Endocrine control of nestling begging behaviour in the pied flycatcher, *Ficedula hypoleuca*

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Nicola M. Goodship Ph.D. Thesis

Endocrine control of nestling begging behaviour in the pied flycatcher, *Ficedula hypoleuca*

Summary

Begging signals given by nestling birds may advertise their condition or quality and parents may respond by allocating their resources in relation to begging intensity. In order for such signals to be honest, they must be costly to produce. The aim of this project was to investigate the role of nestling endogenous testosterone (T) as a potential mechanism to control begging signals in pied flycatchers, *Ficedula hypoleuca*. Androgen levels were analysed from invasive and non-invasive (faecal) samples using T radioimmunoassay. In the laboratory, nestling begging behaviour was measured as: 1) the duration of begging displays and 2) the maximum height of begging stretches. It was found that individual nestlings begging most intensively had the highest circulating levels of T immediately after testing. This relationship was tested experimentally by dosing nestlings with oral doses of T and assessing the effects on nestling begging signals. The results showed that the duration of begging displays by T-dosed nestlings were longer than controls, confirming the causal nature of T in controlling nestling begging signals. A field study investigated the effect of brood reduction on parental provisioning strategies, nestling behaviour and T levels. During brood reduction parents allocated food resources according to rules based more strongly on nestling begging behaviour compared with control days. A partial cross-fostering experiment tested whether nestling T and begging behaviour related to levels of relatedness within the nest. The begging duration of cross-fostered broods was longer than control broods and fostered nestlings increased their faecal androgen levels, although the reliability of this result should be further investigated. A biochemical validation study confirmed that excreted androgen metabolites were measured in the faeces of pied flycatchers. Overall, the results of this project confirmed that T is responsible in part for the control of begging intensity and may be a mechanism that controls begging behaviour in nestling birds.

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List of abbreviations

ACTH:	Adrenocorticotropic hormone
ANOVA:	Analysis of variance
AR:	Androgen receptors
BMR:	Basal metabolic rate
CHD:	Chromobox-helicase-DNA
CNS:	Central nervous system
CRH:	Corticotrophin-releasing hormone
DHT:	5α-dihydrotestosterone
DNA:	Deoxyribonucleic acid
ESSs:	Evolutionary stable strategies
FSH:	Follicle stimulating hormone
GH:	Growth hormone
GLM:	General Linear Model
GnRH:	Gonadotropin-releasing hormone
H:	Hour
[^{3}H]T:	Tritiated testosterone
HPLC:	High performance liquid chromatography
HPA:	Hypothalamo-pituitary-adrenal
HPG:	Hypothalamo-pituitary-gonadal
ICHH:	Immunocompetence handicap hypothesis
ID:	Identification
I.P.:	Intraperitoneal
LED:	Light-emitting-diode
LH:	Luteinizing hormone
Min:	Minute
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PHA:	Phytohaemagglutinin
PNS:	Peripheral nervous system
RBCs:	Red blood cells
PRL:	Prolactin
RIA:	Radioimmunoassay
RMR:	Resting metabolic rate
RPM:	Rounds per minute
S:	Second
S.D.:	Standard deviation
S.E.:	Standard error
Sib:	Sibling
SMGF:	Sheffield Molecular Genetics Facility
T:	Testosterone
T-RIA:	Testosterone radioimmunoassay
TSH:	Thyroid stimulating hormone
UV:	Ultra violet

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Chapter 1

Introduction

1.1 ANIMAL COMMUNICATION

1.1.1 Evolution of animal signals

During development, the young of altricial birds depend on their parents to provide them with resources. To attract the attention of their parents, nestlings beg by calling loudly, gaping and jostling for a position close to the provisioning parent (Kilner & Johnstone 1997). This type of behaviour is an animal signal which is thought to play an important role in resolving parent-offspring conflict (Trivers 1974). Through begging behaviour, offspring may provide parents with information about their condition or quality (Kacelnik et al. 1995; Kilner 1995; Price & Ydenberg 1995; Stamps et al. 1989) and parents may respond by allocating their resources in relation to begging signals (Gottlander 1987b; Kacelnik et al. 1995; Ryden & Bengtsson 1980; Smith & Montgomerie 1991).

Animals use many different types of behavioural, physiological and morphological signals to communicate with one another, and if signals convey reliable information, they are likely to be maintained through natural selection (Searcy & Nowicki 2005). A fundamental concept of animal communication, is that a signal by one individual (signaller), must in some way modify the behaviour of another (receiver) (Krebs & Davies 1987). Maynard Smith & Harper (2003) described their definition of an animal signal as: 'any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved'. If an animal signal is to alter the behaviour of another individual, there must be a benefit to the receiver of the signal to respond to the signaller, otherwise the receiver will cease to respond. An early view on the evolution of animal signals assumed that selection operated at a group level, so that behaviour evolved towards what was best for the group or the species (Searcy & Nowicki 2005). However, the modern view of animal communication believes that selection acts largely at the level of the individual, so that behaviour evolves toward what is best for the individual performing the behaviour, and not towards what is best for the group

(Searcy & Nowicki 2005). Information can also be provided by animal cues. Hasson (1994) defined a cue as any feature in the environment, animate or inanimate, which is used by an individual as a guide to future action. Animal signals are different from animal cues in that signals evolved because of their effects on other individuals, whereas cues may provide information to other individuals, but they do not evolve because of the affect they have (Maynard Smith & Harper 2003). For example, when parent birds provide their young with food, they may use nestling begging signals to allocate resources. Alternatively, parents may use nestling cues, such as nestling mass or sex to decide which nestling to feed (Whittingham et al. 2003).

Animal signals are not always reliable. For example, some insects mimic the warning colours of other distasteful insects, although they themselves are edible (Wilson 1971). Although signallers can communicate either reliable or deceptive information through a signal, signals must be reliable often enough for the receiver's response to the signal to be selected (Maynard Smith & Harper 2003). Whether animal signals from signaller to receiver are reliable or deceptive depends on the honesty of signals. Zahavi (1975) first proposed the 'handicap principal', which suggests that signals are honest because they are costly to make, although not all signals carry the same level of costs (Maynard Smith & Harper 2003). There are two different kinds of cost involved with signalling systems, efficacy costs and strategic costs (Guilford & Dawkins 1991). Efficacy costs are involved in the production of a signal to convey the necessary information to another individual, and once the signal is received there are no more costs involved. Efficacy costs must be paid even when the signaller has no temptation to lie. For example, male nightingales sing loudly overnight to attract female attention, but they lose 5 - 10 % of their body mass in the process (Thomas 2002). Male nightingales may bare efficacy costs in their signal production; the energetic investment involved with singing loudly may be necessary to convey the signal over a long distance, as the females are flying a long way above the males. Alternatively, strategic costs are the additional costs involved with producing a signal beyond that which is necessary to convey information to the receiver; i.e. strategic costs are greater than just the efficacy costs alone that are required to transmit a signal. Strategic costs may also be called 'handicaps' and these are the costs required for honest signalling and to prevent cheating (Maynard Smith & Harper 2003). For example, the tail of a peacock is

energetically costly to develop and display (efficacy costs), but there are additional costs (strategic costs) involved with the display of this signal, as a peacock with a large tail is more vulnerable to predators. When signal production involves strategic costs, it is expected that only good quality individuals are able to bare additional costs and poor quality individuals are unable to lie, due to the costs involved (Maynard Smith & Harper 2003). Therefore, cheating individuals do not occur, or at least they occur only very rarely which maintains the honesty of the signal (Zahavi 1975).

1.1.2 Begging behaviour

Although the majority of studies in the literature on begging behaviour are focussed on nestling birds, the young of other animals also use different types of visual, auditory, tactile and chemical signals to gain parental provisioning (see Kilner & Johnstone 1997 for review). Non-avian examples of visual begging signals are exhibited by the larvae of burying beetles, *Nicrophorus vespilloides*, that raise their head and wave their legs in a rhythmic way (Rauter & Moore 1999; Smiseth & Moore 2004). Tadpoles of poison arrow frogs, *Dendrobates pumilio* are also known to swim vigorously to stimulate the mother to release trophic eggs (Weygoldt 1980). Begging calls that indicate offspring need are used by grey seal pups, *Halichoerus grypus* (Smiseth & Lorentsen 1995) and piglets, *Sus scrofa* (Weary & Fraser 1995). Wilson (1971), described the tactile begging signals produced by agelenid spiderlings, *Coelotes terrestris* that stroke the mother's chelicerae with their palps in order to encourage the mother to regurgitate food (Tretzel 1961). Mice, *Mus musculus* use chemical begging signals before birth by releasing an insulin-like growth factor II (IGF-II) to stimulate the mother to pass more resources to the embryo (Haig & Graham 1991).

Nestling birds also use a range of different begging signals to attract parental attention. For example, nestlings may use begging calls (Price & Ydenberg 1995), different begging postures (Redondo & Castro 1992), coloured gapes (Hunt et al. 2003; Kilner 1997; Kilner & Davies 1998), coloured heads (Boyd & Alley 1948; Nuechterlein 1983) and plumage colouration (Lyon et al. 1994) to gain parental food resources. Two hypotheses have been put forward to suggest why nestlings may use multiple signals. The redundant signal hypothesis (Johnstone 1996; Møller & Pomiankowski 1993) suggests that parents gain information from two or more nestling signals to gain a better

estimate of a single aspect of nestling condition. For example, previous studies have found that different components of nestling begging displays (latency, duration, posture and the duration of begging calls) co-vary in a way that enriches the information content of the signal (Gottlander 1987b; Redondo & Castro 1992; Smith & Montgomerie 1991). Alternatively, the multiple solicitation signal hypothesis suggests, that begging displays may carry multiple messages which may provide parents with different kinds of information about nestling condition or quality (Johnstone 1995; Johnstone 1996).

1.1.3 Parent-offspring conflict

Parental investment was defined by Trivers in 1974 as: 'any investment by the parent in an individual offspring that increases the offspring's chance of surviving, at the cost of the parent's ability to invest in other offspring'. A fundamental concept of parent-offspring conflict is that, as parents and offspring differ in genetic composition (Hamilton 1964), nestlings are selected to demand more than parents are selected to provide, which causes dispute over the division of parental investment (Trivers 1974). Since the pioneering work of Trivers (1974) and Hamilton (1964), there has been much interest in the evolution and maintenance of offspring begging behaviour, which is thought to resolve parent-offspring conflict.

To understand why it is that parents and their offspring are in conflict with each other, it may help to appreciate how genes are passed on between different individuals and spread within the gene pool. Genes may be thought of in anthropomorphic terms as being 'selfish' and are expected to try to pass on as many copies of themselves as possible within the gene pool (Dawkins 1989). From the perspective of kin-selection, parents pass on half of their genes to their offspring and are usually equally related to each of their young, including those which have yet to be born (Trivers 1974). A gene expressed in a parent that influences the distribution of parental resources has an equal chance of being passed to any of the offspring. Therefore, so long as all offspring have an equal fitness (offspring need and reproductive value are the same), parents are expected to provide an equal share of resources to each of their young (Godfray 1995b). From the point of view of an offspring, each individual shares half of its genes with each parent and half with its siblings, as determined by the coefficient of relatedness

(Hamilton 1964). A gene expressed in an offspring which influences the distribution of parental resources will be selected to obtain a disproportionate share of resources (Trivers 1974). This is because the gene is 100 % present in the focal offspring, but there is only a 50 % chance that it is also expressed in each of its siblings. As parents favour an even distribution of resources to offspring both present and future, but present offspring demand a disproportionate share of the resources, a conflict of interest occurs between parents and offspring (Trivers 1974).

Sib-sib conflict also occurs as the focal offspring is selected to demand more resources from the parents than siblings are expected to yield. However, the focal offspring is not selected to demand all parental resources as there is still a 50 % chance that the gene influencing parental resource distribution is also expressed in its siblings. Therefore, the selfishness of the focal offspring is limited, as it still has some genetic interest in the survival of its siblings (Hamilton 1964).

1.1.4 Relatedness

The degree of cooperation between any two individuals depends on the coefficient of relatedness between them, as expressed by Hamilton's (1964) rule:

$$b > rc$$

where the benefits (b) to one individual must outweigh the costs (c) to another individual weighted by the coefficient of relatedness (r). Within a brood of nestlings where siblings compete for a fixed amount of food from parents, it is possible to work out mathematically what share of food each sibling should demand (by begging) using Hamilton's (1964) rule. For example, if there are two equal siblings within a brood X & Y, X should carry on demanding food from the parents until the benefits of the food resources to X are only half those of Y, who gets the remainder (Parker et al. 1989). Theoretically, altruistic behaviour and cooperation declines with decreasing relatedness. As half siblings (sharing genes only from one parent) are less related ($r = 0.25$) than full siblings ($r = 0.5$), each individual is selected to take a greater proportion of parental resources for itself (Godfray 1991; Hamilton 1964).

The degree of relatedness between siblings within a nest, and also between offspring and parents, depends on parentage. For example, in broods where mixed parentage

occurs, the average coefficient of relatedness between different individuals will be lower than in broods where both parents are fully related to all offspring. In many avian species, females participate in extra-pair copulations and consequently nestlings within a brood may be fathered by two or more males (Birkhead & Møller 1992). Extra-pair paternity occurs in pied flycatchers, *Ficedula hypoleuca* and in a closely related species, the collared flycatcher, *Ficedula albicollis* (Alatalo et al. 1989; Brun et al. 1996; Gelter & Tegelstrom 1992; Lifjeld et al. 1991; Ratti et al. 1995; Sheldon & Ellegren 1999). Hence, in both pied and collared flycatcher broods, the genetic relationship between nestlings and the male attending the nest may vary, and there may also be differences of relatedness between nestlings. In other bird species, unrelated offspring can also result from intra-specific brood parasitism, which occurs when females of the same species lay their eggs in the nest of another individual (Berg 2005; Czechowski & Zduniak 2005; Lank et al. 1989) or through inter-specific brood parasitism, which occurs when eggs are laid into the nest of a different species (Chace 2005; Davies et al. 1998; Garamszegi & Aviles 2005; Martin-Galvez et al. 2005).

Parental provisioning and nestling begging behaviour is influenced by the degree of relatedness between different members of a brood (Briskie et al. 1994; Lifjeld et al. 1998; Sheldon & Ellegren 1998). Theoretical models have been developed to predict the amount of paternal care a male should give in relation to his paternity within a brood (see Westneat & Sherman 1993 for a review). Generally, the predictions from theoretical models depend on three main factors: 1) whether there are cues by which males can assess their parentage, 2) whether paternity differs between breeding attempts and 3) whether returns on fitness are higher from other activities (Westneat & Sherman 1993). Cues which indicate the percentage of paternity a male may have in a brood can come from the behavioural cues of mated females or neighbouring males, such as observing extra-pair activities (Sheldon & Ellegren 1998). Alternatively, males may use cues from offspring behaviour (Westneat & Sherman 1993), although other studies have suggested that male birds are unlikely to recognize their own young in a brood of mixed paternity (Burke et al. 1989; Kempenaers & Sheldon 1996; Westneat et al. 1995). Observational studies have previously shown that paternal provisioning is reduced with lower paternity (Dixon et al. 1994; FreemanGallant 1996; Møller & Tegelstrom 1997), but other studies have not found this effect (Wagner et al. 1996;

Westneat et al. 1995; Yezerinac et al. 1996). However, three experimental studies on pied flycatchers (Lifjeld et al 1998), collared flycatchers (Sheldon & Ellegren 1998) and dunnocks, *Prunella modularis* (Davies et al. 1992) have all shown that males reduce their parental effort in response to reduced paternity. In addition, nestling begging behaviour is also influenced by the relatedness of nestmates. An observational study on 11 passerine species found that, whilst controlling for brood size, the loudness of nestling begging calls increased as the relatedness amongst members of the brood declined (Briskie et al. 1994). In the study by Briskie et al. (1994), it was suggested that in species where there is low relatedness within a brood, loud begging nestlings were more likely to attract parental provisioning at the expense of quieter nestmates (Redondo & Dereyna 1988).

1.2 THEORETICAL STUDIES OF PARENT-OFFSPRING CONFLICT

1.2.1 Battleground and resolution models of parent-offspring conflict

There are a number of different models which aim to predict the outcome of resource distribution during parent-offspring conflict (see Godfray 1995b for a review). These models can be classified into two basic types: 1) battleground models (Godfray & Parker 1991) or 2) resolution models (Godfray 1995b). Both types of model are concerned with the outcome of evolutionarily stable strategies (ESSs). The aim of a battleground model is to show the optimum strategy for both parents and young on how resources should be distributed. Any discrepancy between the two optima defines the 'battleground' within which the conflict between parents and offspring is fought (Godfray & Parker 1991). For example, a battleground model by Smith & Fretwell (1974) mathematically investigated the optimum investment per offspring from the point of view of the parent. Smith & Fretwell's (1974) model showed that the optimum investment for a parent is at a level where offspring still gain from the investment, but less investment would mean a reduction in the number of offspring. Thus, the parent balances the benefits of fitter offspring against the reduction in number of offspring. Alternatively, the optimum investment per offspring can be calculated from the point of view of the offspring (Godfray & Parker 1991). Again, the optimum investment into offspring is set by the balance between fitter offspring and the number of offspring. However, whereas a parent is equally related to each offspring and hence should value each offspring identically, a focal offspring is more related to itself than either its

parents or siblings. Therefore a focal offspring is expected to demand more resources than the parental optimum to each offspring (Godfray & Parker 1991). Battleground models indicate the potential of resource allocation between parents and offspring, but they do not make direct predictions about the outcome of parent-offspring conflict (Godfray 1995b). In contrast, the aim of a resolution model is to make a biologically realistic prediction about resource allocation by incorporating assumptions about the phenotypic interaction between the parent and offspring. Resolution models can include some likely behaviour (e.g. nestling begging behaviour) and physiological interactions between parents and offspring, which mean that they can potentially produce testable predictions for empirical studies. For example, in a resolution model by Eshel & Feldman (1991), parental provisioning is determined only by offspring behaviour and the parent's optimum response is to provide resources to increase offspring fitness. Due to their biological complexity, resolution models are mathematically harder to formulate than battleground models (Godfray 1995b).

1.2.2 Resolution models of parent-offspring conflict

The first verbal resolution model of parent-offspring conflict was described by Trivers (1974). In this model, offspring are suggested to 'psychologically manipulate' their parents through begging behaviour in order to gain a greater proportion of parental investment than parents would ideally give. Zahavi (1977) described a different verbal resolution model of parent-offspring conflict in which offspring are suggested to produce noisy begging displays in order to attract the attention of predators, thereby reducing their own fitness levels. As parents are expected to provide resources to reduce conspicuous begging behaviour, Zahavi (1977) suggested that offspring 'blackmail' their parents for additional resources. Following on from these early theoretical treatments of begging behaviour, a number of quantitative resolution models have been developed which have incorporated different aspects of Trivers's and Zahavi's verbal models. Later models have had a tendency to predict the outcome of resource distribution between parents and their offspring as a result of either: 1) scramble competition between siblings (Macnair & Parker 1979; Parker 1985; Parker & Macnair 1979; Stamps et al. 1978), 2) honest signalling of offspring need (Godfray 1991; Godfray 1995b; Grafen 1990) or 3) a combination of offspring competition and need (Parker et al. 2002; Royle et al. 2002).

The first scramble competition models by Parker & Macnair (1979) suggested that parents allocate food resources in relation to offspring begging intensity and that offspring respond to increased resources by begging less. Parker & Macnair (1979) split parent-offspring conflict into two different types: inter-brood (between broods) and intra-brood (within a brood) conflict. During inter-brood conflict, offspring are suggested to manipulate resistant parents for additional food resources at the parents expense of investing in future generations (Parker & Macnair 1979). During intra-brood conflict, the total investment that parents provide to their offspring is fixed and offspring manipulate their parents for limited food resources while parents act passively by feeding offspring displaying the greatest begging intensity (Macnair & Parker 1979). At equilibrium in scramble competition models, parent-offspring conflict is resolved because the costs involved with begging mean that there is a limit to begging behaviour and sibling competition (Parker & Macnair 1979). Further models of sibling competition have shown that competition between siblings within a brood for limited parental food resources can lead to the evolution of begging displays (Harper 1986; Parker et al. 1989). Models of sibling competition yield three predictions: 1) begging intensity should reflect nestling competitive ability, 2) parents should feed nestlings showing greatest begging intensity and 3) begging should be costly.

Alternatively, honest signalling models suggest that nestling begging behaviour indicates levels of offspring condition, and parents are expected to actively distribute food to offspring with the greatest need following assessment of individual begging levels (Godfray 1991; Godfray 1995b). After assessment of condition, parents should allocate food according to the reproductive value of each offspring (Clutton-Brock 1991). At equilibrium in signalling models, the costs of begging behaviour are thought to keep the signal honest (Godfray 1991; Godfray 1995b). There are three main predictions associated with honest signalling models: 1) begging intensity should reflect nestling need, 2) parents should feed nestlings showing greatest begging intensity and 3) begging should be costly.

Some of the key predictions produced by scramble competition and honest signalling models are the same for both types of model (Parker et al. 2002; Royle et al. 2002). Two key predictions shared by both types of model are that parents should provision

offspring in relation to begging intensity and that there are costs involved with begging behaviour (Kilner & Johnstone 1997). The difference between scramble and honest signalling models is defined by how parents allocate food between offspring. In models of scramble competition, parents passively allocate food to offspring displaying the greatest begging intensity, and therefore, offspring have control over food allocation. In honest signalling models, parents actively choose to feed offspring with the greatest need, and therefore, parents have control over food allocation. The reasons why offspring increase their begging behaviour are different according to whether parents are allocating food passively or actively (Royle et al. 2002). When parents allocate food passively, sibling competition can enhance begging intensity and offspring with the greatest need must increase begging behaviour further still to receive parental food. When parents allocate food actively, only offspring need drives begging behaviour with no additional enhancement from sibling competition.

1.3 EMPIRICAL STUDIES OF PARENT-OFFSPRING CONFLICT

1.3.1 Sibling competition

There are a number of empirical studies that support the idea that competition between siblings influences begging behaviour. Empirical studies can be classified as observational or manipulative. While observational studies can be useful to show potential relationships between variables, only manipulative studies can show true cause and effect.

Within broods, the presence of nestmates has been found to increase the overall begging intensity of each nestling (Harper 1986; Price 1996; Price & Ydenberg 1995; Smith & Montgomerie 1991). An observational study by Harper (1986) showed that nestling begging intensity in eight cotingid species (*Cotingidae*) with only one nestling per brood was lower than the begging intensity with two nestlings or more per brood. In two experimental studies which manipulated nestling begging levels in yellow-headed blackbirds, *Xanthocephalus xanthocephalus* (Price 1996; Price & Ydenberg 1995) and American robins, *Turdus migratorius* (Smith & Montgomerie 1991), it was found that nestlings increased their begging intensity in response to hungrier nestmates. In addition, an experimental study by Muller & Smith (1978) found that the broadcast of playback begging calls to broods of zebra finches, *Taeniopygia guttata* increased

nestling begging behaviour. In the hole-nesting starling, *Sturnus vulgaris* it has been observed that nestlings compete with each other for access to the front of the hole, which is the only position in the nest where they can receive food from parents (Kacelnik et al. 1995).

There have been a number of observational studies showing that parents preferentially feed nestlings with the greatest begging intensity in the presence of other siblings. For example, Stamps et al. (1989) found that when nestling budgerigars, *Melospitticus undulates* begged more than their siblings, they received more parental food. Furthermore, there is strong correlative evidence to show that parents preferentially feed offspring that are: 1) closest to the parent, 2) first to beg and 3) highest in the nest (Gottlander 1987b; Greigsmith 1985; Kacelnik et al. 1995; McRae et al. 1993; Ploger & Mock 1986; Ryden & Bengtsson 1980; Smith & Montgomerie 1991; Stamps et al. 1985; Stamps et al. 1989). By contrast, manipulation studies on European starlings, *Sturnus vulgaris* suggest that nestling begging behaviour does not depend on the behaviour of nestmates and is purely a function of individual need (Cotton et al. 1996).

1.3.2 Signalling need

Empirical studies have also provided support for honest signalling models in which parents have an active choice on how food is distributed among their offspring. There have been a number of studies to show that begging behaviour reflects offspring need. For example, manipulative studies on pigeons, *Columba livia* (Mondloch 1995), magpies, *Pica pica* (Redondo & Castro 1992) and yellow-headed blackbirds (Price & Ydenberg 1995) have shown that offspring begging behaviour correlates with need and that parents base food allocation decisions according to begging signals. In addition, several manipulative studies have shown that begging intensity increases with food deprivation (Kilner 1995; Price & Ydenberg 1995; Smith & Montgomerie 1991). Food deprivation in yellow-headed blackbird nests has been found to result in nestlings begging at a higher rate (Price & Ydenberg 1995). In a study which experimentally prevented American robin parents from feeding part of their brood, it was found that food-deprived nestlings increased their begging intensity, which subsequently resulted in these nestlings receiving more food from the parents (Smith & Montgomerie 1991). Nestling begging behaviour has also been found to decrease with food supplementation

in magpies (Redondo & Castro 1992) and yellow-headed blackbirds (Price & Ydenberg 1995).

In addition to these studies, parents have also been shown to respond to need over and above sibling competition. An experimental study on canaries, *Serinus canaria* showed that parents consistently fed nestlings most in need, even when the nestlings were not closest to the parent (Kilner 1995). Other studies have also shown that male and female parents do not feed young of different sizes equally, with females often preferring to feed smaller offspring that may be expected to lose sibling competition (Lessells 2002). For example, observational studies on pied flycatchers (Gottlander 1987b), great tits, *Parus major* (Bengtsson & Ryden 1981), budgerigars (Stamps et al. 1985) and crimson rosellas, *Platycerus elegans* (Krebs et al. 1999) have shown that females preferentially feed smaller young.

Furthermore, nestling begging intensity may not only signal a need for food but may also signal other requirements, such as a need for warmth (Evans 1990; Evans et al. 1994). In a manipulation study, Evans (1990) found that whilst still in the egg, the young of white pelicans, *Pelecanus erythrorhynchos* increased their calling rate as temperature decreased. In addition, after hatching, begging calls in the same species induced parental brooding rather than provisioning (Evans et al 1994).

1.3.3 Other factors affecting parental provisioning

If parents do actively choose which offspring to feed, they are unlikely to rely on begging intensity alone (Kilner & Johnstone 1997). By relying solely on begging intensity to allocate resources, it would be expected that parents would sometimes feed nestlings with low fitness returns. For example, small nestlings may beg more than large nestlings (Bengtsson & Ryden 1983; Price & Ydenberg 1995) but their chances of survival are often lower (Boland et al. 1997; Stoleson & Beissinger 1995). Therefore, in addition to begging signals, parents may also use alternative cues not controlled by nestlings to allocate food resources, such as nestling mass, and parents may successfully raise more young by investing their resources in larger nestlings, especially when food is limited (Lack 1968). Hatching asynchrony is a widespread phenomenon in birds in which the last laid eggs of a brood hatch later than the first

(Mock & Parker 1997). Across a range of passerine and non-passerine species, hatching asynchrony results in a feeding hierarchy within the brood and there can be considerable size differences between nestlings (Krebs 2001; Margalida et al. 2004; Martinez-Padilla et al. 2004; Muller et al. 2003; Price & Ydenberg 1995). For example, in yellow-headed blackbirds hatching is spread over 72 h which results in a size hierarchy between nestlings. In this species, large nestlings receive food from parents more often, even when smaller siblings beg relatively more (Price & Ydenberg 1995). Hatching asynchrony also results in size differences between canary offspring (Schwabl 1996). Kilner (1995) found that larger canary nestlings are able to gain closer access to the parent than smaller siblings, although once nest position was experimentally controlled for, canary parents were found to provision on a basis of need. Several other studies have also found that nestling size can affect nestling proximity to the feeding parent (Löhrl 1968; Mead & Morton 1985; Ryden & Bengtsson 1980). In addition, parents may also bias their provisioning to nestlings of one sex when the fitness returns of sons and daughters differ (Trivers & Willard 1973).

It is often hard to tell from empirical studies whether parents are passively or actively allocating food among offspring. Parents arriving at a nest with food to face a brood of begging young may either passively feed whichever nestling has competed most successfully among siblings (i.e. sibling competition models), or actively feed a particular nestling after assessing offspring condition (i.e. honest signalling models). The difficulty in distinguishing between the two types of feeding patterns lies in the fact that offspring that most successfully compete with other siblings are expected to be the fittest individuals most likely to survive as adults and should therefore be favoured by parents (Kilner & Johnstone 1997). Recent models developed by Parker et al. (2002), have combined offspring competition and need by extending the sibling-competition scramble model by Parker et al. (1989) and compared the results with Godfray's (1995a) model of honest signalling. Parker et al. (2002) models show, that when parents allocate food according to scramble competition between siblings, begging levels and the amount of food gained by offspring will be determined by a combination of offspring competitive ability and true need.

1.3.4 Costs of begging behaviour

Theoretical signalling systems may only be evolutionary stable if the signal carries associated costs (Godfray 1995a; Kilner & Johnstone 1997). The costs associated with offspring begging behaviour may be necessary for the maintenance of honest advertisement as implied by the handicap principal (Zahavi 1977). Several potential costs of begging behaviour have been investigated including: 1) predation risk (Briskie et al. 1999; Haskell 1994; Leech & Leonard 1997; Macnair & Parker 1979), 2) energetic costs (Leech & Leonard 1996; Macnair & Parker 1979), 3) growth costs (Kilner 2001) and 4) physiological costs through endogenous testosterone (T) production (Buchanan et al. 2001; Evans et al. 2000; Folstad & Karter 1992; Johnsen 1998). Previous studies have provided evidence for the costs involved with begging:

1) Empirical studies on nestling western bluebirds, *Sialia mexicanus* (Haskell 1994) and nestling tree swallows, *Tachycineta bicolor* (Leech & Leonard 1997) have shown that noisy begging behaviour attracts unwanted attention from predators. In these studies where playback sounds of begging calls were broadcasted from artificial nests baited with eggs, it was found that more eggs were predated from noisy nests than from silent control nests (Haskell 1994; Leech & Leonard 1997). In addition, if the rate of begging calls broadcasted from a nest was increased, the rate of predation at these nests also increased (Haskell 1994). A study by Briskie et al. (1999) on 24 bird species has also shown that species which are subject to greater levels of nest predation produce calls with a higher frequency (pitch) and lower amplitude (loudness) than species subject to lower rates of nest predation. The results of the Briskie et al. (1999) study suggest that as this type of call structure is difficult for a potential predator to locate, an increased risk of predation has led to the evolution of begging calls that minimize location by predators. Furthermore, the study indicated that attracting predators is a direct cost of begging in nestling birds (Briskie et al. 1999).

