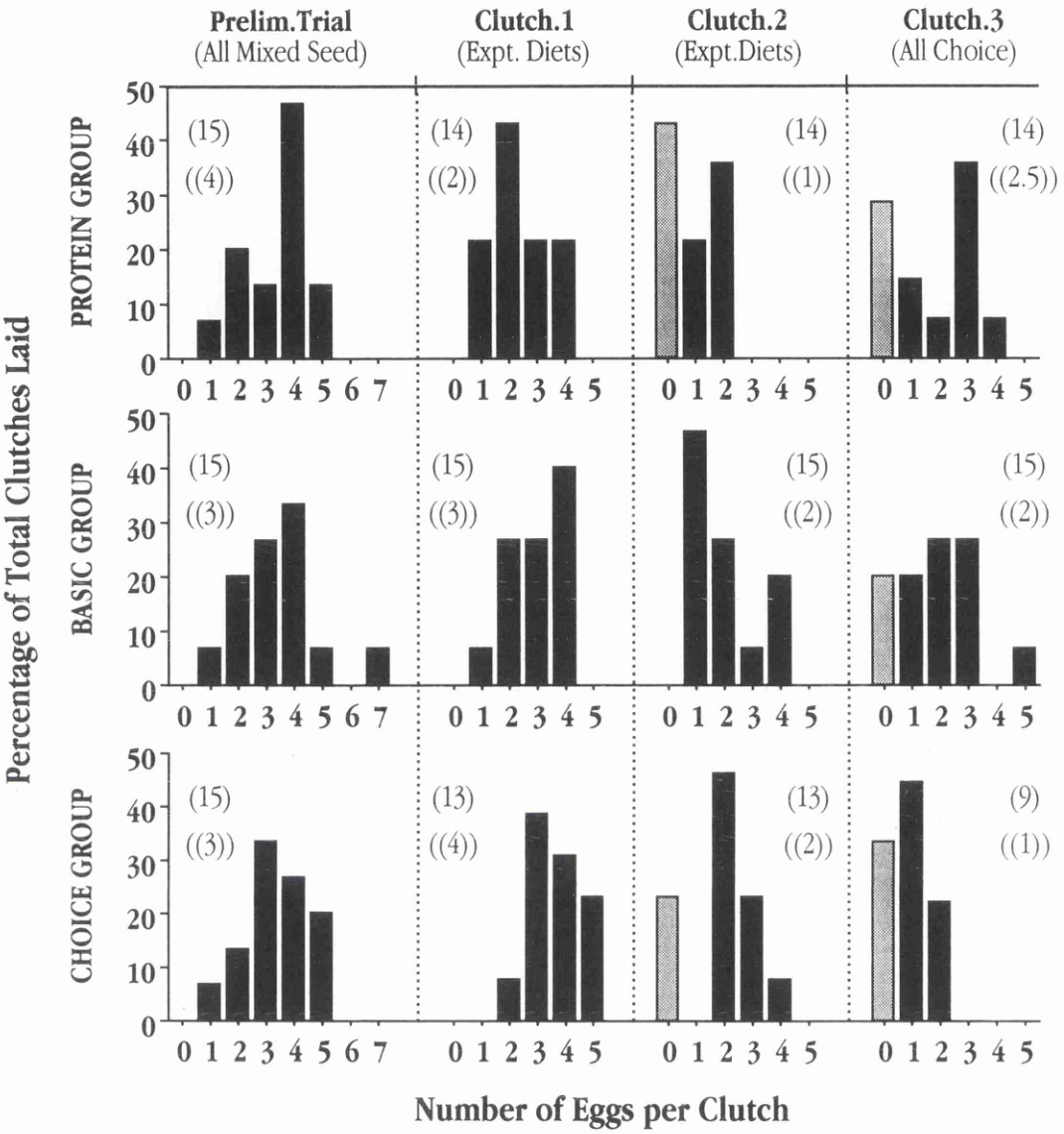


Fig.3.4 Clutch size of experimental groups. (n) = number of pairs. ((n)) = median average.

The number of eggs laid in the *Preliminary* trial (produced while receiving a mixed seed diet) and during experimental breeding attempts are shown in Fig.3.4. One *Protein* bird and two *Choice* birds were excluded from the trial due to illness / injury. In comparison with the *Preliminary* clutches, *Protein* and *Basic* birds showed a tendency towards smaller clutches over first and second experimental breeding attempts, followed by a stabilising of / increase in clutch size when presented with a choice between diets. In comparison with the *Preliminary* clutch, *Choice* birds showed an initial increase in clutch size over the first experimental breeding attempt, followed by a decline in the size of subsequent clutches.

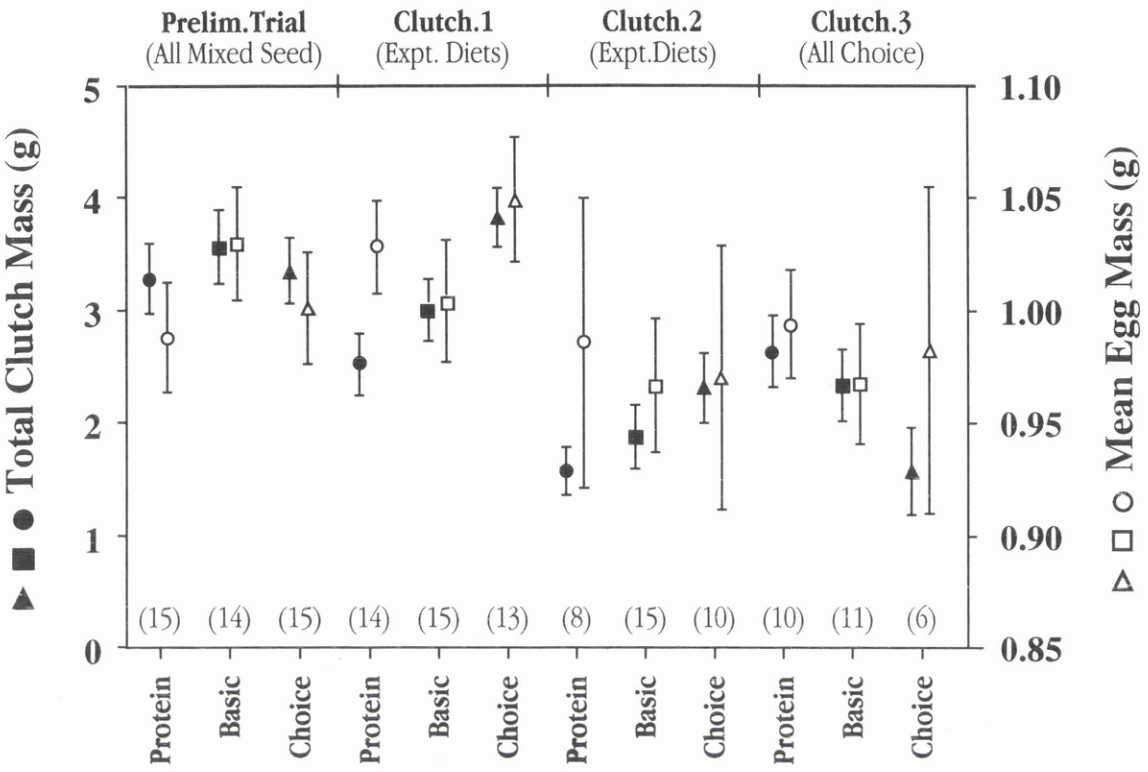


Fig.3.5 Mean clutch mass and mean egg mass of successive clutches. Bars indicate standard errors. (n) = number of pairs.

Mean clutch mass and mean egg mass of successive clutches are shown in Fig.3.5. Repeated measures ANOVA indicated significant trends in clutch mass both over the course of successive clutches ($F_{3,105} = 25.44$, $p = 0.000$), and between dietary groups ($F_{6,105} = 3.22$, $p = 0.006$). This was used as a statistical justification for individual ANOVA analysis both between clutches, and over the course of the trial.

After successful completion of *Clutch.*1, several birds did not lay a replacement clutch. When this happened, the event was scored as a "0 gram" second clutch and the pair excluded from further analysis.

Results of the statistical comparisons of mean clutch mass and mean egg mass are summarised in Tab.3.3. Total mass of *Clutch.1* was significantly greater for *Choice* birds than the other experimental groups. Mean egg mass of *Clutch.1* eggs was greater than those laid in the *Preliminary* clutch in both *Protein* and *Choice* groups.

Mean Clutch Mass		
Preliminary Clutch	*ANOVA* $F_{2,41} = 0.22, p = 0.804$	No sig. diff. between dietary groups
Clutch.1	*ANOVA* $F_{2,39} = 5.55, \mathbf{p = 0.008}$	*Fisher's Pairwise Comparisons* *Choice* > both *Protein* and *Basic*
Clutch.2	*ANOVA* $F_{2,30} = 1.33, p = 0.279$	No sig. diff. between dietary groups
Clutch.3	*ANOVA* $F_{2,24} = 2.12, p = 0.143$	Non-sig. increase in *Protein* and *Basic* Non-sig. decrease in *Choice*
Protein Group	*ANOVA* $F_{3,43} = 4.66, \mathbf{p = 0.007}$	*Fisher's Pairwise Comparisons* *Clutch.2* < all other clutches
Basic Group	*ANOVA* $F_{3,51} = 4.01, \mathbf{p = 0.012}$	*Fisher's Pairwise Comparisons* *Clutch.2* < *Preliminary* and *Clutch.1*
Choice Group	*ANOVA* $F_{3,40} = 6.92, \mathbf{p = 0.001}$	*Fisher's Pairwise Comparisons* *Clutch.2* < *Clutch.1* *Clutch.3* < *Preliminary* and *Clutch.1*

Mean Egg Mass		
Preliminary Clutch	*ANOVA* $F_{2,41} = 0.72, p = 0.492$	No sig. diff. between dietary groups
Clutch.1	*ANOVA* $F_{2,39} = 0.79, p = 0.460$	No sig. diff. between dietary groups
Preliminary v Clutch.1		
Protein	*Paired T-tests* $n = 13, t = 2.22, \mathbf{p=0.047}$	*Clutch.1>Preliminary*
Basic	*Paired T-Tests* $n = 14, t = 0.67, p=0.513$	No sig. change in egg mass between clutches
Choice	*Paired T-Tests* $n = 14, t = 2.68, \mathbf{p=0.019}$	*Clutch.1>Preliminary*

Tab.3.3 Summary of statistical comparisons of mean clutch mass (over the course of the trial), and mean egg mass (within and between dietary groups for *Preliminary* and *Clutch.1* only).

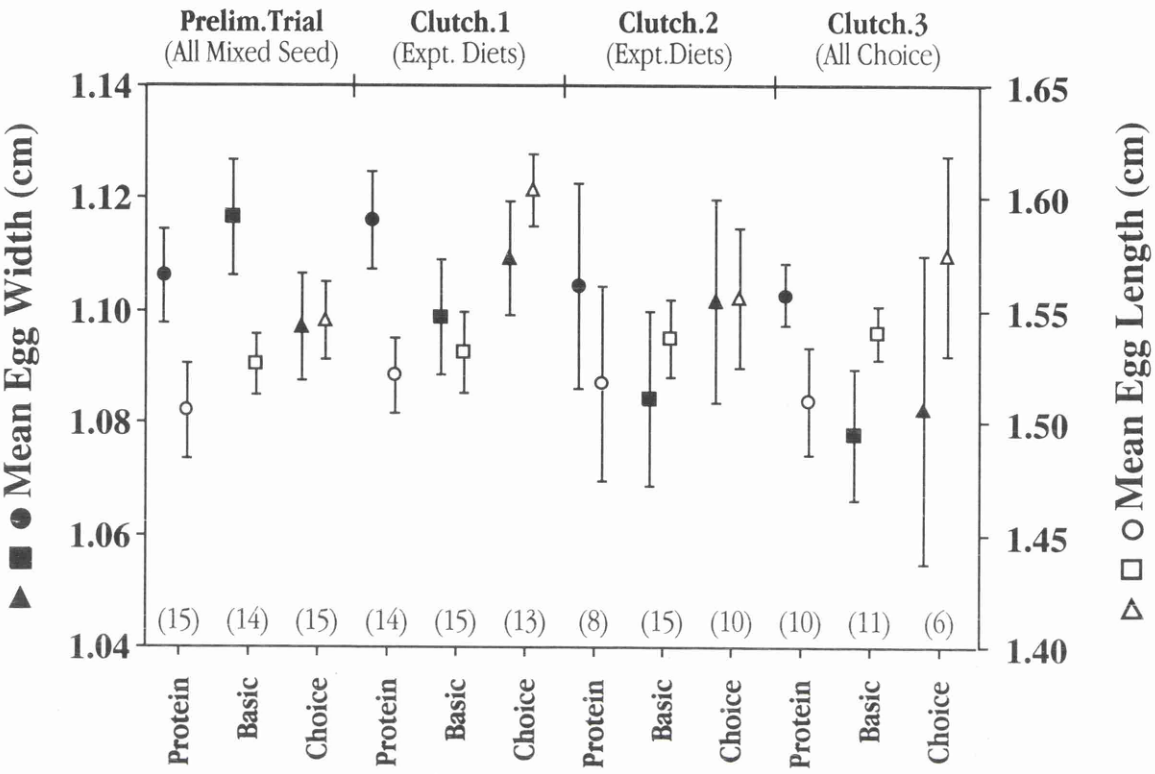


Fig.3.6 Mean width and length of eggs. Bars indicate standard errors. (n) = number of pairs.

Mean width and length of eggs remained fairly constant over the course of the trial (Fig.3.6).

Results of the statistical comparisons of mean egg width and length are summarised in Tab.3.4. *Choice* birds produced eggs in *Clutch.1* which were longer (significantly) and wider (bordering on significance) than those of the *Preliminary* clutch (produced when maintained on a mixed seed diet). *Choice Clutch.1* eggs were significantly longer than those of the other dietary groups.

Mean Egg Width		
Preliminary Clutch	*ANOVA* $F_{2,41} = 1.08, p = 0.348$	No sig. diff. between dietary groups
Clutch.1	*ANOVA* $F_{2,39} = 0.83, p = 0.444$	No sig. diff. between dietary groups
Preliminary v Clutch.1		
Protein Group	*Paired T-tests* n = 14, t = 1.24, p=0.236	No sig. diff in egg width between clutches
Basic Group	*Paired T-Tests* n = 14, t = 1.42, p=0.180	No sig. diff in egg width between clutches
Choice Group	*Paired T-Tests* n = 13, t = 2.11, **p=0.056**	Egg width *Clutch.1>Preliminary* (bordering on significance)

Mean Egg Length		
Preliminary Clutch	*ANOVA* $F_{2,41} = 1.33, p = 0.276$	No sig. diff. between dietary groups
Clutch.1	*ANOVA* $F_{2,39} = 6.44$, **$p = 0.004$**	*Fisher's Pairwise Comparisons* Length *Choice*>other dietary groups
Preliminary v Clutch.1		
Protein	*Paired T-tests* n = 14, t = 0.72, p=0.484	No sig. diff in egg length between clutches
Basic	*Paired T-Tests* n = 14, t = 0.41, p=0.692	No sig. diff in egg length between clutches
Choice	*Paired T-Tests* n = 13, t = 4.64, **$p = 0.001$**	Length *Clutch.1>Preliminary*

Tab.3.4 Summary of statistical comparisons of mean egg width and length. Comparisons are made within and between dietary groups for *Preliminary* and *Clutch.1* only.

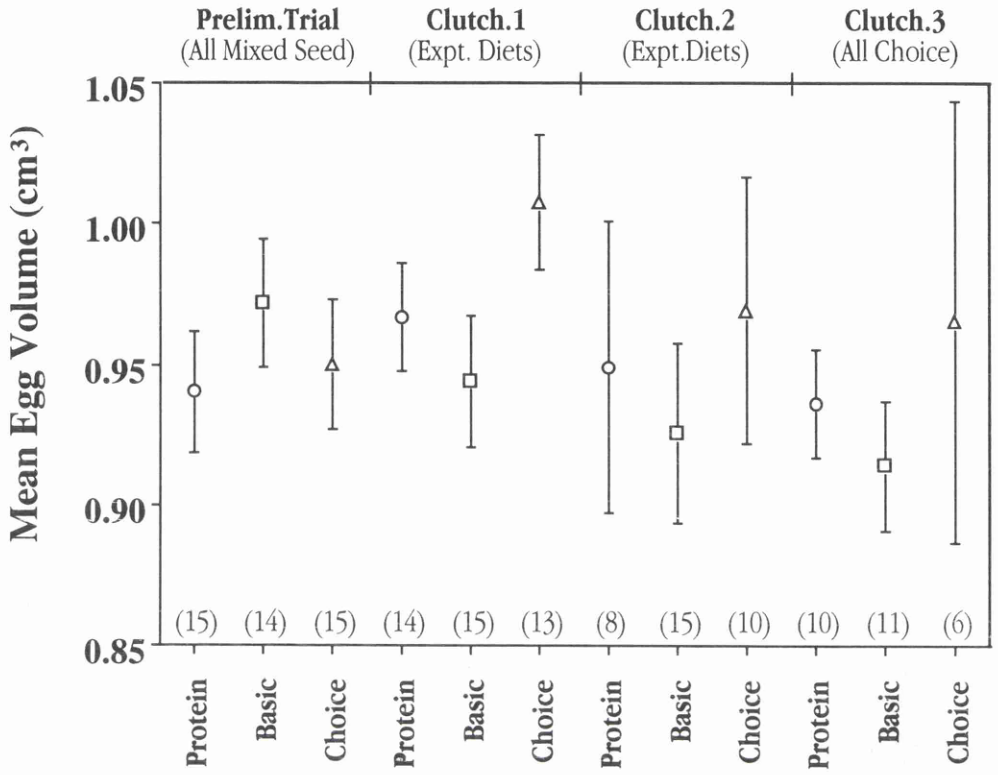


Fig.3.7 Mean volume of eggs. Bars indicate standard errors. (n) = number of pairs.

Mean volume of eggs remained fairly constant over the course of the trial (Fig.3.7). Egg volume was calculated by combination of mean width and length using the formula:

$$\text{Volume} = (0.51 \times \text{Length}) \times \text{Width}^2 \qquad \text{(Hoyt 1979)}$$

Results of the statistical comparisons of mean egg volume are summarised in Tab.3.5. *Choice* birds produced significantly larger *Clutch.1* eggs than during the *Preliminary* clutch. There was no significant difference in mean egg volume between *Preliminary* and *Clutch.1* in the other two dietary groups.

Mean Egg Volume		
Preliminary Clutch	*ANOVA* $F_{2,41} = 0.51, p = 0.606$	No sig. diff. between dietary groups
Clutch.1	*ANOVA* $F_{2,39} = 2.03, p = 0.145$	No sig. diff. between dietary groups
Preliminary v Clutch.1		
Protein	*Paired T-tests* $n = 14, t = 1.23, p=0.240$	No sig. diff in volume between clutches
Basic	*Paired T-Tests* $n = 14, t = 0.76, p=0.463$	No sig. diff in volume between clutches
Choice	*Paired T-Tests* $n = 13, t = 3.20, \mathbf{p=0.008}$	Egg volume *Clutch.1>Preliminary*

Tab.3.5 Summary of statistical comparisons of mean egg volume. Comparisons are made within and between dietary groups for *Preliminary* and *Clutch.1* only.

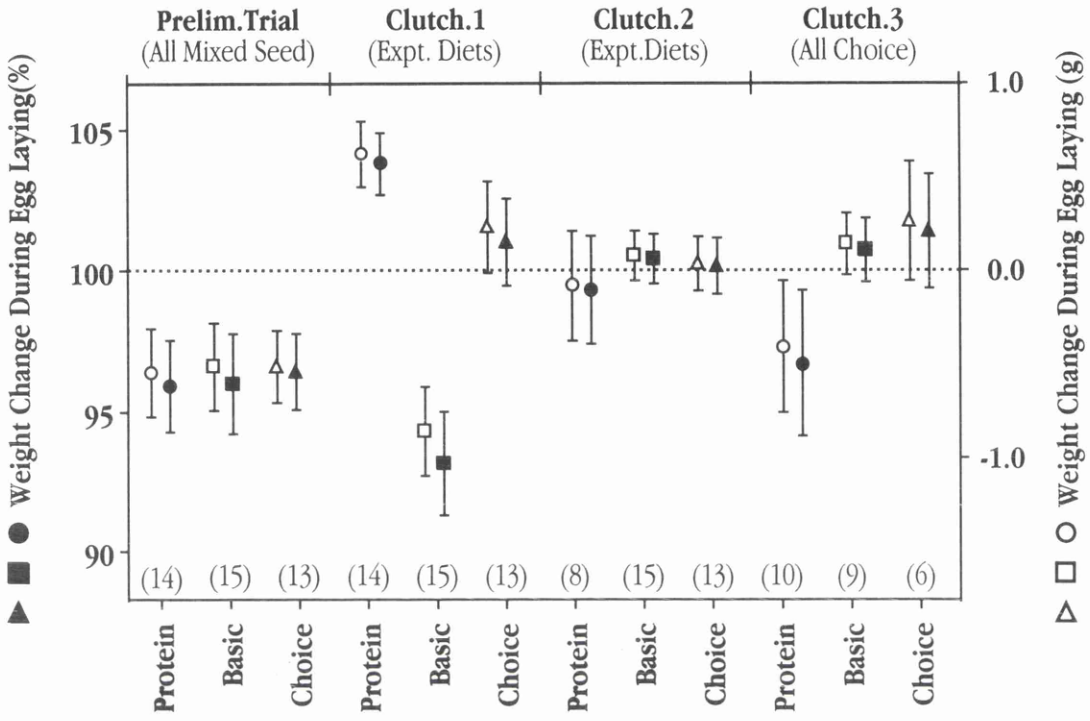


Fig.3.8 Weight change of laying females. Comparisons of pre-laying weight with post-laying weight over successive clutches. Weight change is shown both as +/- grams and as a percentage of body weight at the start of the clutch. Bars indicate standard errors. (n) = number of pairs.

Over the *Preliminary* trial birds of all dietary groups lost a similar amount of weight, approximating to 0.5g or 4% of body weight (Fig.3.8).

Results of statistical comparisons of weight dynamics of breeding females over the course of successive clutches are summarised in Tab.3.6. Females receiving *Protein* or *Choice* diets tended to maintain, or even increase body weight during laying.

Weight Change (+/-g)		
Preliminary	*ANOVA* $F_{2,37} = 0.03, p = 0.974$	No sig. diff between dietary groups
Clutch.1	*ANOVA* $F_{2,39} = 13.25, \mathbf{p = 0.000}$	*Fisher's Pairwise Comparisons* *Basic* losses sig. more weight than both *Protein* and *Choice* birds
Preliminary v Clutch.1		
Protein	*Paired T-Tests* n = 13, t = 4.44, **p=0.001**	Sig.diff in weight change between *Preliminary* clutch (ave. -0.64g) and *Clutch.1* (ave.+0.62g)
Basic	*Paired T-tests* n = 13, t = 1.32, p=0.211	No sig. diff in weight change between clutches
Choice	*Paired T-Tests* n = 11, t = 2.07, **p=0.065**	Borderline diff. in wt change between *Preliminary* clutch (ave. -0.55g) and *Clutch.1* (ave.+0.28g)

Weight Change (%)		
Preliminary	*ANOVA* $F_{2,37} = 0.01, p = 0.995$	No sig. diff between dietary groups
Clutch.1	*ANOVA* $F_{2,39} = 12.54, \mathbf{p = 0.000}$	*Fisher's Pairwise Comparisons* *Basic* losses sig. more weight than both *Protein* and *Choice* birds
Preliminary v Clutch.1		
Protein	*Paired T-Tests* n = 13, t = 4.52, **p=0.001**	Sig.diff in wt change between *Preliminary* clutch (ave. 96.1%) and *Clutch.1* (ave.104.3%)
Basic	*Paired T-tests* n = 13, t = 1.21, p=0.251	No sig. diff in weight change between clutches
Choice	*Paired T-Tests* n = 11, t = 2.22, **p=0.051**	Borderline diff. in wt change between *Preliminary* clutch (ave.96.4%) and *Clutch.1* (ave.102.3%)

Tab.3.6 Summary of statistical comparisons of weight change. Comparisons are made within and between dietary groups for *Preliminary* and *Clutch.1*. Weight change is represented as +/- g and % body weight at the start of the clutch.

3.4 DISCUSSION

3.4.1 *Self-selection of diet in the zebra finch*

Despite the visual and textural similarity of the diets, and the switching of the positions of presentation bowls on a daily basis, zebra finches were able to select between high protein and basic diets (Fig.3.3). Murphy and King (1987) observed similar discriminative ability in white-crowned sparrows, which were able to distinguish between diets, on the basis of sulphur amino acid content, within a single day's feeding. It should be stressed that the mean diet selection outlined in Tab (3.1) does not necessarily represent an optimum balance for any individual bird; this being equi-dependent on the physiological state of the individual. As pointed out by Hughes (1993), care should be taken in generalising about dietary optimality.

Contrary to prediction, the balance of dietary selection did not appear to alter over the course of the trial. Detection of selection trends may have been complicated by the short duration and intensive nature of the trial; the metabolic demands of specific points in the laying cycle possibly having protracted repercussions for subsequent periods.

The selective preference for the basic diet was not so distinct in the experimental groups previously experiencing single diets (Fig.3.3). As predicted, birds previously receiving basic diet selected comparatively more protein than did the birds maintained on the choice diet, however, this may have arisen equally from unfamiliarity of the presentation of a choice of diets. The latter is supported by the observation that birds previously experiencing only the high protein diet, contrary to prediction, did not exhibit an increased preference for the basic choice. However, exposure to high levels of dietary protein encourages an adaptive increase in the enzymes involved in the metabolism of specific amino acids (Fisher 1967). This physiological conditioning may explain the continuing elevated selection of the protein diet by birds with a high protein dietary history.

3.4.2 *Trends in dietary intake and egg production*

The opportunity to select between experimental diets enabled the female finches to lay more and larger eggs than they produced when previously maintained on a mixed seed diet, and more and larger eggs than the birds maintained exclusively on either single experimental diet. These trends are summarised in Tab 3.7.

Clutch Characteristic	
Number of Eggs Laid	Decreased over successive clutches when initially maintained on single diets, but increased when *Choice* presented. Birds maintained on *Choice* initially laid more eggs. Clutch size later decreased over successive clutches when maintained on *Choice.*
Total Clutch Mass	Trends as above.
Egg Mass	Increase in *Clutch.*1 for *Protein* and *Choice* birds. No difference for *Basic.*
Egg Length	Increase in *Clutch.1* for *Choice* birds. No difference for *Protein* and *Basic.*
Egg Width	Increase in *Clutch.1* for *Choice* birds (bordering on significance). No difference for *Protein* and *Basic.*
Egg Volume	Increase in *Clutch.1* for *Choice* birds. No difference for *Protein* and *Basic.*
Body Weight Change of Laying Female	Increase *Clutch.1* for *Choice* and *Protein* birds. *Decrease* for *Basic.*

Fig.3.7 Summary of the effects of dietary regime on egg production.

However, the promotional effects of diet choice on egg production became reduced over successive clutches. Some factor outwith dietary intake may have limited continued egg production (e.g. depletion of endogenous reserves). Alternatively, the choice diets may have become nutritionally limiting and unable to maintain a continually elevated level of egg production. Boiled egg white, while constituting a fairly balanced source of the amino acids required for whole egg formation, may have been limiting in the provision of vitamin D (required for absorbance of dietary calcium for shell formation, Robbins 1993), essential fatty acids, e.g. linoleic acid, (see Carey 1996) or general energy availability for provisioning of the fatty component of egg yolk. The latter suggestion may be supported by the trend of increasing consumption over the course of the trial (Fig.3.2, 3.3).

It is unlikely that the rise in consumption over the course of the trial could be solely attributed to initial unfamiliarity with the presentation of a mash diet, because the birds were maintained on the experimental diets for eight days prior to pairing and so had time to become accustomed to the novel diet. However, it is possible that initial under-eating may have occurred as a result of the high water and fibre content of the experimental diets in comparison with that of the routine mixed seed diet. The role of dietary fibre in birds is not fully understood, though it has been linked to an increase in dietary efficiency (see Carey 1996).

In all experimental groups, the general trend of increasing dietary intake was noted to stabilise, or even significantly fall during the egg laying period (Fig.3.2, 3.3), supporting the previous observations of reduced consumption in breeding zebra finches by Donnan (1993). A decrease in food consumption during egg laying may be regarded as counter-intuitive. However, by the time that the first egg has been laid, all subsequent eggs of the clutch are already partly developed, and the energy and protein demands on the laying female are reduced from this time on (Houston *et*

al. 1995a, but see also Krementz and Ankney 1986). Additionally, behavioural changes associated with breeding may contribute to a reduction in the energy expenditure of the bird. Locomotory activity has been previously noted to be reduced in breeding birds, prompting the suggestion that this may occur in order to conserve energy for egg production (Donnan 1993, Houston *et al.* 1995a), or as a mechanism to limit damage to developing eggs (Fogden and Fogden 1979). In zebra finches comprehensive incubation of the clutch usually commences after the laying of the last egg, or the fourth egg in clutches of more than five (Zann and Rossetto 1991). Therefore, in this series of experiments, the possibility of reduced energy requirement during incubation (arising from lower locomotory activity or body insulation from the nest / close association with the male) cannot explain the observed reduction of food intake.

The reduced activity of the female during the laying period may largely arise from reciprocating the domestically-compromised behaviour of the male. In this study locomotory demands on males were effectively restricted during nest "construction" by provision of a straw lined nest box and nesting activity was confined to lining the pre-formed nest with fresh grass, rather than *bona fide* construction. Pair-bonding encourages physical association of the female with the male and so compromised activity in the male may effectively encourage inactivity in the female. Compromise of natural nesting behaviour may have been further evident in this series of experiments, as a repeated tendency was observed for the continued addition of excessive nesting material subsequent to initiation of the clutch. Immelmann (1962a, from Zann 1996), observed similar behaviour in domesticated zebra finches, often effectively curtailing incubation. I would view this as a stereotypical behaviour, over-compensating for the limited activity requirement for nest formation. Similarly, inactivity of females during the breeding cycle may be an artefact of captivity.

Birds maintained on the protein only diet, though showing an initial increase in the weight of laying females (Fig.3.8) and mean egg mass (Fig.3.5), otherwise responded negatively to the high protein diet, with replacement clutches tending to be small and some birds postponing laying altogether (Fig.3.5). This may have arisen as a consequence of a reduction in energy availability in the diet, arising from the increased cost of metabolising protein for energy. Similar negative effects of excessive protein supplementation have been noted in other species (see Vickery *et al.* 1994). Additionally, allocation of energy reserves to maintenance of nitrogen balance may have further reduced availability of resources for egg production. The faeces of protein diet birds were noted to be more copious than usual, which may have been indicative of the excretion of excess nitrogen.

3.4.3 *Mechanisms for diet selection*

The basic and supplemented diets used in this experiment were specifically formulated to resemble each other visually and texturally (2.3.1) and the position of the diets was changed daily. Thus, the birds had no external criteria on which to base their choice of diet. So, how could they tell the difference?

If the post-ingestive effects of the high protein diet were immediately deleterious, then a preference for the basic diet may have arisen from aversion learning. However, this is unlikely as there was no indication that over the course of the trial the birds were attempting to exclude the protein supplement from their diet - if this had happened, one may have expected that the degree of discrimination to have progressively increased over successive experimental periods.

Though visually and texturally similar, the experimental diets may have differed in both taste and odour. The size of the olfactory bulbs in relation to the size of the forebrain (often taken as an indicator of the development of the sense of smell) of passerines is among the smallest of any birds (Campbell and Lack 1985). Hence discrimination between diets on the basis of odour is unlikely in this case.

Taste, initially thought to be fairly impoverished in birds, has been demonstrated to be an important determinant of dietary selection in chickens, and there is some suggestion that taste sensitivity may vary in response to degree of dietary deprivation (*review* Gentle 1979).