2) More controversial, are energetic costs of begging behaviour which have recorded oxygen consumption measured in a respirometry machine while nestlings are begging at intensities of their own choice. Through this method, some studies have reported that begging is energetically expensive (Macnair & Parker 1979) and that increases in resting metabolic rate (RMR) may be costly in conditions of low food availability

(Leech & Leonard 1996). In contrast, other studies have also reported that there are no significant energetic costs associated with begging behaviour and have suggested that begging is relatively cheap (Bachman & Chappell 1998; Chappell & Bachman 1998; McCarty 1996). However, the results of these later studies may have reported inaccurate measurements of energetic costs of begging (Kilner 2001).

3) In a study in which pairs of nestling canaries were forced to beg for different durations, Kilner (2001) showed that there were short and long term growth costs associated with begging behaviour. In the same study, Kilner (2001) showed that enforced begging had the least effect on older nestlings that were nearly fully grown, and also nestlings that begged most intensively lost the most mass attributed to metabolic expenditure.

4) There have been a number of studies to suggest that there are costs involved with the production of T. In a range of bird species, including red-winged blackbirds, *Agelaius phoeniceus* (Johnsen 1998), circulating T levels have been found to influence male aggressive behaviour towards other males. In the house sparrow, *Passer domesticus* Buchanan et al. (2001) found that an elevation of T within naturally occurring ranges increased basal metabolic rate (BMR) and suggested that T is a potential energetic cost. T levels have also been found to correlate with corticosterone, the avian stress hormone (Evans et al 2000). Finally, the immunocompetence handicap hypothesis (ICHH) states that as T is responsible for the production of signals (e.g. male secondary sexual signals) and is also immunosuppressive, the cost of being able to express signals is decreased immune function (Folstad & Karter 1992; Roberts et al. 2004). If signals depend on T for their development, high levels of T will be needed for the development of a large signal and low quality individuals are expected to be unable to withstand the costs involved with signalling, which keeps the signal honest (Folstad & Karter 1992). If increased T levels in nestling birds produces increased physiological costs, this would infer T-mediation of the cost of nestling begging displays.

A confounding factor concerning the costs involved with nestling begging displays is that individuals may differ in the ease with which they can bear the costs of begging (Kilner & Johnstone 1997). For example, good quality individuals may not suffer such

high costs associated with T production (Folstad & Karter 1992), but also, these costs may not be so detrimental to individuals most able to tolerate high T levels. Different ages and sizes of nestlings within a brood as a result of hatching asynchrony can also affect the impact of costs on begging nestlings. Studies have shown that older, stronger nestlings can afford the costs involved with a greater begging effort for the same level of hunger more than younger nestlings (Godfray 1995a; Kilner 1997; Parker et al. 2002). For this reason experimental manipulative studies are the only real way to test the factors controlling begging signals.

1.4 PHYSIOLOGICAL CONTROL OF BEGGING

1.4.1 The neuroendocrine system

Two main systems of internal communication including: the nervous system and the endocrine system, interact to control physiology and behaviour in animals (Brown 1994). Because these systems are not independent from each other, they are often referred to as the neuroendocrine system (Blalock 1989), but due to their different structural organisations, each system plays a different role in communication.

The nervous system is responsible for maintaining contact between an animal's internal and external environment and for adjusting physiology and behaviour in response to external environmental cues (Benzo 1986). In vertebrates, the nervous system can be subdivided anatomically into the central nervous system (CNS) and the peripheral nervous system (PNS), the latter includes the cranial and spinal nerves that stem from the brain and spinal cord. During development, the CNS develops from a long hollow tube of cells called the neural tube. As the brain develops, the neural tube forms three lobes which eventually form the forebrain, midbrain and hindbrain in adults (Becker & Breedlove 1992). In studies of behavioural endocrinology, most research is focussed on the forebrain, which in birds is composed of the cerebral hemispheres, the thalamus and the hypothalamus (Benzo 1986). The hypothalamus, which is a relatively small region at the base of the forebrain, is the neural control centre for all endocrine systems in vertebrates (Becker & Breedlove 1992). The nervous system is involved with three processes including: sensory input, integration and motor output (Campbell 1993). Sensory input is the intake of external environmental cues through sensory receptors (e.g. visual, auditory or tactile) which then transform environmental stimuli into signals

that are passed along sensory neurons to the CNS for processing. Integration occurs in the CNS and refers to the process by which information from sensory receptors is interpreted in order to conduct a response to the appropriate part of the body. Motor output, is the conduction of signals from the CNS through the motor control system along motor neurons to the appropriate target muscle or gland cells that actually carry out the body's response to the environmental stimuli.

The nervous system controls the release of hormones from endocrine glands (Muller & Nistico 1989). Birds have similar endocrine organs to mammals and lower vertebrates including: 1) the pituitary-hypothalamus complex, 2) gonads, 3) pancreatic islets, 4) adrenal glands, 5) thyroid glands, 6) parathyroid gland, 7) ultimobranchial gland and 8) the endocrine cells of the gut (Scanes 1986). Animal hormones (from the Greek *hormon*, meaning 'excite'), may be defined as chemical messengers that are synthesised in ductless (endocrine) glands and are secreted into the bloodstream (Brown 1994). Hormones produced by the endocrine glands travel in the bloodstream to distant target cells where they act on receptors of the target cells to exert a specific physiological or biochemical regulatory action. Attached to the hypothalamus is the pituitary, which is a very important endocrine gland that stimulates other endocrine glands to synthesise and secrete their hormones. The pituitary gland is divided into three parts: the anterior lobe (*pars distalis*), the intermediate lobe (*pars intermedia*) and the posterior lobe (*pars nervosa*). The anterior and intermediate lobes form a true endocrine gland (called the adenohypophysis), but the posterior lobe is really neural tissue and is an extension of the hypothalamus (Brown 1994). The hypothalamus controls the action of the pituitary gland through the secretion of neurohormones, which are produced and released from specialised neurons in the hypothalamus called neurosecretory cells (Becker & Breedlove 1992). One class of neurohormones, which includes oxytocin and vasopressin, are synthesised in neurosecretory cells and are transported in axons of the neurosecretory cells to the posterior pituitary. Oxytocin (primarily responsible for contractions at birth and lactation processes), and vasopressin (responsible for water reabsorption in the kidneys), are stored in nerve terminals of the posterior pituitary from where they are released directly into the bloodstream. A second class of neurohormones called releasing hormones is secreted from the hypothalamus into specialized blood vessels that supply the adenohypophysis. Releasing hormones

stimulate or inhibit the release of hormones by cells in the adenohypophysis which are released into the blood stream to stimulate other endocrine glands to release their hormones. Corticotrophin-releasing hormone (CRH) controls the release of glucocorticoids through the hypothalamo-pituitary-adrenal (HPA) axis, and gonadotropin-releasing hormone (GnRH) controls the release of sex steroids through the actions of the hypothalamo-pituitary-gonadal (HPG) axis (Becker & Breedlove 1992; Brown 1994). Both CRH and GnRH neurohormones may form part of the neural control required for nestling begging behaviour (Schwabl & Lipar 2002a).

In birds, as in mammals, there are six hormones produced and released from the adenohypophysis including: growth hormone (GH), adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (PRL) (Scanes 1986; Brown 1994). CRH produced by the hypothalamus stimulates corticotroph cells in the adenohypophysis to produce ACTH (figure 1.1) which acts on the adrenal cortex to synthesize and release glucocorticoid hormones (e.g. corticosterone, the avian stress hormone). In addition, GnRH from the hypothalamus stimulates the release of FSH and LH (collectively called gonadotropins) from the adenohypophysis (figure 1.1). The gonadotropins travel from the anterior pituitary in the bloodstream to the gonads where they induce gonadal maturation, maintain gonadal function and stimulate the gonads to produce sex steroids in males and females (e.g. T, progesterone, androstenedione and estradiol). The brain is a target organ for many hormones produced by endocrine glands, which in turn regulates their secretion. After stimulation from pituitary hormones, the target endocrine glands release their hormones into the bloodstream which feed back both to the hypothalamus and the pituitary. In this way, the brain continuously monitors and regulates hormone production by the endocrine glands (Brown 1994).

1.4.2 Steroid hormones

There are five major classes of steroid hormones which include: androgens, glucocorticoids, progestins, estrogens and mineralocorticoids (Kawata 1995). All steroids are synthesised from the common precursor cholesterol, although there is considerable diversity in structure in this class of compounds. T is the primary androgen in both birds and mammals (Marsh 1992). In birds, T may come from

maternal sources or may be endogenously produced, either by the adrenal cortex glands or by the gonads (e.g. Schwabl 1993; Ottinger & Abdelnabi 1997; Adkins-Regan et al. 1990). The adrenal cortex glands primarily synthesise glucocorticoids, although androgens are also produced (Adkins-Regan et al. 1990; Kawata 1995). The gonads are the main source of androgens in adult birds, although there are significant differences between species regarding the gonadal development in young birds (Adkins-Regan et al. 1990; Ottinger & Abdelnabi 1997).

Previous studies have found that androgen production by the gonads of embryos is detectable early in embryonic development in precocial birds (Ottinger & Abdelnabi 1997). For example, gonadal production of steroid hormones has been detected in domestic chicken, *Gallus domesticus* embryos at 3.5 days of incubation (Woods & Erton 1978; Woods & Podczaski 1974) and 5.5 days in the quail (Scheib et al. 1985). It has been found in both chicken and quail embryos, that the adrenal gland contains substantial concentrations of sex steroids, although after hatching the adrenal gland shifts to primarily produce corticosterone in young chickens (Ottinger & Abdelnabi 1997; Tanabe et al. 1979; Tanabe et al. 1986). In quail, steroid hormone levels in the adrenal glands are relatively constant throughout embryonic development, and in the same study it was also noted that the gonadal content of steroids in both males and females during embryonic development reflected the changes observed in the plasma level of steroids. Therefore, it was suggested that the gonads are the source of plasma steroids during development in the quail embryo (Ottinger & Abdelnabi 1997).

In altricial birds, the gonads of embryos are not known to be able to synthesise androgens, and the gonads may only become functional after hatching (Adkins-Regan et al. 1990; Silverin & Sharp 1996). Previous studies have shown that after hatching, nestlings of altricial birds have detectable levels of androgen in circulation (Naguib et al. 2004; Silverin & Sharp 1996; Williams et al. 1987), although relatively few studies have directly investigated the source of androgen levels in altricial birds (Adkins-Regan et al. 1990; Silverin & Sharp 1996). A study on male zebra finches showed that castration greatly reduced circulating androgen levels in adults, but did not reduce androgen levels in nestlings aged 7 – 9 days old (Adkins-Regan et al. 1990). It was suggested that the reason for the difference between adult and nestling castration, is that

nestling male zebra finch sex steroids are predominantly adrenal in origin, whereas adult sex steroids are predominantly gonadal (Adkins-Regan et al. 1990). The results from the study on zebra finches suggested that the shift from adrenal to gonadal production of sex steroids may take place later in development in altricial compared with precocial birds. However, there may be a difference between altricial species regarding the timing of when the gonads are able to produce sex hormones. A previous study on great tits has shown that the gonads of nestlings can produce T as early as 6 days post hatching (Woods et al. 1975). In another study on great tits by Silverin & Sharp (1996), castration of 9 day old male nestlings significantly reduced circulating T levels. Furthermore, in the same study, injections of GnRH into great tit nestlings aged 9 days old, significantly increased circulating levels of LH and T in males, and increased circulating levels of LH and estradiol in females. Injections of GnRH increased plasma levels of LH in great tit nestlings younger than 9 days old, although the earliest age that T levels were affected was at 6 days old, and then only in male nestlings. Therefore, in great tits, the hypothalamus and the pituitary may not become a fully functional unit until nestlings are between 6 – 9 days old (Silverin & Sharp 1996). Another study on male song sparrows, *Melospiza melodia* and swamp sparrows, *Melospiza georgiana* found that nestlings castrated at 21 – 28 days old resulted in low T levels, which suggests that the gonads of these nestlings were producing sex steroids at this age (Marler et al. 1988). Overall, in precocial birds, the shift from adrenal to gonadal production of sex steroids can occur during embryonic development, but in altricial birds, the shift may occur later during the first month after hatching (Adkins-Regan et al. 1990).

Androgens are produced by both sexes in birds. In the gonads of adult male birds, LH acts primarily to stimulate the Leydig cells in the testies to differentiate and produce T, while FSH promotes spermatogenesis. In the gonads of adult female birds, the major site of androgen production are the cells in the walls of each developing follicle (Scanes 1986). In adult domestic fowl, androgen production in the follicles which is stimulated by LH, peaks two or three days before the ovulation of each follicle and ceases just before ovulation (Yu et al. 1992). In young chickens and quail, males have higher levels of androgens than females, and females have higher levels of estrogens than males (Woods & Podczaski 1974; Woods et al. 1975). In chicken embryos, T has been

detected on day 5.5 of incubation, and from day 7.5 through to day 17.5 male embryos have significantly higher plasma T levels than females (Woods et al 1975). However, in altricial birds, including zebra finches and canaries, it has been found that after hatching nestling females have higher androgen levels than nestling males during development (Adkins-Regan et al. 1990; Weichel et al. 1986).

1.4.3 Steroid hormones and begging behaviour

Two types of steroid hormones: endogenously produced corticosterone (Kitaysky et al. 2003; Kitaysky et al. 2001b) and maternally-derived androgens, (Eising & Groothuis 2003; Schwabl 1996a) have been shown to influence begging behaviour in nestling birds. In addition, other studies have found a positive covariation between endogenously produced T and measures of begging behaviour (Goodship & Buchanan 2006; Quillfeldt et al. 2006; Sasvári et al. 1999).

Begging is the first coordinated behaviour performed by altricial birds after hatching (Schwabl & Lipar 2002b). In the very early stages of development soon after hatching, the neuroanatomical substrates required for begging including: the sensory organs, brain, motor control system and muscles, are poorly developed (Starck 1993). In very young birds, begging is a simple reflex that may be controlled by hunger and satiety signals from the hypothalamus or external tactile cues (Schwabl & Lipar 2002b). As nestlings develop and their neuroanatomical substrates mature, begging becomes a more complex behaviour which could be influenced by external signals from parents and siblings as well as internal signals such as hormones produced by the endocrine system (Andrew 1975b; Clifton et al. 1988). In addition, as nestlings get older, experience and learning also influence begging behaviour (Kedar et al. 2000). As begging is essential for nestling growth and survival, it would be expected that selection favours the development of this behaviour and all associated controlling mechanisms.

1.4.4 Endogenous corticosterone

Corticosterone is the dominant glucocorticosteroid in birds (Holmes & Phillips 1976). In a range of avian species, females are known to deposit corticosterone into their egg yolks. For example, female corticosterone levels at the time of laying have been found to correlate with corticosterone in the egg yolks of Japanese quail, *Coturnix coturnix japonica* (Hayward & Wingfield 2004). In an experimental study on chickens, maternal corticosterone has been shown to increase distress vocalizations and pecking response of chicks (Freire et al. 2006). It was found in the study by Freire et al. (2006), that when corticosterone was injected into eggs next to the embryo on day 18 of incubation, chicks hatching from the treated eggs emitted more distress vocalizations and had an increased pecking rate compared with control chicks. Links between nestling begging behaviour and corticosterone have been investigated in studies concerned with endogenous corticosterone production and behaviour (Kitaysky et al. 2001b). In a range of avian species, nestlings are known to produce corticosterone after hatching including: canaries (Schwabl 1999), black-legged kittiwakes, *Rissa tridactyla* (Kitaysky et al. 1999), blue-footed boobies, *Sula nebouxii* (DelaMora et al. 1996) and American kestrels, *Falco sparverius* (Heath & Dufty 1998; Sockman & Schwabl 2001). Experimental studies have shown that corticosterone levels increase with food shortage and are negatively correlated with body condition in black-legged kittiwake (Kitaysky *et al.* 1999) and red-legged kittiwake, *Rissa brevirostris* nestlings (Kitaysky et al. 2001a). Food restriction has also been shown to increase circulating corticosterone levels in blue-footed boobies (DelaMora et al. 1996), and corticosterone levels have been found to be negatively correlated with body condition in 5 day old American kestrels (Sockman & Schwabl 2001). In contrast, other studies have not found a correlation between nestling corticosterone levels and body condition in northern mockingbirds, *Mimus polyglottos* (Sims & Holberton 2000), canaries (Schwabl 1999), or in another study on American kestrels (Heath & Dufty 1998). Recent studies by Kitaysky et al. (2001b; 2003) on black-legged kittiwakes have found that experimentally elevated nestling corticosterone levels increase begging effort and food provisioning rates by parents. Additionally, Kitaysky et al. (2001b) also found that nestlings in broods of two implanted with corticosterone begged more frequently than singletons. From this study, it was suggested that begging behaviour may be regulated by corticosterone, and the costs involved with elevated corticosterone levels (Kitaysky

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allow for raising a full brood (Schwabl et al. 1997), and may thus be a mechanism for brood reduction (Lack 1947). Overall, the differential allocation of maternal T into egg yolks may represent a hormonal mechanism by which mothers influence the survival of their offspring, either by counteracting the competitive hierarchy caused by hatching asynchrony (e.g. Eising et al. 2001) or by enhancing it (e.g. Schwabl et al. 1997).

In addition to yolk T levels being positively correlated with elevated aggression levels (e.g. Schwabl 1993), three studies also have shown that when T levels are experimentally elevated in the egg yolks of canaries (Schwabl 1996a), black-headed gulls (Eising & Groothuis 2003) and zebra finches (Engelhardt et al. 2006), nestlings hatching from T-treated eggs display a greater begging effort than nestlings hatching from control eggs. In canaries, Schwabl (1996a) found that T treatment increased the number of begging bouts, the total time spent begging and the duration of a begging bout within the first hour after hatching. There may be benefits of higher yolk T levels to nestling growth and development, as it has been found that nestlings hatching from T-treated eggs can have a greater body mass and tarsus length (Eising et al. 2001; Engelhardt et al. 2006; Schwabl 1996a) and nestlings also hatch sooner which may substantially decrease hatching asynchrony (Eising et al. 2001; Eising & Groothuis 2003). Due to increased begging behaviour in the first 24 h after hatching from T-treated eggs, canary nestlings may be able to gain more parental food which results in a faster growth rate (Schwabl 1996a). Black-headed gull nestlings hatching from T-treated eggs were found to beg more frequently, be more alert and respond faster to a parent returning to the nest with food during the first week after hatching compared with control nestlings (Eising & Groothuis 2003). In a study by Engelhardt et al. (2006) it was found that T-treatment of zebra finch eggs increased female begging duration, but did not affect begging effort of males. The improved growth of T-treated female nestlings and reduced growth of males in this study, may have been directly due to more persistent begging in female siblings, which would allow them to out-compete their male siblings (Engelhardt et al. 2006).

Female circulating hormone levels depend on a wide array of factors, ranging from the time of day to the stage of the reproductive cycle (Sockman & Schwabl 1999). At present it is not fully understood if mothers actively allocate T into their eggs and

thereby control offspring begging behaviour, or whether T allocation is a passive process of hormone deposition into the egg (Birkhead et al. 2000; Gil 2003). The ability of a female to allocate hormones into egg yolks depends on the timing of copulation relative to egg-laying, and also the timing of androgen deposition in the developing ovarian follicles. Steroid hormones are lipophilic and may pass from adult females into the lipoprotein matrix of egg yolk during vitellogenesis (egg yolk synthesis) (Schwabl & Lipar 2002a). There is some evidence to show that maternal T allocation is a passive process, as yolk T levels correlate with circulating plasma T levels in female canaries (Schwabl 1996b). However, other studies have suggested that females may be able to actively influence the quality of their offspring by varying the amount of androgens within each egg in accordance with perception of their partner's quality (Gil et al. 1999; Gil et al. 2004). For example, a study on captive zebra finches found that adult females deposit relatively more T and DHT into their eggs when paired to an attractive male (Gil et al. 1999). It has been previously shown that the attractiveness of male zebra finches can be manipulated by the addition of colour rings, red rings render males more attractive and green rings reduce male attractiveness (Burley et al. 1982). In the study by Gil et al. (1999), females put significantly more androgens into their eggs yolks when their male partner wore red rings compared when the same male wore green rings. As elevated androgen levels may be costly, for example they suppress the immune system (Evans et al. 2000; Roberts et al. 2004) and reduce survival (Birkhead et al. 1999), only offspring fathered by high quality males may be able to withstand the high concentration of androgens in egg yolks (Gil et al 1999). It has also been found that male song quality can also influence female T deposition into egg yolks. In an experimental study on canaries using play-back song, females exposed to attractive male song (songs were longer, had a greater repertoire and contained special song phrases; Vallet & Kreutzer 1995) deposited more T into their egg yolks than when the same females were exposed to unattractive male song (Gil et al. 2004). However, in another similar study on canaries, females did not lay eggs with more T when exposed to attractive male song (Marshall et al. 2005). Yolk T, whether actively or passively allocated into eggs, may vary adaptively between species with different optimum reproductive strategies (Eising et al. 2001; Schwabl et al. 1997; Whittingham & Schwabl 2002).

1.4.6 Endogenous T

It is not yet known how maternally-derived T in egg yolks relates to offspring endogenous T production. Although yolk T may enhance embryonic development within the egg (e.g. by increasing the mass of the begging muscle, *musculus complexus*) and thus would correlate with nestling begging behaviour post hatching (Schwabl 1996a; Eising & Groothuis 2003), steroids have short half-lives (Goymann et al. 2002a) and yolk T is likely to be fully metabolised within days of egg laying. Therefore, to understand more fully the relationship between nestling circulating T levels and begging, studies are now required to investigate the effects of nestling endogenous T production on behaviour. As mentioned in section 1.4.2, studies on precocial birds have found that embryos within eggs are able to produce their own T prior to hatching (Adkins-Regan et al. 1990; Ottinger & Abdelnabi 1997; Scheib et al. 1985; Woods & Erton 1978; Woods & Podczaski 1974). Other studies have also found that altricial nestlings can produce their own T from a young age after hatching (Adkins-Regan et al. 1990; Marler et al. 1988; Silverin & Sharp 1996). For example, circulating plasma T levels have been measured in nestling European starlings, (Williams et al. 1987), great tits *Parus major* (Silverin & Sharp 1996), European robins, *Erithacus rubecula* (Schwabl & Lipar 2002b) and zebra finches (Adkins-Regan et al. 1990; Naguib et al. 2004). Previous studies have correlated nestling endogenous T with measures of begging effort (Goodship & Buchanan 2006; see chapter 3). Quillfeldt et al. (2006) found that T and corticosterone levels of thin-billed prion, *Pachyptila belcherie* nestlings were positively correlated with measures of begging behaviour. The single offspring in each nest, as is typical of petrels, showed a positive covariation between body condition, begging calls and circulating levels of T. In broods of white storks, *Ciconia ciconia* first hatched nestlings, that have high plasma T levels, respond faster to feeding parents and receive more food than their younger siblings (Sasvári et al. 1999). Circulating T levels have been previously elevated in black-headed gull, *Larus ridibundus* nestlings using T implants (Groothuis & Meeuwissen 1992; Groothuis & Ros 2005). However, it was found from these T manipulation studies that although T-treated nestlings were more aggressive towards other siblings, there was a significant decrease in begging behaviour compared with control birds.

1.4.7 Potential mechanisms of hormones in begging

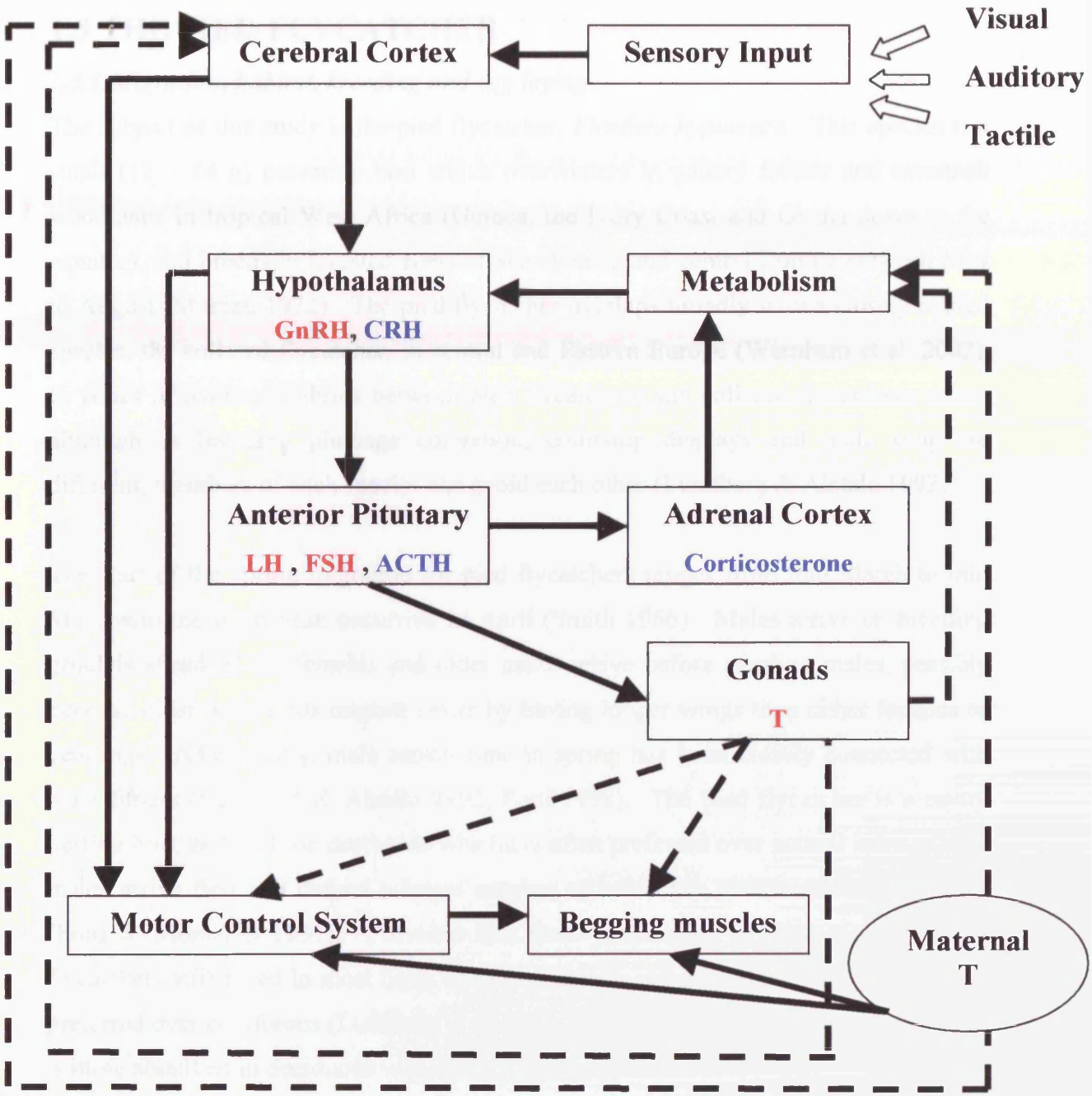
There are several potential mechanisms through which T may control begging behaviour in birds (figure 1.1).

First, Schwabl (1996a) suggested in his study investigating the effect of yolk T on the begging effort of canary nestlings, that T may increase metabolic rate which could cause earlier and intensified begging. However, as yet, there has been no direct study linking T levels with increased metabolic rate incurred during begging behaviour. T has been shown to increase BMR in adult male house sparrows (Buchanan et al. 2001), although another study found that T implants decreased RMR in white-crowned sparrows, *Zonotrichia leucophyrys gambelii* (Wikelski et al. 1999) and had no effect on BMR in white-plumed honeyeaters, *Lichenostomus penicillatus* (Buttemer & Astheimer 2000). Glucocorticoids (e.g. corticosterone) are also known to be important during the development of organs such as the lung and gut at the time of hatching, which in turn may play an important role during nestling respiration and feeding (Scanes et al. 1987). In adult birds, increased levels of endogenous corticosterone also activate stored energy reserves which can allow for increased locomotory activity (Astheimer et al. 1992; Bray 1993; Harvey & Klandorf 1983). As corticosterone and T levels are thought to be correlated in birds (Duffy et al. 2000; Evans et al. 2000; Poiani et al. 2000; Roberts et al. 2004; Schoech et al. 1999; Sockman & Schwabl 2001) and corticosterone and T bind to the same plasma binding globulin (Deviche et al. 2001; Klukowski et al. 1997), a rise in corticosterone levels during an increase in metabolic activity may be positively related to a rise in T levels.

Second, T may also enhance brain function and perception of cues which stimulate begging behaviour (Andrew 1975a; Andrew 1975b; Andrew 1994; Clifton et al. 1988; Godsave et al. 2002). In studies on domestic fowl chicks, T treatment has been shown to enhance attention by increasing binocular fixation and persistence (Andrew 1975a; Andrew 1975b; Clifton et al. 1988). Furthermore, a study on black-headed gulls by Eising & Groothuis (2003), found that in nine out of ten behavioural scores, nestlings hatching from T-manipulated eggs were quicker or more persistent in their begging behaviour than control nestlings. A recent study which investigated the distribution of androgen receptors (AR) in the developing zebra finch brain, found that AR are

expressed in male and female embryos from 7 days old (Godsave et al. 2002). As AR expression begins in the embryo before the gonads are fully functional, androgen production is very low when AR are first expressed in the embryonic hindbrain at 7 days (Galli & Wasserma 1973). Therefore, the major source of androgens available to the embryo for binding to the hindbrain AR, may come from maternal androgens deposited into egg yolks (Gil et al. 1999; Godsave et al. 2002; Schwabl 1993) or from an endogenous source produced by the adrenal glands (Adkins-Regan et al. 1990).

Third, T may increase motor neurone activity and influence the development of muscles used for begging (Godsave et al. 2002; Lipar & Ketterson 2000). For example, the *musculus complexus* in birds is a large, dorsally located neck muscle that receives signals from the brain via the *nucleus supraspinalis,* which is a motor neuron that plays an important role in the nestling begging response (Schwabl & Lipar 2002). The *m. complexus* has two main functions: 1) it is used to break through the shell at hatching (Gross 1985) and 2) it is the main muscle used to bend and extend the neck during begging behaviour (Ashmore et al. 1973). A recent manipulation study on red-winged blackbirds has found that the mass of the *m. complexus* of nestlings hatching from T-treated eggs is greater than that of nestlings hatching from control eggs (controlled for nestling size) (Lipar & Ketterson 2000). Additionally, an injection of flutamide (T antagonist) into egg yolks decreases the relative *complexus* mass of nestlings (Lipar & Ketterson 2000). If larger muscles are stronger, then the increased mass of the *m. complexus* would mean that T-treated nestlings could break through their shells more efficiently and could extend their necks higher during begging (Lipar & Ketterson 2000). In addition to influencing the overall size of the *m. complexus*, T may also affect the motor control system underlying the begging muscle. For example, a study on canary nestlings has found that the *n. supraspinalis* has androgen receptors (Gahr et al. 1996) which may be active during begging. As previously discussed, zebra finch embryos aged 8 days old express AR in motor neurons innervating the upper neck region (Godsave et al. 2002). Furthermore, in other non-avian vertebrates, T has also been shown to influence the development of the musculosketal system (Kawata 1995). The administration of T has been found to increase muscle mass in the forelimbs of leopard frogs, *Rana pipiens* (Sidor & Blackburn 1998) and the sonic muscle in two species of fishes, *Porichthys notatus* (Brantley et al. 1993) and *Cynoscion regalis* (Connaughton & Taylor 1995).



Key

GnRH: Gonadotropin-releasing hormone
CRH: Corticotropin-releasing hormone
LH: Luteinizing hormone
ACTH: Adrenocorticotropic hormone
FSH: Follicle-stimulating hormone
T: Testosterone
– – No empirical evidence for mechanism pathway
——— Empirical evidence exists to suggest possible mechanism

Figure 1.1: Diagram illustrating endogenous testosterone and corticosterone production and how these two hormones may control begging behaviour.

1.5 THE PIED FLYCATCHER

1.5.1 Migration, habitat, breeding and egg laying

The subject of this study is the pied flycatcher, *Ficedula hypoleuca*. This species is a small (12 – 14 g) passerine bird which overwinters in gallery forests and savannah woodlands in tropical West Africa (Guinea, the Ivory Coast and Ghana down to the equator), and breeds in forested areas of Scandinavia and central Europe between May to August (Moreau 1972). The pied flycatcher overlaps broadly with a closely related species, the collared flycatcher, in central and Eastern Europe (Wernham et al. 2002). In zones of overlap, hybrids between pied flycatchers and collared flycatchers occur, although as breeding plumage coloration, courtship displays and male song are different, members of each species can avoid each other (Lundberg & Alatalo 1992).

The start of the spring migration for pied flycatchers ranges from mid March to mid May, with the main peak occurring in April (Smith 1966). Males arrive on breeding grounds ahead of the females and older males arrive before yearling males, possibly because older male birds migrate faster by having longer wings than either females or yearlings. Additionally, male arrival time in spring has been closely connected with male fitness (Lundberg & Alatalo 1992; Potti 1998). The pied flycatcher is a cavity nesting bird, and will use nestboxes which are often preferred over natural holes. Older males arrive first and defend a larger number of nestboxes than males arriving later (Potti & Montalvo 1991). Provided that there are nesting cavities available, pied flycatchers will breed in most types of forest habitats, although deciduous woodland is preferred over coniferous (Lundberg et al. 1981; Silverin 1998a), possibly because food is more abundant in deciduous woodland (Alatalo & Alatalo 1979).

Upon arrival, males begin to defend territories with suitable nesting sites and when females arrive approximately one week later (Silverin 1993), males attract female attention by singing (Lampe & Sætre 1995). When a female approaches a male singing outside a nestbox, the male will repeatedly fly to and from the entrance to the nestbox while emitting a characteristic high-pitched song to encourage the female to perch at the entrance and enter the nest; this behaviour can be repeated several times over. If the female chooses to accept the male, copulations can last from 13 days before, until 2 days after, the first egg has been laid (Von Haartman 1951).

Pied flycatcher eggs are a blue/green colour and clutch size normally varies within the range of 4 – 8 eggs (mean = ± S.D.: 6.29 ± 0.03, n = 1124; von Haartman 1967), with females laying one egg per day, usually early in the morning after sunrise. Females incubate their eggs for two weeks and hatching occurs over 0 – 3 days (mean: 1.25 ± 0.54 days, n = 120; Slagsvold 1986). Eggs laid by the Welsh population of pied flycatchers are similar in dimension, shape and volume to those of the English and European populations (Kern & Cowie 1996). It has been found that egg mass which increases with laying order has a positive effect on nestling growth rate during the early nestling phase (Hillstrom 1999).

1.5.2 Mating systems

Pied flycatchers have both monogamous and polygynous mating systems. Bigyny (1 male : 2 females) often occurs, although under favourable conditions males can attract a third female (Von Haartman 1951). Polygynous species can be split into two different types: 1) monoterritorial males keep females in one single territory and 2) polyterritorial males keep females in separated distant territories (Møller 1986). Pied flycatchers are an example of a polyterritorial species (Møller 1986), and although the distance between territories can vary considerably between populations (Potti & Montalvo 1993; Silverin 1980), the success at becoming polygynous may increase with the distance between two territories as result of decreased female-female aggression (Slagsvold et al. 1992). The first males to arrive at a breeding site may defend several nest holes, but as more males arrive, territory size declines until each male is left defending only one or two nest holes. After pair formation, the male usually stays within his own territory to guard the nest hole from intruders and to protect the female against copulatory attempts from other males (Lundberg & Alatalo 1992). At the start of egg laying, some males may move away to another territory to attract a second female who is likely to be unaware of the presence of the initial female (Alatalo & Lundberg 1984). The occurrence of polygyny in pied flycatchers varies across geographical range, depending on ecological conditions such as habitat type, food availability and competition for nest sites (Slagsvold & Lifjeld 1988). For example, a study in south west Sweden found that 93% of males were polyterritorial (Silverin 1980), in northern Sweden 30% of males were polyterritorial (Nyholm 1984) while a study in central Spain found that only 4.4% of males in the population were

polyterritorial (Potti & Montalvo 1993). The fitness of two females mated with a polygynous male is lower than that of a monogamously mated female (Alatalo & Lundberg 1984) because polygynously mated females receive less food from males during incubation (Lifjeld & Slagsvold 1989a; Lifjeld et al. 1987) and less help with feeding nestlings (Alatalo et al. 1982; Lifjeld & Slagsvold 1989b; Stenmark et al. 1988). Polygynous males are more likely to be cuckolded than monogamous males but polygynous males also produce a greater number of fledging offspring (Lubjuhn et al. 2000).

Although males guard their females, extra-pair copulations occur in pied flycatchers (Alatalo et al. 1989; Brun et al. 1996; Gelter & Tegelstrom 1992; Lifjeld et al. 1991; Ratti et al. 1995), and collared flycatchers (Alatalo et al. 1989; Sheldon & Ellegren 1999), especially at high breeding densities (Bjorklund & Westman 1983). Before egg-laying, the distance between paired males and females has a significant effect on the frequency of extra-pair copulations. For example, a study by Alatalo et al. (1987) found that extra-pair copulations significantly increased if a mated male moved further than 10 m away from the female. When extra-pair copulations occur, the cuckolder tends to be a neighbouring male (Slagsvold et al. 2001). Extra-pair copulations can result in extra-pair paternity, with the consequence that some males feed offspring that are not their own and some offspring within a nest are not full siblings (Alatalo et al. 1989). Heritability can be estimated by comparing morphology between nestlings and adults. For example, tarsus length is known to be highly heritable in pied flycatchers (Alatalo et al. 1989), although there may also be environmental effects on tarsus length (Potti & Merino 1994). A study which compared nestling and adult tarsus length in 1651 nests found that the heritability estimate of the offspring on the male at the nest was 18 % lower than on the female, suggesting that 18 % of all offspring had a father that was not the male feeding the young (Alatalo et al. 1989). Recently, more reliable studies have used genetic techniques such as DNA fingerprinting to estimate relatedness (Brun et al. 1996; Gelter & Tegelstrom 1992; Lifjeld et al. 1991; Ratti et al. 1995). The extent to which extra-pair paternity occurs in pied flycatchers varies between different populations. For example, a study in Sweden on 38 nestlings from 7 broods revealed that 24 % of nestlings in 43 % of broods, were the result of extra-pair copulations (Gelter & Tegelstrom 1992). Alternatively, a study in Norway on 135

nestlings from 27 broods showed that only 4 % of nestlings in 15 % of broods were the result of extra-pair copulations (Lifjeld et al. 1991).

Egg dumping is very rare in female pied flycatchers and therefore females are almost always related to their offspring (Lundberg & Alatalo 1992). A recent study also found that male and female pied flycatchers visit nests other than their own at the time of feeding nestlings. The study suggested that males making visits to other nests late on in the breeding season were unlikely to be looking for extra-pair copulations (as females had already mated), and that it may be possible that both males and females spend time during the nestling stage looking for potential future nest sites (Ottosson et al. 2001).

1.5.3 Diet, nestling growth and begging behaviour

Pied flycatchers feed upon insects gleaned from leaves, taken from the air and picked up off the ground including: lepidoptera (butterflies and moths), diptera (flies and mosquitoes), hymenoptera (ants, bees and wasps) and coleoptera (beetles), (Lundberg & Alatalo 1992). In this study, pied flycatchers in Wales preferred to feed their nestlings on lepidoptera and diptera larvae and similar preferences have been found in other European populations (Moreno et al. 1995; Sanz et al. 2003). In this study, fieldsites were mainly composed of sessile oak trees, *Quercus petraea* upon which many moth larvae feed. Recent studies on climate change have found that increased spring temperatures over the last few decades have advanced the timing of oak leafing in European woodlands, and consequently the time of the main peak of food abundance for birds has also advanced (Penuelas et al. 2002; Sanz et al. 2003; Visser & Holleman 2001). A study on two Mediterranean populations of pied flycatchers, found that the timing of egg laying and clutch size of pied flycatchers has not been affected by the increase in spring temperatures between 1980 – 2000, although the reproductive output of pied flycatchers has been in decline which may be due to the mismatch between the timing of the peak food supply and nestling demand (Sanz et al. 2003). However, a study in Wales has found that the timing of egg laying by pied flycatchers between 1971 - 95 has advanced by 13 days (Slater 1999). Mean first-egg date has been negatively correlated with mean minimum air temperature, although fledging success has not been found to be significantly related to first-egg date (Slater 1999).

Nestlings are fed in the nest usually by both parents, although the amount of care varies with the age of the young and the pairing status of the female (Lundberg & Alatalo 1992). In the Welsh population, nestlings are in the nest for approximately 16 days until fledging, which is similar to the length of the nestling period in southwest Finland (Von Haartman 1954). Nestling periods vary between European populations, ranging from 14.7 to 16.0 days (Jarvinen 1990). In 2004, nestlings in this study from 6 pied flycatcher nests were weighed from day of hatching (day 0) until they fledged from the nest (day 16) and a growth curve of mean nestling mass on each day was calculated (figure 1.2). The young hatch from the egg naked and blind, and in this study they weighed on average 1.60 ± 0.27 g on day 0. However, during the first few days in the nest, nestling growth was rapid and mean nestling mass increased to 14.28 ± 0.32 g by day 10. From day 10 until day 16, mean nestling mass remained constant and slightly declined on the last two days. On average, nestling mass gain in this study increased at 0.95 ± 0.09 g per day between days 0 – 3, 1.75 ± 0.20 g per day between days 3 – 8 and 0.91 ± 0.39 g per day between days 8 – 10.

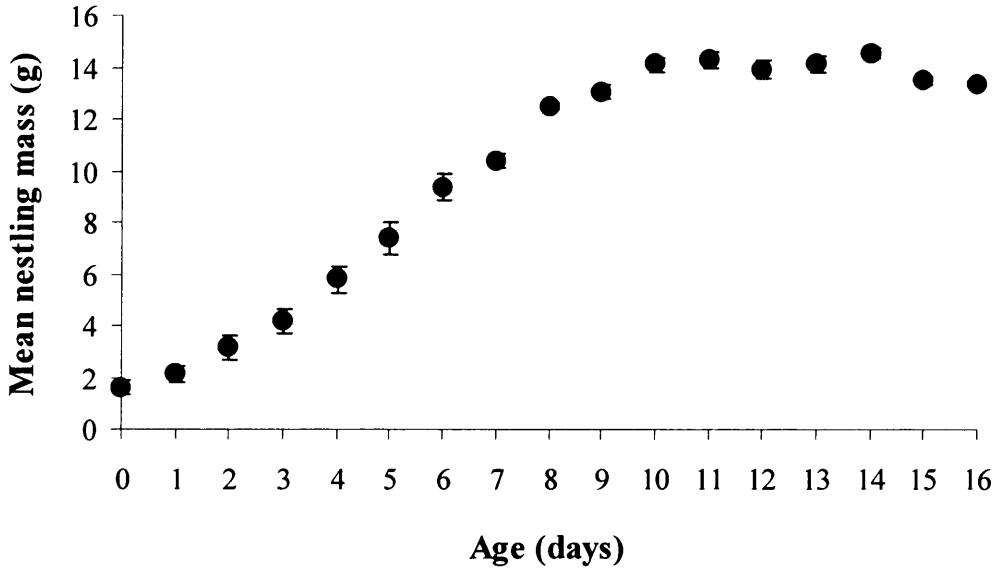


Figure 1.2: Mean ± SE nestling mass from day of hatching (day 0) to fledging (day 16). Data are from 6 pied flycatcher broods measured in Wales in 2004.

The growth curve of the Welsh population in this study is consistent with growth curves from other European pied flycatcher populations (Von Haartman 1954). At about 5 days after hatching, the first feathers can be seen and at about 6 days nestlings start to open their eyes. Young fledge from the nest over 1 – 2 days. A study has reported that during the process of fledging, adult males feed young which have left the nest and adult females feed young that are still in the nest (Curio 1959). Adults continue to feed young after leaving the nest for approximately another 8 days (Curio 1959).

Nestling begging behaviour and parental provisioning has been previously investigated in pied flycatchers (Gottlander 1987b; Ottosson et al. 1997; Wright et al. 2002). In a study which experimentally manipulated brood size, it was found that nestlings from large broods (8 nestlings) begged at higher rates and called at different frequencies compared with nestlings in small broods (4 nestlings), in addition parents provisioned large broods at a greater rate but at a lower visit rate per nestling (Wright et al. 2002). In the study by Wright et al. (2002), nestling begging behaviour was recorded on video camera every 10 min for a total of 90 min inside a test box, a similar protocol was adopted in this study to measure begging behaviour of pied flycatcher nestlings (see chapter 3 and 4). In another study by Gottlander (1987), begging behaviour and parental provisioning was measured in unmanipulated broods and then again in the same broods after nestlings had been food deprived for 90-105 min. In both unmanipulated and manipulated broods parents fed the closest nestlings and also nestlings that begged most intensively. In manipulated broods, heavier nestlings were on average closer to the feeding parent than in the unmanipulated broods (Gottlander 1987). Pied flycatcher provisioning rates have been increased by playing back begging sounds to parents at the nest over the major part of the nestling period (Ottosson et al. 1997). Therefore, by begging more, nestlings can increase parental provisioning, thus potentially compete with future siblings.

1.5.4 Female choice and male plumage

During mate choice, females may use several signals that indicate male quality, paternal ability and the quality of resources held by the male in his territory. Male song quality may be an important signal for female mate choice in pied flycatchers. For

example, it has been shown experimentally that females prefer to mate with males with larger song repertoires and more versatile song (Lampe & Sætre 1995). An observational study has found that song rates are positively correlated with food availability in a territory (Gottlander 1987a), and therefore females may gain direct benefits from choosing a male with a complex song. Male age is also an important factor in female mate choice and experimental studies have shown that females prefer to mate with older more experienced males (Sætre et al. 1994), and that older males have a greater ability to feed young (Sætre et al. 1995). Females mated to older males have also been found to lay larger clutches and produce more fledglings than those mated to younger males (Harvey et al. 1985; Harvey et al. 1988). As song repertoire and versatility is positively correlated with male age (Espmark & Lampe 1993; Lampe & Espmark 1994), song may be a cue used by females to assess male age and experience.

In addition to song, females may also use male plumage colour to assess male quality. Adult pied flycatchers undergo two annual moults: a complete summer moult occurs after the breeding season before the autumn migration (July – August), and a pre-breeding partial spring moult which includes a renewal of most of the body feathers, takes place before the spring migration (January – February) (Lundberg & Alatalo 1992). After the spring moult, adult pied flycatchers are sexually dichromatic with most male birds displaying a conspicuous black and white breeding plumage, while all females are brown and white. However, the colour of the feathers on the head and back of breeding males is extremely variable, ranging from jet black (bright plumage) to brown or greyish brown (dull plumage) and some males have a brown plumage very similar to that of females. After the summer moult, males change black and white feathers for brown and white, and consequently after the summer moult, the sexes are difficult to tell apart. An experimental study has found that during the breeding season females prefer bright rather than dull coloured males (Sætre et al. 1994). In an aviary experiment, it was found that females preferred males that were painted brightly black over dull coloured males of the same age (Sætre et al. 1994). Recent studies investigating plumage reflectance have found that under ultra violet (UV) reflectance of male pied flycatcher plumage is positively correlated with age (Siitari & Huhta 2002) as well as song complexity (Lampe & Sætre 1995). Therefore, as plumage becomes

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Swedish collared flycatchers, and an observational study on pied flycatchers in Norway did not find any evidence that females use patch size as a mate choice cue (Dale et al. 1999).

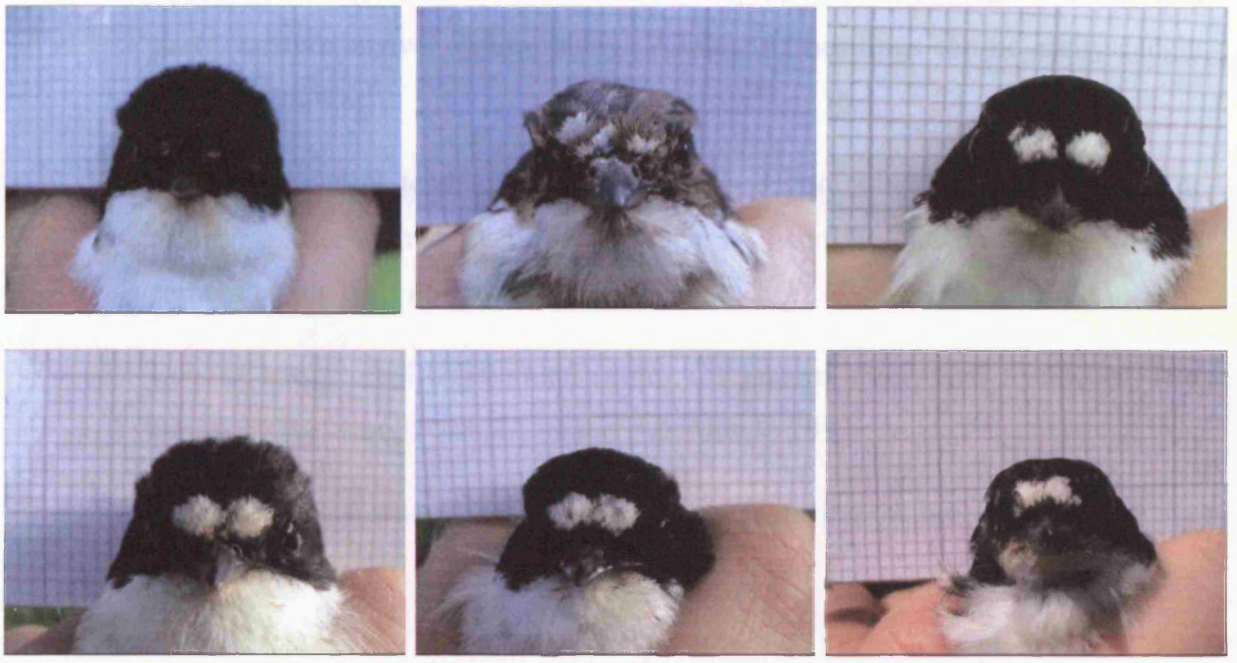

Figure 1.3: Range of male pied flycatcher head patch size seen at Llysdinam, mid Wales.

Studies of male signalling and female mate choice have previously reported a positive relationship between T and plumage patch size. For example, male house sparrows have a chest bib which is used as a signal of dominance, and in this species, Buchanan et al. (2001) identified a positive relationship between circulating T levels and chest bib area size. In collared flycatchers, T levels have been associated with the size of the forehead patch (Garamszegi et al. 2004). The effects of forehead patch size and T levels on habitat choice have been separately compared in pied flycatchers, however, although T levels were correlated with habitat choice there was no apparent effect of forehead patch size (Silverin 1998b). To date, there has not been a study to show that forehead patch size and T levels are related in pied flycatchers.

1.5.5 Study species: the pied flycatcher

Pied flycatchers are in many ways an ideal avian species for biological studies for the following main reasons:

1. Breeding ecology can be easily studied, as pied flycatchers readily accept and prefer nestboxes over natural holes. Adults and nestlings can be easily caught inside nestboxes.

2. They are tolerant to disturbance and adults do not often desert their eggs or nestlings after human disturbance, such as handling nestlings or putting a camera inside their nestbox.

3. Lots of background behavioural ecology is known about pied flycatchers (for summary see Alatalo 1985; Lundberg & Alatalo 1992).

4. Adults are conspicuous and sexually dimorphic during the breeding season.

1.6 AIMS

The overall aim of this 3 year project was to investigate the role of T in controlling begging behaviour in young passerine birds, using nestling pied flycatchers as a study species. The first year of the project was spent learning laboratory techniques and also collecting the first field data during the 2003 breeding season in Mid Wales. Fieldwork involved recording nestling begging behaviour in nestboxes using cameras, and plasma and faecal samples were also collected to assess androgen concentrations through T radioimmunoassay. In the second year, further experimental studies were carried out during the 2004 breeding season, including a T dose and response study. The final year of the project was spent running two biochemical validation studies to measure androgen concentration in the faeces of 4 species of adult passerine birds in captivity.

The following aims and hypotheses were addressed in the data chapters of this thesis:

Chapter 3

Aims

1. To measure begging behaviour in nestling pied flycatchers.

2. To test the effects of hunger on nestling begging behaviour.

3. To correlate begging with circulating levels of endogenous T.

Hypotheses

H_1 Begging intensity increases with food deprivation.

H_2 Begging intensity correlates with circulating T levels.

H_3 Individuals with higher levels of T show increased indices of fitness as a result.

Chapter 4

Aims

4. To elevate nestling circulating T levels through an oral dose of T.

5. To investigate if nestling begging intensity can be increased by elevating T levels.

Hypotheses

H_4 Nestlings dosed with T increase their begging behaviour.

Chapter 5

Aims

6. To investigate sex differences between parents in provisioning rates.

7. To investigate what effect reduced brood size has on parental provisioning rates and nestling begging behaviour.

8. To examine the effect of reduced brood size on brood androgen levels.

Hypotheses

H_5 Parental provisioning rates and nestling begging behaviour decrease with reduced brood size.

H_6 Brood androgen levels decrease with reduced brood size.

Chapter 6

Aims

9. To reduce relatedness between different members of a brood through a partial cross-fostering experiment.

10. To determine what effect relatedness has on parental provisioning rates, nestling begging behaviour and nestling androgen levels.

Hypotheses

H_7 Reducing sibling and parent relatedness increases offspring begging behaviour.

H_8 Nestlings in reduced relatedness broods increase their androgen levels.

Chapter 7

Aims

11. To validate the method used to measure faecal androgens in four species of adult passerines by: 1) identifying and quantifying metabolites of T excreted in faeces and 2) increasing circulating T levels (by injecting with GnRH) and quantifying the rate of T metabolism through blood and faecal samples.

Hypotheses

H_9 Faecal androgen levels are increased through an injection with GnRH

Chapter 8

Aims

12. To examine inter-brood differences in nestling plasma T levels.

13. To examine the effect of nestling age and sex on plasma T levels.

14. To test covariation in nestling cell-mediated immune response and circulating T levels.

15. To test covariation in adult male white forehead patch size with circulating T levels.

16. To investigate adult plasma T levels in different breeding states.

Hypotheses

H_{10} Nestling T levels differ between broods.

H_{11} T suppresses the immune response of nestling birds.

H_{12} Male head patch size correlates with circulating T levels in adult males.

H_{13} Adult T levels vary with breeding state.

Chapter 2

Methods

2.1 STUDY SITES

Fieldwork was carried out between late April and early July in 2003 and 2004 on a free-living nestbox breeding population of pied flycatchers, *Ficedula hypoleuca.* Fieldwork was conducted at two field sites near the Cardiff University Field Centre, Llysdinam, Newbridge-on-Wye, Mid Wales (SO0090758623) (figure 2.1). One field site was located at Ty Mawr farm, 2.3 miles southwest of Newbridge-on-Wye (SN9878357896) (figure 2.2), and a second site situated at the entrance to Ystrad house, 2.2 miles due north of Newbridge-on-Wye (SO00839260856) (figure 2.3).

In March 2002, 199 wooden nestboxes (90 mm long x 110 mm wide x 230 mm deep) with detachable wooden lids, were set up at Ty Mawr (n = 145 nestboxes) and Ystrad (n = 54 nestboxes) field sites. Each nestbox had an entrance hole diameter of 34 mm and was attached permanently to a tree approximately 2 m above ground level. Ty Mawr field site covered a total area of 19 ha and was composed of 3 patches of mixed woodland 100 - 300 m apart separated by fields and grassy slopes, *Arrhenatherum.* The majority of woodland was composed of sessile oak trees, *Quercus petraea* spaced 15 – 30 m apart, intersected by conifer trees, *Pinus sp.* and bordered by two narrow slow flowing brooks. Estyn brook lay on the north side of Ty Mawr farm and Hirnant brook on the south side, there was little undergrowth except for a few small dense areas of stinging nettle, *Urtica dioica* fern and moss. Ystrad field site covered an area of 8 ha and was composed of a narrow strip of more densely packed sessile oak trees (5 - 10 m apart) and a few conifer trees situated beside the fast flowing river Wye. There was dense vegetation undergrowth at Ystrad with an abundance of stinging nettle, fern, bramble, *Rubus sp.* and hawthorn, *Crataegus monogyna* scrub.

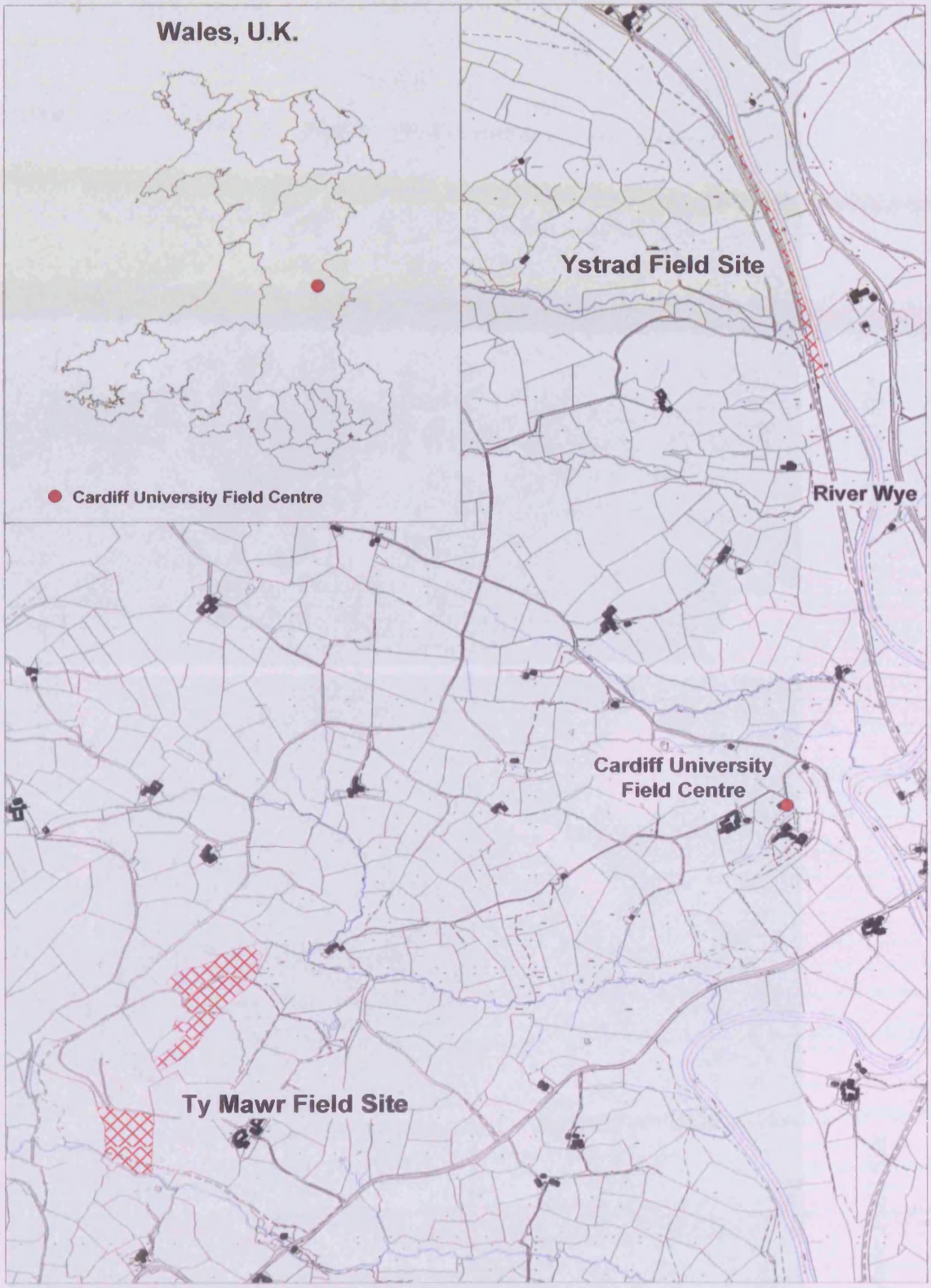

Figure 2.1: Map of study areas

Figure 2.2: Ty Mawr Field Site

Figure 2.3: Ystrad Field Site

In 2003 and 2004, the population of pied flycatchers breeding in the nestboxes at Ty Mawr and Ystrad field sites was studied. Male pied flycatchers arrived at the field sites approximately two weeks ahead of the females in early April. By the end of April / early May, pairing and nest building within the nestboxes was complete and females started to lay eggs, laying 1 egg per day to produce on average a complete a clutch size of 7 eggs. After a 2 week incubation period, pied flycatcher nestlings hatched at day 0 and on average fledged at day 16. Both male and female pied flycatchers helped with the rearing of their young.