It is possible that the finches develop a specific nutrient appetite during breeding, intake of the appropriate dietary balance being mediated through post-ingestive feedback acting directly to alter the "perceived taste" of the selected diet. As satiation for the components of a specific diet is approached, taste aversion arises, encouraging selection of an alternative diet.

Exactly how birds are able to associate specific foods with their ingestive consequences has not been fully resolved, though flavour has been implicated (see Hughes 1993). The physiological mechanisms for modification of perceived taste may be based on detection of chemical imbalance in the blood stream. The anterior prepyriform cortex and medial amygdala of the brain are sensitive to deranged plasma amino acid patterns and the hypothalamus may also play a role (Boorman 1979). Possible chemical stimuli include low concentrations of required amino acids, and / or high levels of phenyalanine (Spargo *et al.* 1979). Changes in the concentration of intracellular amino acids are thought to influence protein synthesis by a variety of mechanisms (Millward *et al.* 1974) and so may rectify temporary nutritional imbalance through utilisation of endogenous reserves. Concurrent influence of dietary intake would present a mechanism by which these reserves may be rapidly replenished.

It is possible that the close similarly between the experimental diets masked the ability of birds to effectively discriminate between them, especially if zebra finches naturally incorporate colour / familiarity into a search-image for a specific food-type. This is likely, as zebra finches have been previously shown to select food items on the basis of colour (V. Olson *pers. comm.*). Additionally, there is evidence that the ability to use taste as a mechanism for maintenance of balanced dietary intake may be reduced in domesticated birds, in comparison with wild counterparts (Kare and Maller 1967). This could be further investigated by dyeing diets of

differing protein content with colours that were selectively neutral. This would enable colour to be used as a potential indicator of dietary quality, and hence might improve the efficiency of diet selection.

3.4.4 *Future studies*

As indicated earlier, the factors influencing diet choice in wild zebra finches may be very different from those in captive birds. When transgenerational links are disrupted, e.g. by introduction to captivity, individuals are often less productive than conspecifics reared under these conditions (see, for example Griffith *et al.* 1989), and the determinants of dietary selection in wild birds might be an extremely interesting field of investigation.

Hughes (1993) outlined previous work on rats, in which dietary intake of the mother was shown to influence food preferences of her offspring both during gestation, and later through the chemical composition of her milk. With reference to Zann (1996), previous studies have recorded different species of grass to represent the predominant food source in the diet of wild zebra finches.

It would be interesting to see whether selection of seeds by zebra finches is similarly influenced by the seeds they receive in their first meals from the crops of their parents, with special reference to unripened grain. Chicks from the same clutch could be cross-fostered to adults with access to specific seed combinations. This would enable examination of the extent to which the diets fed to chicks by their parents might influence their choice of seed later in life, and their selection of seed to feed their own young.

Chapter 4

ENDOGENOUS PROTEIN RESERVES FOR EGG PRODUCTION: A REASSESSMENT OF SPECIFIC PROTEIN STORES IN ZEBRA FINCHES

... abandon all hope, you who enter here...

Dante

SUMMARY

Pectoral muscle is recognised as an important reserve of endogenous protein. Analysis of the sarcoplasm of the flight muscle of zebra finches supported previous observation of protein removal during egg production. However, SDS electrophoresis in combination with amino acid analysis of muscle protein fractions indicated this loss to be of a general nature and not confined to a specific protein. No evidence was found to support the previous findings of a high molecular weight storage protein specific to the amino acid requirements of egg production, and it is suggested that this observation may have arisen as an artefact of the experimental methodology. The theoretical benefits of specific storage proteins for free-living zebra finches are argued to be less advantageous than previously suggested.

4.1 INTRODUCTION

4.1.1 *Protein demands and acquirement during egg formation*

Total clutch mass may approach 30-40% of the body weight of the laying female in zebra finches. Protein typically constitutes 10% wet weight of egg contents (Murphy 1994a), and egg formation may elevate the protein requirement of a bird by as much as 230% of normal daily requirements (Robbins 1981). Egg production is therefore a protein demanding process.

Protein requirement for egg production may be satisfied by modification of dietary intake and/or utilisation of endogenous protein reserves (Houston *et al.* 1995a). Indeed, onset of breeding has been suggested to be linked to attainment of sufficient protein reserves in some species (Jones and Ward 1976, Fogden and Fogden 1979).

4.1.2 *The concept of endogenous stores and reserves*

The importance of the concept and definition of protein reserves is well recognised (*review* Fisher 1967). Reserves have been defined as those tissue proteins open to "reversible depletion" (Allison *et al.* 1964), and more recently, "any tissue components that can be drawn upon to relocate endogenous nutrients when daily intake of nutrients falls below needs" (Murphy 1994a). As such, "reserves" are distinct from (and inclusive of) "stores", the latter defined by Murphy (1994a) as "nutrients... in amounts above those maintained by well nourished... birds". For the purposes of this chapter, protein stores are similarly considered as a discrete component of the total endogenous protein reserve.

Major storage tissues include skeletal muscle, liver, intestine and epidermis (Addis *et al.* 1936, Allison and Wannemacher 1965, Murphy and King 1985, Waterlow *et al.* 1978). In zebra finches, as in most birds, the pectoral muscles may account for 20% of total body weight, and so potentially represent the largest reservoir of endogenous protein.

The importance of skeletal muscle as a potential protein store is well recognised (Houston *et al.* 1995a, 1995c, Jones 1980, Millward 1970, Murphy and King 1985, Swain 1992, Waterlow and Stephen 1968) and the translocation of protein from pectoral muscle to the forming egg has been demonstrated in zebra finches, using labelled amino acids as tracers (Houston *et al.* 1995b).

4.1.3 *The role of muscular sarcoplasm in protein storage*

As a result of investigations into spawning king salmon, *Oncorhynchus tschawytscha*, Greene (1919) first suggested that the muscular sarcoplasm might represent a reservoir of labile protein which may be utilised in times of nutritional stress, without seriously impairing the contractile function of the muscle. Observation of muscle fractions of

healthy and partially starved red-billed quelea, *Quelea quelea,* prompted Kendall *et al.* (1973) to propose a similar mechanism for birds. Loss of protein in quelea manifests as a reduction in sarcoplasm volume, while the myofibrillar component of the flight muscle remains comparatively unchanged. Recent work has indicated that other small passerines may similarly utilise flight muscle sarcoplasm as a protein store to offset the demands of egg production (Swain 1992, Houston *et al.* 1995 a).

The role of the muscular sarcoplasm as a protein store was further investigated by Houston *et al.* (1995c). Crude extracts of the pectoral muscle of zebra finches, sampled before and after laying, were subject to gel filtration with the aim of isolating constituent proteins. Elute absorbance was monitored at 280nm (the wavelength at which protein is detected in solution). Analysis indicated three peaks of absorbance. These were identified as avian haemoglobin, avian myoglobin and a third "*Peak.1*". This was a high molecular weight protein, relative molecular mass (RMM) ca.400,000. The amplitude of this peak declined significantly during the period of egg production.

One of the proposed uses of endogenous protein stores is to provide selective amino acids potentially limiting in the food supply. It was suggested by Houston *et al.* (1995c) that this high molecular weight protein may represent a "specific protein reserve" utilised to offset the demands of egg production. As such, one might expect that *Peak.1* would be enriched in certain amino acids, such as methionine and cystine, previously shown to be disproportionately high in eggs in comparison with plant and animal tissue (Harvey 1970) and potentially limiting in egg production (Fisher 1967).

4.1.4 *Aims*

The first aim of this chapter was first to isolate the *Peak.1* fraction by gel column filtration*, following the methods of Houston *et al.* (1995c).

The second aim was to perform sodium dodecyl sulphate (SDS) electrophoresis** on pectoral muscle extracts derived from breeding and non-breeding birds to determine general trends in the protein profile of flight muscle sarcoplasm in response to egg production.

* In the column, molecules are sorted on the basis of molecular weight. Large molecules are unable to penetrate the interstices of the gel and so follow a direct path through the column and emerge first. Small molecules follow a tortuous path, percolating far into the interstices of the gel, and are eluted later. The amino acid composition of *Peak.1* was determined to ascertain whether this fraction contained elevated levels of amino acids potentially limiting to egg production.

** Heating protein samples in the presence of SDS encourages attachment of charged SDS molecules to the protein residues. The ratio of charge:molecular mass is constant. Samples were loaded into wells at one end of a polyacrylamide gel and an electrical potential set up across the gel, encouraging migration of the charged proteins between two electrodes. All molecules are attracted equally. However, the migration of larger molecules is retarded by the gel. Smaller molecules are less impeded. This enables differentiation of proteins on the basis of size, molecules separating into bands of similar weight. The gel was calibrated by concurrent running of a mixture of standard molecular weight proteins, enabling the molecular weight of the protein bands to be determined.

4.2 METHOD

4.2.1 *Experiment 1: Gel filtration*

4.2.1.1 *Preparation of birds*

Birds were kept in cages measuring 60cm x 50cm x 40cm, under a timer controlled lighting regime of 8 hour dark to 16 hour light. All birds received cuttlebone, water and oyster grit *ad libitum*. Ambient temperature was maintained at 20.5°C (+/-2).

A preliminary investigation was conducted to evaluate the suitability of gel filtration for the effective separation of sarcoplasmic proteins. Five female finches were kept separately from males, and fed on a mixed seed diet with a protein supplement (boiled egg white in *Haithes chick rearing mix*), with the objective of fully replenishing endogenous protein reserves, and bringing them into full pre-breeding condition.

4.2.1.2 *Experimental protocol*

The experimental protocol was based on the original methodology of Houston *et al.* (1995c). After being maintained for one month under these conditions, the birds were sacrificed by CO_2 asphyxiation and pectoral muscles removed. A 0.5g sample of muscle was added to 8ml of 0.25M sucrose in 60mM Tris buffer solution in an ice-packed tube. The mixture was homogenised by Ultra-Turrax (12 x 5 second periods, with 30 seconds of cooling between bursts). The sample was then centrifuged for one hour in an MSE centrifuge at 11,000G. The resulting supernatant represents a crude extract of the sarcoplasm.

A 2ml sample of supernatant was then subjected to filtration using a gel column, (bed dimension 87.5 x 2.6 cm), packed with LKB Ultrogel ACA 34. Sample was washed through with 60mM Tris buffer at pH7.5, pumping at 30ml per hour.

Elute absorbance was monitored at 280nm by an LKB UVCord spectrophotometer, and a permanent record plotted using an LKB Bromma 2210 chart recorder.

4.2.2 *Experiment 2: SDS-Polyacrylamide gel electrophoresis*

4.2.2.1 *Preparation of birds*

Birds were maintained under the conditions outlined in 4.2.1.1. Three groups of five established pairs of finches (15 pairs in total) were selected. Groups were standardised for previous breeding performance (1.1.4) and the birds ranked within each group on the basis of total clutch mass produced. Pairs were separated and the male and female held

in neighbouring cages for two months prior to the trial. Cage partitions allowed visual and vocal communication between partners, but prevented physical contact. During this time all birds were maintained on a mixed seed diet with a protein supplement (boiled hens egg white in *Haithes chick rearing mix*).

One group of females was sacrificed prior to re-introduction to their mates. This group of "***Pre-breeding***" birds represented birds in peak condition. Protein reserves specific to egg production would be expected to be plentiful in this group.

The second group of females was re-united with their original partners and the protein supplement was removed from their diet. These birds were allowed to breed and subsequent clutches were monitored daily. Newly laid eggs were substituted with replicas on the morning of laying to guard against the parents eating them. Birds usually laid at the rate of one egg per day during the laying period. After two successive non-laying days, the clutch was considered complete and the female sacrificed. This group represented females in "***Post-breeding***" condition. Protein reserves specific to egg production would be expected to be depleted in this group.

The third group of females was transferred to individual cages, but were not re-united with their mates. Protein supplement was removed from their diet. Each bird was kept under solitary conditions until the comparatively ranked bird from the *Post-breeding* group had completed its clutch, when it was sacrificed in tandem. This "***Control***" group represented females in which protein stores may have been depleted by routine metabolism and factors other than egg formation.

4.2.2.2 *Experimental protocol*

Supernatant samples were prepared from each of the 15 birds, as outlined previously (4.2.1.2). A Coomassie Blue Protein Assay (Read and Northcote 1981) for total protein content of each sample was carried out in conjunction with the SDS gel electrophoresis, in order that the relative concentration of protein might be compared between dietary groups (Appendix.4.5.1)

SDS gels (10ml of 12% resolving gel and 5ml stacking gel) were prepared following the methodolgy of Harlow and Lane (1988) (see Appendix.4.5.2). Samples were prepared and run following the methodology outlined by Sambrook *et al.* (1989) and the booklet of guidelines accompanying the Mini-Protean II cells.

In each case, 50µl of sample was injected onto 12% SDS-Polyacrylamide gels for electrophoresis in Mini-Protean II cells. Each sample was run on two separate gels. The constituent proteins of the sarcoplasm separated out into about 23 major bands.

Protein bands were stained with Coomassie Blue and their optical density measured using a Fujix BAS 1000 laser scanning densitometer.

Of the 23 protein bands, 3 bands displayed, to some extent, the characteristics predicted for a specific storage protein*. Of these, band number ten (*Band.10*) fitted these predictions most closely. Twenty five samples of *Band.10* were dissected from the dried SDS gels and subjected to amino acid analysis at the Rowett Research Institute, Aberdeen. This analysis incorporated a comparative weight of blank gel, by way of a control, to establish background levels of amino acids present in the gel itself.

* Were a specific protein acting as a store for egg production, presence as a percentage of total protein would be expected to be highest in the *Pre-breeding* group. A similarly high value might be expected in the *Control* group, because if this protein were to represent a store specifically for egg production, it should not be utilised over a short time period for other purposes. Finally, the storage protein should be significantly reduced in the *Post-breeding* birds.

4.3 RESULTS

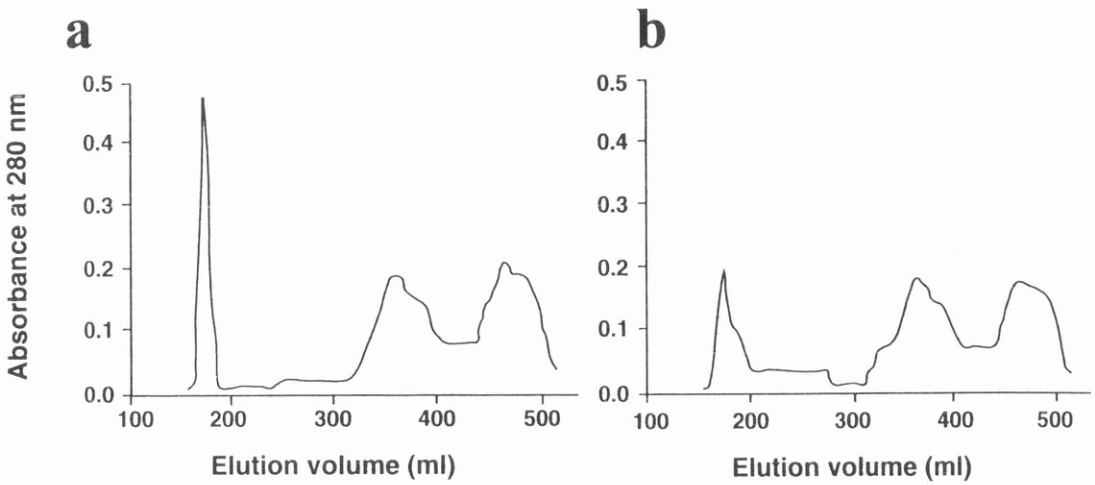
4.3.1 *Experiment 1: Gel filtration*

Gel filtration of flight muscle sarcoplasm extracts by Houston *et al.* (1995c), produced three absorbance peaks (reproduced Fig.4.1a, 4.1b).

Pectoral muscle extracts from three birds in pre-breeding condition were analysed in replicate by gel filtration following the methodology of Houston *et al.* (1995c). Two representative absorbance profiles are shown in (Fig.4.1c). In all analyses, two absorbance peaks were attained, corresponding with *Peak.1* and *Peak.2*. *Peak.3* was never reproduced in the current study.

The amplitude of *Peak.1* was noted to vary considerably. Maximum absorbance at 280nm varied between 0.5 and 1.0 despite the fact that the birds were all in pre-breeding condition. A high degree of variation was also noted in the amplitude of *Peak.1* between replicate runs of the same sample (e.g. 0.67 and 0.48), indicating poor reproducibility of results using this method.

Elutes representing *Peak.1* (n=3) and *Peak.2* (n=1) were isolated using a fraction collector and sent to the Rowett Research Institute for amino acid analysis (Fig.4.2).



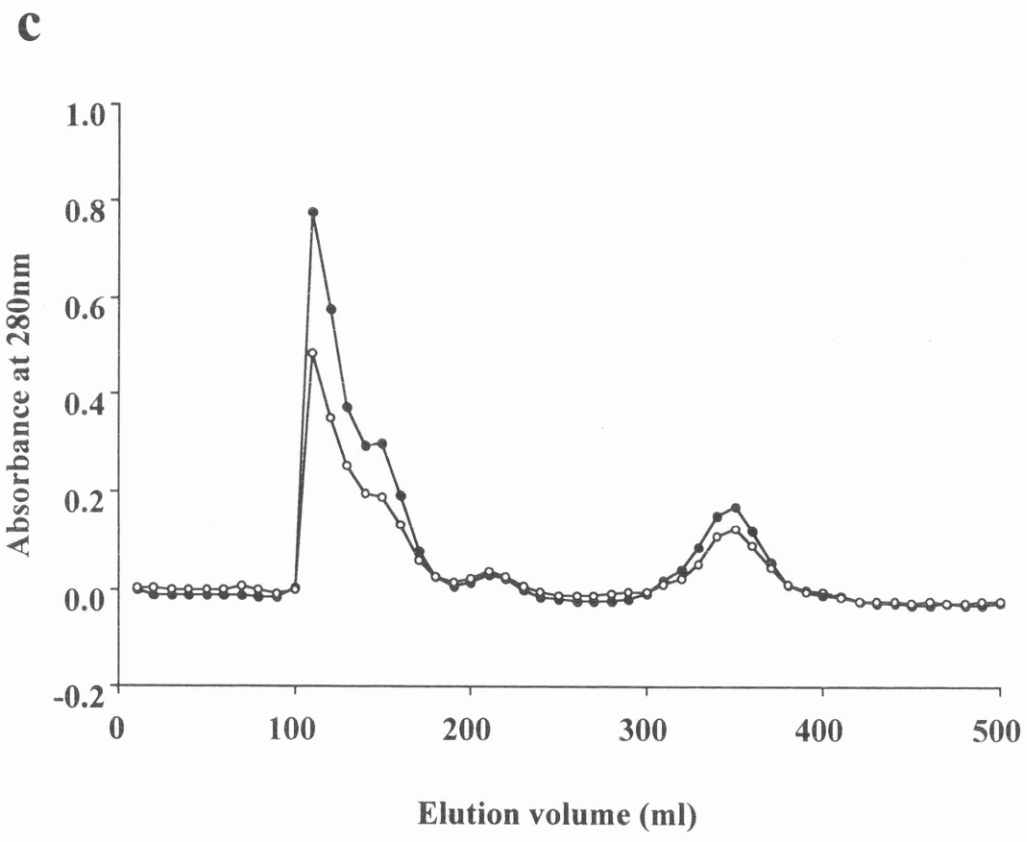


Fig.4.1 Results of gel filtration of water soluble proteins from pectoral muscle extract of a female zebra finch.

(a) Three days before the first egg was laid (from Houston *et al.* 1995c).
(b) From a bird taken after laying the last egg of a clutch (from Houston *et al.* 1995c).
(c) Current results from two representative birds in pre-breeding condition.

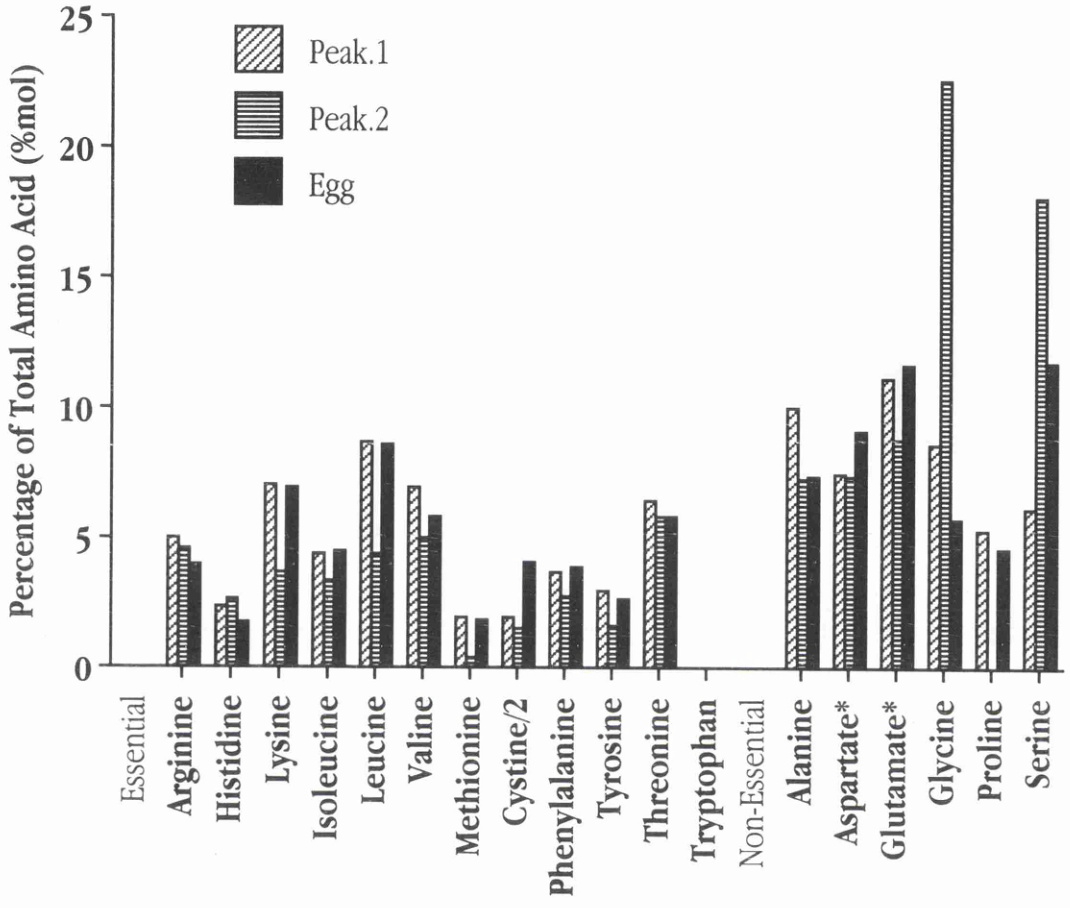


Fig.4.2 Amino acid composition (%mols) of *Peak.1* (n=3) and *Peak.2* (n=1) elution fractions. Amino acid composition of zebra finch eggs is shown for comparison, calculated from Murphy (1994a). * Note : Asparagine and glutamine are deaminated by acid hydrolysis to aspartate and glutamate.

The amino acid composition of *Peak.1* is typical of general protein, with concentrations of sulphur amino acids not especially elevated.

Amino acid composition of *Peak.2* was atypical of protein, and more closely resembles that of free amino acids and small peptides present in the cytosol (the soluble components of the cytoplasm), (G.Lobley *pers. comm.*).

4.3.2 *Experiment 2: SDS electrophoresis*

Given the apparent unsuitability of gel filtration as a mechanism for isolating the component proteins of the crude sarcoplasmic extract, SDS electrophoresis was performed in an effort to discover if specific protein loss was occurring during egg production (Fig.4.3).

Coomassie Blue Protein Assay for total protein content indicated no significant difference in the concentration of sarcoplasmic protein between *Pre-breeding* and *Control* groups. However, protein concentration was significantly reduced in *Post-breeding* birds (ANOVA $F_{2,12}=4.86$, $P<0.05$), in comparison with both *Pre-breeding* (13% lower) and *Control* (11% lower) birds (Fisher's pairwise comparison).

Supernatant samples were run on SDS gels, and the resultant bands stained with Coomassie blue (Fig.4.3a). The densitometer identified up to 30 protein bands, molecular weight markers indicating these to range between RMM 10,000-100,000 (Fig.4.3b). Replicate gels were run for each sample and the results from these averaged.

Due to the large number of proteins present, the small gels proved to be of insufficient length for complete resolution of all bands, and in some cases peaks partly merged into one another. Though the laser scanning densitometer identified up to 30 individual peaks in some runs, these were not fully differentiated in all samples, and so peaks were aggregated into 23 major peaks which were represented discretely in all samples (Fig.4.4). For example, Peak.1 and Peak.2 (Fig.4.3b) did not differentiate in all runs and so were aggregated into "Band 1" (Fig.4.4).

Results were standardised on the basis of total protein content, i.e. *Post-breeding* values were reduced by 11% with respect to *Control* and 13% with respect to *Pre-breeding* values, to enable direct comparison between experimental groups (Fig.4.5).

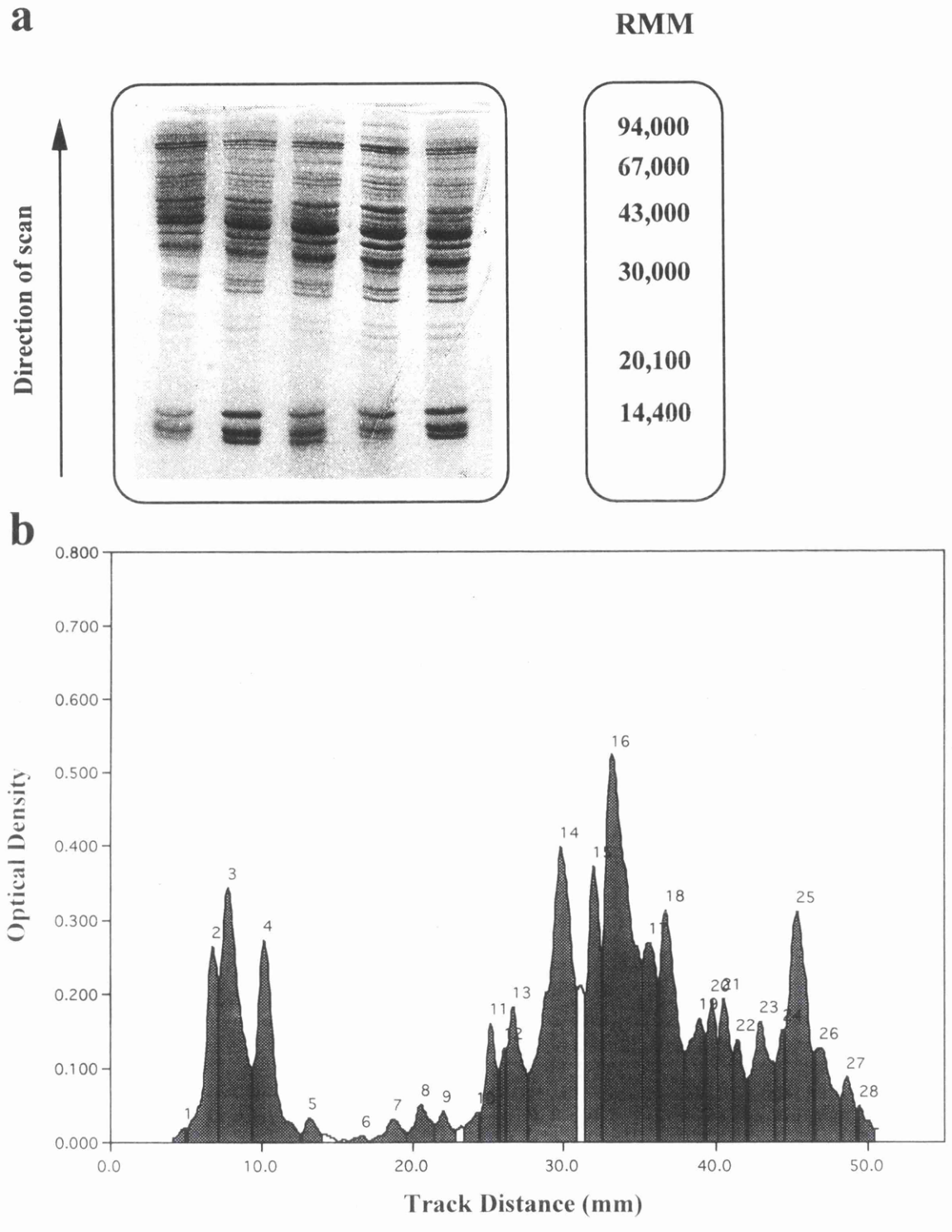


Fig.4.3 Results of SDS gel electrophoresis of water soluble proteins from pectoral muscle of female zebra finches in pre-breeding condition.

(a) Example of SDS gel, showing tracks from five pre-breeding birds.
(b) Example of densitometer scan of a single SDS gel track from a pre-breeding bird.

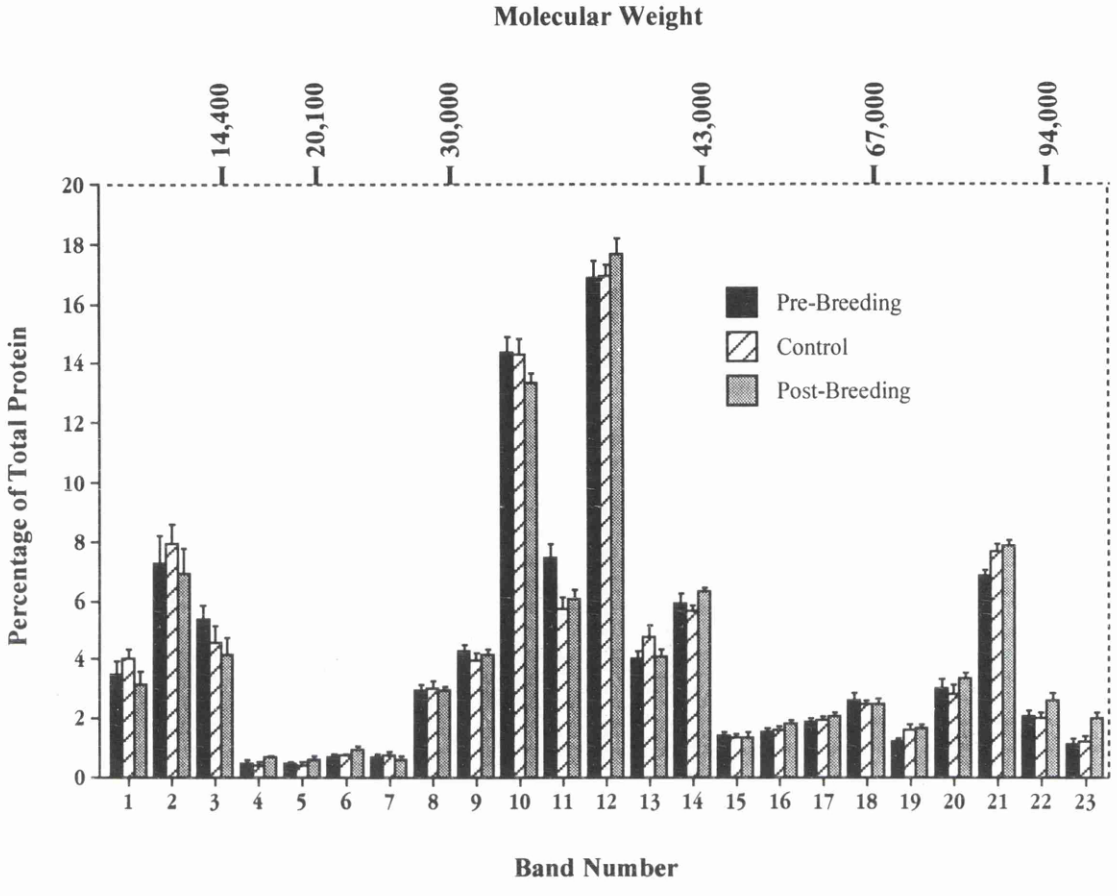


Fig.4.4 Molecular weight profiles of sarcoplasmic proteins of the flight muscle of zebra finches. Distribution as a percentage of total protein content is shown for Pre-breeding, Control and Post-breeding birds. Means are indicated (n=5) +/- standard error.