2.2 MONITORING

2.2.1 Nestboxes

During the breeding seasons of 2003 and 2004, nestboxes (n = 199) were checked for the presence of pied flycatcher nests, typically composed of dry grass and leaves. Once identified, each pied flycatcher nest was monitored throughout the breeding season to record the first egg laying date, clutch size, nestling hatching date, brood size, number of young surviving and fledging date. Nests that were identified in a nestbox as belonging to species other than pied flycatchers, (predominantly blue tits, *Parus caeruleus* and great tits, *Parus major* with a couple of nests belonging to nuthatches, *Sitta europaea*) were left undisturbed for the rest of the breeding season. Pied flycatchers occupied 40.2 % of the nestboxes in 2003 and 30.1 % of the nestboxes in 2004. In total, blue tits, great tits and nuthatches occupied 41.2 % and 29.6 % of the nestboxes in 2003 and 2004 respectively, with the rest of nestboxes in each year remaining unoccupied during the breeding season (figure 2.4). At the end of each breeding season in July, all nests were cleared, and nestboxes remained empty in the field until the following year.

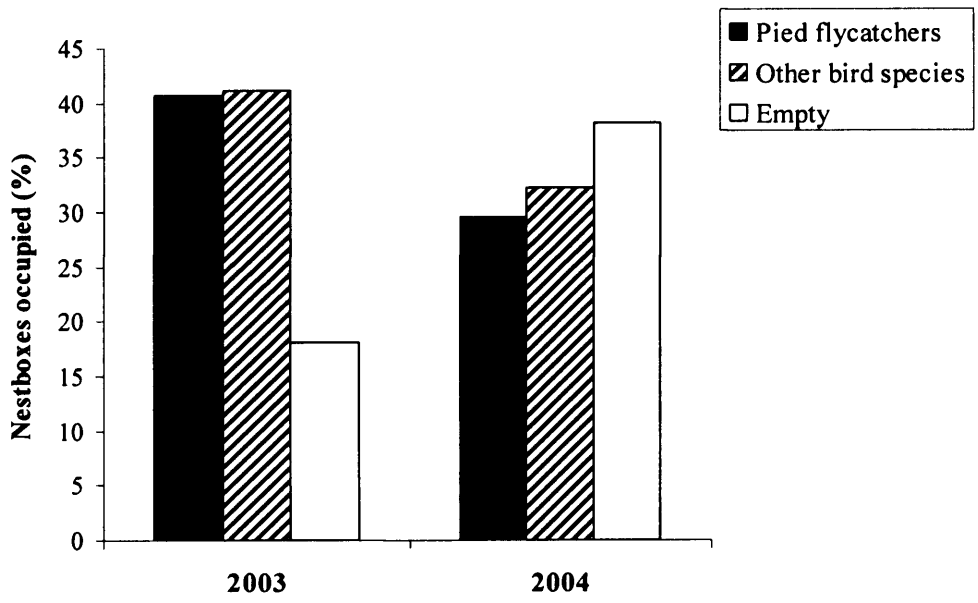

Figure 2.4: Percentage of nestboxes on study field sites in 2003 and 2004 occupied by pied flycatchers (black bars), bird species other than pied flycatchers (striped bars) and unoccupied empty nestboxes (open bars).

2.2.2 Adult pied flycatchers

Adult pied flycatchers were caught between April - June in 2003 (male sample size = 47, female sample size = 68) and in 2004 (male sample size = 16, female sample size = 60). A combination of techniques were used to catch adults including: mist nets, ground trap nets baited with mealworms, nestbox traps and by hand from nestboxes. A playback tape of pied flycatcher calls was used during the 2003 breeding season to attract adults towards the mist nets, but as the tape did not increase the number of pied flycatchers caught, it was not used again in 2004. The majority of adult females in 2003 and 2004 were caught whilst incubating a complete clutch of eggs, either by hand or by using traps in the nestbox (adult females were replaced and stayed in the nestbox after sampling). The majority of adult males in 2003 were caught in nestboxes using traps whilst feeding young. However, in 2004, adult males were mostly caught in mist nets or ground trap nets early in the breeding season before nest building and egg laying started. All adults appeared undisturbed when caught inside nestboxes. No nests in this study were abandoned due to trapping efforts.

Once caught, adults were ringed with one metal ring and three split plastic colour rings. Body mass was measured using a hand held pesola (accuracy 0.25 g), tarsus length

measured using plastic callipers (accuracy 0.1 mm) and wing length measured using a metal wing rule (accuracy 1 mm). To assay circulating plasma testosterone (T) levels (see 2.7.4), a sample of 61 adult males (97% of all adult males caught in 2003 and 2004) and 94 females (73% of all adult females caught in 2003 and 2004) were blood sampled (see 2.7.1). In 2004, adults were aged as 1 year, 2 year or > 2 year according to the extent of white feathers on the wings (Jenni & Winkler 1994). On average, adult birds were held for < 20 min and were released at site of capture after sampling. Whilst in hand, the white forehead patch of each adult male was photographed in front of a piece of graph paper using a digital camera (Sony DCR-TRV25E), to investigate if there is a relationship between plasma T and the size of the white forehead patch. The digital images of male forehead patches were downloaded onto a PC, and the area size of each forehead patch was calculated using the software package, ImageJ 1.30 (http://rsb.info.nih.gov/ij/index.html).

2.2.3 Nestling pied flycatchers

A calendar was kept to follow the number of active pied flycatcher nestboxes, and the number and age of nestlings in each nest on every day during the 2003 and 2004 breeding season. The majority of nestlings in 2003 and 2004 were removed from the nestbox at least once by hand between 6 - 14 days old for the purpose of ringing with a metal ring. Many nestlings were removed twice from the nest; once at day 7 and again at day 11 in order to take blood and faecal samples to measure androgen levels (see 2.7.4). Whilst in the hand, body mass was measured using a hand held pesola (accuracy 0.25 g) and tarsus length measured using a pair of plastic callipers (accuracy 0.1 mm). Between 11 - 12 days, wing length was also measured using a metal wing rule (accuracy 1 mm).

2.3 RECORDING BEHAVIOUR

2.3.1 Field laboratory 'test box'

In 2003 and 2004, begging behaviour of 7 day old pied flycatcher nestlings was recorded in a laboratory at the Cardiff University Field Centre during two separate behaviour studies: 1) nestling hunger manipulation (see 2.5.1) and 2) nestling T manipulation (see 2.5.2). In each study, single nestlings were placed alone on a heated artificial nest, (a heat retaining flask containing a hot gel hand warmer and bird nesting

material) and were covered over with a dark piece of material. The artificial nest was positioned inside a dark (50 lux) wooden 'test box' (33 cm x 31 cm x 60 cm) containing scaled paper and a Sony ^{mini}DV handycam camera (DCR-TRV25E) 0.25 m from the nestling, which was used to record behaviour (figure 2.5). Nestling temperature (always observed in the range of 26 - 31 °C) was monitored without disturbance throughout the time in the test box by using a temperature sensitive probe (Maplin, Barnsley, England), placed under the nestling. Nestling begging behaviour was recorded in the test box during begging trials. In each begging trial, a nestling was stimulated to beg by removing the covering piece of dark material and making 2 standardised "tut-tut'' noises in quick succession while gently waving a metal spatula 3 - 4 cm above the head of the nestling. The spatula was continually waved until begging behaviour ceased (i.e. once > 10 s elapsed after head of the nestling was lowered), and the begging stimulus was terminated by replacing the covering piece of material over the nestling. The apparatus and protocol for quantifying begging behaviour was developed from previously published methodology (Kilner 2001; Wright et al. 2002).

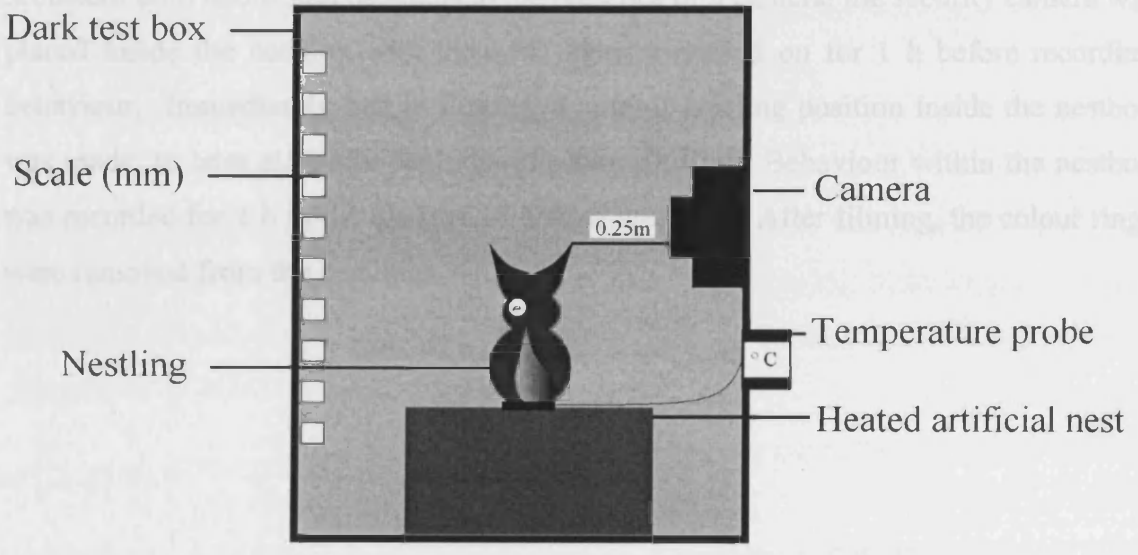

Figure 2.5: Laboratory test box apparatus used to record nestling pied flycatcher begging behaviour.

2.3.2 Field nestboxes

In 2003 and 2004, adult and nestling pied flycatcher behaviour was recorded inside field nestboxes during two behaviour studies: 1) brood size manipulation (see 2.5.3) and 2) relatedness manipulation (see 2.5.4). In both studies, a black and white security camera with 2 red light-emitting-diodes (LED) which was protected by a waterproof housing (76 mm length x 50 mm width x 31 mm depth) was used to film behaviour inside nestboxes. Before the start of the breeding season in early April 2003, small metal plates (80 mm long x 50 mm wide x 1.5 mm thick) were screwed to the inside lid of each nestbox. The security camera was attached to a metal plate on the inside of the lid using a magnet, and once the lid was replaced, the camera captured an aerial view of the nestlings inside the nestbox. Behaviour was recorded onto a Sony ^{mini}DV handycam camera (DCR-TRV25E) that was situated in a waterproof container approximately 2 – 3 m from the nestbox. The Sony camera was connected to the security camera via a waterproof lead (figure 2.6).

To be able to identify individual nestlings on film, each nestling was ringed with 1 split plastic colour ring before the security camera was attached to the lid of the nestbox. To accustom both adults and nestlings to the presence of a camera, the security camera was placed inside the nestbox with the LED lights switched on for 1 h before recording behaviour. Immediately before filming, a note of nestling position inside the nestbox was made, to later aid individual identification on film. Behaviour within the nestbox was recorded for 1 h in the absence of human presence. After filming, the colour rings were removed from the nestlings.

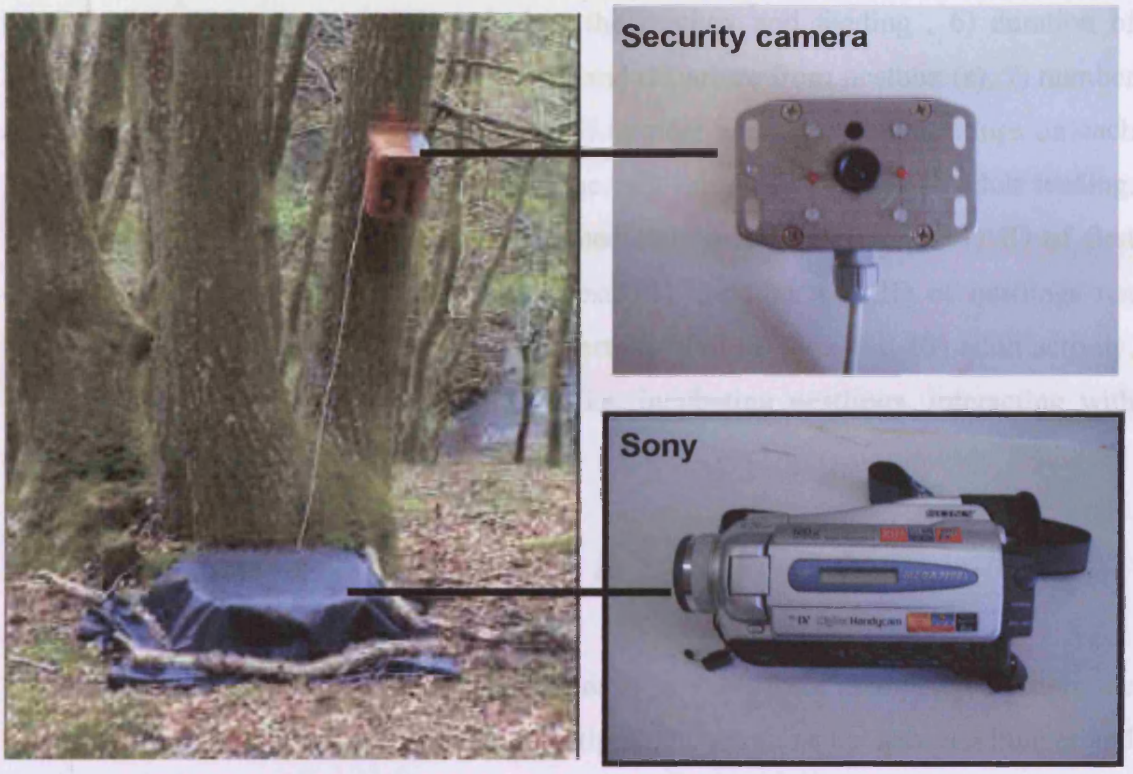

Figure 2.6: Photos showing the types of cameras used to record pied flycatcher behaviour in field nestboxes.

2.4 VIDEO ANALYSIS

2.4.1 Field laboratory test box

In the nestling hunger manipulation and nestling T manipulation studies, nestling begging behaviour recorded in the field laboratory test box was classified as: 1) the duration (s) of the begging display (from the start of the begging stimulus until begging behaviour ceased) and 2) the maximum height (mm) of the begging stretch (maximum distance tip of beak moves during a begging display).

2.4.2 Field nestboxes

In the brood size manipulation and the brood relatedness manipulation studies, adult provisioning and nestling begging behaviour recorded in field nestboxes was classified as: 1) adult male and female brood provisioning rates, 2) the occurrence of an adult feeding call (call made by adults to stimulate nestling begging behaviour), 3) adult latency (s) to feed nestlings (time lapsed from adult arrival at the nest to feeding a nestling), 4) nestlings begging in presence or absence of an adult 5) number and ID of

nestlings begging between adult arrival at the nestbox and feeding , 6) duration of brood begging display between adult arrival and departure from nestbox (s), 7) number and ID of nestlings begging on each visit, 8) number and ID of fed nestlings on each parental visit, 9) ID of highest nestling in the nest immediately prior to adult feeding, 10) ID of nestling nearest to the adult immediately prior to feeding, 11) ID of first nestling to beg upon adult arrival at the nest, 12) number and ID of nestlings not begging at all between adult arrival and departure from nestbox and 13) adult activity, other than feeding, whilst in the nestbox (i.e. incubating nestlings, interacting with another adult or removing faecal sac).

2.5 BEHAVIOUR STUDIES 2003 & 2004

2.5.1 Nestling hunger manipulation

The hunger levels of single nestlings aged 7 days (n = 24) were manipulated during the breeding seasons of 2003 and 2004 to investigate the relationship between hunger and begging intensity. Each nestling was temporarily (< 5 h) removed from a field nestbox (under the Countryside Council for Wales licence, OTH : SB : 05 : 2003) and fed to satiation with Nectarblend rearing mix (Haiths Seeds, Cleethorpes, UK) made up to a standard concentration (Kilner 2001). Once satiated, the nestling was transported to the field laboratory to record begging behaviour during begging trials in the test box (see 2.3.1). Whilst in the test box, each nestling was food deprived for 120 min and stimulated to beg in a begging trial every 10 min. The protocol for quantifying begging behaviour closely followed previous published methodology (Kilner 2001; Wright et al. 2002) and was designed to standardise the degree of hunger between the nestlings by feeding each to satiation and then testing begging behaviour during the short term food deprivation. After 120 min, nestlings were blood sampled (100 μl) to assess circulating T levels (see 2.7.4), weighed with a pesola balance (accuracy 0.25 g) and returned to their natal nestbox in the field. Nestling begging behaviour was analysed (see 2.4.1) at 120 min post food satiation after initial analysis showed that nestlings reached their maximum begging intensity at this time.

2.5.2 Nestling T manipulation

In 2003, a pilot study on nestlings aged 13 - 14 days old (n = 64) was carried out to manipulate circulating T levels with an oral dose of T in peanut oil. Each nestling was temporarily (< 10 min) removed from a nestbox (n = 17), dosed orally with either 0.5 or 1 μg of T (Sigma) in 10 or 20 μl of peanut oil using a blunt tipped syringe, and then replaced back in the natal nestbox. At either 0, 30, 60, 90 and 120 min after dosing, nestlings were blood sampled (approximately 100 μl) to assess circulating T concentration and weighed (accuracy 0.25 g) using a pesola balance.

In 2004, 7 day old nestlings (n = 24) were temporarily removed (< 5 h) from a nestbox, fed to satiation with Nectarblend rearing mix made up to a standard concentration (Kilner 2001) and transported to the field laboratory. At 30 min since food satiation, each nestling was administered with either one oral dose of 4 μg of T diluted in 20 μl peanut oil (T dosed nestlings, n = 13), or just 20 μl of the peanut oil vehicle (control nestlings, n = 11). After dosing, each nestling was placed alone on an artificial nest inside a test box (see 2.3.1) and begging behaviour was recorded and analysed from begging trials at 20, 30, 70 and 90 min after dosing (see 2.4.1). At 90 min after dosing, nestlings were blood sampled (approximately 100 μl) to measure circulating T levels, weighed using a pesola balance (accuracy 0.25 g) and then returned to their natal nestbox in the field. From the pilot study it was found that circulating T levels after an oral dose of T were highest after 30 min. To assess T levels at 30 min after dosing in 2004, an extra 12 nestlings were administered with either one oral dose of 4 μg of T diluted in 20 μl peanut oil (n = 6), or 20 μl peanut oil (n = 6) and blood sampled after 30 min.

2.5.3 Brood size manipulation

In June 2003, the influence of brood size on adult provisioning rates, nestling begging behaviour and nestling androgen levels was investigated. Pied flycatcher broods (n = 8) were filmed in field nestboxes over 2 consecutive days: once before (control day) and once after the size of the brood was reduced (experimental day). Behaviour was filmed using a security camera inside a nestbox and recorded onto a Sony ^{mini}DV handycam camera (see 2.3.2). On the control day, each brood was filmed for 1 h. On the experimental day, 24 h after the first visit, each brood was reduced by temporarily

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kept on ice. As little disturbance as possible was caused to the birds during the collection period by removing the plastic sheet to another room to collect the faecal samples. Within 1 h of collection, faecal samples were transported on ice to a -80 °C freezer where they were stored until transported to the University of Veterinary Medicine, Vienna, for further analysis (see 2.6.2).

At 10 days post injection with [^{3}H]T solution, 1 canary and 1 zebra finch were sacrificed to establish whether radioactivity levels within the body tissues had returned to background levels. The viscera of each bird was extracted from the body cavity, macerated and then 0.1, 0.3 and 1 ml of the homogenate was transferred into scintillation vials in triplicate. After adding 10 ml scintillation fluid to the homogenate, vials were counted on a scintillation counter. Similarly, the radioactivity remaining in the faeces of the pied flycatcher and great tit at 0, 6, 8 and 10 days post injection was analysed to check that radioactivity was at background levels. For each species, 3 faecal samples were transferred into separate scintillation vials for each time period post injection and 10 ml of scintillation fluid was added to the faecal samples before reading on a scintillation counter. It was found that radioactivity in the viscera homogenate and the faecal samples were at background levels after 10 days.

2.6.2 Validation study 1 analysis: Radiolabeled T metabolism

2.6.2.1 [^{3}H] T extraction

Faecal samples collected between 0 - 70 h post injection with [^{3}H]T solution were analysed at the University of Veterinary Medicine, Vienna. To measure radioactivity, each faecal sample was extracted with 1 ml of 60 % methanol by shaking for 20 min. Faecal samples were then centrifuged for 10 min before a 50 µl aliquot of the supernatant was removed, mixed with 4 ml of scintillation fluid (Quicksafe A®, No 100800, Zinsser Analytic, Maidenhead, UK) and counted in a scintillation counter (Packard Tri-carb 2100TR).

2.6.2.2 High performance liquid chromatography (HPLC)

To characterise the excreted metabolites in the faeces of each species, straight-phase high performance liquid chromatography (HPLC) separations were performed following previously published methodology (Palme & Möstl 1997). One methanol

extracted faecal sample containing the highest amount of radioactivity from each [^{3}H]T injected male bird was used to characterise to metabolites. Metabolites were separated by straight phase HPLC (n-hexane/chloroform: 70/30; flow: 2 ml/min) on a Lichrosorb Si 60 column (10 μm, 25 x 0.4cm; Forschungszentrum Seibersdorf, Austria) using a linear methanol gradient from 0 to 60 % in the first 30 min, 6 % from the 30th to 35th min and thereafter up to the 10 % until the 40th min. To determine which fractions contained metabolites of [^{3}H]T radioactivity, 50 μl sub samples of each fraction were mixed with 200 μl of scintillation cocktail (Packard Microscint ^{TM} PS) and counted on a scintillation counter (Packard Top Count) in duplicate.

2.6.2.3 Radioimmunoassay determination of metabolites

Once aliquots had been removed from each HPLC fraction for counting [^{3}H]T radioactivity, the rest of each fraction was dried down in a glass test tube overnight in an oven at 40 °C. Dried fractions within the glass test tubes were transported back to Cardiff University and stored at -20 °C until further analysis. Fractions were analysed over 2 days at Cardiff University. On day 1, 300 μl of dichloromethane was added to each test tube, which was then vortexed at 720 rpm for 60 min to remove the dried hormones from the sides of the glass tubes. The dichloromethane in each glass tube was left to evaporate to dryness in a fume cupboard. Once dry, fractions were reconstituted in 50 μl of assay buffer, vortexed for 30 s and stored at 4 °C overnight. On day 2, each test tube was vortexed at 800 rpm for 60 min before duplicate aliquots (20 μl) of each fraction was analysed by radioimmunoassay to measure androgen levels (see 2.7.4).

2.6.3 Validation study 2 protocol: GnRH-challenge of androgen production

Between May - June 2005, 6 adult male canaries, zebra finches, pied flycatchers and great tits were housed in separate cages with access to food and water *ad libitum*. The animal room was maintained at an ambient temperature of 18 ± 1 °C and a 14L: 10D photoperiod.

As a control measure, birds were injected i.p. with 50 μl PBS using a luer tip Hamilton syringe and x 25 g needle, 5 days before dosing with chicken GnRH-I. All injections were administered between 0900 – 1000 h. Faecal samples were collected at 0, 1, 2, 3,

4, 6, 8 and 12 h post injection on non-absorbent plastic sheets inserted underneath the perches of each cage. The plastic sheet was replaced in each cage 1 h before a collection time. Within 1 h of collection, faecal samples were transported on ice to a -80 °C freezer where they were stored until further analysis. As little disturbance as possible was caused to the birds during the 12 h collection period by removing the plastic sheet to another room to collect the faecal samples.

Prior to the start of the study, a stock solution of GnRH was made by dissolving 1 mg of chicken GnRH-I (Bachem, Merseyside) in 5 ml of PBS. The stock solution was divided into 1 ml aliquots which were stored in a -20 °C freezer until required. On the day of GnRH injection, an aliquot of the stock GnRH solution was thawed and mixed thoroughly before 1 ml was added to 9 ml PBS to make a diluted injection solution (1 μg GnRH / 50 μl PBS). Birds were blood sampled (approximately 100 μl) immediately before and 30 min after an i.p. injection with 1 μg chicken GnRH-I. Faecal samples were collected in the same way as controls at 0, 1, 2, 3, 4, 6, 8 and 12 h post injection. Blood samples were kept on ice until centrifuged in a bench top centrifuge (Boeco) at 1300 rpm for 15 min. Plasma was drawn off using a Hamilton syringe, transferred to a labelled 0.5 ml microcentrifuge tube and stored in at -20 °C until analysed for T by radioimmunoassay. Faecal samples were pooled for each individual at each collection time and androgens were extracted and analysed by radioimmunoassay (see 2.7.4).

2.7 PHYSIOLOGICAL MEASURES

2.7.1 Blood sampling

Adults and nestlings (aged 7 - 14 days) were blood sampled under Home Office licence (PPL 30/1961) for the purpose of analysing T levels (see 2.7.4) and molecular sexing nestlings (see 2.7.5). Blood samples (approximately 100 µl), were taken from each bird by lightly pricking the brachial vein in the wing with a sterile needle, drawing blood into 2 - 3 microcapillary tubes (50 µl capacity) and expelling into 1.5 ml plastic collecting tubes. All birds were weighed after blood sampling with a pesola balance (accuracy 0.25 g). During fieldwork in 2003 and 2004, blood samples were kept at around 0 °C for a maximum of 5 h by transferring the plastic tubes onto ice packs stored inside a cool bag. At the field laboratory, blood samples were centrifuged in a bench top centrifuge (Boeco) at 1300 rpm for 15 min. Plasma was transferred into a labelled 0.5 ml microcentrifuge tube to be later analysed for T. Red blood cells (RBCs) were retained in the original collecting tube for molecular sexing. Between April - July in 2003 and 2004, plasma and RBCs were stored at -4 °C in the field laboratory until transported back to Cardiff University on dry ice, to be stored at -20 °C until further analysis.

2.7.2 Faecal sampling

In addition to blood sampling, non-invasive faecal sampling was also used as a technique to assess androgen levels excreted by adults and nestlings. In the field, nestlings were stimulated to produce a faecal sample directly into a 1.5 ml plastic tube, by holding the nestling above the tube and gently squeezing the abdomen. Adult faecal samples were collected during the 2005 validation studies by placing plastic sheets in the bottom of each bird cage. Whenever possible, directly touching faecal samples by hand was avoided to stop the transfer of hormones and contaminants into the sample. Faecal samples were kept at around 0 °C for a maximum of 5 h in the field by transferring the plastic tubes onto ice packs stored inside a cool bag. Between April - July in 2003 and 2004, faecal samples were stored at -4 °C in the field laboratory until transported back to Cardiff University on dry ice, to be stored at -80 °C until freeze dried. All faecal samples were stored in a freeze dried state until further analysis.

2.7.3 Faecal T extraction

Faecal samples were extracted over 2 days.

Day 1: To extract faecal androgens, freeze dried faecal samples were homogenised with a pestle and mortar, before 0.01 g of the homogenate was extracted with 2.5 ml of 90 % ethanol in a 10 ml conical flask by shaking on an orbital shaker (IKA ® KS 130 basic) for 60 min. The contents of each flask were vortexed, decanted into a test tube and centrifuged at 4500 rpm for 20 min. Approximately 2 ml of supernatant was transferred using a pipette into a second test tube, which was put onto a hotblock under air to dry at 37 °C. The pellet in the first test tube was re-suspended in 1.25 ml 90 % ethanol, vortexed (V400 Multitube vortexer, Alpha Laboratories) for 1 min and centrifuged again at 4500 rpm for 20 min. All supernatant was then added to the second test tube on the hotblock and left to dry (4 - 5 h). Once evaporated, 4 ml dichloromethane was added to the second test tube to remove the dried hormones from the sides of the glass tube, and vortexed for 3 min. Test tubes were stored at 4 °C in a fridge overnight.

Day 2: The contents of each test tube were mixed by vortexing for 3 min and shaking on an orbital shaker for 30 min. The supernatant was transferred to a third test tube and left to dry under air on a hotblock (2 - 3 h). Once evaporated, the dried androgens sticking to the inside of the third test tube were dissolved in 0.3 ml of assay buffer solution, transferred to a 1.5 ml plastic tube and stored at -20 °C until assayed for androgen concentration by T radioimmunoassay (see 2.7.4).

2.7.4 Testosterone radioimmunoassay (T-RIA)

2.7.4.1 Theory underlying radioimmunoassay techniques

Androgen concentrations in plasma and faecal samples taken from birds were measured by direct radioimmunoassay (Parkinson & Follett 1995). Radioimmunoassay (RIA) is a technique which is used to measure small quantities of materials (e.g. hormones) of unknown concentration circulating in the blood (Parkinson & Follett 1995). During a hormone radioimmunoassay, an antigen-antibody complex is formed when a plasma or faecal sample containing the hormone (unlabelled antigen), is incubated in an assay tube with a fixed amount of antibody and some additional hormone labelled with an

isotope (labelled antigen). A separate reference hormone standard, containing: a known concentration of unlabelled hormone, radiolabeled hormone and a fixed amount of antibody, is incubated at the same time. The underlying principle of hormone radioimmunoassay is the competitive inhibition of binding of the labelled antigen to antibody by unlabelled antigen. Both labelled and unlabelled hormones in the sample and standard assay tubes bind and unbind with the antibody (the number of antigens out number antibody binding sites), until a state of equilibrium is reached. Because a fixed amount of antibody is present in each assay tube, the amount of labelled hormone bound to the antibody (the measured radioactivity) is a function of the concentration of unlabelled hormone in either the standard or the sample tube. The higher the concentration of unlabelled hormone, the lower the quantity of labelled hormone bound to the antibody and, therefore, a high activity reading for a given sample will mean a low hormone concentration. By comparing how much each labelled antigen binds to the antibody between sample and standard curve concentrations (which is linear between upper and lower cut off points), the concentration of hormone in the sample can be estimated.

2.7.4.2 T-RIA protocol

Total androgen concentration was measured in faecal and plasma samples collected between 2003 - 2005 by T radioimmunoassay using anti-T anti-serum (code 8680-6004, Biogenesis, UK) and [^{125}I]-T label (code 07-189126, ICN, UK) (Parkinson & Follett 1995) at Cardiff University. In each radioimmunoassay, 50 - 100 extracted faecal samples or plasma samples were processed over 3 days. Also included in each assay were 3 controls (including: blanks, total counts and no hormones), as well as standards of a known T concentration to calculate a calibration curve and inter-assay variation.

Day 1: To set up faecal and plasma samples, 20 µl aliquots of extracted faeces or plasma samples were added to 2 microcentrifuge tubes using a Hamilton syringe. To set up 3 controls, 20 µl of assay buffer solution was added to 12 (3 x 4) microcentrifuge tubes using a Hamilton gun. To set up standard samples for a calibration curve, microcentrifuge tubes were labelled 1 - 12 in triplicate, before 20 µl of assay buffer solution was added to tubes 2 - 11 and 20 µl of assay standard (20 ngml^{-1}) was added to

1) The activity of

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Protocol for blank controls:

- Assay blanks were analysed in duplicate with every T-RIA. Instead of adding anti-T anti-serum to assay tubes on day 1, 50 µl of normal rabbit serum (Lot: 486 82, DA Boldon, England) was added to blanks. Blanks were then assayed in the same way as other samples (see 2.7.4.2).

2) It was demonstrated that T added to the assay medium could be recovered. This confirmed that the T-RIA quantitatively detected the amount of T actually present in the samples.

Protocol for accuracy of extracted samples:

- On day 1 of a T-RIA, 3 assay tubes were prepared for 6 plasma samples and 6 extracted faecal samples taken from the same adult male pied flycatchers. The following reagents were added to each assay tube in duplicate:

  Tube 1: 10 µl of plasma or extracted faecal sample + 10 µl of assay buffer.

  Tube 2: 10 µl of assay standard (20 $ngml^{-1}$) + 10 µl of assay buffer.

  Tube 3: 10 µl of assay standard (20 $ngml^{-1}$) + 10 µl of plasma or extracted faecal sample.
- All tubes were assayed by T-RIA (see 2.7.4.2).
- % recovery for plasma samples was found to be 118.0 %
- % recovery for faecal samples was found to be 117.7 %

3) It was demonstrated that T added to faecal samples before extraction could be later recovered with T-RIA.

Protocol for accuracy of extracted faecal samples before extraction:

- On day 1 of faecal extraction (see 2.7.3) 3 x 10 ml conical flask were prepared for 6 faecal samples taken from adult males. The following reagents were added to each conical flask:

Flask 1: 0.01 g of freeze dried faecal sample + 2.5 ml 90 % ethanol + 20 µl assay standard (15 $mgml^{-1}$).

Flask 2: 0.01 g of freeze dried faecal sample + 2.5 ml 90 % ethanol + 20 µl assay buffer.

Flask 3: 2.5 ml 90 % ethanol + 20 µl assay standard (15 $mgml^{-1}$).

- After extraction, samples in each flask were analysed by T-RIA (see 2.7.4.2).
- % recovery was found to be 68.9 %

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Overall, for the faecal samples, intra and inter-assay variation were higher than desirable but was consistent between assays. However, intra and inter-assay variation for plasma samples were low and within acceptable limits. To be able to compare hormone levels between individuals within each experiment, plasma and faecal samples were analysed by T-RIA together.

2.7.5 Molecular sexing

Nestling pied flycatchers cannot be sexed from external morphology. Instead, molecular sexing from DNA obtained from RBCs can be used. Described very simply, the technique of molecular sexing involves using a pair of primers to label a specific region of DNA (a gene) located on each sex chromosome. Birds have 2 sex chromosomes, females are heterozygous (ZW) and males are homozygous (ZZ). The procedure of polymerase chain reaction (PCR) is used to amplify the target gene on the Z and W chromosomes to make many copies. As the 2 target genes (1 from each sex chromosome) differ slightly in length (Griffiths et al. 1998), gel electrophoresis can be used to separate the products of PCR according to weight, producing 1 or 2 bands of DNA on the gel. After electrophoreses, a gel under ultra violet light will display 1 band of DNA for males and 2 bands for females. The reason why the targeted DNA sequences on Z and W chromosomes differ in length can be explained by the composition of chromosomes. Chromosomes are linear sequences of genes, which in turn are composed of coding (extron) and non-coding (intron) sequences of DNA. Extrons are highly conserved DNA sequences but introns tend to be less conserved and are likely to evolve even between closely related species. The gene targeted for in molecular sexing is the chromobox-helicase-DNA (CHD) binding gene, and in bird sex chromosomes, the CHD gene is present in both the W (CHD-W; Griffiths and Tiwari 1995) and Z chromosomes (CHD-Z; Griffiths and Korn 1997). The CHD gene can be targeted using 2 recently developed primers, P8 (forward primer) and P2 (reverse primer) (Griffiths et al. 1998). These primers target conserved exonic regions and then amplify across an intron within the CHD gene (Griffiths et al. 1998). As these introns are non-coding they are less conserved and their lengths usually differ between the CHD-W and CHD-Z genes. As a result, PCR products vary in size and CHD-W and CHD-Z can be seen as 2 separate bands on an electrophoresis gel (figure 2.7).

Nestling pied flycatchers were molecular sexed at the Sheffield Molecular Genetics Facility (SMGF), University of Sheffield using RBCs. The protocol for molecular sexing using P2/P8 primers was based upon previously published work (Griffiths et al. 1998). Prior to molecular sexing, RBCs were stored in plastic tubes at -20 °C and were transported to Sheffield University on dry ice.

2.7.5.1 DNA extraction

DNA was extracted from RBCs following a protocol based on Bruford et al. (1998). A sterile toothpick was used to transfer approximately 5 µl of RBCs into a sterile 1.5 ml plastic tube on ice containing 250 µl Digsol (20mM EDTA pH 8.0, 120mM NaCl, 50mM Tris.HCl pH 8.0 and distilled H_2O) and 10 µl of 10 $mgml^{-1}$ Proteinase K. Tubes were vortexed for 30 s, placed in a rack, wrapped in tissue and cling film and then incubated in a rotary oven at 55 °C for 3 h. Once digested (the solution was straw coloured), 300 µl of 4M ammonium acetate was added to all tubes. Each tube was vortexed 3 times for 30 s over 15 min and centrifuged at 13000 rpm for 10 min. The supernatant was pipetted into a new sterile 1.5 ml plastic tube (protein pellet thrown away) and 1 ml 100 % ethanol was added to the supernatant. Tubes were inverted and centrifuged at 13000 rpm for 10 min. Ethanol was poured out of each tube and DNA was rinsed (to remove the salt) by adding 0.5 ml 70 % ethanol and inverting each tube a few times. Ethanol was poured off, and the DNA which could be seen as a white pellet at the bottom of each tube, was left to dry by standing the plastic tubes underneath a desk lamp for 1 h. Once completely dry, DNA pellets were re-dissolved in 100 µl TE (10mM Tris.HCl pH 8.0, 0.1 mM EDTA pH 8.0 and distilled H_2O), vortexed for 2 s and placed in a water bath at 37 °C for 30 min (tubes were flicked every 10 min to prevent the DNA pellet sticking to the side). DNA extractions were stored at -20 °C until further analysis.

2.7.5.2 Polymerase-chain-reaction (PCR)

The concentration of DNA was tested on a fluorometer and each DNA extraction was diluted (1:20 dilution, DNA : distilled H_2O) on a microtitre plate to give a standardised DNA concentration of 10 $ngml^{-1}$. P2 and P8 primers were used to target the CHD genes on the sex chromosomes in a PCR. To set up a PCR reaction, 9 µl of 'mastermix' (distilled water, 10 x NH_4 reaction buffer, 2mM dNTP's, 25mM MgCl2,

10μM forward primer P2, 10μM reverse primer P8 and Taq) was added to 1 μl diluted DNA or 1 μl distilled water (control) on a 72-well Terrasaki plate. One drop of mineral oil was added to each well and the plate was covered with a piece of Saranwrap and placed in a PCR machine. After some experimentation with PCR conditions at the SMGF, a new PCR reaction profile was adopted to molecular sex pied flycatchers.

The new PCR reaction profile developed for pied flycatchers in February 2005:

94 °C for 2 min
Followed by 40 cycles of:
94 °C for 45 s
56.5 °C for 45 s
72 °C for 45 s

Followed by 72 °C for 5 min
Followed by 4 °C soak

The reaction profile developed for pied flycatchers used a higher annealing temperature of 56.5 °C in comparison to 48 °C described by Griffiths et al. 1998. With a lower annealing temperature of 48 °C the second band of DNA in females failed to amplify clearly, making it impossible to sex type individuals.

2.7.5.3 Gel electrophoreses

The DNA products of PCR were separated through electrophoreses on a 2 % agarose gel (250 ml 1xTBE, 5 g agarose) stained with 14 μl 1 $mgml^{-1}$ ethidium bromide. On the terrasaki plate containing the PCR products, 1 μl of 100 bp Lambda DNA standard was added to the last well and 14 μl of 1xOrange G loading buffer was added to all wells. Each sample was loaded onto a gel in an electrophoresis tank containing 1 x TBE buffer and the DNA products ran from the –ve to the +ve electrode by electrophoresis at 140 Volts for 2 h. The CHD-W and CHD-Z genes could be seen as separate bands of DNA when viewed under a UV light. As females are heterozygous (ZW) and males are homozygous (ZZ), female nestling pied flycatchers were sexed by

the presence of 2 bands of DNA and males were sexed by the presence of one DNA band (figure 2.7).

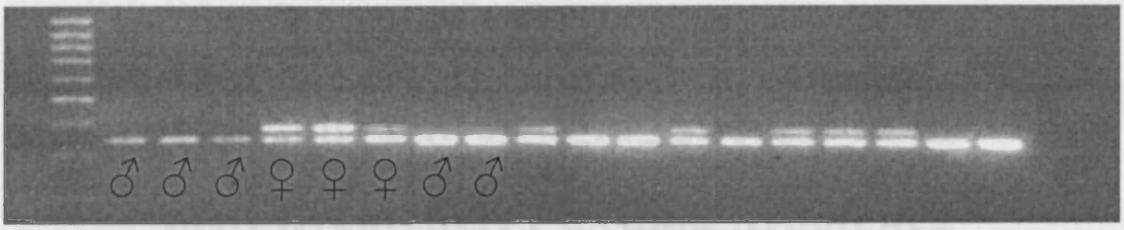

Figure 2.7: PCR products on a 2 % agarose gel under UV light. Female nestlings show 2 DNA bands, males 1 DNA band.

2.7.6 Nestling cell-mediated immune response

The cell-mediated response of 13 nestlings (aged 12 - 14 days) was tested in 2004 using an injection of phytohaemagglutinin (PHA) into the wing web. PHA is a plant lectin which promotes a hypersensitivity reaction and has been widely used to investigate immune function in passerine birds (Buchanan et al. 2003; Morales et al. 2004; Verhulst et al. 2005). To investigate the relationship between T levels and immune function, nestlings were first blood sampled from the branchial vein on the right wing and weighed using a pesola balance (accuracy 0.25 g). The thickness of the left wing web was measured before injection of PHA using a pressure sensitive spessimeter to the nearest 0.01mm (Alpa s.r.l., Milan). Into the left wing web, 50 µl of PHA (Sigma L-8754) (24 mg of PHA in 4.8 ml of 1xPBS) was injected and the response (swelling on the left wing web) was measured 24 h post-injection. Three repeated measurements were taken with the pressure sensitive spessimeter on each occasion. Immune response was determined as the thickness increase (mm) of the left wing web (thickness post injection minus thickness pre-injection).

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3.1 ABSTRACT

Animal signals are hypothesised to be costly in order to honestly reflect individual quality. The evolution of the offspring solicitation signals given by nestling birds are thought to advertise either need or individual quality. We tested the potential role of testosterone (T) in controlling the intensity of these signals by measuring begging behaviour as 1) duration of the begging display and 2) maximum height of the begging stretch and by sampling endogenous T levels in nestling blood. We tested nestling pied flycatchers, *Ficedula hypoleuca* using well-established experimental paradigm involving transient food deprivation to encourage begging behaviour and then blood sampled nestlings at the end of these tests for T levels. Our results show that individual nestlings with the most intense begging displays had the highest circulating levels of T immediately after testing. In addition, we found substantial differences between broods in terms of circulating T. Finally, we found evidence that broods with higher levels of T showed increased fledging success, indicating a benefit for increased T production in nestlings. The potential trade-offs involved in T-mediated begging behaviour are discussed.

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for begging (Lipar and Ketterson 2000). A logical conclusion of such studies is that maternally-derived T controls offspring T production, or some other developmental control mechanism, however no study has yet related maternal T directly with offspring endogenous T after hatching. Furthermore, whilst maternal T may trigger developmental pathways, it seems unlikely that maternally-derived T *per se* controls offspring behaviour. This is because passerine nestlings are known to be able to produce their own T prior to hatching (Adkins-Regan et al. 1990; Ottinger & Abdelnabi 1997; Woods et al. 1975) and in addition, steroids have half-lives of hours, not days (Goymann *et al.* 2002). Circulating plasma T has been measured in nestling European starlings, *Sturnus vulgaris* (Williams *et al.* 1987), great tits, *Parus major* (Silverin & Sharp 1996), European robins, *Erithacus rubecula* (Schwabl & Lipar 2002b) and zebra finches, *Taeniopygia guttata* (Naguib *et al.* 2004). However, these studies did not attempt to relate endogenously produced T with begging behaviour. In a recent study of thin-billed prion, *Pachyptila belcherie* nestlings, both corticosterone and T levels were positively correlated with measures of begging behaviour (Quillfeldt et al. 2006). The single offspring in each nest, as is typical of petrels, showed positive covariation between body condition, begging calls and circulating levels of T. However, at present, it remains unknown what effect T produced by passerine nestlings has on begging behaviour, within and between broods, or what consequences this may have for nestling fitness.

Pied flycatchers represent an excellent study species to test the relationship between begging behaviour and T, as their begging behaviour has previously been studied (Wright *et al.* 2002) and they have reasonably large brood sizes ($\bar{x}$ = 7 nestlings). In adult male pied flycatchers, plasma T has been shown to affect habitat choice (Silverin 1998a) and response to predators (Silverin 1998b). The aim of this study was to test the effects of hunger on nestling begging behaviour and to correlate begging with circulating levels of endogenous T. We tested two hypotheses in pied flycatcher nestlings 1) begging intensity correlates with circulating T levels and 2) individuals with higher levels of T show increased indices of fitness as a result.

3.3. METHODS

3.3.1 Study subjects

We conducted manipulative experiments to determine the relationship between increasing hunger, begging behaviour and plasma T in nestling pied flycatchers during May and June 2003 and 2004. A nestbox (n = 199) population of pied flycatchers was studied at Llysdinam Field Centre in mid Wales, which consists of a 26.96-ha area of oak and coniferous woodland situated in steep sided valleys. Over 2 breeding seasons, broods from 24 randomly selected nestboxes were used in this study. Nestboxes were monitored to record: laying date; hatching date (hatching = day 0); number of eggs hatching; brood size and fledging success. Adults were caught at the nest and ringed and all nestlings were ringed at 7 days with an individual colour ring and an individually numbered metal ring.

3.3.2 Experimental protocol

At 7 days of age, a randomly selected nestling was temporarily (< 5 h) removed from a nestbox (n = 24) and fed to satiation with Nectarblend rearing mix (Haiths Seeds, Cleethorpes, UK) made up to a standard concentration (Kilner 2001). Once satiated, the nestling was food deprived for 120 min, during which time it was transported to the field laboratory to record begging behaviour. The protocol for quantifying begging behaviour closely followed previous published methodology (Kilner 2001; Wright et al. 2002) and was designed to standardise the degree of hunger between the nestlings by feeding each to satiation and then testing begging behaviour during the short term food deprivation.

Each nestling was placed alone on a heated artificial nest (a heat retaining flask containing a hot gel hand warmer and bird nesting material) and covered over with a dark piece of material. The artificial nest was positioned inside a dark wooden 'test box' (33 cm x 31cm x 60cm) containing scaled paper and a Sony ^{mini}DV handycam camera (DCR-TRV25E) 0.25 m from the nestling. Nestling temperature (always observed in the range of 26 to 31°C) was monitored without disturbance throughout the experiment using a temperature sensitive probe (Maplin, Barnsley, England) placed under the nestling.

After 30 min food deprivation, we began recording begging trials every 10 min. In each begging trial, we stimulated the nestling to beg by removing the covering piece of dark material and making 2 standardised 'tut-tut'' noises in succession while gently waving a metal spatula 3 – 4 cm above the head of the nestling. The spatula was continually waved until begging behaviour ceased (i.e. once > 10 s elapsed after head of the nestling was lowered). The begging stimulus was terminated by replacing the covering piece of material over the nestling. In each begging trial we recorded 1) the duration (s) of the begging display (from the start of the begging stimulus until begging behaviour ceased) and 2) the maximum height (mm) of the begging stretch (maximum distance tip of beak moves during a begging display). After 120 min food deprivation and 9 begging trials, each nestling was blood sampled (100 µl) to assess circulating T concentration, weighed (accuracy 0.25 g) then returned to the original nestbox in the field. Siblings from the experimental boxes were also weighed and blood sampled at 7 days post-hatching.

3.3.3 Hormone assays

Blood samples (100 µl) were collected in heparinised capillary tubes (50 µl), expelled into microcentrifuge tubes (1.5 ml) after puncturing the brachial vein with a sterile syringe needle (x 25 g) and stored on ice for a maximum of 3 h before centrifugation. Blood samples were centrifuged for 15 min at 13000 rpm in a bench top centrifuge (Boeco) and plasma was transferred into a microcentrifuge tube (0.5 ml) and stored at – 20 °C until assay. All samples were assayed within 10 months of collection.

Total androgen concentrations were measured in the plasma samples by direct radioimmunoassay using anti-T anti-serum (code 8680-6004, Biogenesis, UK) and [^{125}I]-T label (code 07-189126, ICN, UK) (Parkinson & Follett 1995). Although there is some cross-reaction of the antiserum with other androgens in the plasma, the cross-reactivity is low (22.2 %) and therefore this assay presents a reliable surrogate measure of absolute T levels. It was confirmed that pied flycatcher plasma samples diluted parallel with the standard curve. The samples were run in four assays with 50 % binding level at 0.34 - 0.56 ngtube^{-1} and a detection limit of 0.01 – 0.02 ngml^{-1} for 20 µl of sample, experimental samples were assayed in 20 µl duplicate volumes. The intra-assay coefficient of variation was 4.88 % and the inter-assay variation was 10.34 %.

3.3.4 Statistical analysis

Statistical tests were performed in MINITAB 14.0. We used General Linear Models (GLM) to construct two models relating to individual begging behaviour using: 1) duration of the begging display 2) maximum height of the begging stretch as the dependent variable. These models asked what variables best described variation in nestling begging intensity. For both begging display duration and maximum height of begging stretch we investigated the effects the following variables: nestling T, nestling mass, brood size, sampling date, and year, while the food volume used to satiate nestlings and the time of day at which nestling T was sampled were also entered to control for any variation. Mean T ± S.E. in 12 broods in 2003 was calculated to investigate inter-family variation. Finally we asked what variables predicted variation in nestling fledging success and in this case we included the following variables in our initial model: nestling T, nestling mass, brood size, hatch date and year as independent variables with biologically relevant interactions. Variables were deleted in a stepwise fashion and normality was checked at each step. Where necessary, data were tested for normality using the Anderson-darling test and when data could not be transformed to meet normality, non-parametric statistics were employed.

3.4 RESULTS

3.4.1 *Begging trials*

The mean duration of nestling begging displays increased from 2 s at 30 min since food satiation to 50 s at 110 min since food satiation (ANOVA $F_{9,110} = 11.49$, $p < 0.001$, n = 12). The maximum height of begging stretches also increased over the same time from 7.1 to 44.4 mm (ANOVA $F_{9,110} = 8.91$, $p < 0.001$, n = 12). The duration of begging displays and maximum height of begging stretches reached a plateau after 110 min since food satiation, suggesting that nestlings reached their maximum begging intensity during the 120 min begging trials. Subsequent analyses were therefore conducted using the data gathered at 120 min post satiation.

3.4.2 *T and begging behaviour*

The final GLM for duration of begging displays showed that only year and nestling T explained significant variation in begging duration (table 3.1 (a), figure 3.1). Although the duration of begging displays were shorter in 2004 than in 2003, there was a positive correlation between begging display duration and nestling T within both years (2003: $r = 0.661$, $n = 12$, $p = 0.019$; 2004: $r = 0.731$, $n = 12$, $p = 0.025$). Mean nestling T (0.127 $ngml^{-1} \pm 0.033$) included one outlier sampled in 2003 with a substantially higher T value (0.702 $ngml^{-1}$) compared with other nestlings in the sample. However, handling treatment and all other measured variables of the outlier including mass, brood size, date, volume of food used to satiate and sampling time were similar to the other nestlings. Removal of the outlier from the GLM made the effect of nestling T on the duration of begging displays marginally non-significant ($F_{1,17} = 3.14$, $p = 0.094$).

The final GLM for maximum height of begging stretch showed that nestling mass, nestling T, date and brood size all had a significant positive effect on maximum height of begging stretch (table 3.1 (b)). The positive effect of nestling T was still significant even after the outlier was removed (ANOVA $F_{1,10} = 14.06$, $p = 0.005$). Year was found to have a marginally non-significant relationship with maximum height of begging stretch (ANOVA $F_{1,10} = 4.45$, $p = 0.061$). There were three significant interactions. The significant interaction between nestling T and year, may be explained by the generally shorter begging stretches observed in 2004, compared with 2003 for any given value of nestling T. The relationship between nestling mass and date changed through the season. Nestling mass at 7 days old increased with the date during the early part of the season, but later in the season, nestling mass was not affected by date. It was also found that the variance of nestling mass in larger broods, was greater than that of smaller broods.

Source	d.f.	F	P
(a) Duration of begging display (s)			
Year	1	14.54	0.001
Nestling testosterone	1	31.47	< 0.001
(b) Maximum height of begging stretch (mm)			
Nestling mass	1	54.95	< 0.001
Nestling testosterone	1	21.90	0.001
Date	1	64.00	< 0.001
Brood size	1	32.86	< 0.001
Year	1	4.45	0.061
Nestling testosterone * year	1	9.36	0.012
Nestling mass * Date	1	59.43	< 0.001
Nestling mass * Brood size	1	28.89	< 0.001

Table 3.1: Variables in a general linear model which explain individual variation in (a) duration of begging displays and (b) maximum height of begging stretch by 7 day old pied flycatchers (n = 24) at 120 min food deprivation.

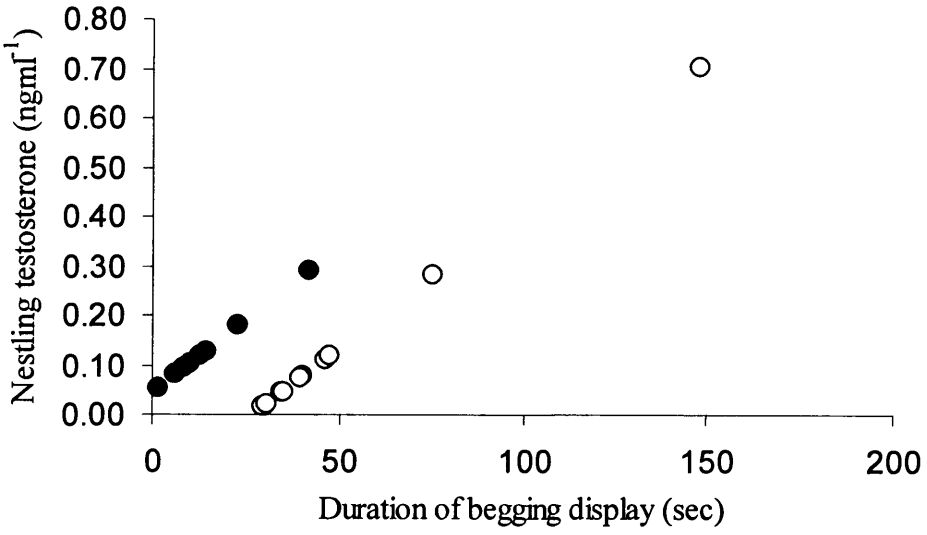

Figure 3.1: The relationship between the duration of begging displays and circulating testosterone levels in 7 day old pied flycatchers. Fitted data are presented for 2003 (open circles) and 2004 (filled circles).

3.4.3 *T and family*

There was significant inter-family variation on nestling T (figure 3.2). It was found that mean brood T level of family F, including the outlier, was significantly higher than mean brood T in all other families (except family I), in the same year (Fisher's pairwise comparisons $F_{11,56} = 5.76$, $p < 0.001$, $n = 68$). T levels of individual nestlings measured during the begging trials were highly correlated with mean brood T levels ($r = 0.913$, $n = 12$, $p < 0.001$).

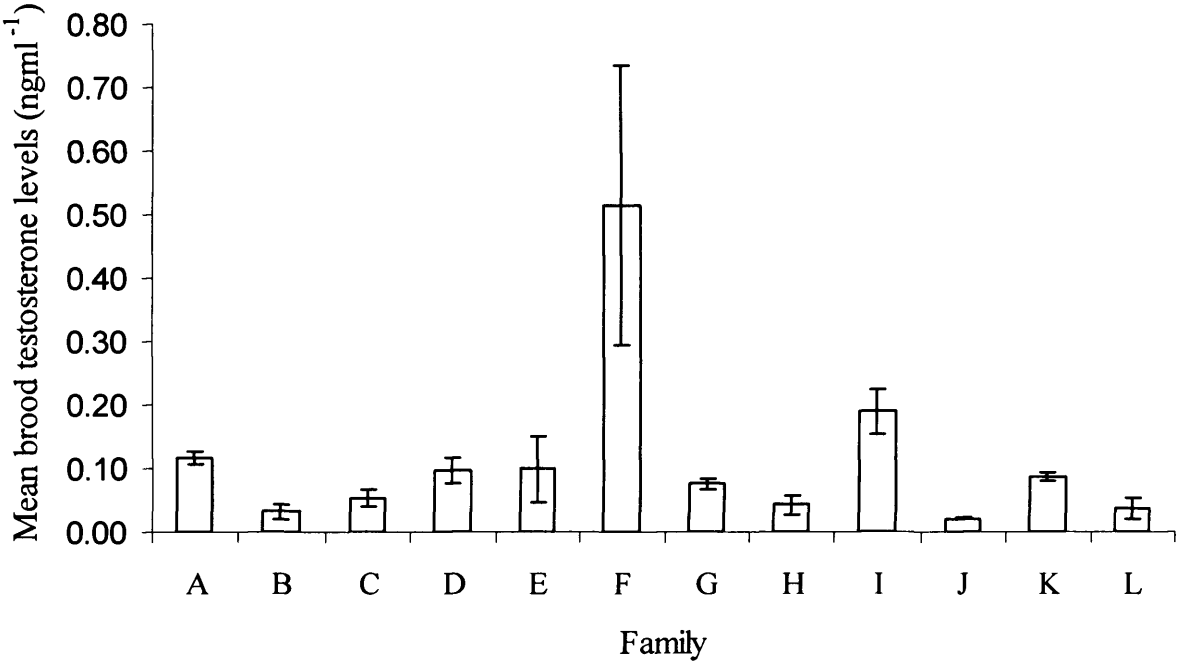

Figure 3.2: The mean ± S.E. testosterone levels of all pied flycatcher siblings (sampled at 7 days old) in 12 individual broods (A - L) in 2003.

3.4.4 *T and fledging success*

We tested the variables predicting significant variation in fledging success and found that mean nestling T was positively associated with fledging success across 2003 and 2004 ($F_{1,21} = 6.08$, $p = 0.022$, $n = 23$). In the final GLM model there was no significant effect of mean nestling mass, brood size, date or year on fledging success. Broods with high T levels at 7 days old were more likely to have a higher proportion surviving until fledging than broods with low T levels (figure 3.3). Although the percentage fledging success was similar between years in our sample of 24 nestboxes used for the begging trials, over the whole nestbox breeding colony at Llysdinam, fledging success was higher in 2004 (mean fledging success = 87%) than in 2003 (mean fledging success = 60%) (Mann-Whitney *U* Test, 2003 n = 82, 2004 n = 59, $P < 0.0001$).

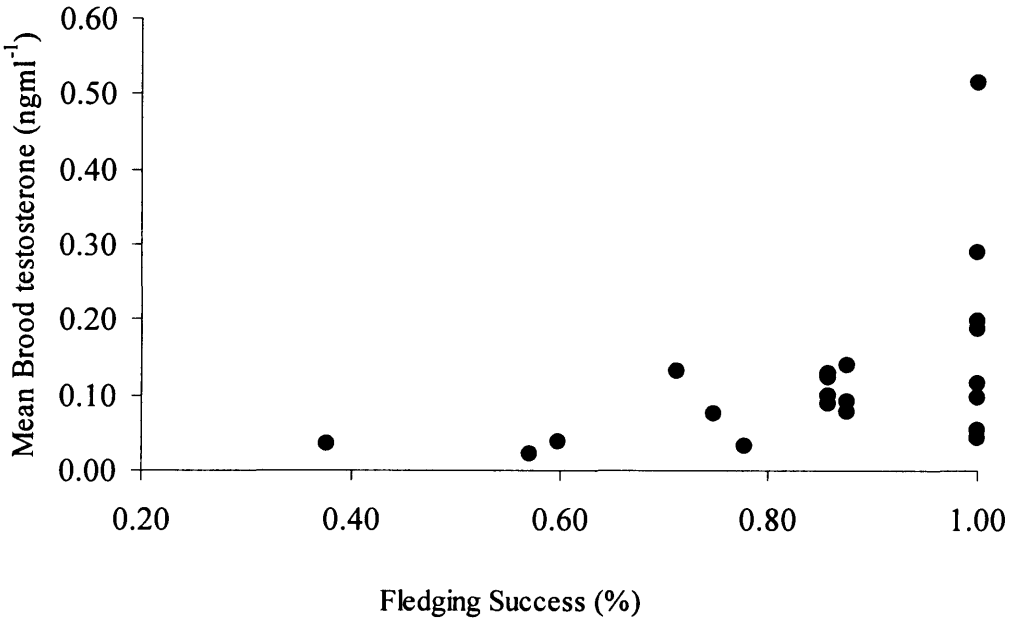

Figure 3.3: Relationship between mean brood testosterone levels and fledging success (n = 23) sampled in 2003 and 2004.

3.5. DISCUSSION

To our knowledge, the results of this study show for the first time, the covariation between nestling begging behaviour and circulating plasma T levels in passerine nestling birds. We found this relationship for two different measures of begging effort 1) duration of the begging display and 2) maximum height of the begging stretch at a standardised time after feeding. Although this study did not address a direct causal relationship, T may affect begging behaviour by increasing metabolism and growth rate (Schwabl 1996a; Smith & Montgomerie 1991), enhancing perception of cues which stimulate begging behaviour (Andrew 1994) or by increasing the mass of the hatching muscle (Schwabl 1996a). Alternatively, there may not be a causal relationship between T and begging behaviour, but T production may be a proxy for indices of condition. Further manipulative work is now needed to determine the causal links between T and begging behaviour.