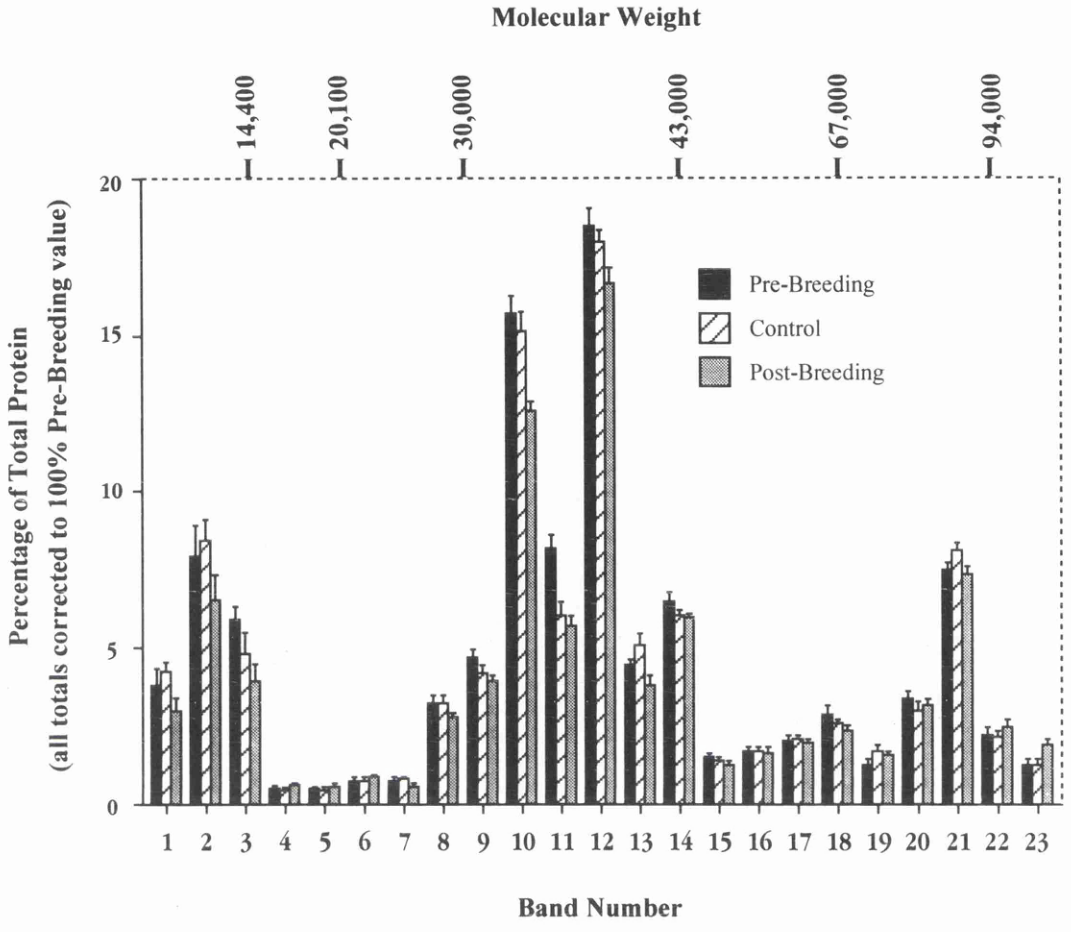


Fig.4.5 Molecular weight profiles of sarcoplasmic proteins of the flight muscle of zebra finches. Relative protein content is shown for Pre-breeding, Control and Post-breeding birds. Means are indicated (n=5) +/- standard error.

Examination of protein profiles from the three groups of birds (Fig.4.5) indicated only three to exhibit the profile predicted as a candidate for a specific storage protein (4.2.2.2). These are highlighted in Tab.4.1. Of these, *Band.10* appeared most reduced in the *Post-Breeding* birds, and so was selected for further analysis.

Band Number		Comparison between treatment groups
1	*ANOVA* $F_{2,12} = 2.81, p = 0.100$	No sig. diff. between treatment groups
2	*ANOVA* $F_{2,12} = 1.58, p = 0.246$	No sig. diff. between treatment groups
3	*ANOVA* $F_{2,12} = 3.66, p = 0.058$	No sig. diff. between treatment groups
4	*ANOVA* $F_{2,12} = 2.31, p = 0.142$	No sig. diff. between treatment groups
5	*ANOVA* $F_{2,12} = 3.68, p = 0.057$	No sig. diff. between treatment groups
6	*ANOVA* $F_{2,12} = 1.87, p = 0.196$	No sig. diff. between treatment groups
7	*ANOVA* $F_{2,12} = 6.74, \mathbf{p = 0.011}$	***Fisher's Pairwise Comparisons Post-B. < Pre-B. and Control***
8	*ANOVA* $F_{2,12} = 3.16, p = 0.079$	No sig. diff. between treatment groups
9	*ANOVA* $F_{2,12} = 5.90, \mathbf{p = 0.016}$	*Fisher's Pairwise Comparisons Pre-B. > Post-B.* and *Control*
10	*ANOVA* $F_{2,12} = 13.36, \mathbf{p = 0.001}$	***Fisher's Pairwise Comparisons Post-B. < Pre-B. and Control***
11	*ANOVA* $F_{2,12} = 14.82, \mathbf{p = 0.001}$	*Fisher's Pairwise Comparisons Pre-B. > Post-B.* and *Control*
12	*ANOVA* $F_{2,12} = 4.85, \mathbf{p = 0.029}$	***Fisher's Pairwise Comparisons Post-B. < Pre-B. and Control***
13	*ANOVA* $F_{2,12} = 5.44, \mathbf{p = 0.021}$	*Fisher's Pairwise Comparisons Control < Post-B.*
14	*ANOVA* $F_{2,12} = 2.61, p = 0.115$	No sig. diff. between treatment groups
15	*ANOVA* $F_{2,12} = 2.41, p = 0.140$	No sig. diff. between treatment groups
16	*ANOVA* $F_{2,12} = 0.05, p = 0.954$	No sig. diff. between treatment groups
17	*ANOVA* $F_{2,12} = 1.01, p = 0.392$	No sig. diff. between treatment groups
18	*ANOVA* $F_{2,12} = 2.57, p = 0.118$	No sig. diff. between treatment groups
19	*ANOVA* $F_{2,12} = 4.13, \mathbf{p = 0.043}$	*Fisher's Pairwise Comparisons Pre-B. < Post-B.* and *Control*
20	*ANOVA* $F_{2,12} = 0.68, p = 0.523$	No sig. diff. between treatment groups
21	*ANOVA* $F_{2,12} = 5.10, \mathbf{p = 0.025}$	*Fisher's Pairwise Comparisons Control > Pre-B.* and *Post-B.*
22	*ANOVA* $F_{2,12} = 0.90, p = 0.431$	No sig. diff. between treatment groups
23	*ANOVA* $F_{2,12} = 6.33, \mathbf{p = 0.015}$	*Fisher's Pairwise Comparisons Post-B. > Pre-B.* and *Control*

Tab.4.1 Summary of statistical comparisons of concentration of specific molecular weight protein bands between treatment groups. Candidates for storage proteins shown in bold-type.

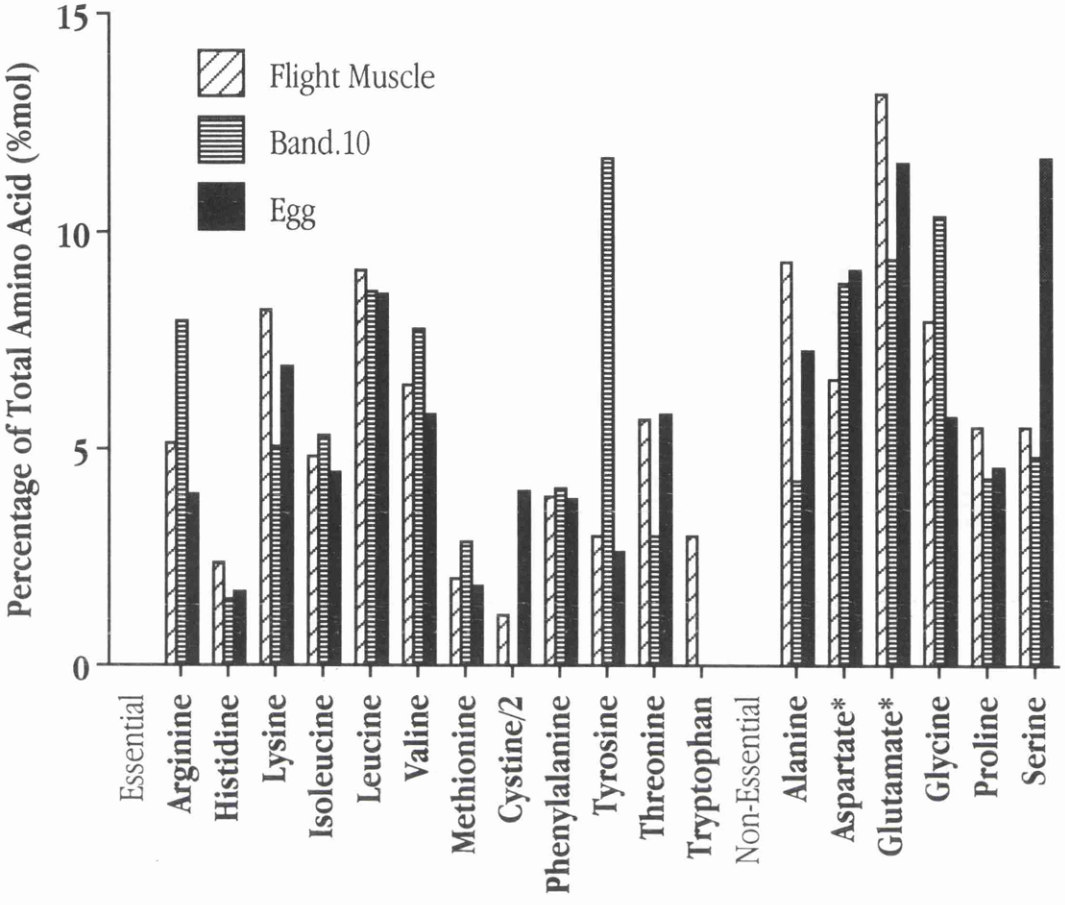


Fig.4.6 Amino acid composition (%mols) of zebra finch eggs (n=4, calculated from Murphy 1994a), whole flight muscle (n=2), and *Band.10* (n=1, from 25 combined samples). *Band.10* samples were dissected from SDS gels of birds from all treatment groups and pooled for analysis. * Note : Asparagine and glutamine are deaminated by acid hydrolysis to aspartate and glutamate.

Amino acid analysis of *Band.10* indicated comparatively elevated levels of glycine (non-essential), arginine and especially tyrosine (essential), in comparison with that of whole flight muscle (Fig.4.6).

There was no elevation in the concentrations of sulphur amino acids, lysine or threonine.

4.4 DISCUSSION

4.4.1 *Experiment 1: Gel filtration*

Fig.4.1a and Fig.4.1b, reproduced from Houston *et al.* (1995c), refer to single samples. The possible variability of results arising from the analysis might thus not be immediately obvious. In the current study, gel filtration of pectoral muscle extracts yielded unsatisfactory results. Replicate analyses of three female finches in pre-breeding condition were highly variable both between birds and between replicate runs. For example, based on data from the current study, Fig 4.1c illustrates a similar difference in Peak.1 amplitude (ABS +/-0.3) between two birds, both of which were in pre-breeding condition.

Amino acid analysis of the fractions representative of the absorbance peaks (Fig.4.2) indicated that the composition of *Peak.1* was typical of general protein, with no concentration of amino acids potentially limiting to egg production. *Peak.2* profile was atypical of protein, and more similar to that of free amino acids and small peptides present in the cytosol (G.Lobley *pers. comm.*) Additionally, electrophoresis of *Peak.1* samples produced a multiplicity of bands, indicating a range of proteins to be present in the fraction. There was no indication of the presence of proteins of molecular weight RMM ca. 400,000.

Finally, the reduction in the maximum absorbance of *Peak.1* between pre-breeding and post-breeding birds indicated by Houston *et al.* (1995c) appears superficially impressive (Fig.4.1a, 4.1b). However, analysis of the areas beneath these profiles indicates there to be little difference between the two sets of results. Absorbance profiles were scanned into a Power Mac, and the areas beneath the curves accurately measured using Adobe Photoshop 3.0. The total area bounded by the 300ml elute mark is, in fact, slightly larger in the post-laying bird than for the pre-laying bird, indicating that there was no net removal of protein previous to this fraction.

It appears that the results of Houston *et al.* (1995c) arose as an artefact of the inability of the gel column to effectively separate the crude protein preparation. The unrefined mixture contained protein chains of different sizes, probably intertwined on introduction to the column. The proteins travelled through the column in an aggregated form, effectively behaving as large chain molecules, and were eluted off simultaneously, close to the exclusion limit of the column. As such, the only resulting differentiation was that of protein (*Peak.1*) from free amino acids and small peptides (*Peak.2*) (Fig.1c). The reason for the non-materialisation of *Peak.3* in the present study is unknown.

With reference to the above, it was thought to be inappropriate to sample more birds, for further investigations, using this methodology.

4.4.2 *Experiment 2: SDS electrophoresis*

A reduction in the weight of the pectoral muscle during egg formation has been recorded in many species, including the zebra finch (*review* Houston *et al.* 1995c). The current study showed that protein concentration of the sarcoplasm was approximately 13% lower in post-breeding female finches compared with those pre-breeding (4.3.2). Despite the small sample sizes involved in this experiment (n=5), this result conforms with previous studies monitoring flight muscle protein loss over the laying cycle, 15% in red-billed quelea (Jones and Ward 1976), and 14% in zebra finches (Houston *et al.* 1995a).

Electrophoresis of muscle extracts of non-breeding and post-breeding finches indicated protein loss to be of a general nature, rather than being confined to proteins of a specific molecular weight (Fig.4.4), with little evidence of any particular band being preferentially targeted as a protein reserve.

Of the proteins isolated by electrophoresis, one RMM ca.35,000 (*Band.10*) exhibited some limited characteristics of a candidate for a specific storage protein. Amino acid analysis of this band indicated elevated levels of valine and tyrosine (essential) and glycine (non-essential), (Fig.4.6). However, Allen and Hume (1997) identified lysine, threonine and methionine as being the most limiting for egg production in the natural seed diet of zebra finches. As such, the *Band.10* protein does not represent a high quality source of the amino acids specific to the requirements of egg production.

4.4.3 *Ecological trade-offs of protein storage*

The various tissues of the body may be ranked according to susceptibility to protein loss (Addis *et al.* 1936), skeletal muscle being amongst the most sensitive. However, efforts to distinguish "labile" protein from "fixed" protein have all failed (Borsook and Dubnoff 1943), and it has been suggested (Fisher 1967) that no part of the body should be excluded from consideration of a potential role in protein storage.

In the case of birds, the opportunity to offset the demands of egg formation by the accruement of a specific protein pre-cursor represents an attractive idea. The advantages for a bird with this ability are immediately obvious. The disadvantages are less obvious, and would act at all times outwith the period of egg production.

Even a small increase in the body weight of zebra finches has been shown to promote a large reduction in flight performance (Metcalfe and Ure 1995). Additionally, Bednekoff (1996) has demonstrated the relationship between reduced flight performance and predation risk to be exponential. Hence, small increases in body mass effectively translate into a greatly increased risk of predation. There is no such thing as a free meal - even after it has been eaten.

As such, a less hazardous long-term strategy may be the assignment of protein reserves to readily depletable components of flight muscle. While remaining labile, these components may actively contribute to the flight performance of the bird. When required in the role of egg formation, these reserves are depleted short term. Negative effects on flight performance may, to some extent, be compensated for by a concurrent reduction in body weight or behavioural adaptation.

In the case of zebra finches, which are almost completely granivorous (Zann and Straw 1984), an apparent imbalance is evident between the amino acid profile of grass seed and eggs. This is solved by utilisation of endogenous protein, offset by ingenious dietary supplementation. Breeding in wild finches has been noted to be closely linked with availability of unripened seed, which they readily consume (Morton and Davies 1983, Watson 1992). Analysis of unripened grass seed by Allen and Hume (1997), impressively showed that the amino acid profile of unripened seed not only closely matches that of eggs, but more closely resembles that of eggs than that of ripened seed. The potential for absorption of potentially limiting dietary amino acids is further increased by the softer nature of the ingestate (Allen and Hume 1997). Thus, in zebra finches, the opportunistic timing of breeding to coincide with the availability of unripe seed may effectively ameliorate the need for storage proteins and the utilisation of endogenous protein reserves.

If occurring at all, utilisation of specific protein stores in the flight muscle is of a more complex nature than previously thought, and will only be determined by detailed biochemical analysis beyond the scope of this study.

4.5 APPENDIX

4.5.1 *Coomassie blue assay for total protein content*

Based on the methodology of Read and Northcote (1981).

A standard curve is constructed using Bovine Serum Albumin (BSA) serial dilutions over the range 100-500µg/ml.

Reaction mixture:
- 50µl standard
- 950µl distilled water
- 1000µl Coomassie reagent (0.06% Coomassie blue in 0.3M perchloric acid).

2ml samples of muscle preparation supernatant were freeze dried. Each sample (3 x groups of 5 birds) was analysed in replicate. Approx. 0.002g of solid was re-dissolved in 2ml of distilled water, from which 300µl sub-samples were taken for analysis.

Reaction mixture:
- 300µl of sample solution
- 700µl distilled water
- 1000µl Coomassie reagent

Samples were left to stand for 2-3 minutes, then read in a spectrophotometer at 620nm and 465nm. 50µl distilled water in a reaction mixture was used as a blank. A control of 0.25M sucrose in 60mM Tris buffer was run to confirm that the buffer would not effect sample absorbance.

The absorbance ratio for each sample was calculated as follows:

$$\text{Absorbance Ratio} = \frac{\text{ABS Sample 620nm - ABS Blank 620nm}}{\text{ABS Sample 465nm - ABS Blank 465nm}}$$

(ABS = Sample absorbance at n wavelength)

Absorbance ratio was plotted against BSA on the standard curve to derive total protein content of the solution.

4.5.2 *SDS-Polyacrylamide gel electrophoresis*

Gel constituents were based on formulations of Harlow and Lane (1988):

12% Resolving Gel (10ml volume)	***Stacking Gel (5ml volume)***
3.3ml double distilled water	3.4 ml double distilled water
4.0ml 30% acrylamide mix	0.83ml 30% acrylamide mix
2.5ml 1.5M Tris (pH 8.8)	0.63ml 1.0M Tris (pH 6.8)
0.1ml 10% SDS*	0.05ml 10% SDS*
0.1ml 10% ammonium persulfate	0.05ml 10% ammonium persulfate
0.004ml TEMED	0.005ml TEMED

Gel casting is based on the methodology of Sambrook *et al.* (1989), and the booklet of guidelines accompanying the Mini-Protean II cells.

50µl of supernatant (sarcoplasmic extract) was combined with an equal volume of sample buffer and placed in a boiling water bath for 3mins. The wells were loaded with 2.5µl of sample and run against a set of markers to indicate the molecular weights of the protein bands. Each sample was run on a replicate gel.

After the run was complete, gels were removed from the cells and incubated in Coomassie Blue stain at 54°C for 15 minutes, then transferred to a destain bath at room temperature for two days. Gels were dry-sealed in plastic and scanned using a Fujix BAS 1000 laser scanning densitometer to determine the extent and optical density of bands

* SDS - sodium dodecyl sulphate.

Chapter 5

EFFECT OF DIETARY HISTORY AND EGG PRODUCTION ON PROTEIN SYNTHESIS IN THE ZEBRA FINCH

If you compare your beloved's appearance today with a photograph taken a month ago, he (or she) may look the same, but many of the individual molecules forming that beloved body are different.

Jared Diamond

SUMMARY

The effect of dietary history and egg production on protein synthesis in female zebra finches was investigated using a "flooding dose" method. Fractional synthesis rate (FSR) was determined for liver, leg muscle and pectoral muscle in laying and non-laying birds. FSR of protein in the liver was elevated during egg production and by previous experience of a high protein dietary supplement. FSR of muscle types did not appear to differ significantly from one another, nor between experimental groups. Modification of liver FSR by previous dietary experience is suggested as a contributory mechanism for the promotional effects of protein supplementation on egg production.

5.1 INTRODUCTION

5.1.1 *Protein synthesis, degradation and turnover*

Body protein exists in a state of flux. It is subject to the dynamic, on-going processes of synthesis and degradation. Turnover is defined as the overall rate at which protein is synthesised and degraded in the body. Even under physiologically stable conditions, synthesis will occur with concomitant degradation - "this state is in the direction of synthesis, far from the thermodynamic equilibrium" (Borsook and Dubnoff 1943). Depending on physiological state, tissue synthesis rate may exceed or fall below degradation rate - the former resulting in a net accumulation of protein, the latter in a net release of amino acids from the tissue, causing atrophy or wasting.

Protein turnover rates differ in the various tissues of the body, which may be ranked according to rates of synthesis and degradation (Addis *et al.* 1936). Turnover in human skeletal muscle, for example, occurs at the rate of 12% per day compared to 48% in the liver (Millward 1970). Additionally, a hierarchy of susceptibility to metabolism is seen within tissue types, abdominal muscle tending to atrophy and hypertrophy more rapidly than limb muscle (Waterlow *et al.* 1978). The potential for anabolism and catabolism has been suggested to be positively related to the rate at which muscle masses develop during growth (Babinski and Onanoff 1888, from Dickerson and McCance 1960).

The metabolic plasticity of protein turnover also varies within tissues. Turnover rates may show marked differences between muscle fractions and, possibly as a result of variations in methodology, have often yielded conflicting or at least confusing results. In skeletal muscle both sarcoplasm and fibrils are depleted faster than stroma (Yamaguchi and Kandatsu 1967); collagen becoming comparatively inert once deposited in the intracellular spaces (Dickerson and McCance 1960). During overnight fasting in the shore lark, *Eremophila alpestris*, sarcoplasm appears to deplete more quickly than fibrils (Swain 1992), while the converse was apparent from Millward's (1970) investigation of starvation and protein deficiency in rats. Similar investigation of rats under starvation conditions (Waterlow and Stephen 1966, see also Waterlow *et al.* 1978), indicated both sarcoplasm and myofibrils to be depleted similarly. In studies of egg production in house sparrows, *Passer domesticus*, no difference in depletion rates between muscle fractions was evident (Jones 1991).

However, it should be remembered that the biochemical processes of protein degradation associated with "mild deficiency" differ markedly from those concurrent with "starvation" conditions.

5.1.2 *Factors affecting protein metabolism*

Tissue protein dynamics are subject to a variety of physiological influences including age and sex, body temperature, immunological state, trauma, hormonal and nervous influence, exercise, nutritional plane and, in birds, moult.

Fractional synthesis rate (FSR) is a measure of the rate of protein synthesis based on the percentage of the free pool of amino acids incorporated into protein, per unit time. In studies on rats, Waterlow and Stephen (1968) recorded FSR to decrease with age and to be lower in females than males. Additionally, older animals exhibit a greater resilience to dietary protein deficiency (Fisher 1967). In the case of zebra finches, young birds fed a protein deficient diet never attain full adult weight (Boag 1987).

Protein synthesis rate is positively correlated with body temperature (El Haj *et al.* 1997). However, in juvenile cod *Gadus morhua* L., cold induced reductions in RNA activity may be ameliorated, in acclimated individuals, by an increase in tissue RNA concentration (Foster *et al.* 1992). In birds, denaturing might be expected to be comparatively rapid due to their body temperature being some 6°C higher than that of humans (Jones 1980), however, this is reduced to some extent, by compensatory modification of protein structure (P.Watt *pers. comm.*).

Under immunological challenge, alteration of protein metabolism is vital to the production of phagocytes, lymphocytes and immunoglobulins (Beisel 1977). During moult, protein demands for the generation of new blood and tissue (Murphy and King 1991a, Ward 1969), have been observed to result in an increase in tissue FSR (Murphy and Taruscio 1995).

The interaction between protein dynamics and nutritional plane is complex, and differentially influenced by long/short term dietary history (Fisher *et al.* 1964, Fisher 1967). In rats, initiation of the feeding response is concurrent with a fall in FSR (Waterlow *et al.* 1978), possible cues include low concentrations of specific amino acids in the blood, protein breakdown, and/or high levels of phenyalanine (Spargo *et al.* 1979). Changes in the concentration of intracellular amino acids are thought to influence protein synthesis by a variety of mechanisms (see Millward *et al.* 1974). The anterior prepyriform cortex and medial amygdala of the brain are sensitive to deranged plasma amino acid patterns and the hypothalamus may also play a role in the detection of amino acid imbalance in the blood (Boorman 1979).

During periods of short-term dietary protein deficiency, negative nitrogen balance may promote increased utilisation of endogenous stores. For example, in response to a fall in blood sugar levels, gluconeogenic amino acids such as alanine are released from skeletal muscle to encourage gluconeogenesis in the liver. Reserves are also used to re-equilibrate disrupted free amino acid profiles subsequent to long-term fasting or ingestion of a meal deficient in protein quantity (Millward 1970, Waterlow and Stephen 1968), or quality i.e. imbalanced complement of amino acids (Boorman 1979).

However, as mentioned previously, it is important to note that the metabolic response to starvation or gross nutrient imbalance may be radically different from that involved in moderate compromise. For example, during short-term depletion, degradation of protein proceeds by ubiquitination of lysine groups of the target protein, promoting recognition and subsequent degradation by proteases, while in nutrient-starved cells, non-ubiquitin degradation occurs (Schlesinger and Hersho 1988). Hence, care should be taken in drawing conclusions from physiological reactions to diets far from the norm. Nutritional studies may be complicated by stress arising from protein:calorie imbalance, unrelated to protein metabolism (Fisher 1967), and ingestion of imbalanced diets may result in a variety of deleterious effects, including toxic shock reaction (*review* Harper *et al.* 1970). This has prompted experiments based on normal and super-normal diets (Fisher *et al.* 1964) and those described as not "unreasonably pathological" (Boag 1987).

5.1.3 *The protein demands of egg production and role of endogenous reserves*

In zebra finches, total clutch mass may approach 30-40% of the body weight of the female. Protein typically comprises 10% of the wet weight of egg contents (Murphy 1994a) and egg formation may elevate the protein requirement of a bird by as much as 230% of normal daily requirements (Robbins 1981). As such, egg formation is a protein demanding process. Synthesis of egg proteins additionally represents an energy requirement, each peptide bond needing the energy liberated from the dephosphorylation of 4.5 ATP molecules.

Protein reserves may be defined as those tissue proteins open to "reversible depletion" (Allison *et al.* 1964). Major storage tissues include the liver, skeletal muscle, intestine and epidermis (Addis *et al.* 1936, Allison and Wannemacher 1965, Murphy and King 1985, Waterlow *et al.* 1978), however, it has been suggested that no part of the body should be excluded from consideration as a protein reserve (Fisher 1967).

The importance of skeletal muscle as a protein store in birds is well recognised, (Houston *et al.* 1995a, Murphy and King 1985, Swain 1992). In zebra finches, the direct transfer of amino acids from muscle to forming eggs has been demonstrated using labelled amino acids (Houston *et al.* 1995b).

After fertilisation, rapid yolk development takes 3-4 days in zebra finches, while formation of the albumen and shell accretion takes an additional day (Haywood 1993). Peak protein requirement of the bird during the laying cycle occurs around the day that the first egg is laid (Donnan 1993, Houston *et al.* 1995a). As such, one might expect peak mobilisation of reserves to occur around this point in the laying cycle.

5.1.4 *Aims*

The aim of this study was to investigate the effects of egg production and dietary experience on protein synthesis in female zebra finches. With reference to the protein demands of egg production, protein deficiency has previously been shown to encourage increased reutilisation by the liver, and a reduction in the synthesis rates of skeletal muscle (Waterlow *et al.* 1978). Fractional synthesis rate (FSR), of liver, leg muscle and pectoral muscle were determined for non-breeding birds, using a "flooding dose" method (5.2.1), and compared with those of laying birds at the peak of protein requirement (the day of laying of the first egg).

Protein may be released from skeletal muscle reserves during the period of egg formation by an increase in protein breakdown, or a reduction in synthesis. It was predicted that, if the latter occurs, a reduction in muscle protein FSR would be observed during egg production. Waterlow and Stephen (1968) recorded a similar response to protein deficiency in rats. It was also predicted that liver FSR in breeding birds would be elevated by the demand for yolk protein production.

With reference to Waterlow *et al.* (1978), FSR of pectoral and leg muscle were compared, to investigate whether differential rates of protein synthesis between muscle types in birds may account for the observation of preferential depletion of the flight muscle during times of protein scarcity observed in several species of birds (see Dickerson and McCance 1960, Piersma 1988).

Additionally, as protein metabolism is dependant on recent dietary history (Fisher *et al.* 1964 and Fisher 1967), a comparison of breeding birds previously receiving protein supplemented diets was made with breeding birds previously maintained on a seed only diet. Exposure to high levels of dietary protein encourages an adaptive increase in the enzymes involved in the catabolism of specific amino acids (Fisher 1967), hence, an elevation of FSR might be expected in protein supplemented birds.

5.2 METHOD

5.2.1 *Justification of "flooding dose" methodology*

After acquisition of an appropriate Home Office licence, (Licence No. 2101. Procedure No.19(b)2), fractional synthesis rates (FSR) of tissues were measured using a "flooding dose" method, following the methodology of Watt *et al.* (1991). A dose of (stable) labelled amino acid (1-13C leucine) was administered, in combination with a large amount of unlabelled leucine, to flood the leucine body free pool. As the body synthesises protein, amino acids are extracted from the free pool, incorporating the label into tissue protein. Blood and tissues are sampled at a pre-determined time after injection. The extent of incorporation of the label into the tissues relative to the labelled free pool indicates the rate of protein synthesis.

There are a number of assumptions inherent in this method (Garlick *et al.* 1980)

- the flooding dose does not interfere with protein synthesis
- the specific activity of the flood does not decline, or it declines linearly with time.

These two assumptions have been previously tested and ostensibly confirmed, by analysis of the incorporation of ^{14}C-leucine from a flooding dose administered to white-crowned sparrows, *Zonotrichia leucophrys gambelii* (Murphy and Taruscio 1995), and it was not thought necessary to repeat this justification in the case of another small passerine.

After injection, time must be allowed for the dose to flood the tissue free pool and for incorporation into tissue protein to take place. However, free pool label immediately begins to be utilised for tissue protein synthesis, and diluted by amino acid derived from protein degradation. If the free pool becomes significantly desaturated with label, reutilisation of non-labelled amino acid will occur, effectively reducing the rate at which label is incorporated into the tissue and resulting in an under-estimation of synthesis rate. Murphy and Taruscio (1995) duo-sampled at 5 and 30min. However, to reduce the number of birds involved in the present study, sampling was performed at a single time point. This was determined by a preliminary trial conducted on five zebra finches (5.2.2).

5.2.2 *Preliminary trial*

Preliminary trials were conducted to determine the optimum interval between injection of the label dose and sampling (5.3.1). Label levels in blood plasma were analysed in five birds, individuals being sampled at 2, 5, 10, 20 and 30 mins after injection (Fig.5.1). Tissue samples of liver, heart, gut, pectoral muscle and ovary were additionally taken from the two birds sampled at 5 and 20 minutes, in order to determine whether any label was incorporated into tissue protein within these times.

5.2.3 *Isotope analysis of experimental diets*

Isotopes may be incorporated into avian tissues from dietary sources (see Hobson 1995). Differential enrichment of body tissues with 1-13C may occur if repeated reutilisation of dietary amino acid gives rise to natural fractionation. As such, the egg white component of the protein supplement might have represented a naturally enriched source of isotope, outwith the flooding dose. For this reason, it was thought necessary to analyse the protein supplemented diet for potentially elevated background levels of 1-13C in comparison with the seed diet (5.3.2).