A relationship between T and begging behaviour would also provide a proximate explanation as to why nestling birds vary in their level of begging effort both within and between nests (e.g. Price & Ydenberg 1995; Lotem 1998). Previous studies have suggested that maternal T may play a role in establishing brood hierarchies (Schwabl 1993; Winkler 1993), although in blue-footed booby chicks, T levels have been found to be similar between members of the same brood (Ramos-Fernandez et al. 2000). Patterns of maternal T deposited into egg yolks have been found to vary with laying order, which may either serve to counteract the competitive hierarchy due to hatching asynchrony (e.g. Eising & Groothuis 2003) or enhance it (Schwabl et al 1997). Interestingly, circulating plasma T is known to be elevated during the first 2 days after hatching in nestling birds (Adkins-Regan 1990; Silverin & Sharp 1996), but further study is required to investigate what effect individual T levels have on maintaining broods hierarchies.

Different environmental conditions change not only absolute T levels but may also change how production of T translates into begging behaviour. Our data demonstrate this effect by a year interaction, where the duration of begging displays/ng T is greater in 2003 than in 2004. Studies in a range of passerine species have found that nestling begging intensity increases when food availability is low (Smith & Montgomerie 1991;

Kilner 1995; Price and Ydenberg 1995; Wright et al. 2002), therefore the longer begging displays in 2003 may have been a response to lower food supplies in that year. Food availability plays a key role in fledging success (e.g. Riddington & Gosler 1995), and our data also show that fledging success for the whole of our nestbox breeding colony of pied flycatchers at Llysdinam was lower in 2003 than in 2004. In nest-bound chicks, food shortages are associated with an increase in circulating levels of corticosterone (e.g. Nuñez-de la Mora et al. 1996; Kitaysky et al. 1999; Kitaysky et al. 2001a). Short-term release of corticosterone in black-legged Kittiwake chicks restores energy reserves through begging behaviour and sibling competition by increasing parental provisioning (Kitaysky et al. 2001b). However, chronic elevation of corticosterone has a detrimental effect on cognitive ability (Kitaysky et al. 2003) and chicks may suppress adrenocortical activity during prolonged food shortage (Kitaysky et al. 2001a). As corticosterone and T bind with the same plasma binding globulin (Deviche et al. 2001), T production may also be affected during times of prolonged food shortage.

The results of this study also suggest that there are considerable differences in T production by nestling birds between broods, at the same age post-hatching. This may go some way to explain the apparent differences between broods of nestling birds in their rates of growth and development (e.g. Nisbet et al. 1998; Podlesak & Blem 2001). Furthermore, our data suggest that these differences may translate into real fitness effects, as broods with higher T production at 7 days post-hatching had a greater fledging success from the nest. Even with this relatively small sample of nests, it is clear that this was not a seasonal effect, as nests fledging earlier in the season did not show increased T production. Further investigation is needed to understand the basis for these inter-family effects.

Signalling models predict that for signals to be honest indicators of need they should be costly to produce or maintain (Kilner & Johnson 1997). Many potential costs of begging behaviour have been investigated including predation risk (Macnair & Parker 1979; Haskell 1994; Leech & Leonard 1997; Briskie et al. 1999), energetic costs (Macnair & Parker 1979; Leech & Leonard 1997) and growth costs (Kilner 2001). Endogenous T production is also thought to mediate a number of potential

physiological costs including increased basal metabolic rate (Buchanan et al. 2001), immunosuppression (Folstad and Karter 1992), aggression (Johnsen 1998) and increased levels of corticosterone (Evans et al. 2000). Good quality individuals may not suffer such high costs associated with T production (Folstad & Karter 1992), or these costs may not be so detrimental to individuals most able to tolerate high T levels. If increased T levels in nestling birds produces increased physiological costs, this would infer T-mediation of the cost of nestling begging displays. Manipulative studies altering the levels of nestling T within physiological ranges are required in order to investigate the many potential costs involved.

In conclusion, we would suggest that there is considerable potential for T to be causally involved in controlling the level of begging effort produced by nestlings both between and within nests in this species. Such endocrine control of offspring behaviour could explain the evolution of begging as a costly signal honestly reflecting need.

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4.1 INTRODUCTION

Much is known about the role of testosterone (T) on adult passerine behaviour. For example, in a range of manipulative studies elevated plasma T levels have been found to increase mate attraction (Wingfield 1984), territorial defence (Hegner & Wingfield 1987; Hill et al. 2005; Wingfield 1984), song rate (Cynx et al. 2005; Dittami et al. 1991; Hill et al. 2005; Stoehr & Hill 2000), mate guarding (Saino & Møller 1995) and inter-male aggression (Wingfield et al. 1990). Additionally, elevated plasma T levels are also known to decrease paternal care (Hegner & Wingfield 1987; Moreno et al. 1999; Saino & Møller 1995; Stoehr & Hill 2000), reduce immunocompetence (Duffy et al. 2000; Evans et al. 2000) and prevent the onset of moult (Dawson 1994). Previous correlative studies have found that nestling T and corticosterone levels are positively related to nestling begging behaviour, suggesting that these steroid hormones may play a role in regulating begging responses (Goodship & Buchanan 2006; Quillfeldt et al. 2006; Sasvári et al. 1999). Manipulation studies are now required to investigate the cause and effect of steroid hormones on nestling begging behaviour. Previous studies on gulls, *Laridae* have found that nestling begging behaviour is increased by elevating nestling corticosterone levels (Kitaysky et al. 2003; Kitaysky et al. 2001b), but other studies have shown that begging behaviour is decreased by elevating nestling T levels (Groothuis & Meeuwissen 1992; Groothuis & Ros 2005). It still remains to be investigated how elevated T levels in nestling passerines affect begging behaviour.

Correlative studies have suggested a potential role for T to control nestling begging behaviour and regulate competition between siblings. For example, nestling T has been positively correlated with aggressive behaviour in nestling nazca boobies, *Sula granti* (Ferree et al. 2004) although other studies in blue-footed boobies, *Sula nebouxii* found that dominant nestlings did not have significantly higher T levels than subdominant nestlings (DelaMora et al. 1996; Ramos-Fernandez et al. 2000). In white storks, *Ciconia ciconia* it has been found that first hatched nestlings have higher plasma T levels, respond faster to the feeding parent and receive more food than their younger siblings (Sasvári et al. 1999). Furthermore, in the same study, the number of younger siblings that died was higher when the difference in T levels between first and later hatched nestlings was greater (Sasvári et al. 1999). In thin-billed prion nestlings, *Pachyptila belcherie* both corticosterone and T levels have been found to correlate with

pied flycatchers,
behaviour. This
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4.2 MATERIAL AND METHODS

4.2.1 *2003 Pilot study*

In 2003, a pilot study was carried out to manipulate circulating plasma T levels of pied flycatcher nestlings aged 13 - 14 days old (n = 64) with an oral dose of T in peanut oil. Manipulating T levels using T implants was avoided in this study due to the small size of nestlings, and instead an oral dose of T was administered in peanut oil. The pilot study investigated a suitable concentration of T to be given in an oral dose that would elevate, but would also keep T levels within a normal physiological range. Each nestling was temporarily (< 10 min) removed from a nestbox (n = 17) and dosed orally using a blunt tipped syringe containing either 0.5 or 1 μg of T (Sigma) in 10 or 20 μl of peanut oil respectively, before being placed back in the nestbox. At either: 0, 30, 60, 90 and 120 min after dosing, each nestling was blood sampled (approx 100 μl) to assess circulating T concentration, weighed (accuracy 0.25 g) and placed back in the nestbox.

4.2.2 *2004 Experimental tests*

In 2004, 24 pied flycatcher nestlings aged 7 days old were temporarily removed (< 5 h) from 18 nestboxes (1 – 2 nestlings removed from each box), fed to satiation with Nectarblend rearing mix (Haiths Seeds, Cleethorpes, UK) made up to a standard concentration (Kilner 2001) and transported to the field laboratory. After 30 min since food satiation, each nestling was administered with either one oral dose of 4 μg of T in 20 μl peanut oil (T dosed nestlings, n = 13) or 20 μl of the peanut oil vehicle (control nestlings, n = 11). When 2 nestlings were removed from the same brood (a pair of nestlings were taken from 3 broods), each nestling was allocated different treatments. After dosing, each nestling was placed alone on an artificial nest inside a laboratory test box and begging behaviour was recorded in begging trials at 20, 30, 70 and 90 min post dosing. In each begging trial begging behaviour was recorded as: (1) the duration (s) of the begging display and (2) the maximum height (mm) of the begging stretch. All data from video recordings were recorded blind to treatment. At 90 min post dosing, each nestling was blood sampled (100 μl) to measure circulating plasma T concentration, weighed (accuracy 0.25 g) then returned to the original nestbox in the field.

From the pilot study, it was found that after an oral dose of T, circulating plasma T levels of pied flycatcher nestlings were highest at 30 min post dosing. To assess T

levels at 30 min post dosing in 2004, an extra 12 nestlings from 3 nestboxes (4 nestlings were removed from each box) were administered with either one oral dose of 4 μg of T diluted in 20 μl peanut oil (n = 6) or 20 μl peanut oil (n = 6) and blood sampled (100 μl) after 30 min. Begging behaviour of nestlings that were blood sampled at 30 min post dosing was not recorded. Blood samples were centrifuged in a bench top centrifuge (Boeco) at 1300 rpm for 15 min at the field laboratory and plasma T was measured by radioimmunoassay at Cardiff University. Nestlings were sexed using molecular techniques at the Sheffield Molecular Genetics Facility, University of Sheffield (see 2.7.5). The protocol for molecular sexing using P2/P8 primers was based upon previously published work (Griffiths et al. 1998) and adapted specifically for pied flycatchers (see 2.7.5.2).

4.2.3 *Statistical analysis*

General Linear Model ANOVA (GLM), one-way ANOVA and Mann-Whitney *U* tests were preformed in MINITAB 14.0. In the 2003 pilot study, GLM ANOVA was used to compare mean nestling plasma T levels between two different doses of T and at 5 different time intervals post dosing. In the 2004 T manipulation study, Mann-Whitney *U* tests were used to compare T dosed and control nestling plasma T values measured at 30 min and 90 min post dosing. One-way ANOVA was used to compare nestling begging behaviour at 4 different time intervals post dosing. GLM ANOVA models were used to investigate variables which may influence variation in: (1) duration of begging display and (2) maximum height of the begging stretch. The variables entered into each GLM included: treatment (control or T-dosed nestlings), nestling sex, nestling mass, brood size and date. Variables were deleted in a stepwise fashion and normality was checked at each step. All data were checked for normality using the Kolmogorov-Smirnov test and where necessary data were transformed to meet normality.

4.3 RESULTS

4.3.1 *2003 Pilot study*

The results of the 2003 pilot study showed that after an oral dose of T (0.5 or 1 μg of T), mean plasma T levels of nestlings were elevated at 30 min post dosing and had declined to background plasma T levels by 120 min post dosing, although the difference between time intervals was non-significant (GLM ANOVA $F_{4, 58} = 1.88$, $p = 0.125$) (figure 4.1). Mean nestling plasma T increased from 0.133 to 0.221 $ngml^{-1}$ between 0 to 30 min post dosing, but decreased to 0.104 $ngml^{-1}$ at 120 min post dosing. There were no significant differences between doses of 0.5 and 1 μg T on mean plasma elevation at each time point post dosing (GLM ANOVA $F_{1, 58} = 0.03$, $p = 0.858$).

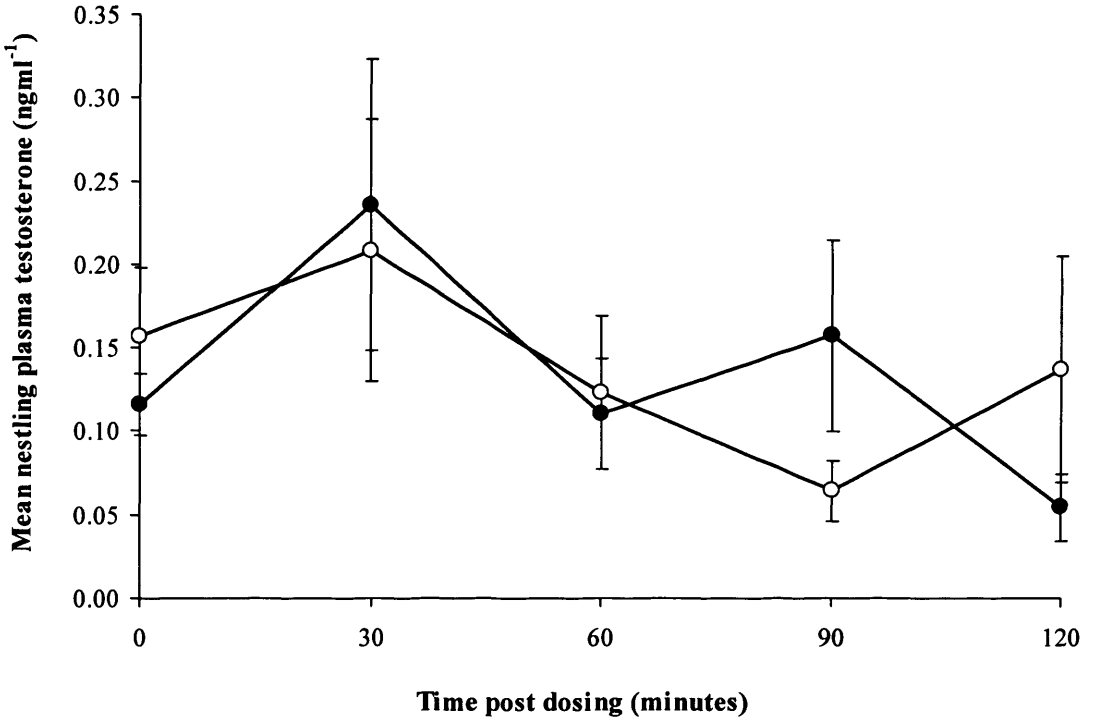


Figure 4.1: Mean ± S.E. plasma T of nestlings (13 – 14 days old) after an oral dose of 0.5 μg (open circles) or 1 μg (filled circles) testosterone diluted in 10 or 20 μl of peanut oil respectively.

4.3.2 *2004 Experimental tests: Plasma T*

The pilot results suggested that the T dose used in 2003 produced only small elevations in circulating T levels. Therefore, a higher T dose was used in 2004. This produced a larger elevation of circulating T levels which were still within a natural physiological range (0.049 – 1.194 $ngml^{-1}$).

In 2004, separate groups of T dosed and control nestlings were blood sampled at either 30 or 90 min post dosing to assess circulating plasma T levels (figure 4.2). Plasma T levels sampled at 30 min post dosing, were significantly higher for T-dosed nestlings (mean = 0.314 ± 0.085 $ngml^{-1}$) than control nestlings (mean = 0.045 ± 0.014 $ngml^{-1}$) (Mann-Whitney *U* test, T dosed nestlings: n = 6, control nestlings: n = 6, p = 0.014). However, caution may be needed when interpreting this result as some of the nestlings blood sampled at 30 min were from the same nest and therefore individual nestlings were not independent from each other.

At 90 min post dosing, the differences in plasma T levels between T-dosed nestlings (mean = 0.322 ± 0.106 $ngml^{-1}$) and control nestlings (mean = 0.125 ± 0.040 $ngml^{-1}$) were marginally non-significant (Mann-Whitney *U* test, T dosed nestlings: n = 13, control nestlings: n = 11, p = 0.068) (figure 4.2). However, as some of the nestlings measured at 90 min post dosing in this study were taken from the same brood (a pair of nestlings were taken from 3 broods), individuals were not independent. Therefore, the analysis was re-run with a reduced data set, only including individuals from a brood once. The results of the reduced data set showed that plasma T levels of T-dosed nestlings were significantly higher than controls at 90 min post dosing (Mann-Whitney *U* test, T-dosed nestlings: n = 13, control nestlings: n = 5, p = 0.027).

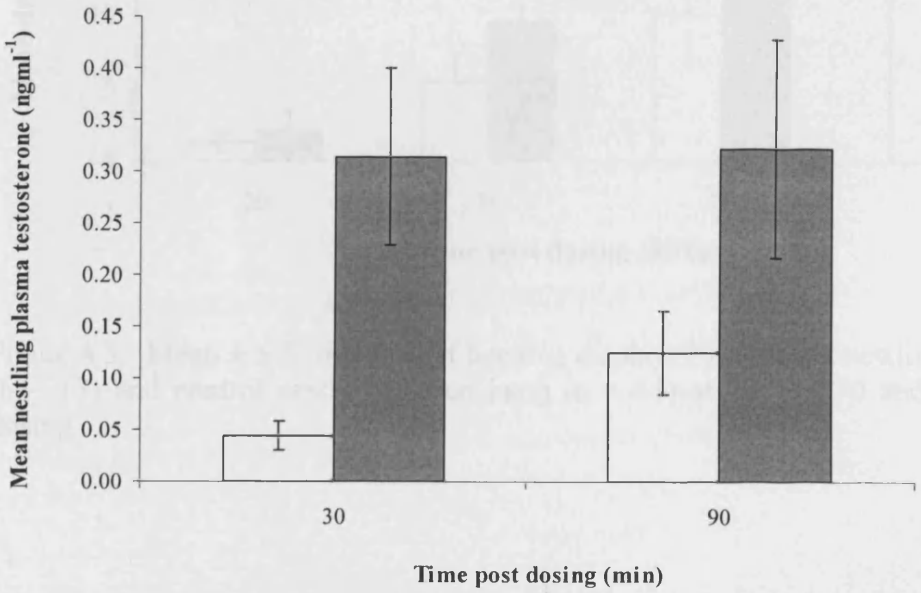


Figure 4.2: Mean ± S.E. plasma testosterone levels of T-dosed nestlings (grey bars) (n = 19) and control nestlings (open bars) (n = 17) sampled at either 30 or 90 min post dosing.

4.3.3 *2004 Experimental tests: Duration of begging display*

The mean duration of T-dosed nestling begging displays increased from 2.2 ± 1.3 s at 20 min post dosing to 28.0 ± 9.1 s at 90 min post dosing (one-way ANOVA $F_{3,48}$ = 9.18, $p < 0.001$) (figure 4.3). The mean duration of control nestling begging displays increased from 1.3 ± 0.7 s at 20 min post dosing to 17.2 ± 5.3 s at 90 min post dosing (one-way ANOVA $F_{3,39} = 4.70$, $p = 0.007$).

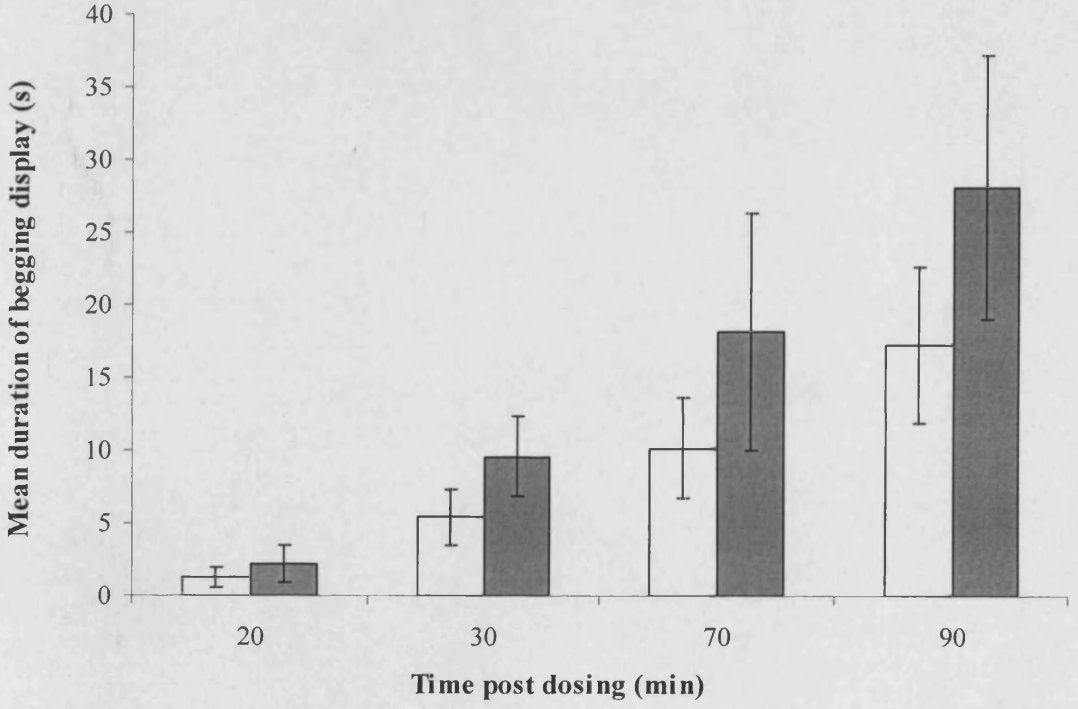


Figure 4.3: Mean ± S.E. duration of begging displays by T-dosed nestlings (grey bars) (n = 13) and control nestlings (open bars) (n = 11) at 20, 30, 70 and 90 min after dosing.

Four GLM models were constructed to investigate the effect of treatment (T-dosed or control) and nestling sex on the duration of individual nestling begging displays at 20, 30, 70 and 90 min post dosing; nestling mass, brood size and date were included in the model as covariates. The final GLM models showed that only date and treatment explained significant variation in begging duration at 30 and 90 min post dosing (table 4.1). The duration of begging displays by T-dosed nestlings were significantly longer than control nestlings at both 30 and 90 min post dosing (figure 4.4). There were significant interactions between treatment and date in the GLM models at 30 and 90 min post dosing. Nestling begging duration positively increased with date for the T-dosed nestlings, but there was a negative effect of date on begging duration for the control nestlings. The duration of nestling begging displays at 20 and 70 min post dosing were not influenced significantly by either treatment, nestling sex, nestling mass, brood size or date ($P > 0.05$).

The GLM models were repeated using the reduced data set with independent nestlings (only including individuals from a brood once) and it was again found that treatment ($F_{1,14} = 7.31$, $p = 0.017$, $n = 18$), date ($F_{1,14} = 6.43$, $p = 0.024$, $n = 18$), and an interaction between treatment and date ($F_{1,14} = 8.39$, $p = 0.012$, $n = 18$) explained significant variation in begging duration 90 min post dosing. However, the GLM model using the reduced data set at 30 min post dosing, showed that there was a marginally non-significant effect of treatment ($F_{1,13} = 3.77$, $p = 0.074$, $n = 18$), and a non-significant effect of date ($F_{1,13} = 2.97$, $p = 0.109$, $n = 18$) and interaction between treatment and date ($F_{1,13} = 4.35$, $p = 0.057$, $n = 18$) on begging duration at 30 min post dosing.

Source	d.f.	F	P
(a) 30 min post dosing			
Treatment	1	4.89	0.040
Date	1	4.48	0.048
Treatment x Date	1	5.34	0.032
(b) 90 min post dosing			
Treatment	1	9.06	0.007
Date	1	10.27	0.004
Treatment x Date	1	10.09	0.005

Table 4.1: Variables in a general linear model which explain variation in the duration of T dosed (n = 13) and control nestling (n = 11) begging displays at (a) 30 min after dosing and (b) 90 min after dosing.

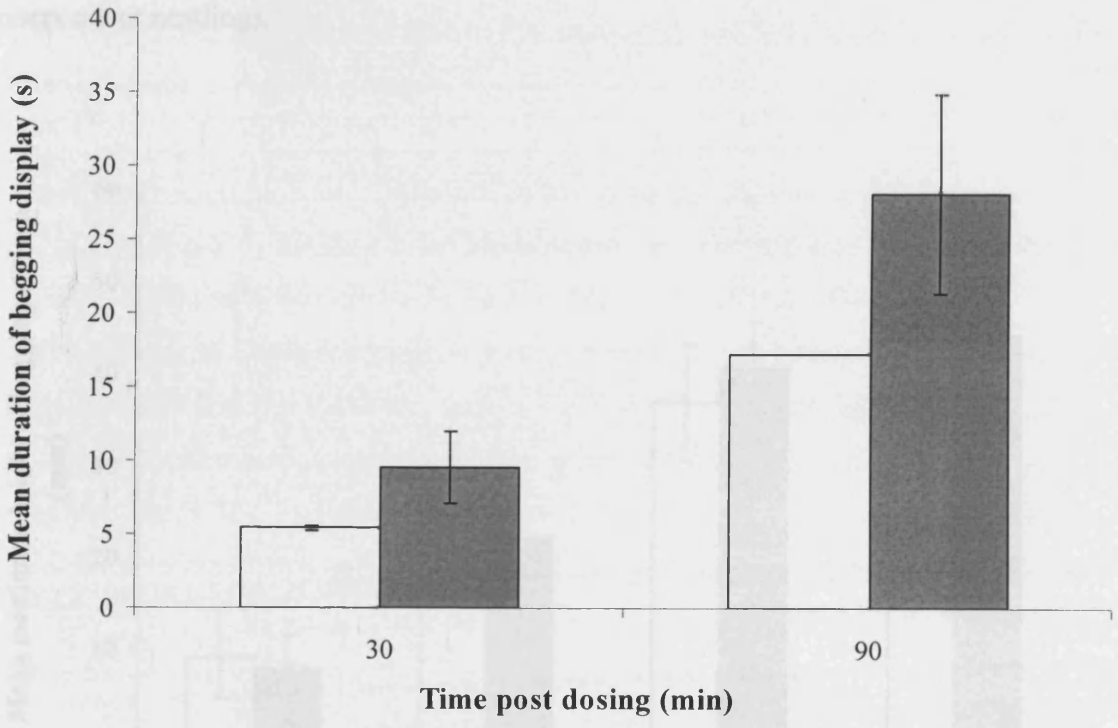


Figure 4.4: Mean ± S.E. duration of begging displays by T-dosed nestlings (grey bars) (n = 13) and control nestlings (open bars) (n = 11) at 30 and 90 min after dosing. Fitted data from GLM model are presented.

4.3.4 *2004 Experimental tests: Maximum height of begging stretch*

The mean maximum height of T-dosed nestlings increased from 7.9 ± 3.7 mm at 20 min post dosing to 44.3 ± 4.7 mm at 90 min post dosing (one-way ANOVA $F_{3,47}$ = 13.04, $p < 0.001$) (figure 4.5). The mean maximum height of control nestlings increased from 9.2 ± 4.4 mm at 20 min post dosing to 35.4 ± 6.5 mm at 90 min post dosing (one-way ANOVA $F_{3,39} = 3.87$, $p = 0.016$).

Four GLM models were constructed to investigate the affect of treatment (T-dosed or control) and nestling sex on the maximum height of nestling begging stretches at 20, 30, 70 and 90 min post dosing; nestling mass, brood size and date were included in the model as covariates. The final GLM models showed that there were no significant effects of treatment, nestling sex, nestling mass, brood size, date or any interactions between variables at any time post dosing on the maximum height of nestling begging stretches ($P > 0.05$). Similar results were obtained using the reduced data set with independent nestlings.

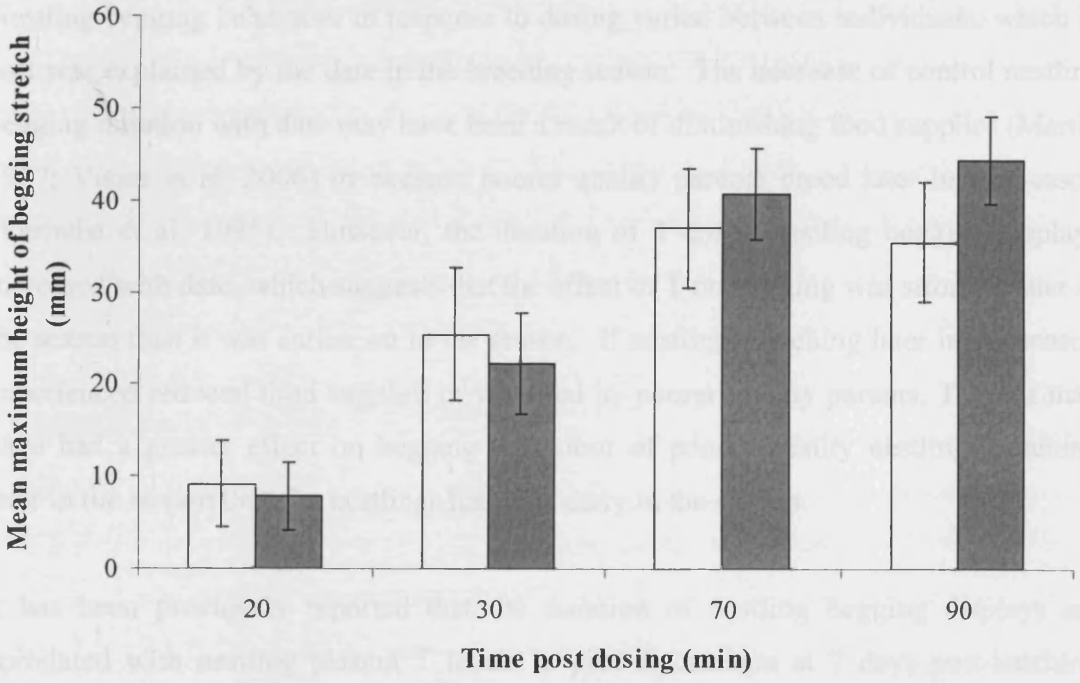


Figure 4.5: Mean ± S.E. maximum height of begging stretch by T-dosed nestlings (grey bars) (n = 13) and control nestlings (open bars) (n = 11) at 20, 30, 70 and 90 min after dosing.

4.4 DISCUSSION

The results of this study show that an oral dose of T elevated circulating plasma T levels and increased begging behaviour in nestling pied flycatchers at 7 days post-hatching. This confirms the causal nature of the positive relationship between circulating levels of T and nestling begging behaviour (Goodship & Buchanan 2006). The begging effort of nestlings, measured as the duration of begging displays, was greater for T dosed nestlings at 90 min post dosing compared with control nestlings, although there was no effect of T on the maximum height of begging stretch. The reason why this particular time interval post-dosing showed a difference in nestling begging duration is not clear, although the pilot study indicated that 30 min post-dosing approximated to the peak of nestling T absorption, a larger T-dose used in the main study showed that plasma T levels were elevated at 90 min post dosing. As there was no effect of T-dosing on the maximum height of nestling begging stretches, it may be suggested that the height of begging is not as T-dependent as begging display duration. Alternatively, the small sample size in this study may not fully show the effect of T-dosing on nestling begging stretches.

Nestling begging behaviour in response to dosing varied between individuals, which in part was explained by the date in the breeding season. The decrease of control nestling begging duration with date may have been a result of diminishing food supplies (Martin 1987; Visser et al. 2006) or because poorer quality parents breed later in the season (Verhulst et al. 1995). However, the duration of T-dosed nestling begging displays increased with date, which suggests that the effect of T on begging was stronger later in the season than it was earlier on in the season. If nestlings hatching later in the season experienced reduced food supplies or were fed by poorer quality parents, T levels may have had a greater effect on begging behaviour of poorer quality nestlings hatching later in the season than for nestlings hatching early in the season.

It has been previously reported that the duration of nestling begging displays are correlated with nestling plasma T levels in pied flycatchers at 7 days post-hatching (Goodship & Buchanan 2006). The results of this manipulation study provide further support for the hypothesis that T may be a mechanism which regulates begging behaviour in this species. Observation studies have also shown a positive relationship

between nestling endogenous T and measures of begging behaviour in thin-billed prions (Quillfeldt et al. 2006) and white storks (Sasvári et al. 1999). In a range of passerine species, including pied flycatchers, it has been shown that parents preferentially feed nestlings displaying the greatest begging intensity (Gottlander 1987b; Kacelnik et al. 1995; Kilner 1995; McRae et al. 1993; Ryden & Bengtsson 1980; Smith & Montgomerie 1991; Whittingham et al. 2003). Furthermore, many studies have also shown that parental provisioning rates to the brood as a whole increase when nestling begging intensity increases, including the duration of nestling begging displays (Ottosson et al. 1997; Price & Ydenberg 1995; Smith & Montgomerie 1991; Wright et al. 2002). By begging for food from parents, nestlings increase their growth and development (Price 1998). As T increased nestling begging behaviour in this study, nestling T production may influence nestling growth rates in pied flycatchers.

The results of the pilot study indicated that nestling plasma T can be elevated through an oral dose of T. The results from the experimental study where nestlings were dosed with a higher concentration of T than in the pilot study, also confirmed that circulating T levels were within natural physiological ranges. The results from this study confirm the potential use of oral-dosing techniques to manipulate hormone levels over the short-term, providing that the dose level and the time scale of T absorption in relation to the behaviour of interest can be quantified. Two previous studies on black-headed gulls have manipulated nestling plasma T levels using T pellets implanted subcutaneously into the neck (Groothuis & Meeuwissen 1992; Groothuis & Ros 2005). In contrast to the results of this study, T implants were not found to increase begging behaviour in black-headed gull nestlings, but T implants did strongly facilitate territorial defence and aggressive pecking behaviour (Groothuis & Meeuwissen 1992; Groothuis & Ros 2005). Three other studies which do support the findings of this study, manipulated T content of egg yolks in canaries, black-headed gulls and zebra finches and found that nestlings hatching from T-treated eggs begged more intensely than controls (Eising & Groothuis 2003; Engelhardt et al. 2006; Schwabl 1996a). In a semi-precocial species such as black-headed gulls, nestlings often perform aggressive displays and defend small territories around the nest (Groothuis & Ros 2005). This kind of aggressive behaviour is not performed by altricial pied flycatcher nestlings. The contradiction in results

between these different studies suggest that T may have a different functional role on begging behaviour in altricial and semi-precocial birds, and/or that pre-hatching and post-hatching treatment with T has different effects on begging behaviour.

Previous studies on passerine species have shown that nestlings can produce their own T from a young age (Adkins-Regan et al. 1990; Silverin & Sharp 1996; Woods et al. 1975). For example, nestling great tits, *Parus major* can produce gonadal T at 6 days post hatching (Silverin & Sharp 1996; Woods et al. 1975), and prior to that age, T is likely to be produced by the adrenal cortex glands (Adkins-Regan et al. 1990; Tanabe et al. 1986). There are several potential mechanisms through which nestling T may control begging behaviour. 1) First, it has been suggested that T may increase metabolic rate which could increase begging behaviour, although as yet, there is no direct evidence for this mechanism (Schwabl 1996a). However, corticosterone may be important for nestling respiration during begging (Schwabl & Lipar 2002a), and as corticosterone and T levels are thought to be correlated in birds (Evans et al. 2000; Roberts et al. 2004) and corticosterone and T bind to the same plasma binding globulin (Deviche et al. 2001; Klukowski et al. 1997), a rise in corticosterone levels during an increase in metabolic activity may be positively related to a rise in T levels. 2) Second, T may enhance brain function and perception of cues which stimulate begging behaviour (Andrew 1975b; Andrew 1994; Clifton et al. 1988; Godsave et al. 2002). In studies of domestic fowl chicks, T treatment has been shown to enhance attention by increasing binocular fixation and persistence (Andrew 1975b; Clifton et al. 1988). Furthermore, Eising & Groothuis (2003) found in black-headed gulls that T-manipulated nestlings were quicker and more persistent in their begging behaviour than control nestlings. 3) Third, T may increase motor neurone activity and influence the development of muscles used for begging (Godsave et al. 2002; Lipar & Ketterson 2000). For example, a study on red-winged blackbirds, *Agelaius phoeniceus*, found that T injected into egg yolks increased the mass of the *musculus complexus*, the main muscle used for begging, while injection of flutamide (T antagonist) decreased relative *complexus* mass (Lipar & Ketterson 2000). In addition, T may effect the motor control system underlying the begging muscle. For example, a study on canaries found that the *nucleus supraspinalis*, a motor neuron which plays an important role in nestling begging response (Schwabl & Lipar 2002a), has androgen receptors (Gahr et al. 1996).

Furthermore, a study on zebra finches has found that androgen receptors are expressed in several parts of the motor system in the developing embryo which may later be active during nestling begging behaviour (Godsave et al. 2002). 4) Finally, T may act indirectly through a combination of these mechanisms to affect begging intensity. This study however confirms that T is causally related to begging behaviour, and not merely a correlated variable.

Theory suggests that signalling systems may only be evolutionary stable if the signal carries associated costs (Godfray 1995a). There are costs involved with nestling begging behaviour which may maintain the honesty of the signal (Zahavi 1977), these costs include: predation risk (Briskie et al. 1999; Haskell 1994; Leech & Leonard 1997; Macnair & Parker 1979), energetic costs (Leech & Leonard 1996; Macnair & Parker 1979), growth costs (Kilner 2001) and physiological costs through endogenous T production (Buchanan et al. 2001; Evans et al. 2000; Folstad & Karter 1992; Johnsen 1998). There are several sources of physiological costs associated with endogenous T production, they include: increased levels of aggression (Johnsen 1998), increased metabolic rate (Buchanan et al. 2001), increased corticosterone levels (Evans et al. 2000), reduced immunocompetence (Folstad & Karter 1992) and growth costs (Groothuis & Ros 2005). If T production results in a physiological cost, low quality individuals are expected to be unable to withstand the costs involved with signalling, which keeps the signal honest (Folstad & Karter 1992).

In conclusion, this study has shown that nestling begging was positively influenced by T as the duration of nestling begging displays were longer by T dosed nestlings than by controls. This manipulative study confirms T is responsible in part for the control of begging intensity in nestling birds and is not merely a correlated variable. This study suggests that T may be a mechanism which controls begging behaviour in nestling birds.

Chapter 5

Parental provisioning, nestling begging behaviour and testosterone during brood reduction in pied flycatchers, *Ficedula hypoleuca*

5.1 INTRODUCTION

Altricial nestling birds are unable to forage for themselves and they attract parental food provisioning by begging (e.g. Kilner & Johnstone 1997; McRae et al. 1993). Due to conflicts of interest between parents and offspring (Hamilton 1964; Trivers 1974) and intra-brood competition between siblings (Macnair & Parker 1979), the begging behaviour of a given nestling is expected to depend on the behaviour of its parents and siblings (Harper 1986; Parker & Macnair 1979). In theory, parents are expected to provide most resources to nestlings with the greatest fitness returns (Clutton-Brock 1991). Signalling models suggest that nestling begging behaviour indicates cryptic levels of offspring condition, and that following assessment of individual begging levels, parents respond by actively distributing food to nestlings with the greatest need (Godfray 1991; Godfray 1995a). Alternatively, scramble competition models suggest that nestlings compete with nestmates through begging behaviour for limited food resources, and that parents respond by passively feeding nestlings displaying the greatest begging intensity (Harper 1986; Parker & Macnair 1979; Rodriguez-Girones 1999). Both types of model assume that parents feed nestlings in relation to their begging intensity and that begging is costly (Kilner & Johnstone 1997). However, in signalling models parents control food allocation among nestlings, whereas in models of scramble competition the nestlings have control.

When distributing food among nestlings, parents may respond to several different signals that indicate nestling condition or competitive ability (Whittingham et al. 2003). Parents may respond to signals controlled by nestlings such as begging behaviour (e.g. McRae et al 1993; Gottlander 1987), or parents may respond to other types of cues not under nestling control such as nestling size (Bengtsson & Ryden 1981; Price & Ydenberg 1995) and sex (Lessells 2002; Trivers & Willard 1973). Past studies which have demonstrated that nestling begging behaviour influences the distribution of food within a nest, have suggested that siblings compete for parental food provisioning. For example, studies on a range of passerine species have shown that parents preferentially feed nestlings that are: 1) highest in the nest (Kacelnik et al. 1995), 2) occupy a key position within the nest in close proximity to the feeding parent (Gottlander 1987b; Kacelnik et al. 1995; Kilner 1995; McRae et al. 1993; Ryden & Bengtsson 1980; Smith & Montgomerie 1991; Whittingham et al. 2003) and 3) are first to beg (Price &

1991). In additio
reduce their provis

Hormonal mecha
competition. For
corticosterone (Ki

Groothuis 2003; Schwabl 1996a) have been shown to enhance begging behaviour. Nestling begging behaviour has been positively correlated with nestling endogenous testosterone (T) levels in pied flycatchers, (Goodship & Buchanan 2006; see chapter 3), thin-billed prions, *Pachyptila belcheri* (Quillfeldt et al. 2006) and white storks, *Ciconia ciconia* (Sasvári et al. 1999). Furthermore, in chapter 4 it was shown that experimentally elevated T levels in nestling pied flycatchers increases nestling begging duration. Maternally derived T has been shown to enhance nestling begging behaviour, which is expected to increase success in sibling competition (Schwabl 1996a) and may also give a competitive advantage to nestlings by increasing the size of the *musculus complexus*, the neck muscle used for hatching and begging (Lipar & Ketterson 2000). In a recent study, circulating plasma T levels in nestling zebra finches, *Taeniopygia guttata* have been correlated with experimentally increased brood size (Naguib et al. 2004). The correlation between nestling T and brood size may have been a result of enhanced sibling competition in the larger broods (Naguib et al. 2004). However, apart from the study by Naguib et al. (2004), little is known about the effects of brood size and sibling competition levels on the variation of nestling T levels.

The present study on pied flycatchers experimentally reduced brood size. The aims of this study were to: 1) investigate parental provisioning strategies in relation to nestling begging signals and other cues, 2) test the effects of brood reduction on parental provisioning strategies, 3) test the effect of brood reduction on nestling behaviour and 4) test the effect of brood reduction on nestling T levels. It was predicted that brood reduction would cause a decrease in nestling begging behaviour, androgen levels and consequently parental provisioning rates.

5.2.2 Video analysi

Parental provisioni
recorded for 1 h in
was classified as:
to the brood h^{-1}), 3
within a brood h^{-1})

feed nestling per visit to the brood (s), 6) feeding call rate h^{-1} (a call made by parents to stimulate nestling begging behaviour), 7) percentage of total parental feeds given to nestlings that extended their neck highest in the nest immediately prior to feeding, 8) percentage of total parental feeds given to nestlings that held their beak closest to the parent immediately prior to feeding and 9) percentage of total parental feeds given to nestlings that were first to beg on parent arrival.

Nestling begging behaviour was classified as: 1) total number of times each brood begged h^{-1}, 2) total number of times each brood begged in parental absence h^{-1}, 3) total number of times each brood begged in parental presence h^{-1}, 4) brood begging duration per parental visit (s) and 5) percentage of brood that begged per parental visit.

5.2.3 Statistical analysis

Wilcoxon matched-pairs signed-ranks tests, Spearman rank correlations, General Linear Models (GLM) and nested ANOVA and were preformed in MINITAB 14.0. Wilcoxon matched-pairs signed-ranks tests were used to compare provisioning rates between adult males and females paired at the same nest on control and experimental days. In addition, Wilcoxon matched-pairs signed-ranks tests were also used to compare parental provisioning or nestling begging behaviour between control days and experimental days. Spearman rank correlations were used to correlate nestling begging signals. GLM ANOVA was used to investigate the influence of mean brood androgen levels, mean brood mass and brood size on parental provisioning rates. Nested ANOVA was used to investigate any differences in parental provisioning rates to male and female nestlings (nestling sex nested within brood). When parametric tests were used, the normal distribution of residuals was checked using the Kolmogorov-Smirnov test and all percentage data were arcsine square root transformed.

5.3 RESULTS

5.3.1 Parental provisioning rates on control days

On control days, when nestlings were 6 days old, parents visited their brood (n = 8) at an average rate of 41.9 ± 4.8 visits h^{-1}. On each visit, parents always fed 1 - 3 nestlings, although the modal number of nestlings fed per visit was 1. Mean nestling provisioning rate by parents (number of feeds to each nestling within a brood h^{-1}) occurred at an average rate of 6.7 ± 0.8 feeds $nestling^{-1}h^{-1}$. Brood provisioning rate by both parents (number of feeds to the brood h^{-1}) occurred at an average rate of 43.8 ± 4.7 feeds $brood^{-1}h^{-1}$ on control days with adult females provisioning their brood (mean = 22.5 ± 2.1 feeds $brood^{-1}h^{-1}$) at an equal rate to adult males (mean = 21.3 ± 3.0 feeds $brood^{-1}h^{-1}$) paired at the same nest (Wilcoxon matched-pairs signed-ranks test W = 22.5, p = 0.575, n = 8). A GLM ANOVA model with brood provisioning rate by parents as the dependent variable, showed that within control broods, there was no significant effect of variation in brood size ($F_{2,3}$ = 1.40, p = 0.372, n = 8) date ($F_{1,3}$ = 3.94, p = 0.141, n = 8) or time of day at which videos were recorded ($F_{1,3}$ = 4.80, p = 0.116, n = 8) on parental provisioning rates. Both parents produced a feeding call upon arrival at the nest to stimulate nestling begging behaviour and on average feeding calls were made on 20.2 ± 5.5 % of parental visits.

5.3.2 Parental provisioning rules on control days: nestling begging signals

This study examined if parents responded to nestling begging signals when allocating food resources. Nestlings that either extended their neck highest in the nest, held their beak closest to the parent, or were first to beg, gained more than 50 % of total parental feeds to the brood (figure 5.1; highest nestling mean = 77.5 ± 3.3 % of total parental feeds; closest nestling mean = 74.6 ± 2.0 % of total parental feeds; first to beg nestling mean = 60.4 ± 4.9 % of total parental feeds). Within nests, Wilcoxon matched-pairs signed-ranks tests were used to demonstrate that the results for the highest (W = 36.0, p = 0.014, n = 8) and the closest nestlings (W = 36.0, p = 0.014, n = 8) were statistically significant, whilst nestlings that were first to beg tended to be fed more (W = 25.0, p = 0.076, n = 8). Within nests there were significant correlations between nestling begging signals: the number of parental feeds given to nestlings that extended their neck highest in the nest was positively correlated with the number of feeds given to nestlings that held their beak closest to the parent (Spearman rank correlation r_s =

0.929, p = 0.001, n = 8) and to nestlings that were first to beg (Spearman rank correlation $r_s = 0.922$, p = 0.001, n = 8). In addition, the number of parental feeds given to nestlings that held their beak closest to the parent was positively correlated with the number of parental feeds given to nestlings that were first to beg (Spearman rank correlation $r_s = 0.826$, p = 0.011, n = 8). Wilcoxon matched-pairs signed-ranks tests were used to show that there were no significant differences between male and female parents, paired at the same nest, in the percentage of total feeds given to nestlings that extended their neck highest in the nest (W = 18.0, p = 1.000, n = 8), held their beak closest to the parent (W = 14.0, p = 0.624, n = 8) or were first to beg (W = 5.0, p = 0.151, n = 8).

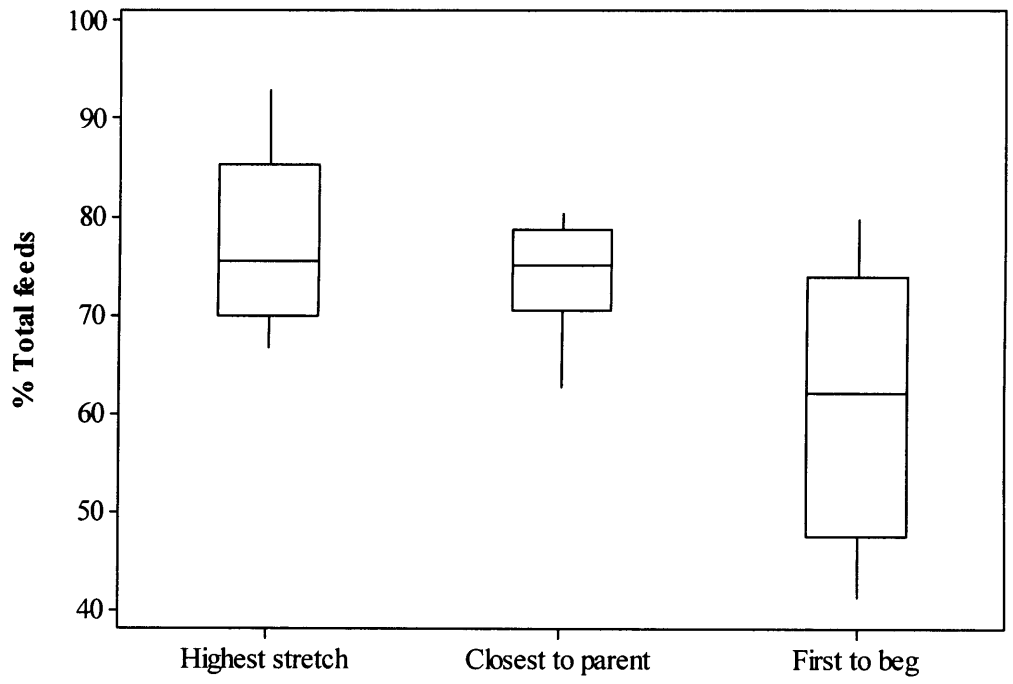


Figure 5.1: Boxplots with interquartile ranges showing the percentage of total parental feeds to nestlings that extended their neck highest in the nest (highest stretch), held their beak closest to the parent (closest to parent) and were first to beg (first to beg). Data are from 8 pied flycatcher broods on control days.

5.3.3 Parental provisioning rules on control days: nestling sex, mass and androgen

On control days, parental allocation responses to cues of nestling sex, mass or androgen levels were investigated. The mean sex ratio (3.0 ± 0.3 males: 3.6 ± 0.2 females) was not significantly different in 8 broods (Wilcoxon matched-pairs signed-ranks test W = 4.0, p = 0.208, n = 8; table 5.1). Nestling sex was not found to influence parental provisioning rates; the percentage of total feeds by adult males were allocated evenly between male and female nestlings ($F_{8,37} = 0.40$, p = 0.911, n = 8) in a nested ANOVA (nestling sex nested within brood). Similarly, adult females fed nestlings of each sex equally (nested ANOVA $F_{8,37} = 0.42$, p = 0.899, n = 8). Two GLM models (one for adult males and one for adult females) were constructed to test if mean brood faecal androgen levels, mean brood mass or brood size influenced brood provisioning rates by adults on control days. There were no effects of these variables on either adult male or female brood provisioning rates ($p > 0.05$).

Brood	Number of nestlings in brood on control day			Number of nestlings in brood on experimental day		
	Male	Female	Total	Male	Female	Total
A	3	4	7	2	2	4
B	4	4	8	3	2	5
C	2	4	6	1	2	3
D	2	4	6	0	3	3
E	2	4	6	1	2	3
F	3	3	6	3	0	3
G	4	3	7	2	2	4
H	4	3	7	2	2	4

Table 5.1: Number of male and female nestlings in 8 broods (A – H) on control and experimental days.

5.3.4 Parental provisioning rates on experimental v control days

The results of this study show that when brood size was reduced (3 nestlings removed from each of 8 broods), adult females reduced provisioning rates to their brood, although adult males did not alter their provisioning rates significantly (table 5.2, figure 5.2). Overall, brood provisioning rate h^{-1} by parents decreased by 22.6 % compared with the same broods on control days. Mean nestling provisioning rate by parents (number of feeds to each nestling within a brood h^{-1}) increased on experimental days, and on average each nestling received 2 extra feeds h^{-1} compared with control days. Parental latency to feed and the feeding call rate h^{-1} were not altered by the reduction in brood size (table 5.2). A GLM ANOVA model with brood provisioning rate by parents as the dependent variable, showed that there was no significant effect of brood size ($F_{2,3}$ = 3.16, p = 0.182, n = 8), which was expected since the variation was low, date ($F_{1,3}$ = 2.12, p = 0.241, n = 8) or time of day at which videos were recorded ($F_{1,3}$ = 0.35, p = 0.594, n = 8) on brood provisioning rates on experimental days.

5.3.5 Nestling begging behaviour on experimental v control days

Broods begged on 100 % of parental visits on experimental and control days. Brood begging duration per parental visit was of a similar length on experimental days (mean = 12.2 ± 1.9 s) compared with control days (mean = 11.7 ± 1.7 s) (Wilcoxon matched-pairs signed-ranks tests W = 12.0, p = 0.441, n = 8). In addition, the percentage of each brood that begged per parental visit was similar between experimental days (mean = 66.2 ± 4.5 %) and control days (mean = 60.5 ± 6.7 %) (Wilcoxon matched-pairs signed-ranks tests W = 7.0, p = 0.272, n = 8). It was noted that broods occasionally begged when parents were absent from the nest. As a percentage of the total number of times that broods begged h^{-1}, begging in the absence of parents on experimental days (mean = 2.8 ± 1.0 %) compared with control days (mean = 4.9 ± 2.7 %) was not significantly different (Wilcoxon matched-pairs signed-ranks tests W = 11.0, p = 1.000, n = 8).

Parental provisioning behaviour	Mean ± S.E. Control Day	Mean ± S.E. Experimental day	W	p
Adult female brood provisioning rate h^{-1}	22.5 ± 2.1	14.1 ± 1.9	35.0	0.021
Adult male brood provisioning rate h^{-1}	21.3 ± 3.0	19.8 ± 2.0	13.0	0.675
Brood provisioning rate h^{-1}	43.8 ± 4.7	33.9 ± 3.0	32.0	0.059
Mean nestling provisioning rate h^{-1}	6.7 ± 0.8	9.5 ± 0.8	0.0	0.022
Mean latency to feed per visit (s)	4.3 ± 0.9	4.9 ± 0.8	10.0	0.294
Feeding call rate h^{-1}	8.5 ± 2.6	10.6 ± 2.4	8.0	0.353

Table 5.2: Mean ± S.E. parental brood (n = 8) provisioning behaviour on control and experimental days. Wilcoxon matched-pairs signed-ranks tests show differences between control and experimental days.

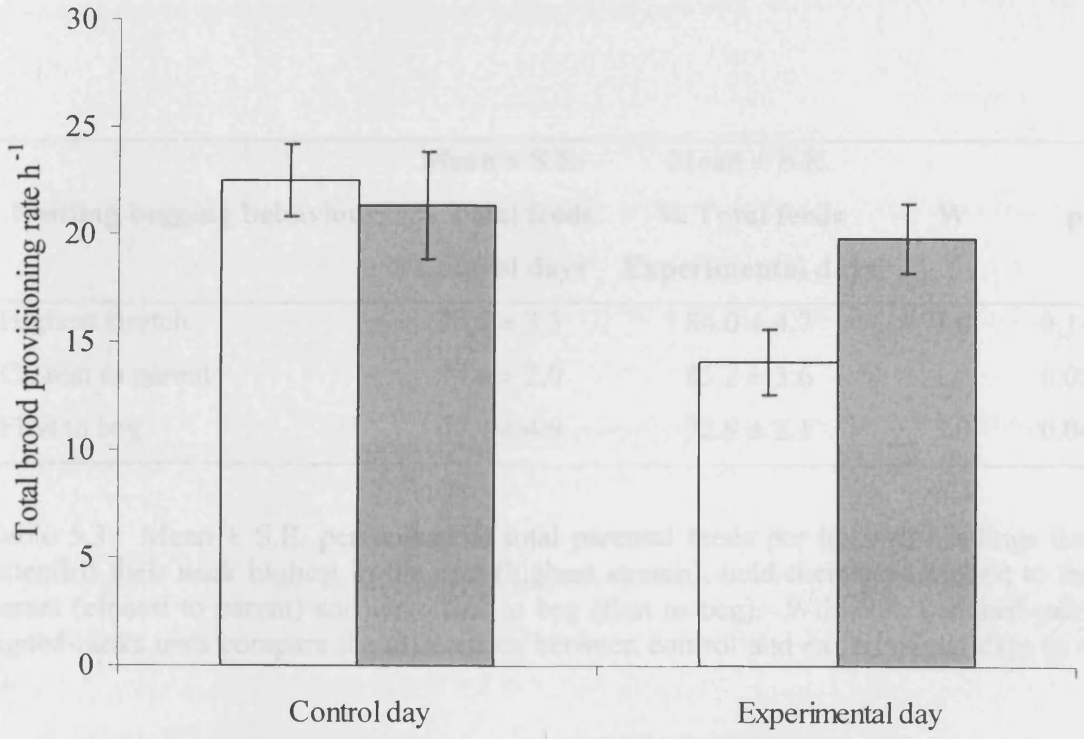


Figure 5.2: Mean ± S.E. total brood (n = 8) provisioning rate by adult females (open bars) and adult males (grey bars) on control and experimental days.

5.3.6 Parental provisioning rules on experimental days: nestling begging signals

Parents altered their provisioning behaviour to individual nestlings in response to a reduced brood size. Nestlings that were first to beg or held their beak closest to the parent gained a greater percentage of total parental feeds on experimental days compared with control days (table 5.3). However, nestlings that extended their neck highest in the nest were not fed significantly more by parents on experimental days compared with control days (table 5.3). The percentage of total feeds allocated to nestlings according to begging strategy on experimental days was compared between adult male and females paired at the same nest (figure 5.3). Compared with adult males, females tended to allocate more of their feeds to nestlings that were first to beg (Wilcoxon matched-pairs signed-ranks test $W = 4.0$, $p = 0.059$, $n = 8$) or held their beak closest to the parent (Wilcoxon matched-pairs signed-ranks test $W = 5.0$, $p = 0.080$, $n = 8$), although the difference was not statistically significant. There were clearly no significant differences between adult males and females in the percentage of feeds given to nestlings that extended their neck highest (Wilcoxon matched-pairs signed-ranks test $W = 10.0$, $p = 0.294$, $n = 8$).

Nestling begging behaviour	Mean ± S.E. % Total feeds Control days	Mean ± S.E. % Total feeds Experimental days	W	p
Highest stretch	77.5 ± 3.3	84.0 ± 4.7	7.0	0.141
Closest to parent	74.6 ± 2.0	85.2 ± 3.6	1.0	0.021
First to beg	60.4 ± 4.9	72.9 ± 2.1	3.0	0.042

Table 5.3: Mean ± S.E. percentage of total parental feeds per hour to nestlings that extended their neck highest in the nest (highest stretch), held their beak closest to the parent (closest to parent) and were first to beg (first to beg). Wilcoxon matched-pairs signed-ranks tests compare the differences between control and experimental days ($n = 8$).

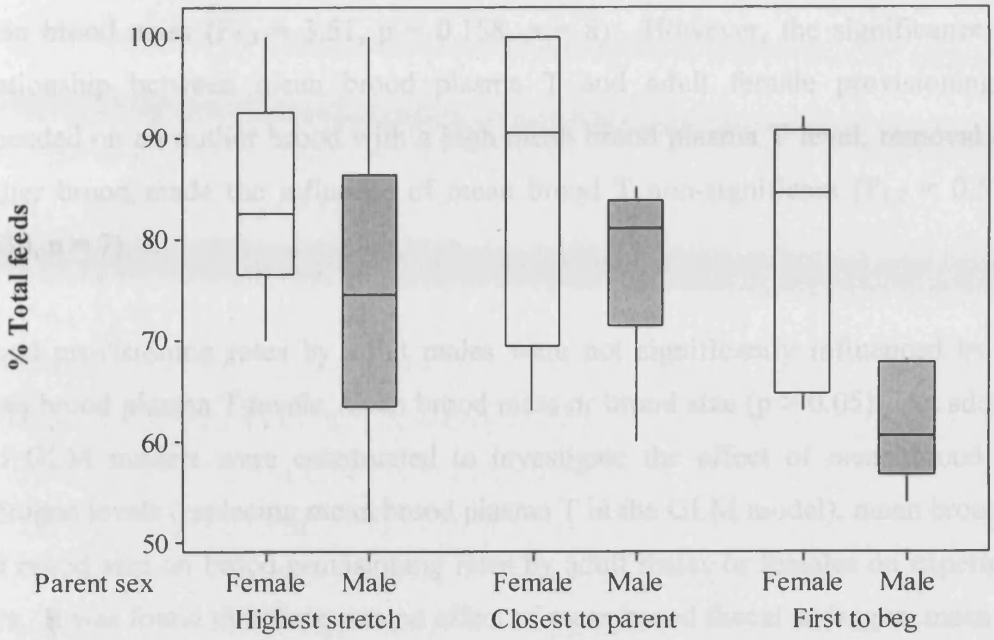
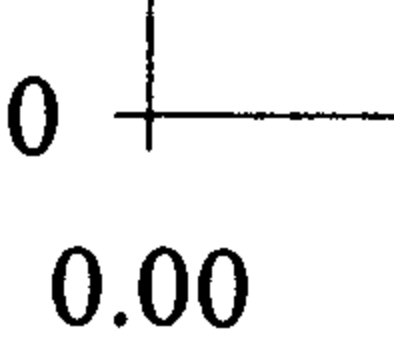


Figure 5.3: Boxplots with interquartile ranges showing the percentage of total feeds by adult females (open bar) and males (grey bar) to nestlings that extended their neck highest (highest stretch), held their beak closest to the parent (closest to parent) and were first to beg (first to beg) on experimental days (n = 8).