5.2.4 *Conditions of experimental birds*

Birds were kept in cages measuring 60cm x 50cm x 40cm, under a lighting regime of 8 hour dark to 16 hour light using timer controlled "daylight" lighting. All birds received cuttlebone, water and oyster grit *ad libitum*. Ambient temperature was maintained at 20.5°C (+/-2).

Fifteen established pairs of finches were selected, and divided into three groups of five. Groups were standardised for female body weight and previous breeding performance (1.1.4), and the females of each group ranked accordingly.

Birds were kept in pair groups for one month prior to the trial. Cage partitions allowed visual and vocal communication between partners, but prevented physical contact. All males were maintained on a seed diet (*Haithes foreign finch mix*) with protein supplement (boiled hens eggs in *Haithes chick rearing mix*). Groups of females received specific diets for one month prior to pairing, as follows:

Seed-Diet Non-Breeding	Mixed seed.
Seed-Diet Breeding	Mixed seed.
Protein-Diet Breeding	Mixed seed, plus high-protein supplement, (boiled hens egg white in plain rusk, 2.3.1 for details).

Zebra finches have previously been shown to be able to self-select a dietary protein balance conducive to increasing egg production (*Chapter 3*). With reference to the dangers of accidental imbalance of diet (5.1.2), the protein supplement was made available in the form of a separate food source, from which the *Protein-Diet Breeding* birds were able to feed at will.

5.2.5 *Trial outline*

Seed-Diet Breeding and *Protein-Diet Breeding* birds were re-united with partners and each pair transferred to a separate cage. Pairs were provided with hay lined nest boxes and fresh grass to encourage nesting activity. Diet supplements were removed from cages and all birds were maintained on a mixed seed diet, hence any differences in protein synthesis rates could only have resulted from past dietary history.

On the morning that the first egg was laid (Day.1), FSR was measured in each female (5.3.3). On each occasion that a *Seed-Diet Breeding* female laid, FSR of the comparatively ranked *Seed-Diet Non-Breeding* female was also measured. FSR increases in response to feeding, and so females were isolated to food-free cages six hours prior to administration of the flooding dose.

The size of the flooding dose used in the current study was based on the guidelines of Garlick *et al.* (1994), and scaled for body weight, corresponds with the amount used by Dr Peter Watt (University of Dundee), in similar experiments previously performed on Japanese quail, *Coturnix coturnix japonica* (Boon *et al.* 1996). The flooding dose comprised 1.1mg leucine and 0.29mg 1-13C leucine (a stable isotope) in 50µl distilled water. The injectate was administered intra-peritoneally (ventral to the stomach) and allowed to circulate for the pre-determined time of 10 mins (see 5.3.1). Birds were kept in the dark during this period, following the protocol of Murphy and Taruscio (1995).

Blood sampling began at 10 min, by venipuncture of the wing vein, and bleeding into a heparinised capillary tube. After collection of approx. 60µl of blood, the bird was quickly CO_2 asphyxiated, and the head removed. Internal organs were dissected out, at room temperature: pectoral muscle, heart, leg muscle, gut (flushed with water), liver, ovary and oviduct (if visible). The total time taken for this procedure was set at 8min. 20sec. All tissue samples were frozen simultaneously at this time, by immersion in liquid nitrogen.

Tissue incorporation of leucine was determined by isolating protein bound leucine from the samples using a preparative gas chromatograph prior to mass spectrometer analysis. See *Appendix 5.5.1* for full method.

Tissue free pool amino acids were determined following the method described in *Appendix 5.5.2.*

Blood samples were spun in a haematocrit centrifuge for 5 min to separate cells and plasma, and analysed following the method described in *Appendix 5.5.3.*

5.2.6 *Calculation of fractional synthesis rate*

Once preparation of the tissue and blood samples was completed, they were passed to Dr Ken Smith (University of Dundee) who performed the mass spectrometer analysis of the samples and calculation of the fractional synthesis rates, as follows:

$$ks = (Sb / (Sf \times t)) \times 100$$

ks = fractional synthesis rate (% / time)
Sb = protein bound enrichment of the amino acid (atom % excess)
Sf = free amino acid pool enrichment (atom % excess)
(averaged enrichment, assuming linear reduction from peak)
t = time from injection to freezing (18min 20sec)

5.2.7 *Complications of methodology*

- The terminal nature of the experiment encouraged the use of only a small number of birds. Preliminary trials of Murphy and Taruscio (1995) involved 48 birds. Rather than repeat this thorough but expensive methodology, it was supported by taking blood samples from five birds and tissue samples from just two birds.

- Intramuscular injection into the pectoral muscle, as performed by Murphy and Taruscio (1995), represents the most effective method of delivery of the label, the tissue sample being taken from the muscle on the other side. This was felt to be unacceptably stressful for these small birds and so the peritoneum was selected as the site for injection.

- The peritoneum is a heterogeneous region, and though some effort was taken to standardise the injection site, slight variations may have given rise to a "first pass effect" (FPE). FPE occurs when the injected dose passes through the liver, prior to flooding the body free pool of amino acid. When this occurs, the rapid rate of protein synthesis in the liver would be expected to result in a disproportionate incorporation of tracer in the liver tissue. Removal of FPE birds from the analysis greatly reduced the variability in FSR within the experimental groups. However, the compromise of four samples by FPE necessitated the assignment of three additional birds to each experimental group, disrupting their original standardisation.

- The most effective killing method is by direct immersion of the live bird in liquid nitrogen, as used by Murphy and Taruscio (1995). This is illegal in Britain, and I found it ethically unacceptable. The method of killing deployed, though fairly rapid, represented an additional variable to the calculation of FSR.

- Once exposed, internal organs should have been immediately flushed with ice-cold saline to effectively reduce synthesis rates.

- Dissection time was protracted by the removal of several organs. Gut, ovaries, oviduct and heart were removed in addition to liver, leg and pectoral muscle. Some processes, for example removal and flushing of the gut, were especially time consuming.

5.3 RESULTS

5.3.1 *Preliminary trial*

Analysis of blood samples taken from the five birds constituting the preliminary experimental group indicated detectable label enrichment of blood plasma to occur within 2 minutes of injection. Enrichment increased rapidly over the first 5 minutes and peaked before 10 mins (Fig.5.1). After this time, plasma enrichment continued to decline, approximately linearly.

Liver, heart, gut, pectoral muscle and ovary tissue samples, taken after 5 and 20 minutes, all contained tissue protein with incorporated label. This indicated that within 5 minutes of injection, measurable levels of label had already been incorporated into new tissue protein as a result of ongoing protein synthesis.

Label levels in the blood plasma were found to be slightly higher than those of the free pool of the tissues, indicating that the administered dose did not completely equilibrate between plasma and tissue free pool. The inability of the flood dose to fully saturate the tissue free pools of amino acids represents a commonly encountered limitation of the use of the flooding dose method in the calculation of absolute protein synthesis rates (e.g. Rennie *et al.* 1994). However, assuming that this limitation applies equally to all experimental groups, the flooding dose technique represents a useful mechanism for comparison of relative FSR.

5.3.2 *Isotope content of experimental diets*

Analysis of seed and protein supplemented diets indicated similar levels of background enrichment in 1-13C. In comparison with a CO_2 standard, the high protein diet recorded an enrichment of $\delta 28.62_{PDB}$, compared with an enrichment of $\delta 31.24_{PDB}$ in the seed diet. Hence, there was no indication that natural fractionation of 1-13C had resulted in an accumulation of the isotope in the egg component of the high protein diet (see 5.2.3). In fact, the background level of isotope was slightly lower in the protein supplement than in the seed diet. Therefore, any additional increase in the isotope enrichment of the tissues of protein supplemented birds could only have been derived from an increased incorporation of label from the flooding dose.

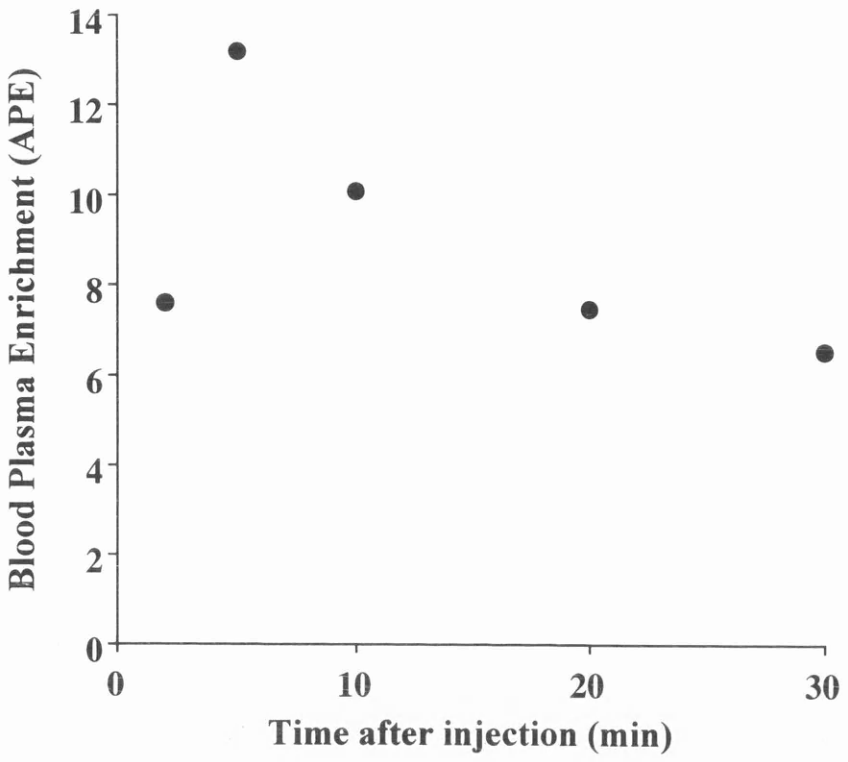


Fig.5.1 Results of the preliminary trial to determine time course for sampling. Blood plasma *atoms percentage excess* (APE) after injection of flooding dose. Points refer to individual birds.

In order to determine the optimum time interval between injection of the tracer and sampling, the concentration of tracer in blood plasma was monitored at 2, 5, 10, 20 and 30 minutes after administration (Fig.5.1). Ideally, sampling should take place after tracer levels have saturated the plasma free pool, but before the level of saturation declines significantly.

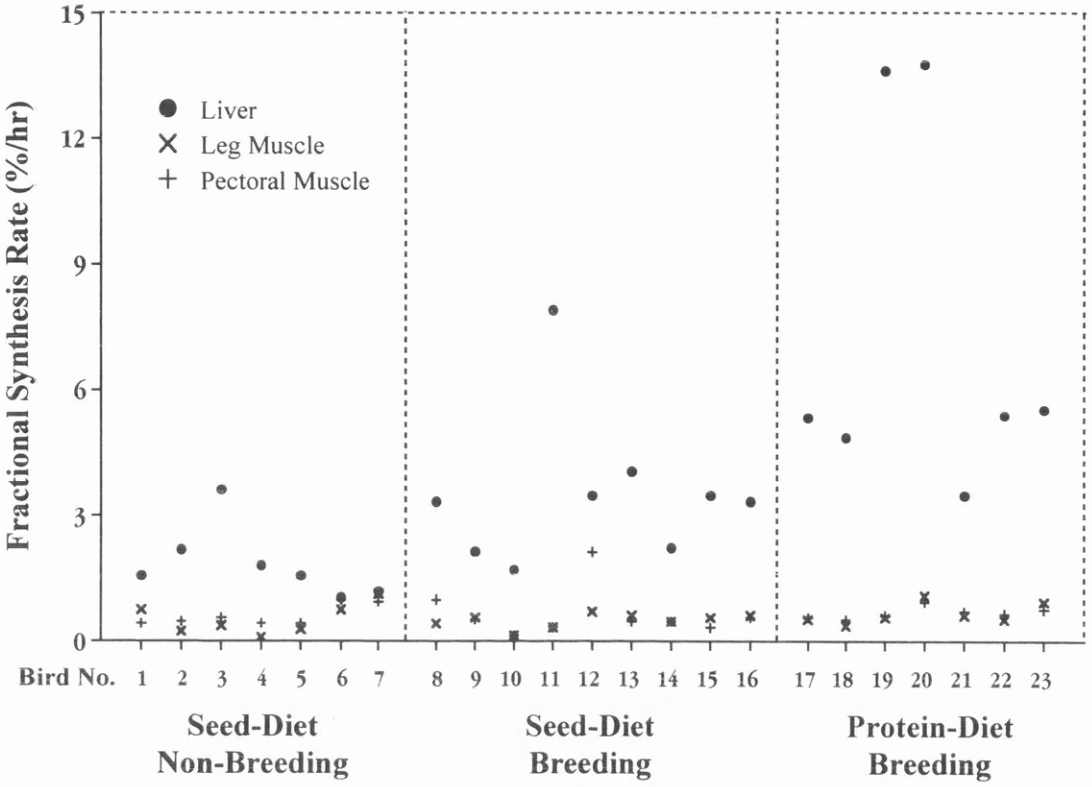


Fig.5.2 Fractional synthesis rate (FSR) of protein in female zebra finches. FSR for liver, leg muscle and pectoral muscle are shown. Sample size = 23 birds (three tissues types from each).

5.3.3 *FSR in experimental groups of female zebra finches*

FSR was determined for liver, leg muscle and pectoral muscle in each experimental group (*Seed-Diet Non-Breeding, Seed-Diet Breeding* and *Protein-Diet Breeding*), (Fig.5.2). Analysis of FSR raw data indicates that label accumulation in the liver of birds 3, 11, 19 and 20 is elevated in excess of 3 standard deviations above the group mean. Statistically, these observations represent samples from a separate population.

With reference to (5.2.7), elevation of FSR of the liver in excess of three standard deviations above the group average was assumed to be indicative of a "first pass effect" (FPE), and liver enrichments of these birds were removed from subsequent analysis, (Fig.5.3.b). FPE enrichment of the liver did not appear to have a measurable effect on the incorporation of tracer into the leg and pectoral muscle, therefore these birds were not excluded from the analysis of muscle tissue enrichment (Fig.5.3.a).

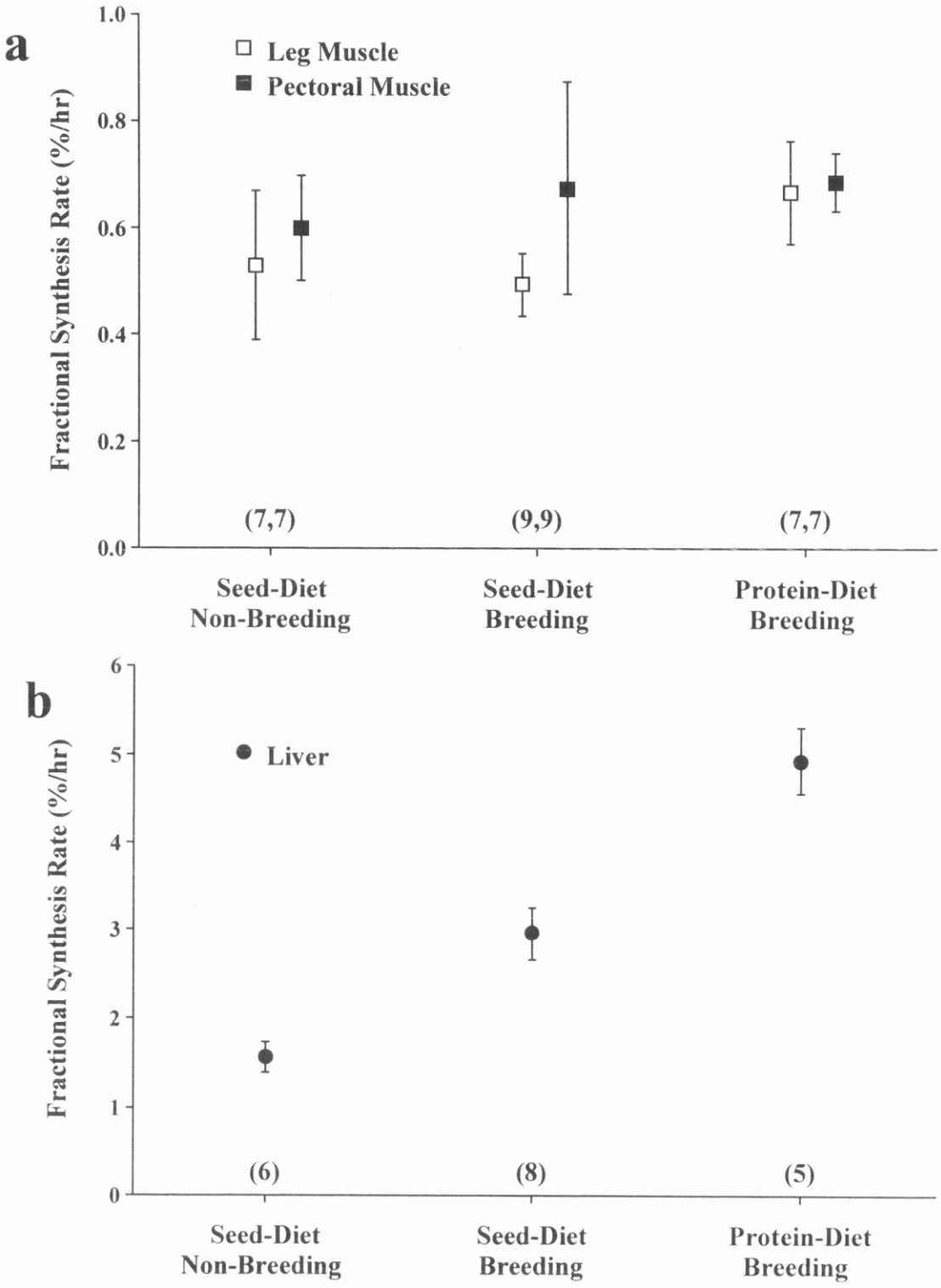


Fig.5.3 Protein synthesis rates in female zebra finches. A comparison of *Seed-Diet Non-Breeding, Seed-Diet Breeding* and *Protein-Diet Breeding* birds. (n) = sample size. Bars show standard errors.

a) Fractional synthesis rate of leg muscle and pectoral muscle.
b) Fractional synthesis rate of liver.

Fractional Synthesis Rate (%/hr)		Tukey's Pairwise Comparisons
Leg Muscle	*ANOVA* $F_{2,20} = 0.86, p = 0.438$	*No significant difference between experimental groups*
Pectoral Muscle	*ANOVA* $F_{2,20} = 0.09, p = 0.914$	*No significant difference between experimental groups*
Liver	*ANOVA* $F_{2,16} = 29.28, \mathbf{p = 0.000}$	*All experimental groups sig. diff.* *Seed-Diet Non-Breeding* < *Seed-Diet Breeding* < *Protein-Diet Breeding*

Tab.5.1 Summary of statistical analysis of fractional synthesis rate (FSR) in female zebra finches. A comparison of *Seed-Diet Non-Breeding, Seed-Diet Breeding* and *Protein-Diet Breeding* birds.

Pooling data from the experimental groups, in order to compare FSR between tissue types, resulted in non-normal data distributions. Hence Mann-Whitney U-Tests were used to provide a non-parametric comparision of the median FSR values of tissue types. FSR for pectoral muscle tended to be slightly, but not significantly, higher than that of leg muscle (0.55 and 0.65%/hr, respectively), (Fig.5.3a), (Mann-Whitney U-Test, C.I. -0.2179,0.0945, p=0.5169).

FSR was higher in liver than in either leg muscle (Mann-Whitney U-Test, C.I. 1.430,3.070, p=0.000) or pectoral muscle (Mann-Whitney U-Test, C.I. 1.319,2.993, p=0.000). Lowest liver FSR was recorded in *Seed-Diet Non-Breeding* birds (1.5%/hr), intermediate in *Seed-Diet Breeding* birds (3.0%/hr), and highest in the *Protein-Diet Breeding* birds (4.9%/hr), (Fig.5.3b).

5.3.4 *FSR analysis of other tissues*

Preparation of individual tissue samples for analysis in the mass spectrometer using the preparative gas chromatograph was extremely time consuming. Unfortunately this meant that complete analysis of all the tissue types collected was not possible. Therefore, no data for FSR are available for gut, ovaries, heart and oviduct.

5.4 DISCUSSION

5.4.1 *Limitations of methodology*

"Methodology dominates understanding of protein metabolism" (Houlihan *et al.* 1995). The comparative ease of application of the flooding dose method, especially in the analysis of free-moving animals, represents its major advantage over the alternative method of continuous infusion. The impracticality of continuous infusion is obvious in the case of the small and easily stressed zebra finch. However, the flooding dose method often gives rise to elevated estimates of synthesis rates, possibly due to engorgement of tRNA synthetase with tracer, or endocrine effects (Rennie *et al.* 1994). Variability of results is also greater, the dose within some tissues being far short of saturating the free pool (Rennie *et al.* 1994). As such, it has been suggested (Waterlow and Stephen 1968) that the flooding dose method, while suitable for comparative purposes, is not a reliable indicator of the absolute rate of protein synthesis.

With reference to the above, results from the preliminary trial indicated non-saturation of all of the tissue free pools. Though not affecting the purposes for which this experiment was performed, the aforementioned limitations restrict these results to comparative estimates, rather than a legitimate evaluation of absolute protein synthesis rates. Increased saturation of the tissue free pool may have been achieved by increasing the size of the flood dose, however, due to differences in protein transfer within the various body tissues, complete saturation of all tissue free pools is not likely to be feasible.

This study investigates only protein synthesis, not degradation, hence predictions regarding protein turnover cannot be made. Degradation rate may be determined separately using, for example, the 3-methylhistidine excretion technique (Taruscio and Murphy 1995), though the heterogeneous incorporation of 3-methylhistidine within different tissues types can lead to complications (P.Watt *pers. comm.*). However, previous studies indicate that muscle growth / wastage are moderated mostly by variation of synthesis rates (Millward 1970, Millward *et al.* 1976, Jones 1980), catabolism being comparatively stable. Though, in moulting birds, elevation of synthesis rate has been indicated to be closely matched by that of degradation, (Taruscio and Murphy 1995).

5.4.2 *General observations on FSR in female zebra finches*

Of the tissues analysed, FSR of the liver was the highest (1.5-4.9%/h), (Fig.5.3b), with FSR of leg muscle (0.55%/h), and pectoral muscle (0.65%/h), much lower (Fig.5.3a). These synthesis rates are similar to those obtained for the white-crowned sparrow (Murphy and Taruscio 1995), liver 3%/h and 5%/h, and pectoral muscle 0.5%/h and 0.6%/h (non-moulting and moulting birds respectively).

It might be expected that synthesis rates of finch tissues would be higher than those of sparrows, as protein synthesis is subject to scaling (Houlihan *et al.* 1995), and the body weight of zebra finches (15g) is less than that of white-crowned sparrows (25g). Also, as zebra finches undergo continuous moult (Zann 1985), an additional elevation of baseline synthesis rates might be anticipated. Non-saturation of the amino acid free pool may have contributed a conservative bias to the estimates of FSR in the current study. Additionally the unfamiliar kinetics of precursor-product transition may have resulted in an over-estimation of free pool labelling.

Though the importance of the rapid metabolism of protein by the liver cannot be overstated, comparatively small variations in turnover of skeletal muscle have large implications for whole body protein metabolism, due to the size of the muscular protein reserve; Millward (1970) suggesting that "skeletal muscle... must play the dominant role in the overall protein metabolism of the body". During short-term dietary protein depletion, a synthesis deficit in combination with the high turnover rate of the liver results in rapid depletion of its protein reserves. However, over time, this contribution of protein is a fraction of that released from the comparatively slow breakdown of muscle (see Fisher 1967).

Previous studies of depletion of protein reserves in undernourished fowl, *Gallus gallus* (Dickerson and McCance 1960) and moulting great crested grebes, *Podiceps cristatus*, (Piersma 1988), indicated an increased susceptibility to breakdown of pectoral muscle in comparison with sartorius / leg muscle. If accelerated breakdown of the pectoral muscle arose as a result of a high rate of protein turnover combined with a synthesis deficit, one might expect to observe a higher rate of synthesis to occur in pectoral muscle than leg muscle.

No significant difference in protein synthesis rate was observed between muscle-types in the current study, indicating that, in the case of the zebra finch, differential depletion of pectoral and leg muscle may not occur (at least not as a result of differential synthesis rates). This result might support the previous observations of Houston *et al.* (1995a), who recorded a similar depletion (12-15% lean dry weight), of both leg and pectoral muscle of female zebra finches over the course of the laying period.

5.4.3 *FSR of skeletal muscle: effects of egg production and dietary history*

The protein demands of moult have been suggested to be met by mobilisation of the protein reserves of skeletal muscle (Ward 1969, Piersma 1988), and moult has been shown to promote an increase in FSR of pectoral muscle (Murphy and Taruscio 1995). The protein requirements for egg production have similarly been shown to be linked to degradation of the skeletal muscle (Houston *et al.* 1995a). However, in the current study, egg production had no significant effect on the protein synthesis rates of leg or pectoral muscle (Fig.5.3a).

An alteration in muscle synthesis rates should not, perhaps, be ruled out by these results. The small sample sizes used in this experiment, combined with the limitations of the protocol may have masked subtle changes in synthesis rates. The elevation in synthesis rate observed by Murphy and Taruscio (1995) was fairly small (1.3 fold), and though their result was obtained using a similar number of experimental birds, it is possible that differentiation was facilitated by moult representing a less variable protein demand than egg production.

Birds with a history of dietary protein supplementation recorded slightly (but not significantly) elevated FSR in skeletal muscle. Refinement of experimental technique, with special attention to improvement of label distribution and saturation of the free pool by the flooding dose would be required to confirm this. However, it would appear likely that other physiological mechanisms, e.g. increased dietary efficiency (El-Wailly 1966), or elevated breakdown rate may play a more important part in the provisioning of protein for egg formation during the breeding cycle.

5.4.4 *FSR of liver: effects of egg production and dietary history*

FSR of the liver of breeding birds was significantly higher than that of non-breeding birds. Additionally, FSR of the liver of breeding birds previously experiencing a protein supplemented diet was significantly higher than that of those previously maintained on a seed only diet (Fig.5.3b).

Egg formation is under hormonal control, mediated by the nutritional plane of the bird. Yolk proteins are synthesised in the liver, and deposited into follicles in response to increased levels of follicle stimulating hormone (FSH). This process, initiated by the hypothalamus, is probably the most important rate-limiting factor in egg formation (Carey 1996).

An increase in the FSR of the liver of breeding birds may arise as a result of the protein requirement for egg yolk formation. Additionally, Murphy and Taruscio (1995) recorded an increase in whole body protein synthesis during moult in excess of 8.5 times the requirement for keratin replacement alone, and suggested that this apparent inefficiency was required to satisfy the range of metabolic demands imposed by moult. It might similarly be expected that the elevation of liver FSR in breeding finches might be associated with a whole range of metabolic modifications (e.g. enzyme and hormone production, formation of the oviduct) concurrent with egg production.

The elevation of FSR of the liver in birds previously experiencing a protein supplemented diet may have arisen though a number of mechanisms. Exposure to dietary protein supplements encourages an adaptive increase in the enzymes involved in the metabolism of specific amino acids (Fisher 1967). Additionally, the elevation of non-essential amino acids in the diet may contribute to the production of enzymes to aid acquisition of limiting amino acids (Harper *et al.* 1970). The net

effect of these processes represents an increased synthetic potential for protein, including yolk protein. The increase in FSR of the liver may result in an increased availability of protein for the formation of egg yolks, or augmentation of endogenous protein reserves. This may represent a mechanism by which protein supplementation of the diet of zebra finches promotes the formation of larger eggs and clutches, as observed in previous studies (*Chapter 3*, Selman and Houston 1996, Williams 1996).

Williams (1996) reported an increase in both yolk protein and albumen content of enlarged eggs arising from birds maintained on a protein supplemented diet. As most albumen is synthesised in the magnum of the oviduct (White and Merrill 1988), one might expect elevated levels of protein synthesis to be recorded in the oviduct samples taken during this experiment. However, the transient nature of the oviduct in zebra finches may complicate this analysis, degradation of the oviduct initiating prior to completion of the clutch in some species of birds (e.g. Houston *et al.* 1983).

The role of skeletal muscle protein metabolism in the provision of protein for egg production requires further investigation. This may be achieved by direct monitoring of release of labelled amino acids (e.g. 3-methylhistidine excretion technique), or by an examination of the physiological associates of protein degradation, e.g. proteasome and ubiquitin.

5.5 APPENDIX

Based on the methodologies outlined in Watt *et al.* (1991) and Garlick *et al.* (1994).

5.5.1 *Determination of label incorporation into tissue protein*

Step. 1 Freeze tissue in liquid nitrogen and grind to a fine powder using mortar and pestle.
Transfer 100-200mg of powdered tissue to an eppendorf with a 3ml capacity.
Transfer remainder to cold screw-top vial for storage (-70°C).

Step. 2 Add 3ml 0.2M perchloric acid (PCA) containing
internal standards (10µl each, and 30µl Norleucine).
Vortex, centrifuge (4°C, 20 min, 2800G). Pour off supernatant.
Supernatant and pellet may now be frozen and stored.

Note: Keep supernatant for tissue free-pool analysis,
and remember to assay protein content of tissue.
See Appendix 5.5.2.

Step. 3 Wash pellet with 5ml 0.2M PCA. Vortex, centrifuge (4°C, 20 min, 2800g).
Discard supernatant. (repeat)

Dissolve pellet in 3ml 0.3M NaOH, 37°C 60 min, remove 50µl for BCA assay). ***Removes RNA.***

Add 2ml 1M PCA, ice cool (4°C 10 min), vortex, centrifuge (4°C, 20 min, 2800g). Discard supernatant.

Add 5ml, 2MPCA, incubate (70°C for 60 min), vortex, centrifuge (4°C, 20 min, 2800g).
Discard supernatant. ***Removes DNA.***

Add 3ml, 6M HCl, transfer to hydrolysis tube and heat, (110°C for 15-18 hours).
(Loosen tops after 15mins to prevent explosions! Re-tighten well to prevent evaporation.)

Step. 4 Remove HCl under stream of nitrogen at 120°C, (approx.1hr). Add 1ml distilled water.

Set up filtration columns (use 5ml Gilson pipette tips) for Dowex H+ resin filtration.
Insert pipette filter into end of tip, and fill with double distilled water (ddW). Allow to drain.
Pipette Dowex slurry into column until 2ml mark is reached.
Prewash with 2M NH_4OH (9ml). ***Removes any pre-present amino acids,***
Neutralise with double distilled water (6-9ml).
Prime with 1M HCl (6ml). ***Initiates resin binding sites for amino acids.***

Add sample, and rinse hydrolysis tube with 1ml HCl.
Resin is then washed with the following - 1MHCl (1mlx2), ddW (1mlx2, then 3ml), 2M NH_4OH (1mlx2). ***Latter substitutes H+ for amino acid, removing it from binding site.***
Amino acids are then eluted with 2M NH_4OH (3ml) into glass centrifuge tube. (Touch litmus paper to column tip to check that total elution volume has been passed - should test alkaline).

Ammonia is evaporated off in turbo-vap, and the sample reduced to 1ml under N_2 (70°C for approx 30min).

Sample is frozen in liquid nitrogen and dried overnight in rotary evaporator.

Step. 5 Prepare standard solution to test Preparation Gas Chromatograph (Prep GC).
5mg Leucine, 5mg Valine, 5mg Isoleucine, 100µl Pyridine, 100µl MTBSTFA*.
Mix in eppendorf. Incubate for 60 min at 70-80°C.

Derivitise samples just prior to introduction to Prep GC.
Add 100µl 5M Pyridine and 100µl MTBSTFA to each eppendorf, in fume cupboard.
Incubate for 60min at 70-80°C.

Prep GC set to 280°C max temp
air and hydrogen mixture
to reach max temp over 40 min
total run time 1 hour
Leucine peak appears after 17-25 min (depending on column).

80µl sub-samples injected into Prep-GC.
Chart recorder used to monitor elution of amino-acid peaks.
Leucine peak collected in U-shaped tube under liquid nitrogen.
Tube plugged with tissue and labelled.

Step.6 500µl of pH 2.2 lithium citrate buffer introduced down side of tube.
Vortex to dissolve all amino acid residue.
Re-plug tube with towel and transfer to oven at (90°C for 30min).

Transfer contents of U-tube to X-tube and rinse with 500µl citrate buffer.
Add anti-bumping bead and place on hot-block (130°C for 30 min).
Cool to room temp - then put on ice.

Add 25mg ninhydrin to sample and controls. Screw tops on tubes and evacuate.
Place in water bath at (90°C for 30 min). Remove and allow to cool.
Add Helium (packing agent) to approx. atmospheric pressure.

Step.7 Transfer samples to mass spectrometer.
Sample run is initiated with a set of low voltage standards.
Run is interspersed with standard dilutions of Leucine.
Samples are run in blocks of eight, separated by CO_2 gas standard tubes.

* N-Methyl-N-T-Butyldimethylsilyl Triflouroacetamide

5.5.2 *Determination of tissue free pool label concentration*

Prepare supernatant following steps outlined Step.1-Step.2 in Appendix.5.5.1

Step. 3 Remove 500µl. of supernatant.
Neutralise, dropwise, with (approx.100µl) 1M $KH(CO_3)$ till pH 7 is passed.
Place sample on ice (for 20min) to encourage precipitate.
Centrifuge at 4°C for 5 min at 2800G.
Run through Dowex H+.
(Removes salts which may interfere with derivitisation).

Transfer elute to Turbo-Vap (70°C under nitrogen) for 20-40mins to reduce volume to 1ml.
Transfer to eppendorf and rinse tubes with 500µl distilled water.

Transfer supernatant to eppendorf. ***Contains free amino acids.***
(Plug may be thrown away).

Acidify (approx. pH2) by dropwise addition of 1M HCl.

Dry in rotary evaporator (six-eight hours).

Step. 4 Derivitise samples. Add 50µl of Pyridine and 50µl of MTBSTFA*.
Vortex. Incubate for 60min at 60-70°C.

Transfer 50µl sub-samples from eppendorfs to automatic sampler glass tubes with micro-inserts.
Cap and crimp seal using callipers.

Step.5 Transfer to automatic sampler mass spectrometer.

* N-Methyl-N-T-Butyldimethylsilyl Triflouroacetamide

5.5.3 *Determination of blood plasma label concentration*

Step.1 Defrost pre-spun blood sample in capillary tube.
Using crystaseal as a plunger. Expel 50µl plasma into eppendorf.

No internal standard or urease need be added (when dealing with avian samples).
Incubate at room temperature (for 20mins).

Deproteinise with 100% ethanol, ratio to sample vol 3:1 (ie.150µl).
Spin in microfuge for 5 min. Collect supernatant.
Neutralise blood CO_3^- ions with 100µl 1M HCl.
Vortex. Dry in Turbo-vap or similar.

Step. 2 Derivitise samples. Add 50µl of Pyridine and 50µl of MTBSTFA*.
Vortex. Incubate (for 60min at 90°C).

Step.3 Transfer 50µl sub-samples from eppendorfs
to automatic sampler glass tubes with micro-inserts.
Cap and crimp seal using callipers.

Step.4 Transfer to automatic sampler mass spectrometer.

* N-Methyl-N-T-Butyldimethylsilyl Triflouroacetamide

Chapter 6

IMMUNOLOGICAL TRADE-OFFS DURING EGG PRODUCTION AND THE ROLE OF DIETARY PROTEIN HISTORY

"... here come germs ..."

N. Fiend

SUMMARY

The repercussions of dietary history and breeding status on immuno-competence were investigated. It was predicted that, were a nutrient allocation trade-off between egg and antibody production to occur, this might be alleviated by protein supplementation of the diet. Female zebra finches of differing dietary histories were immunised with sheep red blood cells (SRBCs) to present them with a potential resource allocation trade-off between egg and antibody production. Egg productivity did not differ between birds maintained, prior to breeding, on seed or protein supplemented diets. However, primary immune response was reduced in breeding birds maintained on a seed diet in comparison with both non-breeding birds and protein-supplemented breeding birds. Hence, females from a good nutritional background were able to maintain egg productivity, without reducing the strength of their immune response. When subjected to concurrent immunological challenge, rather than increasing short-term reproductive capacity, high quality nutrient reserves may promote the immuno-competence of the individual and consequently improve survivorship if exposed to a pathogenic challenge. It is suggested that the dietary protein supplementation observed in wild finches prior to breeding might potentially be regarded as much an immunological measure as a mechanism for increasing egg production.

6.1 INTRODUCTION

6.1.1 *Effect of protein supplementation of the diet*

In laboratory studies of breeding zebra finches, dietary protein supplementation promotes an increase in numbers and/or dimensions of eggs laid (*Chapter 3*, Selman and Houston 1996, Williams 1996). Brood size and its interaction with reproductive effort and parental mortality are key components of life history strategy, which predicts a conflict between clutch size (immediate reproductive effort) and long-term fitness of the parent (Charnov and Krebs 1973, from Stearns 1976).

6.1.2 *Metabolic changes during egg production*

The production of eggs and associated metabolic modifications (e.g. development of the oviduct) are protein demanding processes. The laying female may satisfy this protein requirement by modification of dietary intake and/or mobilisation of endogenous reserves.

During egg production, allocation of blood to the newly developed oviduct effectively reduces blood availability to the rest of the body. Liquid components of the blood are readily replaced (being mostly water), but synthesis of new red blood cells (erythropoiesis) takes more time. Therefore, a reduction in haematocrit (6.2.3) may be expected during the egg laying period. Recovery of pre-breeding haematocrit values might be expected to be limited by nutrient availability for synthesis of the cellular components of blood. Excessive reduction in haematocrit may be regarded as undesirable, with potential repercussions for efficiency of oxygen uptake and transfer. High haematocrit values have been previously correlated with indicators of fitness in birds (Saino *et al.* 1997).

The roles of protein in the immune response are manifold. Protein is vital to the formation of defence immunoglobulins, proliferation of phagocytes and lymphocytes and evolution of the inflammatory and acute phase responses. Additionally, anti-microbial agents (e.g. complement), and cytokines (e.g. interferon) are also protein. During infection, protein metabolism is modified under hormonal control (*review* Marsh 1992), testosterone often being implicated in suppression of the immune response in combination with increasing protein metabolism (see, for example, Veiga *et al.* 1998). In the case of severe infection, utilisation of endogenous protein in excess of stores may manifest as muscle wastage (Beisel 1977). As such, both egg and antibody production represent multiple and potentially competing demands for protein.

Additionally, an egg represents not only a nutrient store for the growing chick, but also a basic immune system. Transfer of gammaglobulins, including anti-viral antibodies, from the circulation of the female to the yolk of the developing egg has been shown to occur, especially during the rapid growth phase of the oocyte (Brambell 1970). Thus, egg production represents an additional immunological productivity burden for the

laying female. This may partly explain the observations of McCurdy *et al.* (1998), who reported a higher incidence of certain blood parasites in female birds of a variety of species, in comparison with males.

Behavioural changes associated with reproduction may compound immunological compromise during the breeding period. Incubating birds may be subjected to an increased risk of contagion from prolonged exposure to nests infested with parasites, while allocation of time to nest building / feeding nestlings might reduce that available for self-maintenance. The close association of communal nesters, such as the zebra finch, may further facilitate the transfer of pathogens and parasites.

6.1.3 *Consequences of protein limitation for parents and offspring*

The combination of these requirements makes breeding a risky time for birds. Previous studies have shown a positive correlation between clutch size and the incidence of malarial parasites in great tits (Oppliger *et al.* 1997). Collared flycatchers, *Ficedula albicollis*, and zebra finches similarly exhibit reduced immune responsiveness in combination with increased reproductive effort (Nordling *et al.* 1998). In zebra finches, immuno-suppression has been observed to be correlated with both reproductive status and brood size (Apanius *et al.* 1994). Protein limitation of the immune response as a result of the competing demands for egg production might represent a mechanism to explain these observations: protein-restricted bobwhites, *Colinus virginianus*, having been shown to exhibit a depressed immune response (Lochmiller *et al.* 1993).

Previous studies (Selman and Houston 1996, Williams 1996) show that endogenous protein reserves of female finches have a considerable effect on their ability to produce eggs. However, no consideration has been made of concurrent effects on their immunological status. Could these reserves additionally be used to reduce the immunological compromise associated with breeding?

Dietary protein supplementation encourages an enhancement of immune response in chickens, and these effects remain evident even after a period of maintenance on a low protein diet (Fisher 1967). More recently, Saino *et al.* (1997) recorded a similar elevation of immuno-competence in association with dietary protein supplementation in barn swallows, *Hirundo rustica*. However, previous studies of zebra finches found no evidence of protein supplementation enhancing immune response of post-laying parents (Deerenberg 1996).

Incidentally, some previous studies have indicated a reduction in immunocompetence in association with protein supplementation, but it has been suggested that this may have arisen from an imbalance of amino acids rather than an excess (*see* Fisher 1967). However, protein:calorie imbalance has been shown to reduce primary immune response of chickens to sheep red blood cells (SRBCs), (Glick *et al.* 1981).

Immunisation with SRBCs is a standard procedure for the presentation of a novel, non-pathogenic immunological challenge, to elicit a generic antibody response in birds (see above, Deerenberg 1996, Lochmiller *et al.* 1993, Sorvari and Sorvari 1997). The

intensity of subsequent antibody activity may be determined by a haemagglutination assay on a small (50µl) sample of blood taken post-immunisation, and may be regarded as a indicator of the capacity of the immune system to mount an antigenic response. Full details of this procedure are given in Appendix 6.5.

6.1.4 *Aims*

It was the aim of these experiments to investigate the effect of endogenous protein reserves on resource allocation in female zebra finches. Prior to initiation of the trial, experimental groups of finches were maintained either on a seed only or protein-supplemented diet for three months, with the objective of fully replenishing endogenous protein reserves. All birds were then placed on a seed only diet and presented with a potential trade-off between egg production and immunological response to a non-pathogenic challenge (immunisation with SRBCs). Clutch and egg size were monitored post-immunisation, and compared between birds of differing dietary history. Blood samples were taken after a pre-determined period and antibody activity and haematocrit measured.

After completion of the first clutch, the birds were re-immunised and eggs removed to encourage laying of a replacement clutch, in combination with a secondary immune challenge. It was predicted that the potential for protein limitation of egg and / or antibody production might be elevated during the second period of laying and immunisation, were body reserves of protein depleted as a result of investment in the primary clutch / primary immune response.

Females from inadequate nutritional backgrounds have previously been shown to produce fewer eggs (Selman and Houston 1996, Williams 1996) and incur increased loss of muscle (Selman and Houston 1996) and body weight (*Chapter 3*, Selman 1994, Williams 1996) during laying, in comparison with birds experiencing a history of dietary protein supplementation. It is hypothesised that the demands of egg formation may conflict with the nutrient requirement for self-maintenance, including immuno-competence (as measured by antibody responsiveness to SRBCs) and ability to synthesise new cells (as measured by recovery of haematocrit). In the case of birds with inadequate nutritional reserves, this might be expected to enforce a trade-off in nutrient allocation, resulting in a reduction in egg, immunological, or cellular productivity. It is predicted that any limiting consequences of this trade-off should be reduced by augmentation of the endogenous reserves of females, through supplementation of the diet with a high quality source of protein prior to breeding.

Finally, the potential of the dietary experience of the female to influence the immuno-competence of her offspring was examined. During egg production, the composition of oviducal secretions are modified by diet (Gerber and Carr 1930), consequently, it is not unreasonable to postulate that maternal diet may have some influence on egg composition. This may, in part, explain previous observations of a reduction in the immune response of nestlings from replacement and late hatching clutches (Sorci *et al.* 1997).

First laid eggs of the primary clutch were fostered to surrogate parents for incubation and chick rearing. Chicks were immunised one month post-fledging with SRBCs and after a pre-determined period a blood sample was analysed to see whether the pre-breeding diet of the parent had any effect on the immunocompetence of the offspring. (Response to SRBCs is mostly IgM mediated. IgM is not transferred from the parent to the egg, and so this immunisation would be expected to elicit a primary immune response in the chick).

6.2 METHOD

6.2.1 *Preliminary trial*

A preliminary trial was conducted to determine the time taken, after immunisation, for zebra finches to achieve a peak intensity of antibody response. Due to the limited availability of female birds, these preliminary trials were conducted on males, and it was assumed that the timing of the immune response would be similar between the two sexes.

After acquisition of an appropriate Home Office licence, (Licence No. 2101. Procedure No.19(b)1), groups of 5-6 males were immunised with sheep red blood cells (SRBCs), and blood samples taken 3, 5, 7, 10, 12, 19 and 38 days later, to determine the intensity profile of the primary response. Details of the preparation of SRBCs and haemagglutination technique are given in Appendix 6.5, and was based on the methodology outlined by Hudson and Hay (1989).

Thirty eight days after primary immunisation, a second dose of SRBCs was administered. Blood samples were taken at 2, 4, 6, 8 and 11 days later, to determine the intensity profile of the secondary response.

6.2.2 *Experimental conditions*

Birds were kept in cages measuring 60cm x 50cm x 40cm, under a lighting regime of 8 hour dark to 16 hour light using timer controlled lighting. Illumination included ultra-violet components, mimicking the spectrum of natural daylight (*Lightbox* "Daylight" lighting). All birds received water, mixed seed, cuttlebone and oyster grit *ad libitum*, (the latter avoiding potential calcium limitation of egg production). Ambient temperature was maintained at 20.5°C (+/-2).

Three groups of fifteen established pairs of finches (45 pairs in total) were selected. Groups were standardised for body weight of the females and previous breeding performance (see 1.1.4 for details of standardisation procedure).

For three months prior to the trial, males and females of all pairs were separated by cage partitions. These allowed visual and vocal contact between partners, but prevented physical contact. All males were maintained on a seed diet with protein supplement (boiled hens egg in a commercial high protein chick rearing mix). Females received the following diets:

Non-Breeding Group	Mixed seed diet / white rusk supplement.
Seed-Diet Breeding Group	Mixed seed diet / white rusk supplement.
Protein-Diet Breeding Group	Mixed seed diet / protein supplement, (based on boiled hens egg white, see 2.3.1).

6.2.3 *Experimental protocol*

Day.1 Females were weighed (+/- 0.01g). Any dietary supplements were removed and cages provisioned with straw-lined nest boxes and fresh grass to encourage the birds to breed.

Females were immunised intraperitoneally, ventral to the stomach, with a washed suspension of $5x10^7$ SRBCs in 100µl of phosphate buffered saline: this dose corresponding with that administered to zebra finches in the previous study of Deerenberg (1996). Females were then re-paired with their partners.

Further details of preparation of SRBCs are given in Appendix (6.5).

Nest boxes were monitored daily, and newly laid eggs were removed* and replaced with replicas to guard against the parents eating them. Eggs were weighed (± 0.001g) and length and width measured (± 0.1mm).

Immune response was measured by taking blood samples at time intervals pre-determined to coincide with peak response intensity (see preliminary trial).

Day.10 A 50µl blood sample was taken from each female by venipuncture of the wing vein with a sterile hypodermic needle. Blood was collected into a heparinised capillary tube and sealed at one end with *Cristaseal* (*Hawksley*), prior to centrifugation for 5 minutes in a *Haematocrit Centrifuge.* The haematocrit of the sample was measured, and the serum sample analysed for anti-SRBC antibodies using the haemagglutination technique (6.5).

Haematocrit is a measurement of blood cellularity. Capillary tubes containing blood samples were spun in a centrifuge to separate the cells from the plasma and the haematocrit score was calculated as follows:

$$\text{Haematocrit} = \frac{\text{length of packed red cells in tube}}{\text{total length of blood sample in tube}}$$

After completion of the clutch, protein supplement was returned to the diet of the *Protein diet* group up until Day.20. This aimed to replenish endogenous stores depleted by the first challenge. *Seed diet* and *Non-breeding* birds were maintained on a seed only diet.

Day.20 Females were immunised with SRBCs for a second time and plaster eggs removed and fresh nesting material supplied to encourage laying of a second clutch. Nest boxes were monitored daily and newly laid eggs were replaced with plaster replicas, as before.

Day.27 A second blood sample was taken, and analysed as before. Females and males were then separated and the diets of all birds were supplemented with a commercial high protein mix (*Haithes chick rearing mix*).

*** The first laid eggs from each clutch were transferred to the nests of foster parents for incubation and chick rearing. On hatching, all cages containing foster pairs were provisioned with a high protein chick rearing mix as a supplement to the basic seed diet. Thus, any differences in the immunocompetence of the foster chicks could only have been due to nutritional differences derived from differential egg provisioning.**

6.3 RESULTS

6.3.1 *Preliminary trial*

A preliminary trial was conducted, on male zebra finches, to determine the time for antibody levels in the blood to reach their peak after immunisation (Fig.6.1). When analysing the results of the haemagglutination assay, test wells in which any visible haemagglutination had occurred were recorded as a positive result. Therefore, as successive wells contained 50% serial dilutions of antibody, the resultant scale on which antibody activity is recorded is $\log_2$.

The primary response did not reach peak intensity until approximately 10 days after immunisation, with mean titre still exceeding 50% peak intensity after 19 days. Contrary to expectation, the intensity of the secondary response was not measurably greater than that of the primary. This may have occurred if the secondary immunisation was delivered prior to the primary response declining sufficiently, resulting in a poorly defined response to the additional immunological challenge. However, in comparison with the primary, the secondary response peaked earlier, approximately 7 days after the second immunisation, and was apparently of shorter duration, falling below 50% peak intensity within 11 days.

Based on these results, I decided to measure primary response 10 days after immunisation, and secondary response after 7 days.

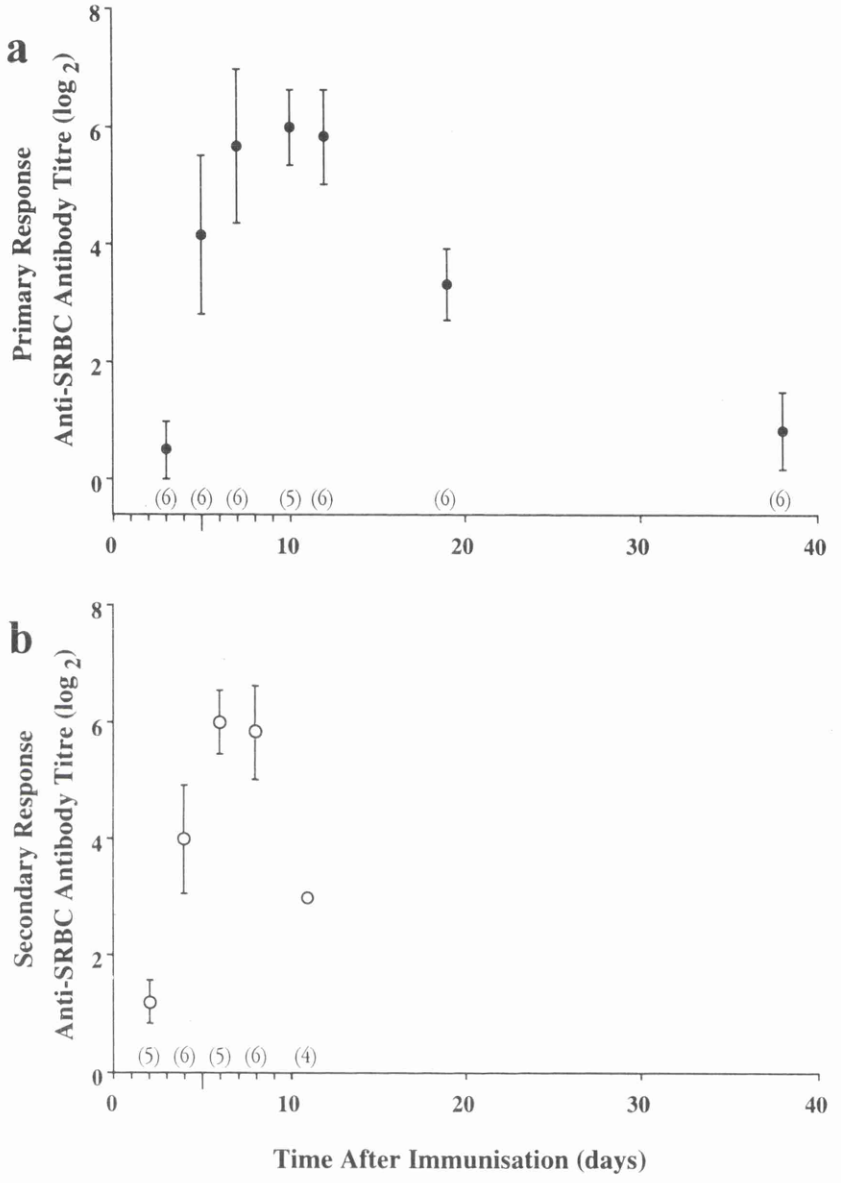


Fig.6.1 Results of the preliminary trial to determine time course and intensity of antibody response to immunisation with SRBCs (using male zebra finches). Bars indicate standard error. (n) = sample size.

a) Primary Response. (Peak antibody activity Day.10).
b) Secondary Response. (Peak antibody activity Day.7).

6.3.2 *Experimental trial*

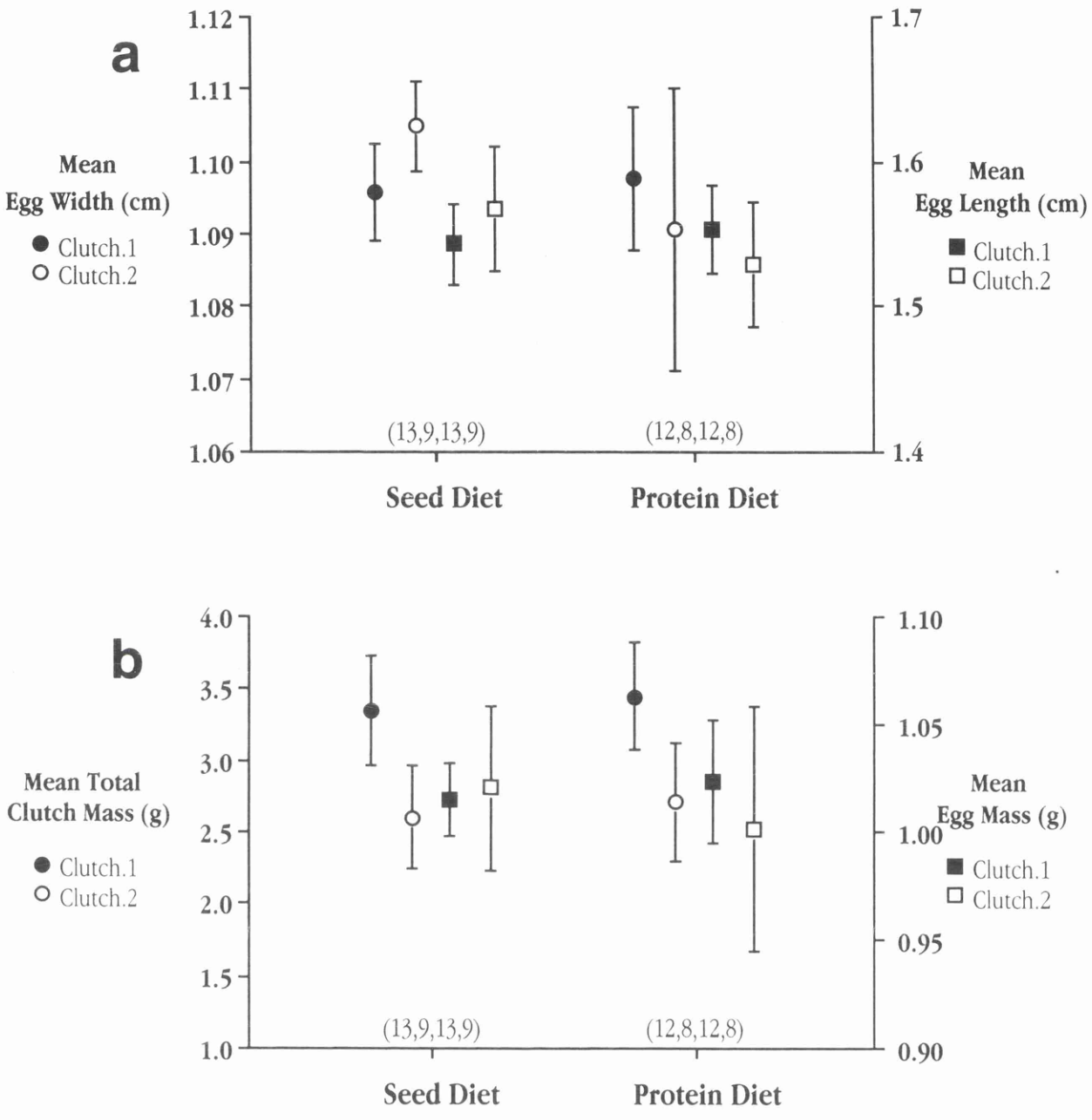


Fig.6.2 Clutch characteristics of breeding groups. Means are shown. Bars indicate standard error. (n) = sample size.

a) Mean egg width and length.
b) Mean clutch mass and egg mass.

Though total clutch mass of second clutches tended to be smaller than that of first clutches, there were no significant differences in total clutch mass nor egg mass/length/width between dietary groups, nor between successive clutches (Fig.6.2). See Tab.6.1 for statistical summary.

Comparison of Egg / Clutch Size between Seed and Protein Diet Groups.		**2 Sample t-test**
Egg Width	*Clutch.1*	t = 0.17, 18 df, $p = 0.87$
	Clutch.2	t = 0.70, 8 df, $p = 0.51$
Egg Length	*Clutch.1*	t = 0.24, 22 df, $p = 0.81$
	Clutch.2	t = 0.65, 14 df, $p = 0.53$
Egg Mass	*Clutch.1*	t = 0.25, 18 df, $p = 0.80$
	Clutch.2	t = 0.28, 12 df, $p = 0.79$
Total Clutch Mass	*Clutch.1*	t = 0.19, 22 df, $p = 0.85$
	Clutch.2	t = 0.20, 14 df, $p = 0.85$

Comparison of Egg / Clutch Size between Clutch.1 and Clutch.2		**Paired samples t-test**
Egg Width	*Seed*	n = 9, t = 1.52, $p = 0.167$
	Protein	n = 8, t = 0.08, $p = 0.940$
Egg Length	*Seed*	n = 9, t = 0.23, $p = 0.821$
	Protein	n = 8, t = 2.15, $p = 0.068$
Egg Mass	*Seed*	n = 9, t = 0.47, $p = 0.651$
	Protein	n = 8, t = 1.06, $p = 0.325$
Total Clutch Mass	*Seed*	n = 9, t = 1.59, $p = 0.151$
	Protein	n = 8, t = 2.31, $\mathbf{p = 0.054}$

Tab.6.1 Summary of statistical comparisons of egg and clutch size. Comparisons are between experimental groups, and between successive clutches.

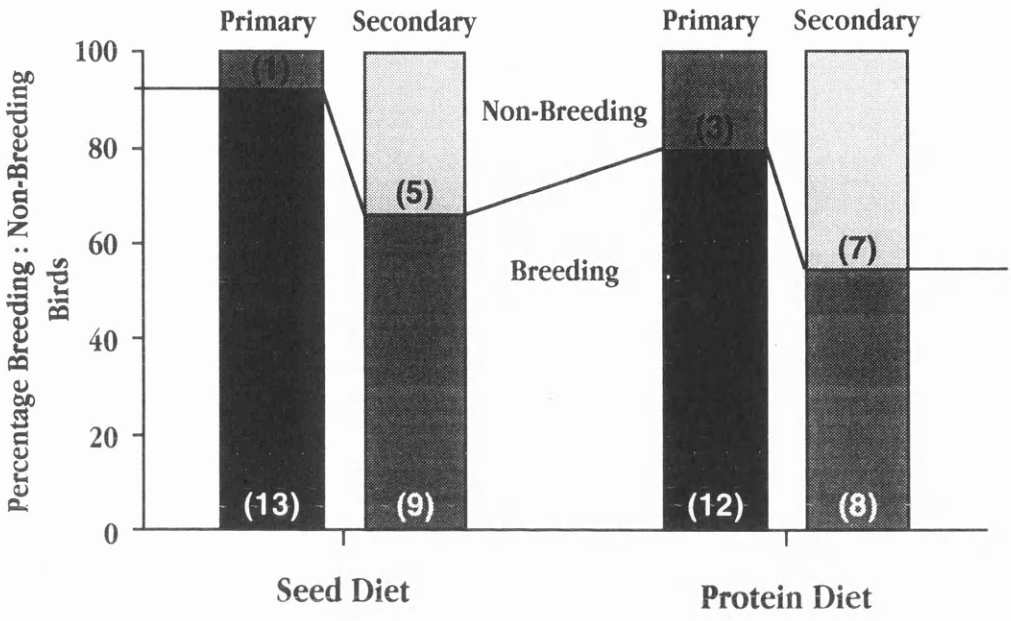


Fig.6.3 Percentage of zebra finch pairs which successfully laid eggs over the course of the primary and secondary immune responses. (n) = sample size.

Though all pairs had previously bred successfully, not all females produced clutches within the time constraints of the experiment (Fig.6.3).

In most cases, non-breeding during the primary immune response was associated with individuals that appeared visibly stressed by the immunisation procedure (e.g. skulking / feather fluffing). These females were excluded from subsequent analysis, this reaction being regarded as independent of the physiological condition of the bird.

Non-breeding during the secondary response may have been associated with wing damage arising from blood sampling. Again, this reaction was regarded as being independent of the physiological condition of the bird, and these females were also excluded from subsequent analysis.

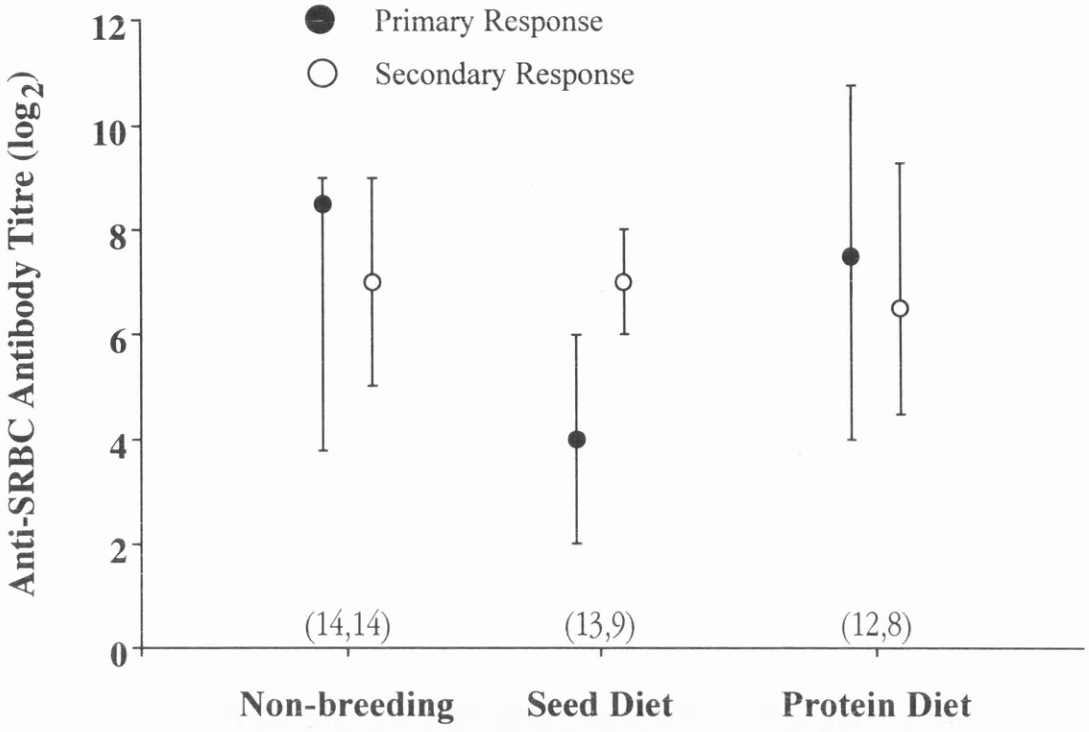


Fig.6.4 Intensity (titre) of antibody response to SRBCs in experimental groups of female zebra finches. Primary response measured after 10 days. Secondary response measured after 7 days. Median titres (log_2) are shown. Bars indicate interquartile range. (n) = sample size.

Parametric analysis of the primary immune response indicated a significant difference between experimental groups (ANOVA $F_{2,36}$=3.44, p=0.043), with *Seed diet* birds significantly lower than both *Non-breeding* and *Protein diet* (Fisher's pairwise comparisons). However, this observation is compromised by the strongly skewed (non-normal) distribution of the *Non-breeding* primary response (Fig.6.4), necessitating non-parametric comparisons.

There was no significant difference in the intensity of the primary response between *Non-breeding* and *Protein diet* groups (Mann-Whitney: W=163.0, CI.-2.998, 1.999, p=0.9793). Primary response of seed diet birds was significantly lower than *Protein diet* birds (T-Test: t=2.35, 22 df, p=0.028), and lower, bordering on significance, in comparison with *Non-breeding* birds (Mann-Whitney: W=141.5, CI. 0.002, 6.000, p=0.0504).

There was no significant difference in the intensity of the secondary response between experimental groups (ANOVA $F_{2,37}$=0.18, p=0.840). Comparison of the primary and secondary response indicated no significant difference in *Non-breeding* (Paired samples T-Test: n=14, t=0.14, p=0.890) and *Protein diet* birds (Paired samples T-Test: n=8, t=0.21, p=0.838), but a significant increase in *Seed diet* birds (Paired samples T-Test: n=9, t=3.25, p=0.012).

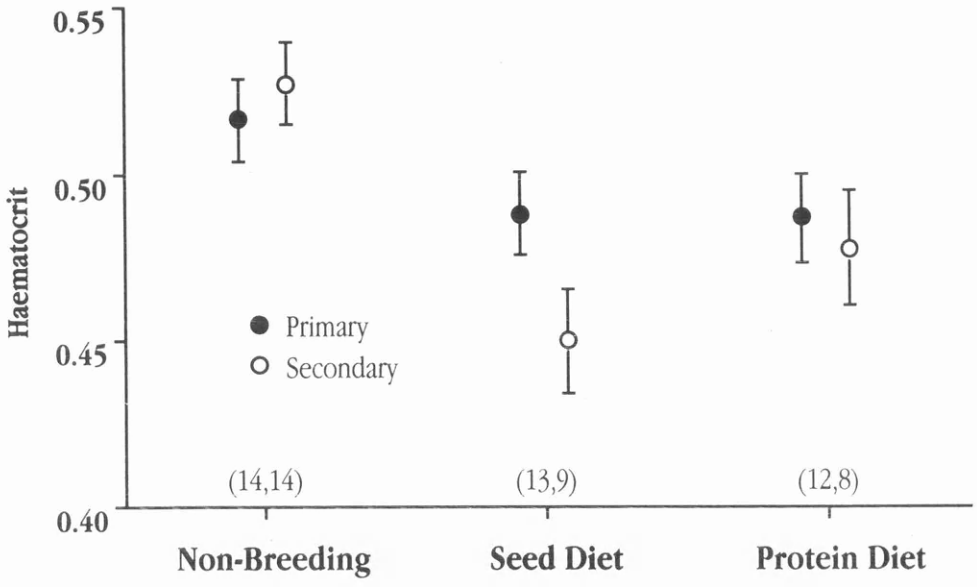


Fig.6.5 Haematocrit measurements of blood samples taken from all experimental birds post-primary and post-secondary response. Means are shown. Bars indicate standard error. (n) = sample size.

Haematocrit of blood samples taken after the primary immunisation was lower in both of the breeding groups than the *Non-breeding* group, however these differences were not significant (ANOVA $F_{2,36}$=1.81, p=0.178), (Fig.6.5).

Haematocrit of blood samples taken after the secondary immunisation differed significantly between groups (ANOVA $F_{2,28}$=7.88, p=0.002), with both *Seed diet* and *Protein diet* breeding birds significantly lower than the *Non-breeding* group, but no significant difference between *Seed diet* and *Protein diet* groups (Fisher's pairwise comparisons).

There was no significant difference in haematocrit between primary and secondary measurements within treatment groups, *Non-breeding* (T-Test: t=0.64, 25 df, p=0.53), *Seed diet* (T-Test: t=1.90, 16 df, p=0.076), *Protein diet* (T-Test: t=0.41, 14 df, p=0.68).

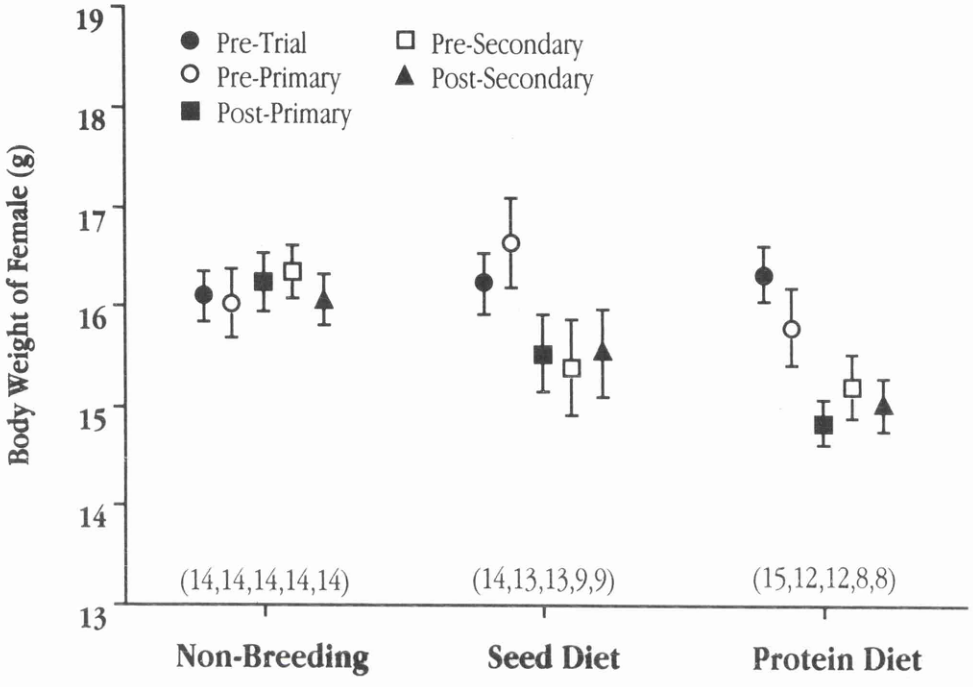


Fig.6.6 Variation in body weight of female zebra finches throughout the course of the trial. Means are shown. Bars indicate standard error. (n) = sample size.

Prior to the start of the feeding trial, all experimental groups were standardised for body weight (and previous breeding success) of the female (Fig.6.6). Body weight of *Non-breeding* birds remained fairly constant over the course of the trial.

Both breeding groups showed similar (generally non-significant) fluctuations in weight over the course of the trial. Body weight fell by about one gram during the primary response/clutch, and then remained fairly stable throughout the second clutch. See Tab.6.2 for statistical summary.

Comparison of body weight between experimental groups.		**Fisher's Pairwise Comparisons**
Pre-Trial	*ANOVA* $F_{2, 36}=0.09$, $p=0.918$	No significant differences
Pre-Primary	*ANOVA* $F_{2, 37}=0.89$, $p=0.421$	No significant differences
Post-Primary	*ANOVA* $F_{2, 33}=4.76$, $\mathbf{p=0.015}$	Non-breeding > Seed Diet, Protein Diet
Pre-Secondary	*ANOVA* $F_{2, 33}=5.26$, $\mathbf{p=0.010}$	Non-breeding > Protein Diet
Post-Secondary	*ANOVA* $F_{4, 57}=2.78$, $p=0.079$	No significant differences

Comparison of body weight between Clutch.1 and Clutch.2		**Fisher's Pairwise Comparisons**
Protein Diet	*ANOVA* $F_{4, 50}=4.42$, $\mathbf{p=0.004}$	Pre-Trial > Post-Prim., Pre-Sec., Post-Sec. Pre-Prim. > Post-Prim.
Seed Diet	*ANOVA* $F_{4, 53}=1.83$, $p=0.137$	No significant differences
Non-Breeding	*ANOVA* $F_{4, 64}=0.24$, $p=0.917$	No significant differences

Tab.6.2 Summary of statistical comparisons of body weight. Comparisons are between experimental groups, and between successive clutches.

6.3.3 *Consequences of trade-off for egg/chick quality*

Studies of the immune response of chicks hatching from the first eggs of the primary clutch were unfortunately not feasible. The first laid eggs of the 25 primary experimental clutches were removed from their nests on the morning of laying and transferred to the nests of incubating foster pairs. Only six of these eggs (four from seed diet parents, two from protein-supplemented parents) hatched successfully. Whether the poor hatchability of these eggs was a result of poor fostering or a direct result of poor egg quality due to the trade-off scenarios experienced by the parents, is not clear.

6.4 DISCUSSION

6.4.1 *Immunological consequences of dietary history and egg production*

Egg production has been shown to have a detrimental effect on antibody production in the female zebra finch. The primary immune response of breeding birds was significantly reduced in comparison with that of non-breeding birds maintained on a similar diet (Fig.6.4). Additionally, haematocrit of breeding birds was significantly reduced in comparison with that of non-breeding birds (Fig.6.5). These results would appear to indicate that egg production may limit nutrient availability for self-maintenance (i.e. immuno-competence and recovery of red blood cell count).

In the current trial, the dimensions and number of eggs laid by protein-supplemented birds were similar to those of birds receiving the seed only diet (Fig.6.3a, 6.3b). However, concurrent with egg production, protein-supplemented birds were able to maintain a level of immune responsiveness undiminished by comparison with that of non-breeding (seed-diet) birds (Fig.6.4). Additionally, the recovery of haematocrit by breeding, protein-supplemented birds appeared to be improved in comparison with that of breeding seed-diet birds (Fig.6.5). This might further indicate that a range of protein dependent metabolic processes are less limited during the period of egg laying in birds endowed with high quality endogenous reserves.

Secondary immunisation in combination with production of a replacement clutch did not produce detectably different levels of antibody activity between experimental groups (Fig.6.4). However, the shorter duration of the secondary response (Fig.6.1b) in comparison with the primary response (Fig.6.1a) may have contributed to increased variability in the degree to which blood samples were representative of peak antibody activity. Also, were the secondary immunisation delivered prior to the primary response declining sufficiently, a poorly defined antibody response might similarly be expected. This, in combination with (possibly) greater variation in the health status of the individuals still recovering from the primary immunisation, may have masked any differentiation between experimental groups. Further work, probably requiring larger numbers of birds, would be required to elucidate this.

6.4.2 *Comparison with previous studies*

The effects of protein supplementation of the diet on antibody response of brood-rearing zebra finches has been previously investigated by Deerenberg (1996): the primary immune response was determined from blood samples taken six and nine days after immunisation; a higher antibody intensity being observed after nine days than after six. The current trial (Fig.6.1a) indicated that the peak primary antibody response might not occur until ten days after immunisation, which may explain Deerenberg's observation of increased activity on the ninth day.

Deerenberg provided (an unspecified) protein supplement to an experimental group of finches, onwards of three days after hatching of the clutch, but did not record any significant increase in antibody responsiveness in these birds. A possible explanation for the difference in these results from those of the current study is that the protein demands of chick-rearing in finches are not as great as those of egg production. As such, it would appear that protein limitation is most prevalent during the period of egg formation, but lessens once the eggs have been laid. This might support previous observations of the great tit, *Parus major*, in which parasite prevalence was positively correlated with clutch size, but not brood size (at 14 days), suggesting that females endured higher costs during clutch production, than during the rearing of nestlings (Oppliger *et al.* 1997).

6.4.3 *Body weight dynamics in response to clutch production*

Body weight of both breeding groups dropped by approx. 1g over the course of the first clutch, then remained fairly constant for the remainder of the trial, a similar trend being previously observed in breeding birds maintained on a basic diet (*Chapter 3*). This may have resulted from a reduced investment of nutrients in the smaller (Fig.6.3b) replacement clutch. However, it may equally indicate an increased investment of endogenous reserves in the production of the first clutch in comparison with the second.

In the absence of immunological challenge, previous studies (*Chapter 3*, Selman and Houston 1996, Williams 1996) indicate zebra finches with a history of dietary protein supplementation are able to maintain or even increase their body weight in combination with laying a clutch of eggs. The ability to maintain body weight appears to be reduced when birds are faced with the additional challenge of mounting an immune response in combination with egg production (Fig.6.6). This may be indicative of an increased utilisation of endogenous reserves when subjected to multiple physiological demands for protein.

6.4.4 *Consequences of productivity trade-off*

To summarise, protein supplementation of the diet of zebra finches has previously been shown to promote egg productivity and weight maintenance during the laying cycle (*Chapter 3*, Selman and Houston 1996, Williams 1996). However, when laying females are concurrently faced with an immunological challenge, protein supplementation appears to enable maintenance of immunological response, while promotion of egg productivity and weight maintenance appear to be compromised.

Resource allocation and life-history strategy have been the source of much scientific investigation, (*review* Stearns 1976). This study suggests a trade-off between assurance of long-term self-survival, and short-term maximisation of breeding potential. In captive birds, the consequences of such a trade-off are fairly limited, with food freely available and zero predation risk. Hence, the promotion of egg production by protein supplementation of captive birds may be a consequence of a nutrient excess.

Wild birds, however, are subject to an elevated risk of natural mortality, arising from susceptibility to potentially debilitating infections (e.g. Oppliger *et al.* 1997), and an increased risk of predation (individuals of poorer health being prone to selection by predators). If this elevated risk of mortality results in an increased nutrient requirement for self-maintenance, one might expect a reduction in optimal clutch size, with any nutrient reserves gained from experience of a high quality diet less comprehensively allocated to increasing egg productivity. The results of this trial would suggest that when subjected to concurrent immunological challenge, high quality nutrient reserves may promote the immuno-competence of the individual, rather than increasing their short-term reproductive capacity. As such, the selection of unripe grain by zebra finches prior to breeding (Allen and Hume 1997, Morton and Davies 1983, Zann 1996) might potentially be regarded as much an immunological measure as a mechanism for increasing egg production.

6.4.5 *Future studies*

Protein restriction encourages a slowing of moult in white-crowned sparrows, *Zonotrichia leucophrys gambelii* (Murphy and King 1991b). If the reduction of immune responsiveness and delayed recovery of haematocrit, observed in breeding seed-diet birds in this study, is symptomatic of general protein limitation, it seems reasonable to assume that other protein demanding metabolic processes, e.g. moulting, may be adversely effected. In many species of birds, completion of breeding is followed by a period of atrophy of the flight muscle (often attributed to provisioning of protein for moult), prior to migration (for example Baggot 1975, Gaunt *et al.* 1990, Piersma 1988, Ward 1969), while in grey-backed camaroptera, *Camaroptera brevicaudata,* moult may be interrupted if suitable breeding conditions prevail (Fogden and Fogden 1979). While the imposition of slowed moult may not be life threatening in the case of the zebra finch, which moults continuously (Zann 1985), delay of moult may have more serious consequences for migratory species. King and Murphy (1984) speculated that plumage fault-bars in white-crowned sparrows may result from stress incurred during handling, rather than nutritional inadequacies. It would be interesting to see whether an interaction occurred between immunological status and moult, specifically whether fault-bars are accrued over periods of immunological challenge.

The poor hatchability of the eggs arising from immunised females is also a point of interest. Whether this resulted from poor fostering, or a direct compromise of egg quality arising from the nutrient trade-off scenarios experienced by the parents is not clear. However, the repercussions of parent / offspring nutrition for life history strategy would represent an interesting field for further investigation.

6.5 APPENDIX

6.5.1 Details of methodology

Preparation of SRBCs for immunisation

5ml SRBCs decanted from mixed 50% V/V suspension in Alsevers solution,
Supplier : *Scottish Antibody Production Unit, Carluke. (SAPU).*
Spin at 1000g for 5 min to produce 1ml packed volume.
Remove supernatant.

Make up to 10ml with filter sterile phosphate buffered saline (PBS). Resuspend cells gently.
Spin at 1000g for 5 min. Remove supernatant. Repeat.

Make up to 10ml with PBS.
0.1ml of suspension should contain approximately $5x10^7$ SRBCs

Immunisation and blood sampling

Based on methodology of Deerenberg (1996)

Resuspending cells immediately prior to injection.
Inject bird, ventral to the stomach, with 0.1ml of suspension (contains approx. $5x10^7$ SRBCs),
At the designated time after immunisation,
60µl of blood is collected in a heparinized capillary tube by puncture of the brachial vein.
Tubes are sealed at one end with Cristaseal, and spun for 5 min in a haematocrit centrifuge.
Haematocrit measurements taken (if required).
Capillary tube is scored (using a diamond pen) on the serum side of the cell/serum interface, and snapped.
Required volume of serum may be drawn directly from capillary tube, using a Gilson pipette.

Haemagglutination assay for anti-SRBC antibodies

Based on methodology of Hudson and Hay (1989)

A 96 well (8x12) round-bottomed well haemagglutination plate is prepared for immunological assay.
25µl of PBS is added to all test wells.
25µl test serum is added to the first well of each row, and mixed.
25 µl of solution removed and transferred to next well.
This process is repeated to obtain a serial dilution along the row.
25 µl PBS is added to each test well (to bulk out sample and facilitate easy reading).
25µl of a 2% vol/vol suspension of SRBCs is added to each well
(approx. 0.25ml packed SRBCs in 10ml - this is a fourfold dilution of the innoculum).
Cover tray with a strip of sealing film.
Mix contents of wells by gently moving plate in a circular motion, whilst holding it flat on the bench.
Leave undisturbed, at room temperature.

Plates were left to develop overnight, after which antibody titres were recorded.
Agglutinated cells remain as a sheet.
Non-agglutinated cells settle as a button in the centre of the wells.
Test wells in which any visible haemagglutination had occurred were recorded as a positive result.

Chapter 7

BILL COLOUR AS AN INDICATOR OF NUTRITIONAL PLANE AND FITNESS: COMPUTER ANALYSIS OF BILL COLOUR IN FEMALE ZEBRA FINCHES

"... minuteness, neatness, wonderful colours and exquisite patterns..."

Richard Zann (1996)

SUMMARY

The potential of bill colour as an indicator of physiological state in female zebra finches was investigated. Carotenoids are suggested to represent a possible link between pigmentation and physiological state. Female finches of different dietary histories were presented with a physiological "challenge" (egg and antibody production in combination with blood sampling), followed by a period of "recovery" (cessation of laying, immunisation and blood sampling in combination with maintenance on a protein supplemented diet). Computer analysis was used to obtain an objective measurement of bill colour over the course of the trial. Bill coloration did not differ significantly between birds maintained on different diets - indicating that bill colour per se might not be an indicator of nutritional plane. However, throughout the trial, physiological challenges provoked a reduction in bill yellowness and brightness, combined with an increase in redness - traits regarded as "unfavourable" in females. Physiological recovery encouraged a reversal of these trends. Deviation from original bill coloration was least, and hence most preferable, in birds previously experiencing a dietary protein supplement. The use of computer software packages in colour assessment, and the role of bill colour as an honest indicator of condition, are discussed.

7.1 INTRODUCTION

7.1.1 *The importance of colour in birds*

The diversity of colour in the bird world is matched by the diversity of the ecological and physiological roles of pigment. Zebra finches are sexually dichromatic, and visual displays during courtship are structured to maximise exhibition of areas of sexually significant pigmentation (Morris 1954), bill wiping and fencing forming an important part of these.

7.1.2 *Bill colour preferences: arbitrary choice, or health evaluation?*

While some intersexual overlap of bill colour is evident between the sexes in zebra finches (Burley and Coppersmith 1987), bills of males are generally darker and redder: females lighter and yellower (Burley *et al.* 1992). A directional preference in mate choice has been demonstrated in females, for males with redder bills, extending to bills reddened beyond the normal phenotypic range of the birds (Burley and Coppersmith 1987). However, Collins *et al.* (1994) reports that female preference was primarily not for male bill colour, but for high song rates, which is a heritable characteristic (Houtman 1990, from Collins *et al.* 1994) that is strongly correlated with bill colour. Males exhibit a stabilising preference for female bill pigmentation central to the phenotypic range, i.e. midway between the naturally occurring extremes of red and yellow (Burley and Coppersmith 1987).

The role of bill colour as an indicator of fitness has been the subject of some debate. Bill colour has been ascribed a high degree of heritability in zebra finches (Price 1996). A tendency towards genetic rather than environmental control might reduce potential for honest indication of selective advantage. Conversely, previous work of Houtman (1990, from Collins *et al.* 1994), indicated that bill colour was not heritable and was positively correlated with the number and weight of offspring. However, Burley *et al.* (1991) recorded an increase in ectoparasite loads in red-billed males, in direct contradiction to predictions of fitness.

Burley and Coppersmith (1987) initially interpreted bill colour preference to be independent of condition. However, later studies noted an association between rapid change in bill colour and subsequent death in females (Burley *et al.* 1992). Bill colour was also noted to be affected by egg production, overcrowding and breeding season (Burley *et al.* 1992). Additionally, oral pigmentation in nestling barn swallows, *Hirundo rustica*, has been shown to decrease in response to immunological challenge (A.Møller *pers. comm.*).

Hence, it would appear that bill colour preferences displayed by zebra finches might arise as a result of bill pigmentation being indicative of physiological well-being, rather than arbitrary favouritism. Male finches have been shown to select females

who's egg producing potential has been elevated by previous experience of a high protein diet (Monaghan *et al.* 1996). Could the males be responding to aspects of female bill colour indicative of physiological condition?

To prove this to be so, it would be necessary to establish a causal metabolic link between bill pigmentation and physiological state. Physiological state may influence bill colour indirectly e.g. the protein demands of egg production / immunological response may act to limit the metabolic processes responsible for turnover and transport of pigment. This may be supported by the observations of Burley *et al.* (1992), in which colour loss in wild-caught birds was alleviated by supplementation of the diet with protein (though similar supplementation did not effect bill colour dynamics of laboratory bred birds). Alternatively, it is possible that bill coloration may be directly linked to health state, as a result of the physiological roles of carotenoid pigments.

7.1.3 *Carotenoids and their physiological roles*

Carotenoids, deposited in fatty droplets of the feather rudiment, are transferred to the keratin of growing feathers during cornification (Desselberger 1930, from Fox and Vevers 1960), and are responsible for the plumage coloration of many species of birds (see Fox and Vevers 1960). Carotenoids are amongst the most widespread of animal pigments (Fox and Vevers 1960), and although the pigments responsible for coloration of bill keratin in zebra finches have not yet been isolated, carotenoids are likely components. For example, dietary supplementation with xanthophylls (see below) has been shown to increase beak pigmentation in chickens (Pinchasov *et al.* 1992).

Carotenoids are polyunsaturated, bicyclic hydrocarbons, the structure of which enables the molecule to selectively absorb photons between the wavelengths of 320-550nm (Latscha 1990) - the reflectance of light outside this range giving rise to their characteristic yellow-red colour. Carotenoids are, for example, responsible for the yellow colour of hens eggs (mostly lutein), the red gape of swallow nestlings (lutein), (A.Møller *pers. comm.*), and the redness of pheasant wattles (astaxanthin) (Goodwin 1952). Carotenoids are also responsible for coloration outside of yellows and reds, e.g. thin layers of carotenoids can overlay blue structural components to produce green coloration (Fox and Vevers 1960).

Carotenoids are synthesised by fungi, algae, some micro-organisms and higher plants. Though unable to affect their *de novo* synthesis, birds are able to accumulate and modify dietary carotenoids, and more extensibly their oxidised counterparts, xanthophylls. Carotenoids and xanthophylls will be collectively termed "carotenoids" for the purposes of this study. Goodwin (1952) suggested that 15-25% of dietary intake of carotenoids may be deposited in the body, and in the case of the granivorous finch, seeds represent a rich source of carotenoid pigments (Fox 1976).

Incidentally, carnivores rely wholly upon the accumulated carotenoids of herbivorous prey to satisfy their requirements (Fox and Vevers 1960).

Modification of carotenoids first occurs during absorbance across the intestinal mucosa (Goodwin 1952). Carotenoids are transported in free form or in association with plasma-lipo-proteins for storage/modification in the liver, integument or fat (Brush and Power 1976). Translocation of endogenous stores provides yolk pigmentation during egg formation (Goodwin 1952), and plumage production (Brush and Power 1976).

In plants, carotenoids are fundamentally associated with photosynthetic apparatus, (Latscha 1990), their role being the neutralisation of triple oxygen and free radicals arising from photosynthesis. Similarly, once incorporated into the animal body, carotenoids act to ameliorate the auto-oxidative effects of free radicals arising from neutrophil combat of phagocytized bacteria (Bendich 1989). The role of carotenoids as precursors of vitamin A is well known, however less than 10% of the 600+ naturally occurring carotenoids can be utilised for this purpose (Olson 1989).

The physiological plasticity of carotenoids and their degradation products, is evident in the variety of functions with which they are associated. These include protection from irradiation, light perception, growth, immune response, wound healing and allosteric effectors in the modification and stabilisation of protein (Latscha 1990).

Well-provisioned endogenous stores of carotenoids may thus be regarded as an endowment of fitness upon an individual, as a result of their potential application to disease resistance and repair of damaged tissue. However, it should be stressed that carotenoids are responsible for a whole range of colours. Brighter pigmentation does not necessarily indicate the presence of more carotenoid, and may equally arise from different carotenoids, or a differential ability in the birds to display what carotenoids they have. Degree of coloration has previously been suggested to be limited by dietary availability (Hill 1994), however Hudon (1994) argues that the ability of birds to express coloration is moderated by their physiological status (specifically body condition and restriction of mobility in captivity). These hypotheses, which are not necessarily exclusive, were respectively termed the "foraging hypothesis" and the "health hypothesis" (Linville and Breitwisch 1997).

7.1.4 *Bill colour as an indicator of fitness*

An ability to invest resources in ornamentation, in addition to sustainment of basal requirements may be regarded as an indicator of fitness (e.g. Møller *et al.* 1996). With reference to the physiological value of carotenoids, an ability to sustain carotenoid levels sufficient for phenotypic embellishment may be regarded as an indicator of good health. For example, A.Møller (*pers. comm.*) suggested that barn-swallow nestlings advertise their fitness with red gapes (derived from dietary lutein), and attract increased attention from parents. If highly pigmented bills indicated good health in the case of zebra finches, one would expect directional preference for strongly pigmented birds of both sexes. Though evident in the

selection of males by females, this has been demonstrated to not be the case in selection of females by males: males displaying a preference for females with bill colours central to the natural range rather than those with extremely coloured bills (Burley and Coppersmith 1987, Price 1996).

In zebra finches, there is apparently a fundamental difference between the sexes in the perceived value of bill coloration. Allocation of carotenoids to bill (and plumage) coloration by females may be superseded by the requirement for self-maintenance and provisioning of endogenous stores for egg production. This being so, it is possible that bill colour in females is less indicative of the quality of the individual, though the possession of endogenous reserves of carotenoids sufficient to influence bill coloration might still be expected to indicative of good health.

7.1.5 *Aims*

The aim of this investigation was to determine the relationship between bill pigmentation and physiological condition of female zebra finches. A computer package was used to obtain objective measurements of bill. However, due to the equivocal nature of the relationship between bill colour and physiological state, no assumptions were made as to what aspects of bill colour were indicative of selective preference, internal health or breeding potential in the birds.

If bill colour represented an accurate indicator of physiological state, one might predict that:

- birds of differing nutritional plane should exhibit different bill colours.
- bill colour should tend towards less preferential qualities when an individual was subjected to physiological challenge, and more preferential qualities during periods of physiological recovery.
- the immuno-competence and / or egg producing ability of a female bird should correlate with some factor of bill pigmentation.

The first of these predictions was tested by comparison of bill colour of birds maintained on seed diets with those maintained on protein supplemented diets. Protein supplementation of the diet of zebra finches prior to breeding has been shown to promote increased immuno-competence (*Chapter 6*) and the laying of larger eggs and clutches (*Chapter 3*, Selman and Houston 1996, Williams 1996), while minimising weight loss incurred by the laying female (*Chapter 3*, Selman and Houston 1996, Williams 1996). A capacity to store protein endogenously enables promotion of egg production for some time after the cessation of dietary supplementation (Selman and Houston 1996, Williams 1996).

As such, augmentation of endogenous reserves by elevation of nutritional plane may be regarded as an endowment of increased "fitness". Therefore, if bill colour is an accurate indicator of physiological state, elevation of nutritional plane should be

concurrent with a related modification in bill pigmentation. Also, if preferences exhibited by males are linked to the fitness of the female, one might expect females with a dietary history of protein supplementation to exhibit more preferential bill colours than those sustained on a seed only diet.

The second of these predictions was tested by monitoring bill colour in four experimental groups of birds which differed in their pre-breeding dietary experience. These groups were subjected to successive periods of physiological *challenge** and *recovery***.

* *Challenge.* In the case of this study, a combination of egg production, and presentation of an immunological challenge were assumed to represent a challenge to physiological well-being, potentially depleting endogenous reserves of protein and carotenoids. Blood sampling (with associated stress arising from handling) represented an additional potential compromise to health. It was predicted that, under such conditions, bill pigmentation should tend towards "less healthy" / "less favourable" characteristics.

** *Recovery.* Removal of these challenges, combined with presentation of a protein rich dietary supplement was regarded as conducive to physiological "recovery", representing an opportunity for the replenishment of endogenous reserves. It was predicted that, under such conditions, bill pigmentation should tend towards more "healthy" / "favourable" characteristics.

Experimental groups were as follows:

- ***Control*:**
  Maintained on a seed diet under constant conditions.

- ***Seed-Diet Non-breeding:***
  Maintained on a seed diet. Exposed to repeated immunological challenge, followed by recovery period.

- ***Seed-Diet Breeding:***
  Maintained on a seed diet. Exposed to repeated immunological and egg productivity challenge, followed by recovery period.

- ***Protein-Diet Breeding:***
  Maintained on a protein-supplemented seed diet prior to pairing, after which, maintained on a seed diet. Exposed to repeated immunological and egg productivity challenge, followed by recovery period.

Finally, having determined which characteristics of bill pigmentation might be associated with physiological well-being, egg and antibody productivity of individuals were compared with bill colour, to investigate whether bill colour represented an accurate indicator of a females egg laying / antibody producing capacity.

NOTE: Due to practical limitations on space during this experiment, control birds were kept in a separate room under similar environmental conditions. Unfortunately, during the course of the experiment, a Megabacterium infection became established in these birds, and several of them died. As such, data from this group could not be used in this analysis.

7.2 METHOD

7.2.1 *Experimental conditions*

Bill colour of female finches was monitored while they were subject to the immunological protocol outlined in *Chapter 6*. Basic experimental conditions are repeated here, for clarity. See 6.2 for full details of immunological procedures.

Birds were kept in cages measuring 60cm x 50cm x 40cm, under a lighting regime of 8 hour dark to 16 hour light using timer controlled "daylight" UV lighting (*Lightbox*). All birds received mixed seed, cuttlebone, water and oyster grit *ad libitum*. Ambient temperature was maintained at 20.5°C (+/-2).

Three groups of fifteen established pairs of finches (45 pairs in total) were selected. Groups were standardised for body weight of the females and previous breeding performance (1.1.4). For three months prior to the trial, males and females of all pairs were separated by cage partitions. These allowed visual and vocal contact between partners, but prevented physical contact. All males were maintained on a seed diet with high protein supplement (boiled hens egg in *Haithes chick rearing mix*).

Females received specific diets *ad libitum*, as follows:

Seed-Diet Non-Breeding Group	Mixed seed diet / white rusk supplement.
Seed-Diet Breeding Group	Mixed seed diet / white rusk supplement.
Protein-Diet Breeding Group	Mixed seed diet / protein supplement based on boiled hens egg white, (2.3.1).

7.2.2 *Presentation of immunological challenge*

After acquisition of an appropriate Home Office licence, (Licence No.2101 Procedure No.19(b)1), female zebra finches were immunised intraperitoneally with a suspension of $5x10^7$ sheep red blood cells (SRBCs) in 100µl of phosphate buffered saline. Immunisation with SRBCs is a standard procedure for the presentation of a novel, non-pathogenic immunological challenge, eliciting a generic antibody response in birds (see, for example Deerenberg 1996, Glick *et al.* 1981, Lochmiller *et al.* 1993, Sorvari and Sorvari 1997). The intensity of the antibody response may be determined by a haemagglutination assay on a small (20 µl) sample of blood serum taken subsequent to immunisation. Full details of this procedure are given in Appendix 6.5.

7.2.3 *Experimental protocol*

Day.1 Females were weighed (+/- 0.01g) and bills photographed. Any dietary supplements were removed leaving mixed seed as the only food source. Cages were

cleaned and provisioned with straw-lined nest boxes and fresh grass. Females were immunised intraperitoneally with SRBCs and then re-paired with their partners.

Nest boxes were monitored daily. Newly laid eggs were removed, weighed (+/- 0.001g) and length/width measured (+/- 0.01mm), and replaced with replicas.

Day.10 A 50µl blood sample was taken from the females by venipunture of the wing vein. This was analysed for anti-SRBC antibodies.

After completion of the clutch, protein supplement was returned to the diet of the *Protein-Diet Breeding* group up until Day.20, with the aim of replenishing endogenous stores depleted by the first challenge. *Seed-Diet Breeding* and *Seed-Diet Non-breeding* birds were maintained on a mixed seed diet.

Day.20 Females were photographed and immunised with SRBCs for a second time. Plaster eggs were removed and fresh nesting material supplied to encourage laying of a second clutch.

Nest boxes were again monitored daily. Newly laid eggs were removed, weighed and measured, and replaced with replicas.

Day.27 Prior to this point in the trial, the experimental protocol was regarded as representing a challenge to the physiological well-being of the female.

A second blood sample and third photograph were taken. Females and males were then separated and the diets of all birds were supplemented with a commercial high protein mix (*Haithes chick rearing mix*). The cessation of blood sampling, and egg and antibody production, in combination with the availability of a high protein dietary supplement were regarded as being beneficial to the physiological well-being of the female, in comparison with the previous protocol.

Day.57 After one month on the supplemented diet, all breeding females were photographed for the last time.

	Challenge.1	**Challenge.2**	**Recovery**
Seed-Diet Non-Breeding	Primary response Blood sample	Secondary response Blood sample	All groups provided with a high quality diet (males removed).
Seed-Diet Breeding	Primary response Blood sample First clutch	Secondary response Blood sample Replacement clutch	
Protein-Diet Breeding	Primary response Blood sample First clutch	Secondary response Blood sample Replacement clutch	

Tab.7.1 Summary of challenge / recovery scenarios imposed on female finches.

7.2.4 *Photographic set-up*

Preliminary trials indicated that a number of steps needed to be taken in order to standardise photographic conditions as much as possible (7.3.1). Birds were photographed against a black background, using a Canon T90 body equipped with a close-focus lens, in combination with two synchronous flashes. Due to the small depth of field of the lens, birds were hand-held in close alignment with a standard colour scale, approximately 40cm from the camera. Positions of camera, flashes and colour scale were maintained by their direct attachment to a T-shaped wooden frame (Fig.7.1). Photographs were taken using Kodak Elite II slide film and processed commercially.

7.2.5 *Computer software package colour analysis*

Mounted slides were scanned into the Power Mac image editing package Adobe Photoshop 3.0, using a Nikon LS1000 35mm Film Scanner. Scanner colour defaults were re-set prior to scanning each slide to prevent settings from "wandering". Once previewed, the area incorporating the bill and standard colour scale was highlighted and scanned. The resulting image was stored using an Iomega 100 zip drive.

The bill area was selected for analysis using the *wand* tool. This selects areas of connected, colour related pixels. A *tolerance* level of "30" for the wand was found to be the most appropriate for this purpose. The selected area was extended until the whole bill was incorporated, care being taken to exclude any areas that showed glare as a result of reflection of the flash. The wand was similarly used to select areas on the standard colour scale (7.3.1).

Colour profiles of bill and colour scale were graphically represented using the *Image-Histogram* option. The mean value of the distribution was recorded in each case. Analysis was carried out in two different colour modes: Red, Green, Blue (RGB) and L*a*b Color (LAB).

Though RGB is perhaps the more familiar spectrum, LAB mode was adopted for the purposes of this study. LAB colour has the advantage of greater reproducibility between devices, and was simpler to interpret in the case of this trial.

LAB mode measures three separate components of colour:

- ***Lightness*** is a measure of picture brightness and is independent of hue. Low values indicate a dark bill; high values, a light bill.

- **Channel *a*** is a measure of colour on a scale from green to red. High values indicate red pigmentation, and for the purposes of this study, it is regarded as a measure of "***Redness***"

- **Channel *b*** is a measure of colour on a scale from blue to yellow. High values indicate yellow pigmentation, and for the purposes of this study, it is regarded as a measure of "***Yellowness***"

Examples of LAB bill colour *Image-Histograms* are shown in Fig.7.2. Typical *Lightness, Channel a* and *Channel b* scores are indicated for a red-billed and a yellow-billed female zebra finch.

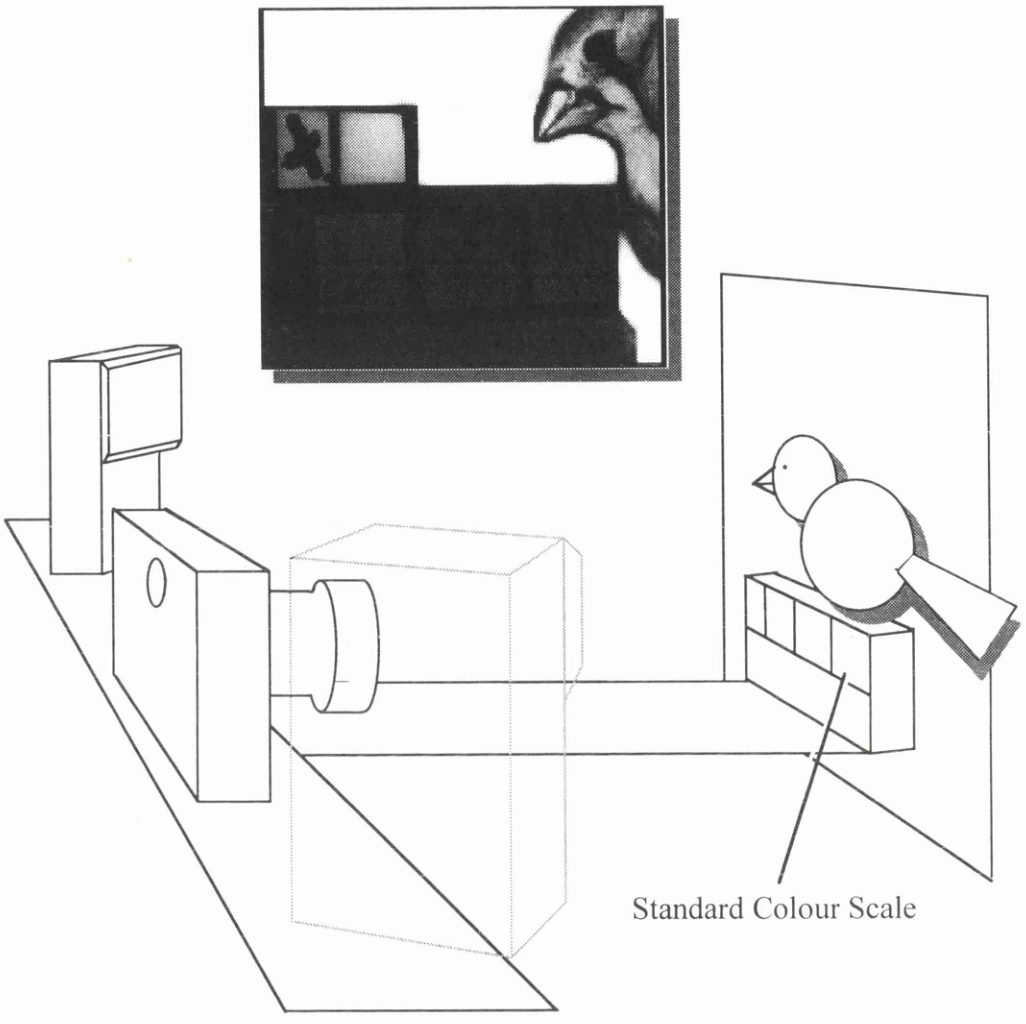


Fig.7.1 Set-up of equipment for photographing finches. Inset is an example of a photograph taken using this set-up, with the black background removed, for clarity.

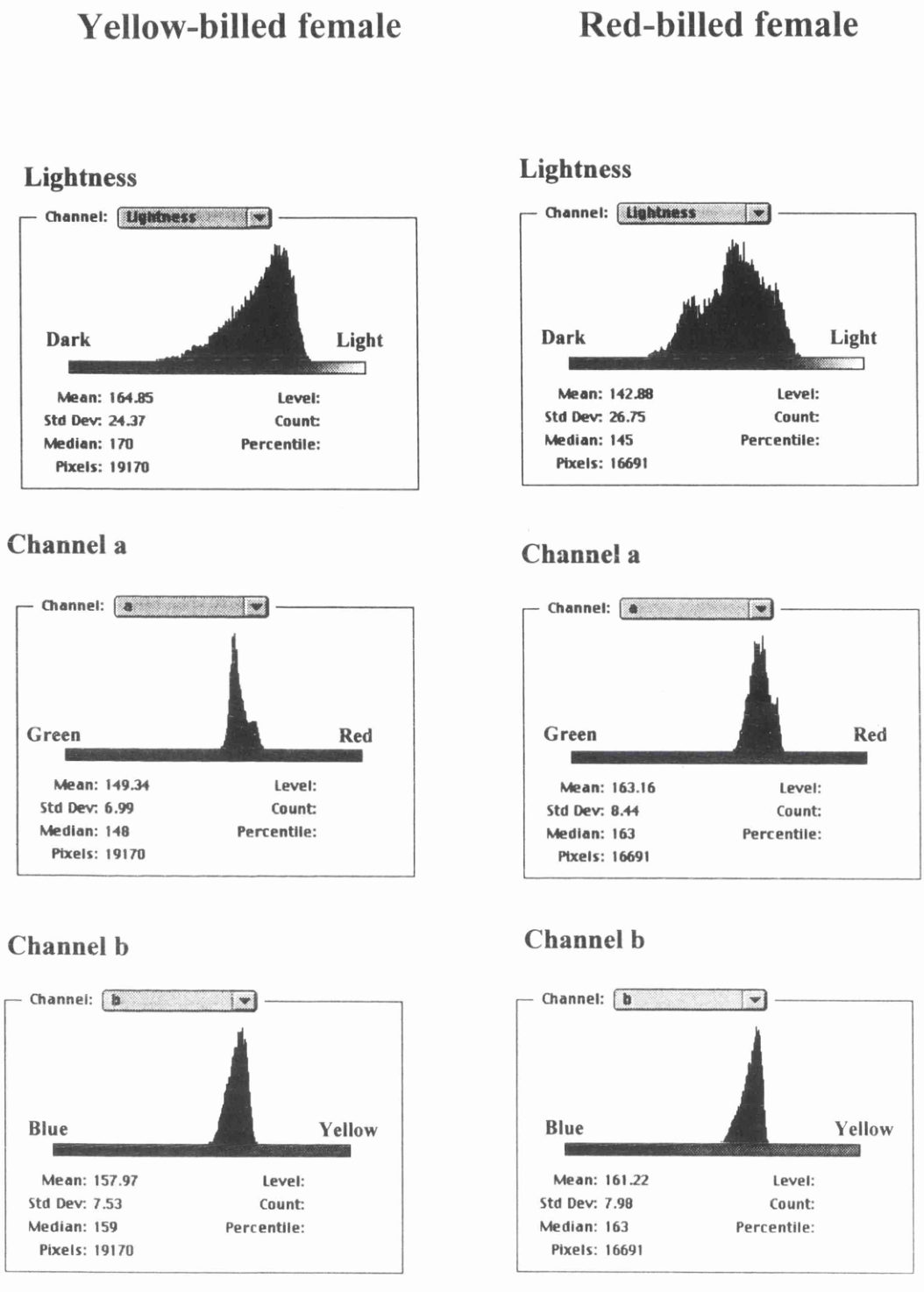


Fig.7.2 Results of computer analysis of bill colour. Representative colour histograms are shown for a yellow-billed and a red-billed female zebra finch. These birds are towards opposite extremes of the natural range of phenotypic pigmentation.

7.3 RESULTS

7.3.1 *Preliminary trial*

Preliminary trials showed great variation in the reproducibility of colour scores for the standard colour scale between different films, and to a lesser extent even between slides on the same film. This indicated the necessity of standardising all bill colour measurements, to account for background variation in colour between slides/films.

Therefore, a range of colour standards were incorporated into each photograph (Fig.7.1). A bright red standard resembling the natural colour of the bill was shown to match the background variation between the slides most closely. (A yellow standard was also tested, but this did not match the background variation of bill colour as closely, possibly due to a greater susceptibility to flash reflectance). As a result, all colour component scores of subsequent slides were standardised against that of the red standard of the first slide to remove deviations in colour characteristics arising from photographic/development variation, using the following equation:

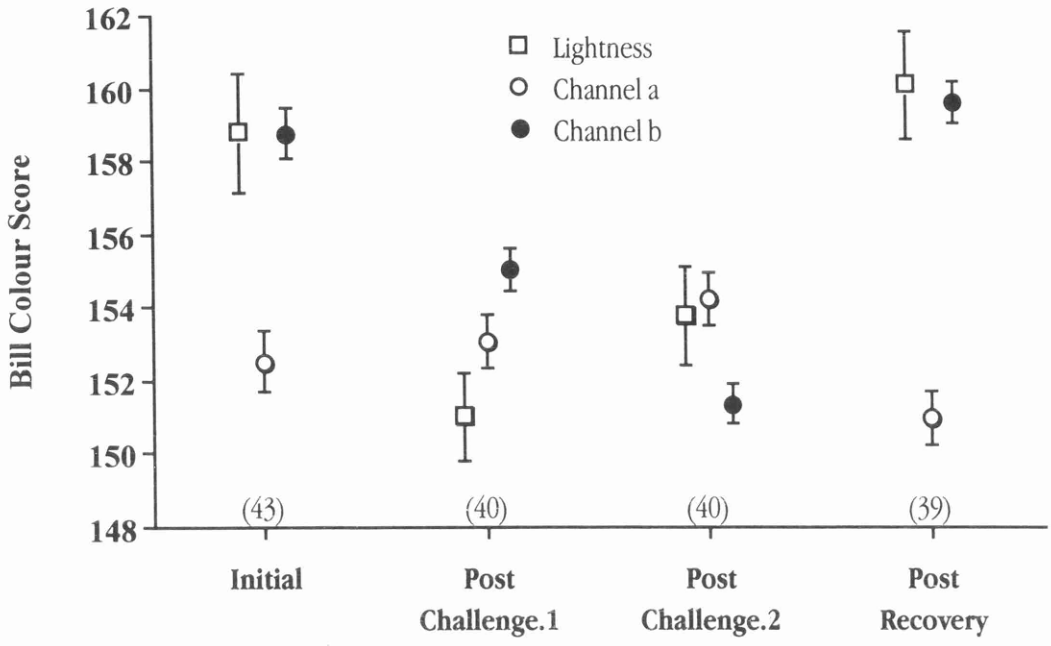
$$\text{Standard colour score}_n = (\text{Red standard}_1 - \text{Red standard}_n) + \text{Mean colour score}_n$$

Colour score = *Lightness, Channel a (Redness), Channel b (Yellowness).*
n = slide number

7.3.2 *Response of bill colour to challenge and recovery*

As detailed above, analysis of bill colour using this computer package produces three individual sets of data - *Lightness, Channel a,* and *Channel b.* Though of interest individually, it was decided to additionally attempt to combine these factors to produce a single composite factor representing overall bill colour. The basis for this was to produce a composite bill colour factor that reflected physiological condition of the bird. i.e. a high composite score would indicate a bird in good condition, a low score would indicate a bird in poor condition. For this reason, *Lightness, Channel a,* and *Channel b* were monitored over successive *challenges* and *recovery* to determine how each individual colour factor was effected by prevailing physiological conditions.

Bill colour at the start of the trial (Day.1) was compared with that after the first challenge (Day.20) and the second challenge (Day.27), and after a one month recovery period on a protein supplemented diet (Day.57). Combining results from all dietary groups, marked trends in colour component scores were observed over the course of the trial (Fig.7.3).



Bill Colour Score		Fisher's Pairwise Comparisons
Lightness	*ANOVA* $F_{3,158} = 8.69$, $p = 0.000$	*Initial > Post Challenge 1* *Post Challenge 1 < Post Recovery* *Post Challenge 2 < Post Recovery*
Channel a (Redness)	*ANOVA* $F_{3,158} = 3.06$, $p = 0.030$	*Post Challenge 2 > Post Recovery*
Channel b (Yellowness)	*ANOVA* $F_{3,158} = 37.77$, $p = 0.000$	*Initial > Post Challenge 1* *Initial > Post Challenge 2* *Post Challenge 1 > Post Challenge 2* *Post Challenge 1 < Post Recovery* *Post Challenge 2 < Post Recovery*

Fig.7.3 Response of bill colour (*Lightness, Channel a, Channel b)* to physiological challenges and recovery. These data represent a combination of all three experimental groups. Mean colour scores and standard errors are shown. (n) = sample size.

LAB Colour Score	Challenge Response	Recovery Response
Lightness	Decrease (sig.)	Increase (sig.)
Channel a (Redness)	Increase (non-sig.)	Decrease (sig.)
Channel b (Yellowness)	Decrease (sig.)	Increase (sig.)

Tab.7.2 Summary of general trends in colour component scores.

The general trends of the colour component scores are summarised in Tab.7.2. It may be seen that physiological *Challenge* is associated with increasing *Redness* scores and decreasing *Lightness* and *Yellowness* scores. During physiological *Recovery* these trends are reversed.

On the basis of these trends, component colour scores were combined to produce two composite colour factors, high scores of which were correlated with physiological recovery: low values with physiological challenge.

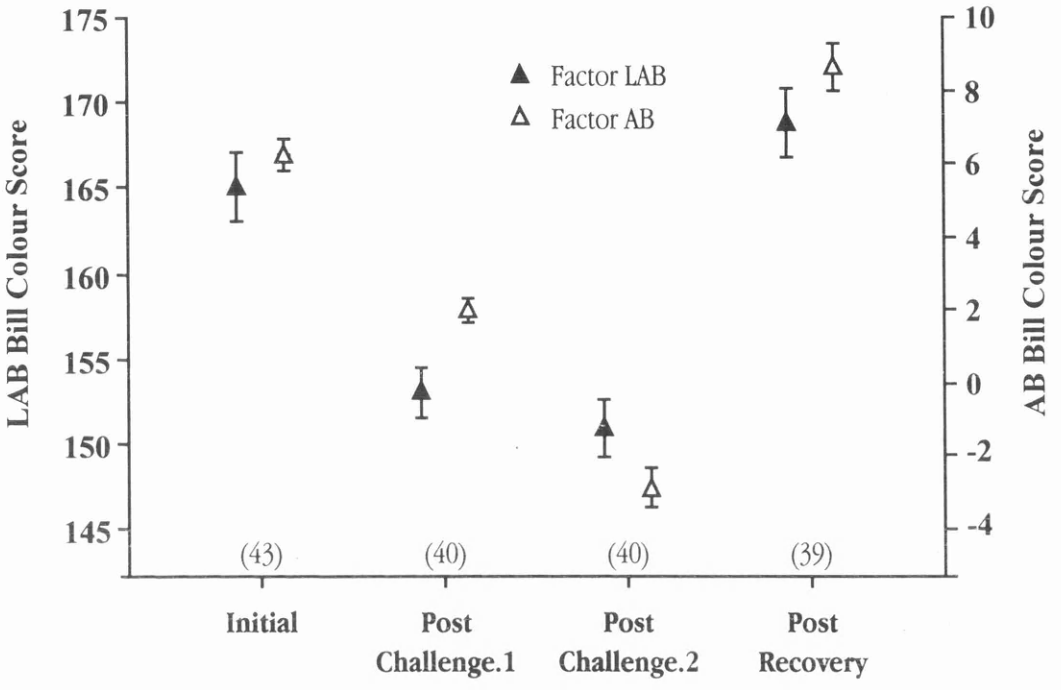
Factor AB = (*Channel b - Channel a*).

Factor AB represents a comparison of the degree of yellow pigmentation of the bill, with the degree of red pigmentation of the bill. A high value correlates with physiological recovery.

Factor LAB = (*Channel b - Channel a*) + *Lightness*

Factor LAB is an integral comparison of all three colour components. It represents a comparison of light/yellow pigmentation with dark/red pigmentation. A high value correlates with physiological recovery.

Trends in *Factor AB* and *Factor LAB* arising from combination of colour components (Fig.7.3) are shown in Fig.7.4. It should be noted that, due to the differences in scale of the two sets of data, Factor LAB is related to the left-hand scale, Factor AB to the right-hand scale.



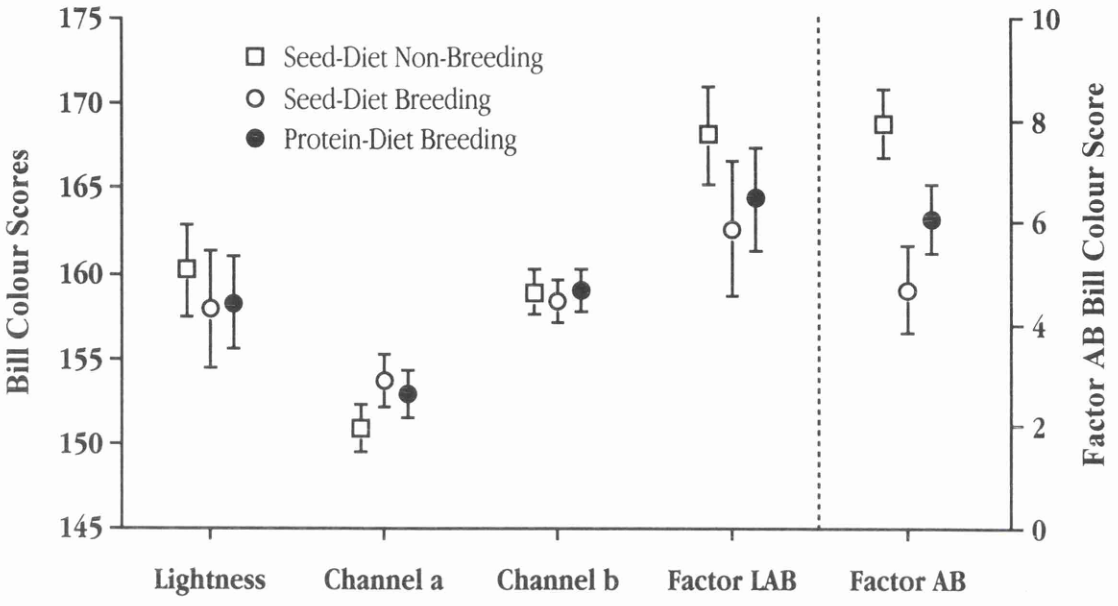
Bill Colour Score		Fisher's Pairwise Comparisons
Factor AB	*ANOVA* $F_{3,158} = 98.73$, $p = 0.000$	*All data sets significantly different from each other*
Factor LAB	*ANOVA* $F_{3,158} = 24.64$, $p = 0.000$	*Initial > Post Challenge 1* *Initial > Post Challenge 2* *Post Challenge 1 < Post Recovery* *Post Challenge 2 < Post Recovery*

Fig.7.4 Response of bill colour (*Factor AB, Factor LAB*) to physiological challenges and recovery. High values correlate with physiological recovery. These data represent a combination of all three experimental groups. Mean colour scores and standard errors are shown. (n) = sample size.

7.3.3 *Effect of nutritional plane and egg production on bill colour dynamics*

Having determined that measurable changes in bill colour occurred over the course of the experiment (7.3.1), data were divided according to experimental group, to investigate the impact of nutritional plane on initial bill colour.

On Day.1 of the trial, finches were photographed prior to pairing and/or immunisation (Fig.7.5). Despite maintenance on different experimental diets for the three months previous to this (7.2.1), analysis indicated no significant difference in any of the colour components between the groups maintained on the seed diet and the protein supplemented group. The only exception to this was *Factor AB* (ANOVA $F_{2,40}=4.82$, $p=0.013$). *Factor AB* was significantly elevated in the *Seed-Diet Non-Breeding* group in comparison with the *Seed-Diet Breeding* group (Fisher's pairwise comparisons). As, up until this point, both these groups had been maintained under identical experimental and dietary conditions, this difference can only reflect a *de novo* contrariety between groups in this aspect of bill colour. Protein supplementation had no apparent effect on initial bill colour.



Bill Colour Score		Fisher's Pairwise Comparisons
Lightness	*ANOVA* $F_{2,40} = 0.17, p = 0.843$	*No significant difference between experimental groups*
Channel a (Redness)	*ANOVA* $F_{2,40} = 0.85, p = 0.435$	*No significant difference between experimental groups*
Channel b (Yellowness)	*ANOVA* $F_{2,40} = 0.08, p = 0.926$	*No significant difference between experimental groups*
Factor LAB	*ANOVA* $F_{2,40} = 0.71, p = 0.500$	*No significant difference between experimental groups*
Factor AB	*ANOVA* $F_{2,40} = 4.82, \mathbf{p = 0.013}$	*Seed-Diet Non-Breeding Group > Seed-Diet Breeding Group*

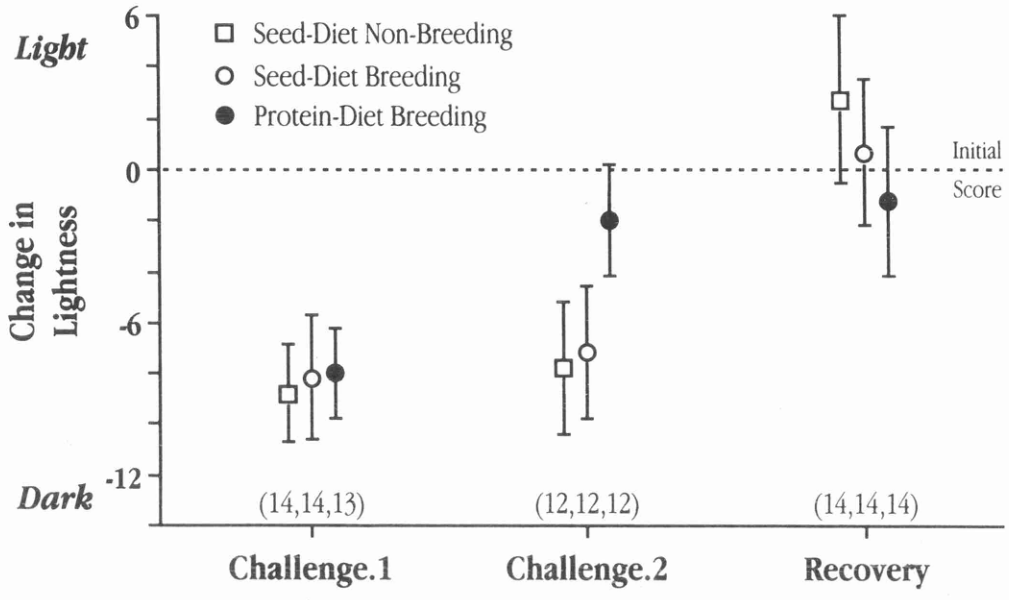
Fig.7.5 Analysis of *Initial* bill colour (Day.1), prior to pairing and / or immunisation. Mean colour scores for individual colour components and combined factors are shown. Right hand axis refers to *Factor AB* only. Standard errors are shown. Sample sizes are as follows, *Seed-Diet Non-Breeding* (n=14), *Seed-Diet Breeding* (n=14), *Protein-Diet Breeding* (n=15).

7.3.4 *Effect of physiological challenge and recovery on change in bill colour*

Having determined that physiological challenges and recovery produced measurable changes in bill pigmentation (7.3.1), comparisons were made between experimental groups, to see whether the degree of change in bill colour was affected by the dietary history and / or breeding status of the birds.

Changes in individual bill colour characteristics were determined by subtracting colour scores *Post Challenge.1, Post Challenge.2* and *Post Recovery* from the initial colour scores (Day.1 of the trial).

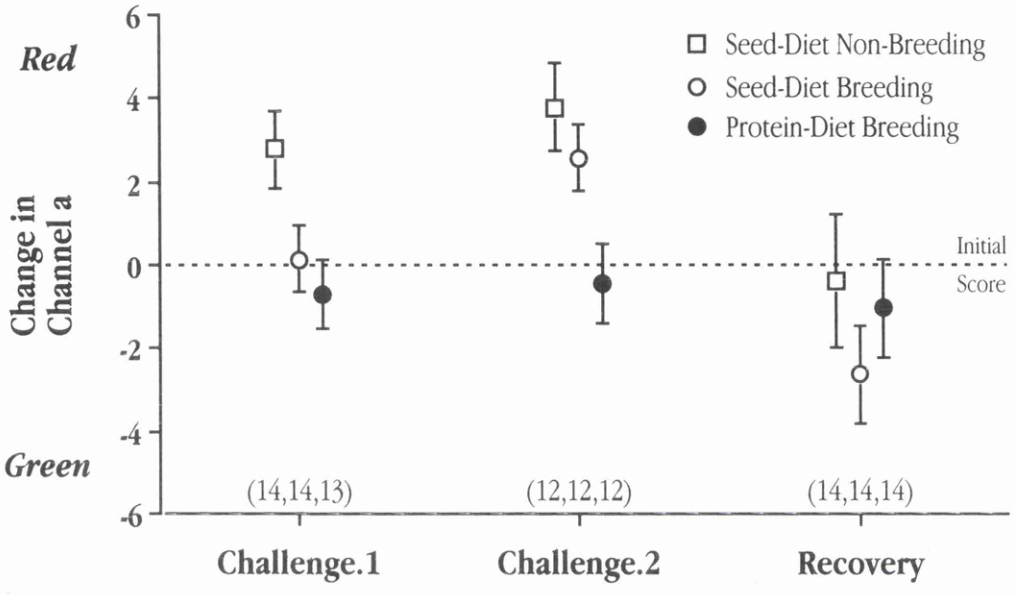
Changes in *Lightness* (Fig.7.6), *Channel a (Redness)* (Fig.7.7) and *Channel b (Yellowness)* (Fig.7.8), and combined colour factors, *Factor AB* (Fig.7.9) and *Factor LAB* (Fig.7.9), were compared between experimental groups.



Change in Lightness		**Fisher's Pairwise Comparisons** (High values correlate with recovery)
Challenge.1	*ANOVA* $F_{2,37} = 0.05$, $p = 0.954$	*No significant difference between experimental groups*
Challenge.2	*ANOVA* $F_{2,37} = 1.73$, $p = 0.191$	*No significant difference between experimental groups*
Recovery	*ANOVA* $F_{2,36} = 0.45$, $p = 0.644$	*No significant difference between experimental groups*

Fig.7.6 Change in bill colour *Lightness* in response to physiological challenges and recovery. Mean colour scores and standard errors are shown. (n) = sample size

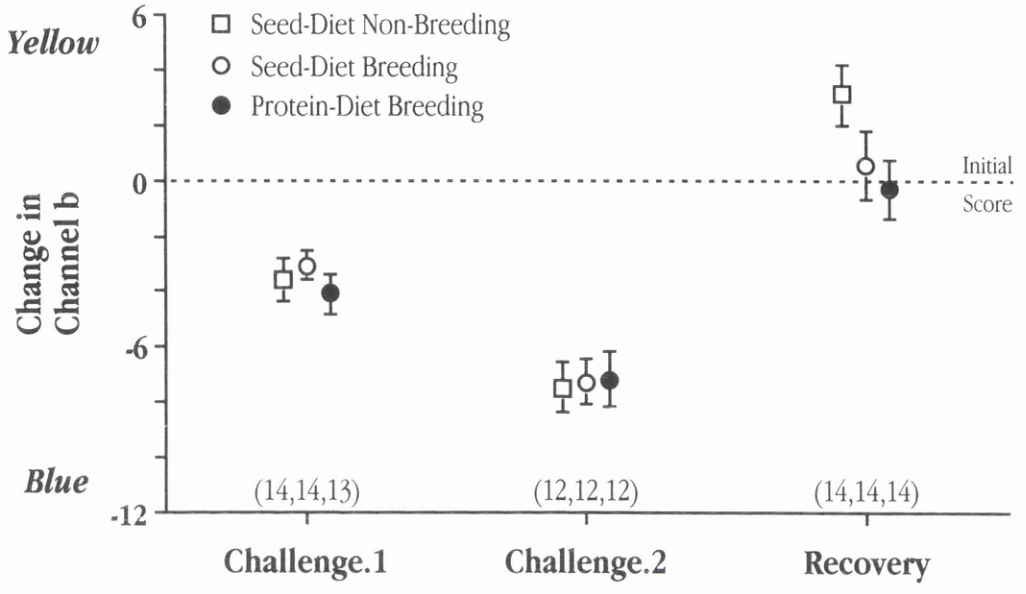
Paired samples t-test indicated that *Recovery* scores did not differ significantly from *Initial* scores.



Change in Channel a (Redness)		**Fisher's Pairwise Comparisons** (High values correlate with challenge)
Challenge.1	*ANOVA* $F_{2,37} = 4.68$, $\boldsymbol{p = 0.015}$	*Protein-Diet Breeding and Seed-Diet Breeding maintain score < Non-Breeding Seed-Fed*
Challenge.2	*ANOVA* $F_{2,37} = 5.27$, $\boldsymbol{p = 0.010}$	*Protein-Diet Breeding maintain score < Non-Breeding Seed-Fed and Seed-Diet Breeding*
Recovery	*ANOVA* $F_{2,36} = 0.73$, $p = 0.491$	*No significant difference between experimental groups*

Fig.7.7 Change in bill colour *Channel a* (*Redness*) in response to physiological challenges and recovery. Mean colour scores and standard errors are shown. (n) = sample size.

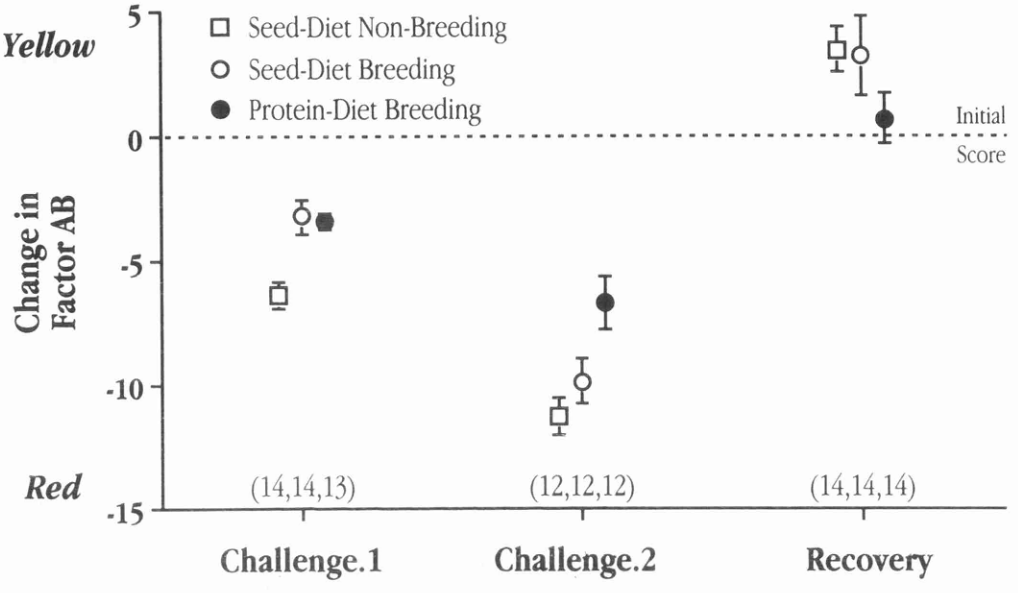
Paired samples t-test indicated that *Post Recovery* bill redness scores exceeded *Initial* scores in the *Seed-Diet Breeding* group (n=12, t=2.21, p=0.049).



Change in Channel b (Yellowness)		**Fisher's Pairwise Comparisons** (High values correlate with recovery)
Challenge.1	*ANOVA* $F_{2,37} = 0.56, p = 0.574$	*No significant difference between experimental groups*
Challenge.2	*ANOVA* $F_{2,37} = 0.03, p = 0.972$	*No significant difference between experimental groups*
Recovery	*ANOVA* $F_{2,36} = 2.50, p = 0.096$	*No significant difference between experimental groups*

Fig.7.8 Change in bill colour *Channel b* (*Yellowness*) in response to physiological challenges and recovery. Mean colour scores and standard errors are shown. (n) = sample size.

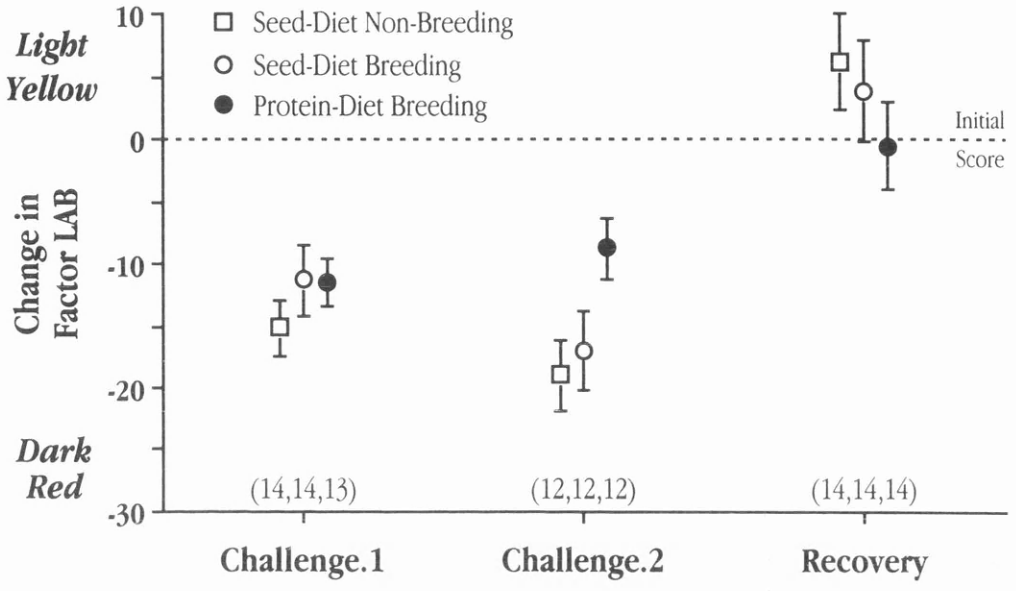
Paired samples t-test indicated that *Post Recovery* bill yellowness scores exceeded *Initial* scores in the *Seed-Diet Non-Breeding* group (n=13, t=2.75, p=0.018).



Change in Factor AB		Fisher's Pairwise Comparisons (High values correlate with recovery)
Challenge.1	*ANOVA* $F_{2,37} = 11.11$, $\boldsymbol{p = 0.000}$	*Protein-Diet Breeding and Seed-Diet Breeding maintain score > Non-Breeding Seed-Fed*
Challenge.2	*ANOVA* $F_{2,37} = 6.76$, $\boldsymbol{p = 0.003}$	*Protein-Diet Breeding maintain score > Seed-Diet Breeding and Non-Breeding Seed-Fed*
Recovery	*ANOVA* $F_{2,36} = 1.75$, $p = 0.188$	*No significant difference between experimental groups*

Fig.7.9 Change in bill colour *Factor AB* (*Red-Yellow*) in response to physiological challenges and recovery. Mean colour scores and standard errors are shown. (n) = sample size.

Paired samples t-tests indicate *Post Recovery* scores to exceed *Initial* scores, in both *Seed-Diet Non-Breeding* (n=13, t=3.74, p=0.003), and *Seed-Diet Breeding* (n=12, t=2.02, p=0.068) groups.



Change in Factor LAB		**Fisher's Pairwise Comparisons** (High values correlate with recovery)
Challenge.1	*ANOVA* $F_{2,37} = 0.92$, $p = 0.407$	*No significant difference between experimental groups*
Challenge.2	*ANOVA* $F_{2,37} = 3.92$, $\mathbf{p = 0.029}$	*Protein-Diet Breeding maintain score > Seed-Diet Breeding and Non-Breeding Seed-Fed*
Recovery	*ANOVA* $F_{2,36} = 0.85$, $p = 0.436$	*No significant difference between experimental groups*

Fig.7.10 Change in bill colour *Factor LAB* (*Dark/Red-Bright/Yellow*) in response to physiological challenges and recovery. Mean colour scores and standard errors are shown. (n) = sample size.

Paired samples t-test indicated that *Recovery* scores did not differ significantly from *Initial* scores.

7.3.5 *Physiological correlates of bill colour*

Immuno-competence, average egg mass, total clutch mass and change in weight of the female over the laying period were regressed against both *Initial* Factor LAB bill colour and change in Factor LAB bill colour (*Post Challenge.2* - *Initial* bill colour) to examine the potential of bill colour / change in bill colour as indicators of the physiological state of the female.

Despite the fact that *Protein-Diet Breeding* birds generally displayed significantly higher levels of immuno-competence than *Seed-Diet Breeding* counterparts (Fig.6.4), no significant correlation was found between the bill colour of an individual and its immune response. Similarly, regression of the physiological factors listed above against either of the measures of bill colour did not produce any significant correlations.

Results of regression analyses are summarised in Fig.7.11.

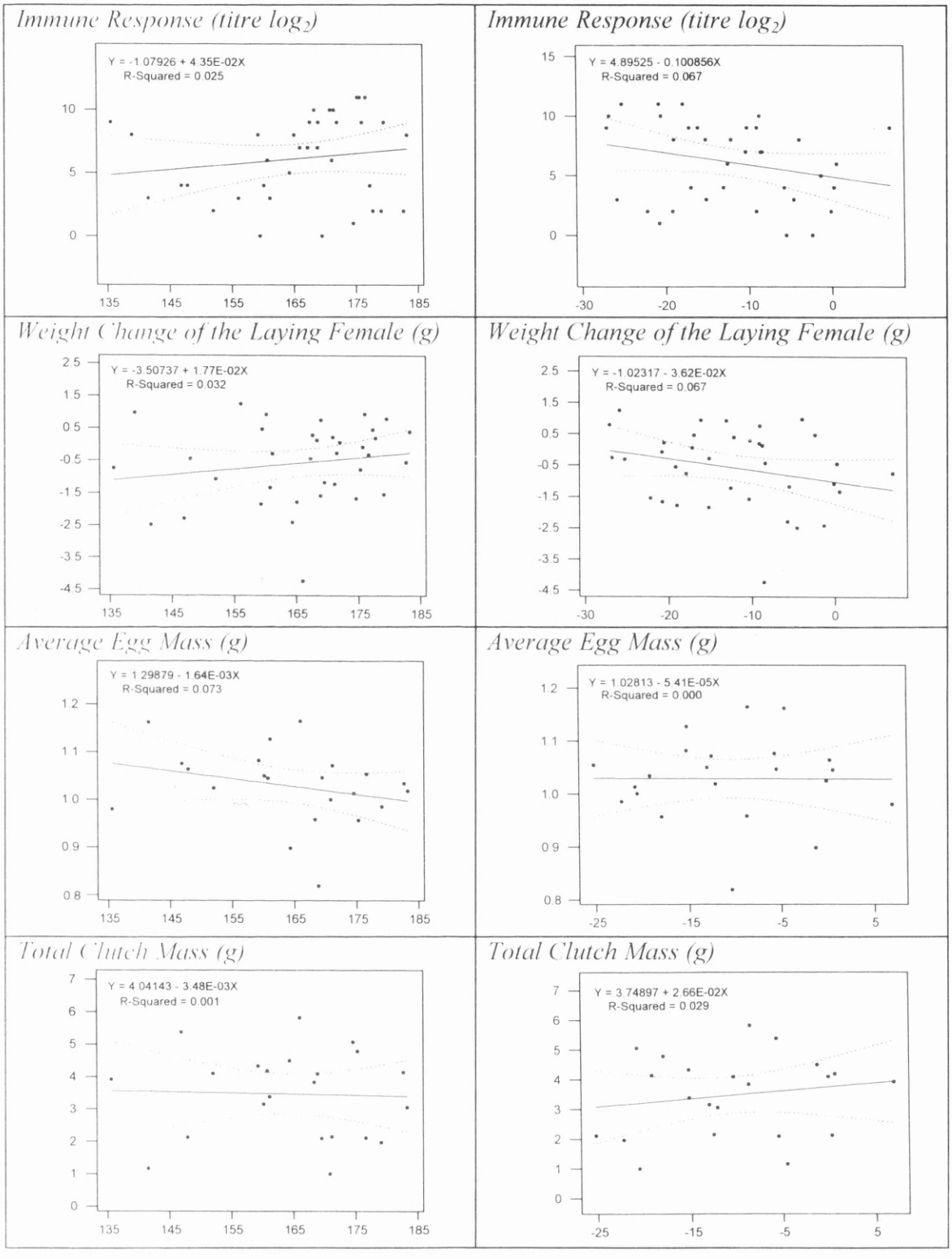


Fig.7.11 Summary of regression analyses of physiological correlates of LAB bill colour.

7.4 DISCUSSION

7.4.1 *Bill colour - an indicator of fitness?*

Monaghan *et al.* (1996), suggested that bill colour was an unreliable indicator of nutritional state, due to the time scale over which change was manifest being "relatively long". However, in the current study, bill colour was demonstrated to produce a measurable response to physiologically challenging conditions within a period of one month (Fig.7.3, 7.4). Under recovery conditions, measurable contrasting trends in bill colour similarly occurred within a month (Fig.7.3, 7.4). Hence, bill colour dynamics may be more rapid than previously assumed.

Despite being maintained on specific diets for three months prior to the initiation of the trial, no difference was recorded in any bill colour component between seed diet and protein supplemented groups at the start of the trial (Fig.7.5). Differences in bill pigmentation between groups may have been obscured by large *de novo* differences in bill pigmentation between individuals, the broad range in phenotypes resulting, in part, from the nutritional experience of the individuals as chicks (de Kogel and Prijs 1996). It is also possible that the nutritional planes of the birds maintained on the different diets were not sufficiently different to result in a measurable contrast in bill colour, neither dietary regime being "unreasonably pathological". Otherwise, this result might indicate that bill colour *per se* is not an accurate indicator of the nutritional plane of female zebra finches.

However, it is interesting to note that during the recovery period, birds previously maintained on a seed diet occasionally achieved colour scores significantly higher than those at the start of the trial. This effect was most pronounced for the bill colour measurement *Factor AB*, (Fig.7.9), with both seed maintained dietary groups attaining significant / close to significant score increases, above those recorded at the start of the experiment. This may indicate that initial bill colour in seed diet groups was sub-optimal, and that supplementation of the diet with protein during the recovery phase of the trial prompted an "over-compensatory" recovery of pigmentation. To fully investigate this, it would be necessary to have determined bill colour of the individual birds prior to the introduction of the experimental diets. Unfortunately, no measure of bill colour was taken at this time.

Therefore, although bill colour may present some indication of nutritional plane of an individual, in the case of this experiment, any significant effect was largely masked by the natural variation within and between experimental groups. As such, the degree to which bill colour indicates nutritional plane in the female zebra finch remains equivocal.

7.4.2 *Bill colour change - a better indicator of fitness?*

Over the course of repeated *challenges* (egg production, elicitation of an immunological response and blood removal), bill colour of female zebra finches became darker (Fig.7.6), redder (Fig.7.7) and less yellow (Fig.7.8). The overall effect of these changes (Fig.7.9, 7.10) was to direct bill colour away from the initial phenotypic mean of the groups. Males have been previously shown to prefer females towards the centre of the phenotypic range (Burley *et al.* 1992), and so these changes might be expected to reduce the favourability of the females.

During the *recovery* period, when all birds were separated from their partners and maintained on a protein supplemented diet, a reversal of these trends was evident (Fig.7.6 - 7.10). Females' bill colour scores tended to revert back towards their initial values and, with reference to the above, this may be expected to promote selection by males.

Though all dietary groups exhibited similar trends over the course of *challenge* and *recovery* periods (Fig.7.6 - 7.10), the magnitude of these changes was significantly reduced in birds previously receiving protein supplements. This enabled protein supplemented birds to maintain the most stable bill colour profiles over the course of the trial, with minimum deviation from the initial phenotypic mean of the group. Previous studies indicate that one might expect such birds to enjoy preferential selection from male finches and possibly increased survivorship (Burley *et al.* 1992). However, elucidation of the physiological mechanisms by which protein limitation may influence bill colour are beyond the scope of this study.

It would appear that bill colour dynamics may represent an accurate and rapid indicator of the changing physiological state of the female. However, stability of bill colour is a characteristic that is not immediately advertisable. As such, its use as a signal of fitness might be questioned, and would certainly only be appropriate in the context of time. Were the dynamics of bill colour to represent a mechanism by which the male could assess the ongoing physiological condition of a female, this may delineate the importance of pair-bond maintenance for the male, potentially enabling him to time breeding attempts to coincide with optimal condition of the female.

Zebra finches are ostensibly monogamous in the wild and pair bond formation is extremely strong (Zann 1996), so partners would at least have the opportunity to monitor each other over a period of time. However, though both male and female are responsible for the formation and maintenance of the pair bond, it is generally believed that, of the two, it is the selection of the male by the female that is the ultimate determinant of whether a pairing will occur (Immelmann 1962b, from Zann 1996). Hence, similar investigation of the bill colour of male finches would be valuable.

7.4.3 *Correlation of bill colour with productivity*

No specific requirement for carotenoids has been demonstrated in birds. It is possible to hatch "healthy" chicks from colourless, carotenoid free eggs (Palmer and Kempster 1919, from Goodwin 1983). However, under natural conditions eggs contain rich deposits of protein and carotenoids (lutein and zeaxanthin), (Fox and Vevers 1960), and egg production has been previously linked with a reduction in immuno-competence of the laying female (Nordling *et al.* 1998, Oppliger *et al.* 1997, but see also Apanius *et al.* 1994). As such, one might have expected the *Seed-Diet Non-Breeding* birds to have maintained preferential bill colour scores in comparison with *Seed-Diet Breeding* birds. Surprisingly, however, there was no evidence of this in the current study. Dietary supply of carotenoids may not have been limiting for egg production during this trial, seed representing a rich dietary source of carotenoids (Fox 1976). However, this still does not fully explain the difference between the observations of the current study and those of Burley *et al.* (1992), who observed diminishment in bill colour of female zebra finches (also maintained on mixed grass seed diet) concurrent with repeated laying.

Despite comparison of a variety of measures of physiological well-being and bill colour (7.3.5), at the level of the individual, bill colour did not appear to represent an accurate indicator of any of the specific aspects of fitness measured. The structure of this experiment, however, does not lend itself to the analysis of individual factors, as multiple, interacting determinants were applied in both challenge and recovery scenarios. Combination of these individual factors to yield a single measure of "fitness" is difficult, and the value of such an estimation would be limited due to the fact that other, possibly important, correlates of fitness were not measured in this experiment, the most obvious being egg quality in the form of carotenoid content of the yolk and chick survival.

7.4.4 *Why bills?*

Plumage is a well recognised display mechanism, however, it may be prone to some delay as an indicator of internal health. The bill is one of the few surfaces of the bird that is not obscured by feathers. This, combined with its location on the body and potential for incorporation into visual display, promotes candidacy as a signaller of internal heath.

The eye and the tarsus are the only other areas of the bird not obscured by feathers. Coloration of both have been observed to fade on introduction of the birds to captivity (Zann 1996, Brush and Power 1976) indicating a susceptibility to variations in dietary pigment. The fact that these areas represent the entirety of the visible surface of the body of a bird, may endow them with an increased importance as signallers of internal heath. In the light of this, the experimental preference of specific leg band colours in zebra finches (Zann 1994) may be of more than artificial interest.

7.4.5 *Ecological validity of using caged birds for this study*

Birds are able to effectively assimilate pigments from diets outwith normal experience (Kritzler 1943, Hudon 1994). Those used in this study were free to select at leisure from an *ad libitum* mixture of seed, which did not include the major dietary component of wild finches; the grass seed *E. crus galli*, (Zann and Straw 1984). While the superficial role of pigmentation may be adequately provided by substitute diets, this may not necessarily be true of any homeostatic attributes of specific carotenoids (see Hudon 1994). The inconsistency of the effect of protein supplementation of the diet on bill colour between wild and captive populations of zebra finches (Burley *et al.* 1992) may be further evidence of this.

Additionally, Hudon (1994), citing the large body of German literature in this field, suggested that restriction of mobility, arising from captivity, was a major determinant of the ability of birds to display pigmentation. Care should therefore be taken in extrapolating observations of pigmentation in captive birds to those in the wild.

Finally, care should be taken in interpreting data arising from bill colour analysis. All observations are confounded to some extent by the fact that the visual range of birds extends into the ultra-violet. Plumage characteristics in the ultraviolet waveband (300-400nm) have been shown to be important distinguishing features of sex in birds (*review* Guilford and Harvey 1998), and ultra-violet vision is involved in mate-selection by zebra finches (Bennett *et al.* 1996). Hence, any observations we make regarding bill colour may be compromised by our limited visual spectrum.

7.4.6 *Observations arising from the use of a computer software package for the assessment of bill colour*

The use of the computer package for the measurement of bill colour had a number of advantages and disadvantages compared with the traditional method of colour card comparison.

Advantages

- Handling time required for photography of the birds was low, and did not impinge on time required for accurate colour assessment.
- Evaluation of colour by the computer was not subjective. This makes colour judgements independent of the observer, additionally allowing colour evaluation to be made by the experimenter without risk of observer bias.
- Package features included a magnification facility allowing minute details of the bill to be examined. Heterogeneous colour features e.g. "pigment pools" too small to be seen by the naked eye became clearly visible.

Advantages (continued...)

- Analysis of colour components included detailed data on relative absorbance across whole of spectrum. There may be a potential here for standardisation of the display with respect to measurement of pigments of specific absorbance in the bill.

Disadvantages

- The package is comparatively complex and time-consuming to operate.
- The accuracy of the package is compromised by the quality of the photograph and its development. Despite efforts to standardise lighting levels and photographic set-up, colour levels varied between (and to a much lesser extent within) films meaning that standardisation of images was necessary.
- Reflection of flash from the bill caused some problems, and although areas of flash reflectance are easy excised by the software package, they do represent a loss of data. Reflection was minimised by holding the head of the bird in a downwards pointing position.
- The package reduces colour to a variety of components. This is beneficial for specifics, but makes overall effects more complicated to elucidate.

7.4.7 *The heterogeneous nature of bill colour*

Magnification of the bill area using the computer package revealed the heterogeneous nature of bill pigmentation. The command *Windows-Palettes-Show Channels* allows the scanned image to be viewed with shades of grey representing colour components, enabling the examination of the distribution of specific colours within the bill.

Small, bright red pools of pigment become visible on even the yellowest bills - a feature not previously reported. *Lightness* and *Yellowness* are distributed fairly evenly over the bill. *Redness*, however, tends to be very much concentrated in the upper mandible, proximal to the head. This area also tends to achieve high yellowness scores.

This may indicate that bill redness/yellowness is the product of differing concentrations of a single pigment, the range of hues arising from variation in a single pigment can be marked (Goodwin 1983). Alternatively, bill colour may arise from the interaction of two or more pigments, and the optical properties of the over/under-lying structures. Additionally, red pigmentation may be due to the presence of xanthophylls, the breakdown products of carotenoids (e.g. astaxanthin). If degradation arose due to utilisation of carotenoids, for example, through implementation in an immune response, this may suggest a mechanism by which physiological challenge may directly cause bill reddening.

Chapter 8

GENERAL DISCUSSION

8.1 DISCUSSION

8.1.1 *Basis of the investigation*

Egg formation places nutritional demands on a bird in addition to those of routine metabolism. The reluctance of zebra finches to supplement their grass-seed diet with insects during the breeding season led Zann and Straw (1984), to suggest that egg production might not be limited by dietary protein availability. However, the opportunistic timing of breeding to coincide with flushes of unripe grain, which the finches readily consume, has been shown to represent an ingenious mechanism by which the finches acquire a dietary balance of amino acids similar to that of the eggs that they are to produce (Allen and Hume 1997).

8.1.2 *The selection, storage and utilisation of protein in zebra finches*

Diet selection in birds is based on a variety of criteria (see Gentle 1979, Zann 1996). However, preferences for specific food-type characteristics should correlate with a preconceived assessment of the nutrient content of the diet, rather than arbitrary preference, if diet selection is effectively to satisfy an individual's nutrient requirement. Investigation of the self-selective ability of zebra finches (*Chapter 3*), indicates that, given the choice between two diets ostensibly differing only in protein content, the birds were able to self-select a balance between the two, and subsequently lay larger eggs and clutches than birds maintained on either of the single diets. It is suggested that the finches developed a "perceived taste", moderated by specific nutrient appetite (3.4.3), enabling them to select between the diets according to their requirements. Satisfaction of a specific nutrient appetite, which may differ radically between individuals, is suggested to be the underlying determinant of diet choice: selection of seeds on the basis of other factors (e.g. handling time) being more a function of the capability or skill of the individual to satisfy this appetite, than primary determinants of diet selection in themselves.

Biochemical analysis of flight muscle sarcoplasm lead Houston *et al.* (1995c) to suggest the existence a high molecular weight protein, possibly acting as a "specific protein reserve" for egg production, which might be utilised without compromising the contractile ability of the flight muscle. Replication of the study of Houston *et al.* (1995c), in combination with additional biochemical analyses indicated that though protein is removed from the sarcoplasm of the flight muscle during egg production, utilisation is not confined to any specific molecular weight protein (*Chapter 4*). The suggested ecological advantages of specific storage proteins are also disputed, on the basis that at all times outwith the period of egg production, support of an otherwise non-functional protein deposit would represent an energetic burden to the bird.

Carey (1996) attributed synthesis of yolk proteins in the liver to be possibly the most important rate limiting factor in egg formation. Examination of fractional synthesis rate (FSR) of protein, indicated increased FSR in the liver in association with both breeding (compared with non-breeding birds), and a history of dietary protein supplementation (compared to birds maintained on a seed diet), (*Chapter 5*). Exposure to elevated levels of dietary protein has been previously shown to result in compensatory metabolic responses, and an adaptive increase in the FSR of the liver, is suggested to be a contributory mechanism for the observed increase in egg productivity associated with protein supplementation of the diet. Transfer of specific amino acids from the pectoral muscle to the forming egg has previously been shown to occur during laying (Houston *et al.* 1995c). However, FSR in muscle tissue remained fairly similar between breeding and non-breeding birds. Therefore, in order for a net release of protein from muscle reserves to be achieved during egg formation, an elevation in protein breakdown rate might be expected in these tissues. However, further physiological analysis of protein degradation would be required to confirm this.

With reference to the opening statement of this chapter, it should be remembered that "routine metabolism" is a physiological concept, rather than everyday reality. In the wild, and to a lesser extent under laboratory conditions, variation in physical and biotic aspects of the environment may have a direct impact on the physiological state of an animal. Increased reproductive effort has been previously indicated to compromise the ability of birds to mount an immunological response (Nordling *et al.* 1997, Oppliger *et al.* 1997), possibly as a result of protein limitation (Lochmiller *et al.* 1993). While the protein demands of egg production are, to some extent, fairly predictable for the bird, protein allocation for possible immunological response may be less so. Immunological responsiveness of female zebra finches maintained on a mixed seed diet decreased during the period of egg formation (*Chapter 6*). Protein supplementation of the pre-breeding diet of females enabled egg production to continue (undepleted) while maintaining a level of immuno-competence comparable with that of non-breeding birds. Similar protein supplementation of brood-rearing finches had not measurable impact on immno-responsivness (Deerenberg 1996), possibly indicating the protein demands of egg production to exceed those of brood rearing. It is suggested that laboratory observations of increased egg laying arising from protein supplementation may not be indicative of the allocation trade-off experienced by wild birds. In the wild, increased risk of mortality in combination with a reduced nutritional plane may constrain optimal clutch size below that of the lab environment, with any additional protein reserves being more important for the long-term self-maintenance of the female than short-term reproductive benefit. In the light of this result, amino acid supplementation of the diet through consumption of unripe grain by wild finches (Allen and Hume 1997), might be regarded as much a counter infection measure, as a mechanism for increasing clutch size.

The potential of bill colour as an external indicator of internal physiological state of female zebra finches was examined (*Chapter 7*), with the suggestion that carotenoid pigments may represent a direct causal link between bill pigmentation and fitness of

the individual. While bill pigmentation was demonstrated to change significantly in response to physiological challenges, and these trends to reverse under conditions conducive to physiological recovery, bill colour was not observed to be correlated with any of the measures of health or fitness on an individual level. Contrary to prediction, the demands of egg production also appeared to have little effect on bill colour. However, protein supplementation of the diet of breeding birds enabled them to maintain bill coloration closest to the phenotypic average of the group over the course of the trial, a trait previously indicated to result in preferential selection by males (Burley and Coppersmith 1987). It is possible that bill colour dynamics, rather than bill colour *per se*, may represent an indicator of the internal physiological state of the female. However, were a male to gain advantage from selection of this, it would be necessary for him to monitor pigment change of the female over a period of time.

8.1.3 *Conclusions*

It is hoped that the simple experiments outlined in this study offer some indication of the diversity and close interaction of some of the aspects of protein in the physiological ecology of the zebra finch. The diversity of the physiological roles of protein, and the variety of factors influencing its utilisation make the potential for study in this area almost limitless.

The importance placed on the acquisition of a suitable dietary source of protein is evident from the fact that zebra finches orientated their entire breeding cycle around the ephemeral availability of a food source (unripened seed) from which they can obtain the correct amino acid balance for egg production. The efficiency of this ingenious mechanism of dietary supplementation must surely have contributed to their success; zebra finches being the most widespread and numerous of Australian Estrildines (Zann 1996).

The apparent discrepancy between the amino acid composition of diet and eggs (Donnan 1993, Houston *et al.* 1995a, Zann and Straw 1984) thus alleviated, the extent of dietary protein limitation on egg production in wild zebra finches may not be as great as previously though. In light of this, the contribution of endogenous protein stores and the promotion of egg production in laboratory birds observed during protein supplementation trials (Selman and Houston 1996, Williams 1996) may be an artefact of the protein impoverished ripened seed diets used to maintain captive birds, and hence not applicable to zebra finches in their natural environment.

The importance of protein limitation on egg production has been recognised for some time, however, this represents just one facet of the total metabolic response to dietary protein. Modification of protein synthesis by protein supplementation may be expected to have repercussions for a range of protein demanding metabolic processes, egg production, immunological response and erythropoiesis being amongst those investigated in this study.

Of the topics studied in this thesis, specific nutrient appetite, protein metabolism and requirement, immuno-competence and bill colour, as well as being connected through the common link of protein, have been observed to display great variation between individuals. Though general trends may be observed in groups of zebra finches, application of these trends to individuals of such "character" should be attempted with care.

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