5.3.7 Parental provisioning rules on experimental days: nestling mass and T

As expected, mean brood mass was significantly greater on experimental days (mean = 11.6 ± 0.2 g) when nestlings were 7 days old compared with mean brood mass on control days (mean = 9.9 ± 0.3 g) when nestlings were 6 days old (Wilcoxon matched-pairs signed-ranks test W = 0.0, p = 0.014, n = 8). However, mean brood faecal androgen levels were similar between experimental and control days (Wilcoxon matched-pairs signed-ranks test W = 11.0, p = 0.363, n = 8).

Nestling plasma T was also measured on experimental days (nestling plasma T was not measured on control days). Two GLM models (one for adult males and one for adult females), were constructed to test if mean brood plasma T, mean brood mass or brood size influenced brood provisioning rates by adults on experimental days. It was found that there was a significant positive effect of mean brood T ($F_{1,3}$ = 9.75, p = 0.052, n = 8) and a non-significant positive effect of brood size ($F_{2,3}$ = 7.90, p = 0.064, n = 8) on



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significantly alter nestling plasma T levels. Further studies using a larger sample size and a longer period of brood reduction are needed to fully investigate the effect of reduced brood size on nestling plasma T levels.

Previous studies have shown that parents respond to changes in nestling begging behaviour by altering their food distribution to individual nestlings within a brood (Kilner 1995; Redondo & Castro 1992). The results of this study suggest that parents responded to brood reduction by more often feeding nestlings showing the greatest begging display. Parents gave a greater percentage of their feeds to nestlings that held their beak closest to the provisioning parent or were first to beg during brood reduction compared with unmanipulated broods (table 5.3). A possible reason why parents changed their provisioning rules during brood reduction is that they may have had more time to assess nestling behaviour. An indication that parents had more time for each nestling during brood reduction may be illustrated by the result that parental provisioning rate to each nestling increased on experimental days. On average, each nestling within a brood was given two extra feeds per hour during brood reduction compared with unmanipulated broods.

Parents have been found to respond to changes in nestling begging intensity and sibling competition by altering provisioning rates to broods as a whole. For example, previous studies on nestling American robins (Smith & Montgomerie 1991) and great tits, *Parus major* (Neuenschwander et al. 2003) have shown that begging intensity increases when sibling competition is increased and that parents respond to higher levels of begging behaviour by increasing their provisioning rates. The results of this study found that although brood begging duration and the percentage of the brood that begged per adult visit was not changed by brood reduction, adult female provisioning rates to their brood decreased when brood size was reduced. These results may suggest that when the size of a brood was reduced, adult females either responded to changes in other types of nestling begging behaviour that were not measured in this study, or that females responded directly to the perception of a reduced reproductive output. In a similar study on a population of pied flycatchers in north Wales, Wright et al. (2002), also experimentally manipulated brood size to create small broods (4 nestlings) and large broods (8 nestlings), each being roughly two nestlings either side of the mean brood

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whereas males did not alter their parental effort (Wiehn & Korpimaki 1997). Wiehn & Korpimaki (1997) suggested from their study on kestrels that while females are able to adjust parental provisioning, male parental effort is fixed at a level where male survival is not jeopardized.

Parental favouritism may be related to nestling cues such as sex, size or age (Lessells 2002; Whittingham et al. 2003). In this study it appeared that males and females provisioned nestlings equally, regardless of their sex and size, but age differences between nestlings were not investigated (nestlings were all the same age). Previous studies have found that parents can differentially provision sons and daughters within a brood (Droge et al. 1991; Lessells 2002). For example, a study on eastern bluebirds, *Sialia sialis* found that adult males provisioned daughters more than sons which may because sons are more philopatric than daughters and may compete with their fathers for mates (Droge et al. 1991). In a food deprivation study, large pied flycatcher nestlings have been found to be able to out compete smaller nestmates for key nest positions and thereby gaining more parental food (Gottlander 1987b). However, in the same study, nestling size was not correlated with gaining the best nest position under natural conditions in the field. Similarly, tree swallow nestlings are most likely to be fed by parents as a result of their own begging behaviour rather than through a cue of nestling sex or size (Whittingham et al. 2003), and in another study on the same species, nestlings of different sizes were fed equally (Leonard & Horn 1996). Male and female parents may also use different feeding rules to provision their offspring. For example, female canaries pay less attention to nestling begging posture as nestlings grow older, whereas provisioning by males remains constant over the same range of nestling ages (Lessells 2002). In this study, there were no significant differences between males and females in their provisioning rates to nestlings that were either highest in the nest, closest to the parent or first to beg within unmanipulated broods. However, during brood reduction, adult females tended to give a greater percentage of their feeds to nestlings that were first to beg or held their beak closest to the parent compared to adult males paired at the same brood, suggesting that there may be some sex differences in provisioning rules between adults in response to the reduction in brood size.

It was noted in this study that broods occasionally begged when parents were absent from the nest. Overall, nestlings begged more in parental absence between parental feeding visits in unmanipulated broods, than when sibling competition was reduced during brood reduction, although the difference was not significant. Previous studies have shown that nestling tree swallows beg in parental absence both between feeding visits and immediately after parents leave the nest, which may influence parental feeding decisions (Leonard et al. 2000). In addition, it has been suggested that nestling begging in the absence of parents may form a means of communication between siblings to reduce sibling competition by negotiating which nestling will be fed on the next feeding visit (Roulin et al. 2000).

In conclusion, this study found that parents responded to nestling begging behaviour and preferentially fed nestlings that extended their neck highest in the nest, held their beak closest to the provisioning parent, or were first to beg. Adult females altered their provisioning rates in response to brood reduction, but adult males did not decrease their provisioning rates significantly. Parents altered their provisioning rules during brood reduction, and food distribution within a brood was more strongly based on nestling begging behaviour. Nestling sex, mass or faecal androgen levels were not found to influence parental provisioning rates. During brood reduction, female provisioning rates were positively related to mean brood T levels, although further work using a larger sample size is needed to test the reliability of this result. A reduction in brood size was not found to influence nestling faecal androgen levels, but this may have been because brood size was not reduced for a long enough period.

6.1 INTRODUCTION

The level of cooperation which is optimal for participants, depends on the degree of relatedness between them (Hamilton 1964). As first expressed by Hamilton's (1964) rule, the coefficient of relatedness (r) between any two individuals will determine how altruistic those individuals are when they compete for limited resources. Cooperation is expected to be greater between closely related individuals than between distantly related individuals. Mendelian heredity shows, that about half of all rare genes (r = 0.5) are usually shared between a parent and an offspring and also between any two offspring with the same genetic parents. Even when parents and offspring are fully related, conflicts of interest occur over the optimum division of food resources, as a given offspring is 100 % related to itself but only 50 % related to its parents and siblings, therefore each offspring has a greater invested interest in itself (Trivers 1974). Theory suggests that offspring are selected to demand more resources than parents are selected to provide (Trivers 1974). In addition, each offspring is selected to demand a greater share of resources than it is selected to yield to other siblings, although the selfishness of each offspring is limited as it still has some genetic interest in the survival of its siblings (Macnair & Parker 1979). However, if a female participates in an extra-pair copulation with another male, a situation may arise that full siblings share the same parental resources with half siblings that have genes only from the mother (r = 0.25). It is expected that as relatedness within a brood declines, so too does the degree of cooperation between individuals, and offspring should try to take more of the food resources for themselves. Alternatively, if offspring share a high degree of relatedness with other siblings they are expected to share more parental resources (Godfray 1991; Hamilton 1964).

In many species of birds, extra-pair copulations result in extra-pair paternity, which means that some males feed offspring that are not their own and that broods are a mixture of full and half siblings (Birkhead & Møller 1992). For example, extra-pair copulations occur in pied flycatchers, *Ficedula hypoleuca* (Alatalo et al. 1989; Brun et al. 1996; Gelter & Tegelstrom 1992; Lifjeld et al. 1991; Ratti et al. 1995), and in a closely related species, the collared flycatcher, *Ficedula albicollis* (Alatalo et al. 1989; Sheldon & Ellegren 1999). Paternity studies using genetic techniques of DNA fingerprinting to investigate relatedness in pied flycatchers, have found that the degree

to which extra-pair paternity occurs varies between populations (Brun et al. 1996; Gelter & Tegelstrom 1992; Lifjeld et al. 1991; Ratti et al. 1995). For example, a study in Sweden on 38 nestlings from 7 broods, showed that 24 % of nestlings (n = 9) in 43 % of broods (n = 3) were a result of extra-pair copulations (Gelter & Tegelstrom 1992). However, a study in Norway on 135 nestlings from 27 broods, found that only 4 % of nestlings (n = 6) in 15 % of broods (n = 4) were a result of extra-pair copulations (Lifjeld et al. 1991). Unrelated offspring may also arise through brood parasitism, where females of the same or different species lay their eggs in another nest, leaving the host to care for the young (Briskie et al. 1994; Lank et al. 1989). However, as egg dumping is very rare in female pied flycatchers, females are almost always related to their offspring (Lundberg & Alatalo 1992).

By begging, nestling birds gain food resources from parents and many studies have shown that parents feed nestlings displaying the greatest begging intensity (Gottlander 1987b; Kacelnik et al. 1995; Price & Ydenberg 1995; Ryden & Bengtsson 1980; Smith & Montgomerie 1991; Whittingham et al. 2003). Previous studies have found that nestling begging behaviour and paternal provisioning rates are influenced by the degree of relatedness between different members of a brood (Briskie et al. 1994; Lifjeld et al. 1998; Sheldon & Ellegren 1998). For example, an observational study by Briskie et al. (1994) found that the loudness of nestling begging behaviour increased as relatedness among brood members declined in different passerine species. However, in contrast, an observational study on tree swallows, *Tachycineta bicolor* found that nestling begging behaviour did not differ with respect to paternity (Whittingham et al. 2003). Observational studies have also shown that paternal provisioning is reduced with lower paternity (Dixon et al. 1994; FreemanGallant 1996; Møller & Tegelstrom 1997), but other studies have not found this effect (Wagner et al. 1996; Westneat et al. 1995; Whittingham et al. 2003; Yezerinac et al. 1996).

Experimental studies, investigating the influence of group relatedness on offspring begging and adult provisioning behaviour, have manipulated relatedness between individuals through cross-fostering, playback and mixed paternity experiments. For example, a cross-fostering experiment on great tits, *Parus major* (Kölliker et al. 2000) found that the origin of nestlings influences their begging behaviour (Kölliker et al.

2000). A cross-fostering experiment on burying beetles, *Nicrophorus vespilloides* (Lock et al. 2004) also found that the intensity of offspring begging behaviour is positively influenced by how closely related individuals are. In burying beetles, adult provisioning tended to be more strongly correlated with offspring begging displays within biological families, than between unrelated individuals (Lock et al. 2004). Studies using playback calls have shown that parents are able to discriminate between the calls of related and unrelated offspring (Hatchwell et al. 2001; Insley et al. 2003), although in some species, recognition ability may differ between male and female parents (Insley et al. 2003; Kölliker et al. 2000). Paternity has been experimentally manipulated in a range of avian species including: dunnocks, *Prunella modularis* (Davies et al. 1992), pied flycatchers (Lifjeld et al. 1998), collared flycatchers (Sheldon & Ellegren 1998) and European starlings, *Sturnus vulgaris* (Wright & Cotton 1994), and these studies have found that males reduce their parental effort in response to reduced paternity.

Previous experimental studies investigating hormonal mechanisms controlling begging intensity in young birds, have identified that steroid hormones including corticosterone and steroid androgens, in particular testosterone (T), positively affect nestling begging behaviour (Eising & Groothuis 2003; Kitaysky et al. 2001b; Schwabl 1996a). In addition, it was found in chapter 4 of this thesis, that experimentally elevated T levels in nestling pied flycatchers increased begging intensity. It may be suggested from these studies that if androgens are involved with regulating nestling begging displays, and begging behaviour increases when brood relatedness is reduced, nestling androgen levels may also be negatively correlated with brood relatedness. Manipulative studies are now required to investigate the effect of brood relatedness on nestling T levels.

The aim of this study was to investigate the effect of reduced brood relatedness on nestling begging behaviour, nestling androgen levels and adult provisioning rates in pied flycatchers. Brood relatedness was reduced through a partial cross-fostering experiment in order to create broods composed of true and foster siblings. It was predicted that nestlings in partially cross-fostered broods would beg more than control broods to attract a greater share of food resources from adults. In addition, nestlings in partially cross-fostered broods were expected to have higher androgen levels compared

with control broods because of associated increases in begging behaviour. If parents are able to detect the presence of offspring that are not their own, parents may be expected to either decrease their provisioning rates in response to reduced brood relatedness, or to selectively chose their own related offspring.

6.2 MATERIAL AND METHODS

6.2.1 Experimental protocol

In June 2004, 12 pied flycatcher broods were randomly assigned into experimental broods (n = 6) and control broods (n = 6). All nestlings investigated in this study were aged between 9 and 11 days old.

On day 1, before brood manipulation, all nestlings in experimental and control broods were faecal sampled, by collecting a faecal sac volunteered by the nestling, to assess excreted androgen levels. To manipulate experimental broods, half of the nestlings within a brood were cross-fostered into a paired brood for 24 h (A – F) matched for nestling age (table 6.1). Within each experimental brood during manipulation, half of the brood was composed of siblings remaining in their natal brood (home-sibs), and the other half was composed of unrelated foster siblings (foster-sibs). To manipulate control broods (G – L), all of the nestlings within a brood were picked up and replaced back in the same nestbox (table 6.1).

On day 2, 24 h after broods had been manipulated, experimental and control broods were filmed for 40 min using a security camera inside a nestbox. After filming, all nestlings within each brood were weighed (accuracy 0.25 g), blood sampled (approximately 100 µl) and faecal sampled. Foster nestlings in experimental broods were transferred back again to their natal nestbox. Blood samples were centrifuged in a bench top centrifuge (Boeco) at 1300 rpm for 15 min at the field laboratory. Androgen levels in plasma and faecal samples were assayed by radioimmunoassay at Cardiff University (see 2.7.4).

During manipulation, nestling begging behaviour and androgen levels were compared between experimental and control broods. Within experimental broods during manipulation, begging behaviour and androgen levels were compared between home-

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0.834, n = 6).

Adult provisioning behaviour during manipulation	Mean ± S.E. Control broods	Mean ± S.E. Experimental broods	W	p
Total adult visit rate 40 min^{-1}	26.2 ± 3.7	34.0 ± 2.4	29.0	0.125
Mean percentage of visits on which zero nestlings were fed (%)	0.8 ± 0.8	5.6 ± 2.3	28.5	0.073
Mean number of nestlings fed per visit	1.18 ± 0.04	1.13 ± 0.04	32.0	0.294
Mean nestling provisioning rate 40 min^{-1}	4.9 ± 0.5	4.7 ± 0.6	41.5	0.748
Brood provisioning rate 40 min^{-1}	28.5 ± 4.7	35.7 ± 2.9	32.0	0.296
Mean latency to feed per visit (s)	1.3 ± 0.2	1.4 ± 0.2	37.0	0.810
Mean adult feeding call rate 40 min^{-1}	1.3 ± 0.7	1.2 ± 0.7	40.0	0.931
Mean percentage of adult feeds to nestlings highest in the brood (%)	60.0 ± 11.1	44.1 ± 8.2	45.0	0.378
Mean percentage of adult feeds to nestlings closest to the adult (%)	63.0 ± 3.4	47.1 ± 6.9	48.5	0.148
Mean percentage of adult feeds to nestlings first to beg (%)	46.2 ± 6.5	35.1 ± 5.1	47.0	0.229

Table 6.2: Mean ± S.E. adult provisioning behaviour to control broods (n = 6) and experimental broods (n = 6) during manipulation. Mann-Whitney *U* tests compare provisioning differences between control and experimental broods.

6.3.2: Nestling begging behaviour: control v experimental broods

During manipulation, broods begged on 100 % of adult visits to control and experimental broods. Mean brood begging duration was longer in experimental broods compared with control broods (table 6.3; figure 6.1). However, the mean percentage of the brood that begged per adult visit and the mean begging effort of fed nestlings were not significantly different between experimental and control broods (table 6.3).

Nestling begging behaviour during manipulation	Mean ± S.E. Control broods	Mean ± S.E. Experimental broods	W	P
Mean brood begging duration per adult visit (s)	5.0 ± 0.4	7.2 ± 0.6	25.0	0.031
Mean percentage of brood that begged per adult visit (%)	0.80 ± 0.05	0.72 ± 0.03	48.0	0.173
Mean begging effort of fed nestling	2.2 ± 0.2	2.3 ± 0.1	34.0	0.468

Table 6.3: Mean ± S.E. nestling begging behaviour in control broods (n = 6) and experimental broods (n = 6) during manipulation. Mann-Whitney *U* tests compare begging behaviour differences between control and experimental broods.

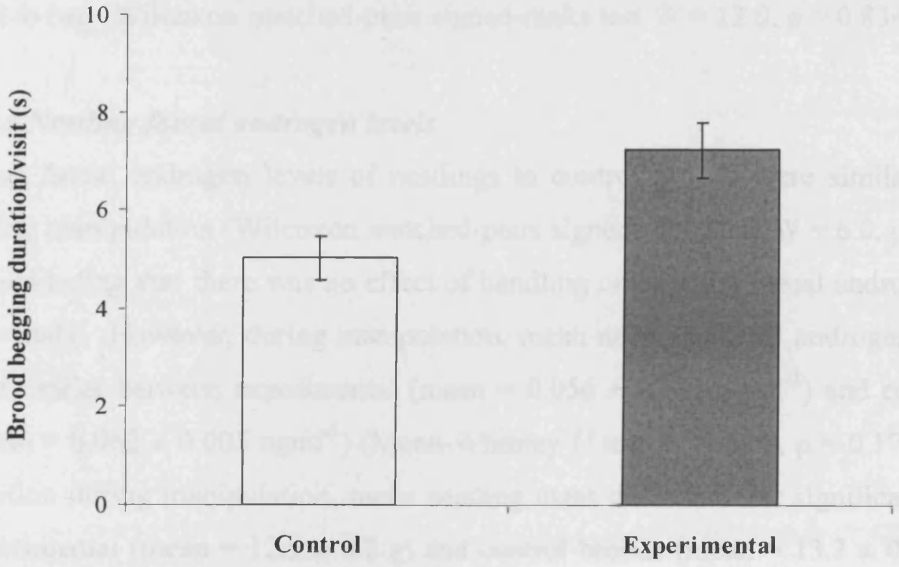

Figure 6.1: Mean ± S.E. brood begging duration per adult visits in control (n = 6) and experimental (n = 6) broods during manipulation.

6.3.3: Nestling begging behaviour: experimental broods

Within experimental broods during manipulation, the percentage of the brood that begged on each adult visit was equally composed of home-sibs (mean = 35.2 ± 2.3 % of brood begging / visit) and foster-sibs (mean = 37.1 ± 3.0 % of brood begging / visit) in the same brood (Wilcoxon matched-pairs signed-ranks test W = 11.0, p = 1.000, n = 6). Furthermore, there were no significant differences between home-sibs and foster-sibs in

of the number of times nestlings were either: highest in the brood just before feeding (Wilcoxon matched-pairs signed-ranks test W = 6.0, p = 0.402, n = 6), closest to the feeding adult (Wilcoxon matched-pairs signed-ranks test W = 17.5 , p = 0.173, n = 6) or first to beg (Wilcoxon matched-pairs signed-ranks test W = 12.0, p = 0.834, n = 6).

Between experimental broods during manipulation, the percentage of home-sibs begging in a brood on each adult visit (mean = 35.2 ± 2.3 % of brood begging / visit) was similar to the percentage of away-sibs begging in a different brood on each adult visit (mean = 37.0 ± 3.1 % of brood begging / visit) (Wilcoxon matched-pairs signed-ranks test W = 8.0, p = 0.675, n = 6). Additionally, there were no significant differences between home-sibs and away-sibs in different broods in terms of the number of times nestlings were either: highest in the brood just before feeding (Wilcoxon matched-pairs signed-ranks test W = 6.0, p = 0.402, n = 6), closest to the feeding adult (Wilcoxon matched-pairs signed-ranks test W = 6.5, p = 0.715, n = 6) or first to beg (Wilcoxon matched-pairs signed-ranks test W = 12.0, p = 0.834, n = 6).

6.3.4 Nestling faecal androgen levels

Mean faecal androgen levels of nestlings in control broods were similar before and during manipulation (Wilcoxon matched-pairs signed-ranks test W = 6.0, p = 0.787, n = 6), indicating that there was no effect of handling on nestling faecal androgen levels in this study. However, during manipulation, mean nestling faecal androgen levels were also similar between experimental (mean = 0.056 ± 0.021 $ngml^{-1}$) and control broods (mean = 0.062 ± 0.005 $ngml^{-1}$) (Mann-Whitney *U* test W = 22.0, p = 0.171, n = 6). In addition during manipulation, mean nestling mass did not differ significantly between experimental (mean = 12.9 ± 0.2 g) and control broods (mean = 13.3 ± 0.4 g) (Mann-Whitney *U* test W = 42.0, p = 0.689, n = 6).

Within experimental broods during manipulation, foster-sibs had a higher mean faecal androgen level compared with home-sibs in the same brood, although the difference was marginally non-significant (Wilcoxon matched-pairs signed-ranks test W = 1.0, p = 0.059, n = 6) (figure 6.2). However, this result was influenced by an outlier foster-sib in brood A, removal of this nestling from the analysis made the difference between home-sibs and foster-sibs in the same brood non-significant (Wilcoxon matched-pairs

signed-ranks test W = 5.0, p = 0.295). In brood B, the concentration of faecal androgens could only be detected in one home-sib nestling, the androgen levels of the other home-sib nestlings in brood B were below the detection limit of the T radioimmunoassay.

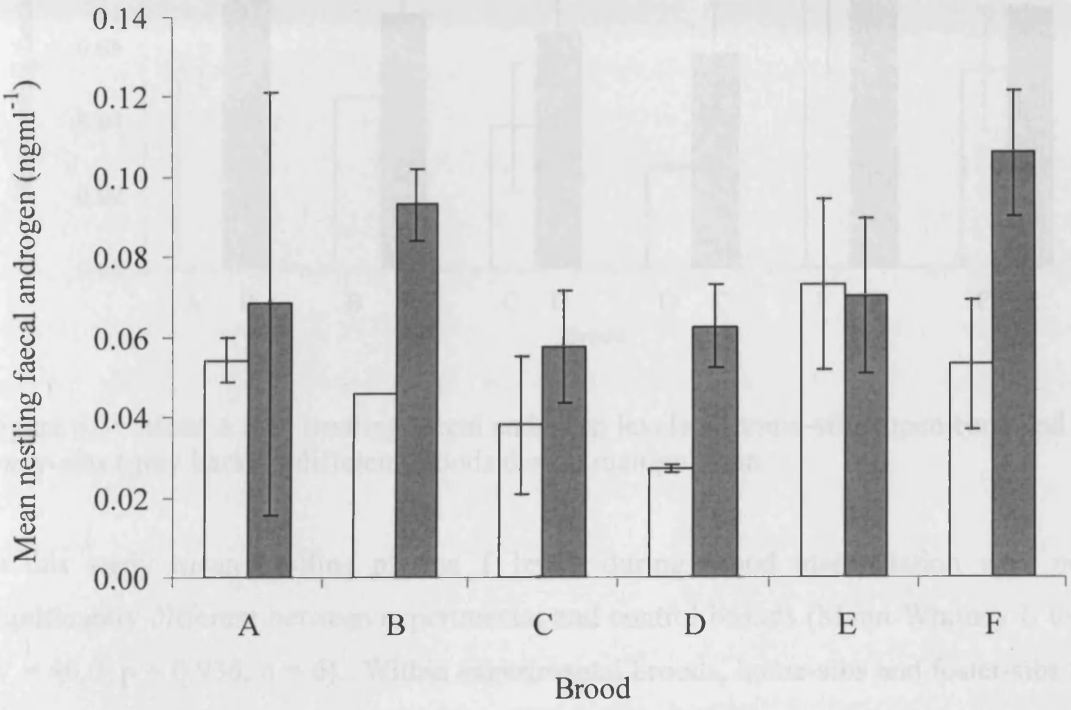

Figure 6.2: Mean ± S.E. nestling faecal androgen levels of home-sibs (open bars) and foster-sibs (grey bars) in the same brood during manipulation.

Before manipulation, home-sibs and away-sibs in the same brood had similar mean faecal androgen levels (Wilcoxon matched-pairs signed-ranks test W = 13.0, p = 0.675, n = 6). However, between experimental broods during manipulation, away-sibs had higher mean faecal androgen levels compared with related home-sibs in their natal brood (Wilcoxon matched-pairs signed-ranks test W = 0.0, p = 0.036, n = 6) (figure 6.3). Removal of an outlier away-sib in brood A, made the difference between home and away sibs in different broods during manipulation non-significant (Wilcoxon matched-pairs signed-ranks test W = 3.5, p = 0.173, n = 6).

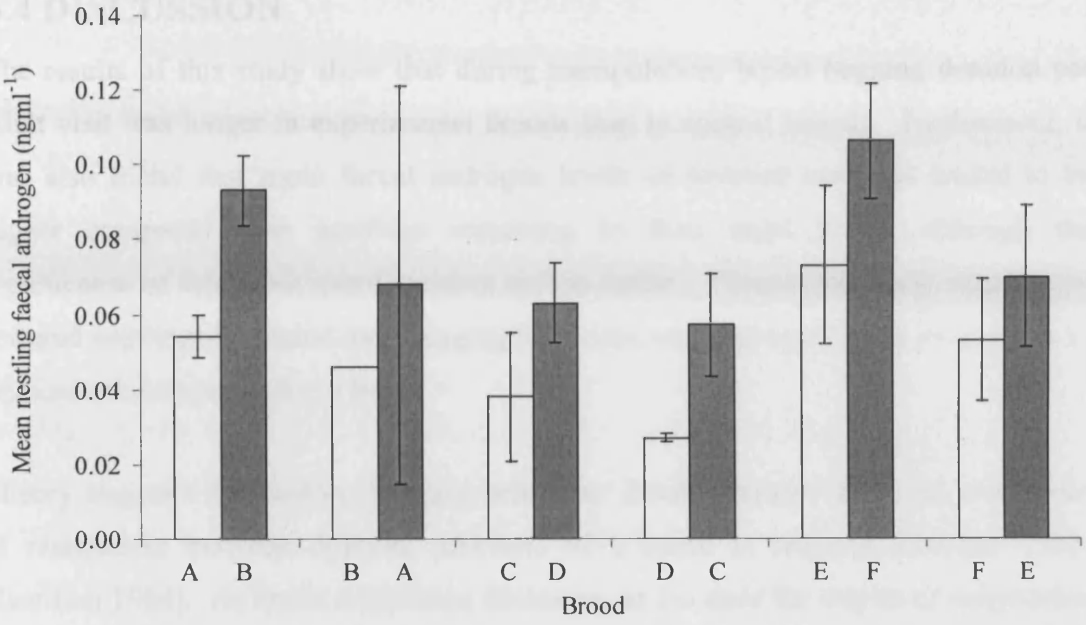

Figure 6.3: Mean ± S.E. nestling faecal androgen levels of home-sibs (open bars) and away-sibs (grey bars) in different broods during manipulation.

In this study mean nestling plasma T levels during brood manipulation were not significantly different between experimental and control broods (Mann-Whitney *U* test W = 40.0, p = 0.936, n = 6). Within experimental broods, home-sibs and foster-sibs in the same brood had similar mean plasma T levels (Wilcoxon matched-pairs signed-ranks test W = 0.91, p = 0.405, n = 6). Between experimental broods, home-sibs and away-sibs in different broods had similar levels of mean plasma T (Wilcoxon matched-pairs signed-ranks test W = 12.0, p = 0.834, n = 6).

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brood begging duration was longer in experimental broods than in control broods, it may be suggested that nestlings were able to recognise the presence of unrelated nestmates within experimental broods. A number of studies on a range of species have shown that young birds are able to recognise unrelated nestmates (Nakagawa & Waas 2004). For example, experimental studies on blue penguins, *Eudyptula minor* (Nakagawa et al. 2001), bank swallows, *Riparia riparia* (Beecher 1988) and spectacled parrotlets, *Forpus conspicillatus* (Wanker et al. 1998) have shown that nestlings respond more to playback calls of siblings than to playback calls made by unrelated nestlings of the same species. However, apart from brood begging duration, other measures of nestling begging behaviour recorded in this study, such as the percentage of the brood that begged per adult visit and the begging effort by the fed nestling, were not significantly different between experimental and control broods. Additionally, within experimental broods, the mean percentage of the brood that begged per adult visit was equally composed of home-sibs and foster-sibs, indicating that unrelated foster nestlings begged as often as nestlings remaining in their natal brood.

During manipulation in this study, a comparison of mean faecal androgen levels of related siblings in different experimental broods showed that fostered siblings (away-sibs) had higher androgen levels than siblings remaining in their natal brood (home-sibs). This may suggest that nestlings that were fostered into a different brood increased their T levels, whereas nestlings remaining in their natal brood did not increase their T levels. However, the faecal androgen results from the small sample of broods used in this study are unreliable, as the significance of the results depended on a high faecal androgen value of one fostered nestling. Furthermore, there were no significant differences in either mean faecal androgen levels or mean plasma T levels between experimental and control broods. It has been previously shown in chapter 3 of this project that the duration of nestling begging displays are correlated with nestling plasma T levels in pied flycatchers at 7 days post hatching. Furthermore, it was also found that enhanced nestling T levels increase the duration of nestling begging displays (chapter 4). As mean brood begging duration was longer in experimental broods in this study, it may be suggested that increased androgen levels of fostered nestlings increased the duration of brood begging displays, although further manipulation studies

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7.1 INTRODUCTION

Traditionally, avian androgen levels in adults and nestlings have been determined from blood samples by measuring the concentration of androgens in plasma (Adkins-Regan et al. 1990; Buchanan et al. 2001; Naguib et al. 2004; Silverin 1998b). Although blood sampling does provide a repeatable estimate of hormone levels, a disadvantage of this method is that birds must be caught and handled, which may influence the basal level of hormones measured in plasma, e.g. corticosterone is sensitive to handling, as well as having a detrimental effect on the wellbeing of the animal. For example, handling and bleeding are known to be stressful procedures, and in a range of avian species corticosterone levels rise within minutes of capture (Beuving & Vonder 1978; Harvey et al. 1980; Wingfield et al. 1982; Wingfield et al. 1992). As circulating corticosterone and androgen levels are thought to be correlated in birds (Duffy et al. 2000; Evans et al. 2000; Poiani et al. 2000; Roberts et al. 2004; Schoech et al. 1999; Sockman & Schwabl 2001) the effects of handling may influence the level of androgens measured in plasma samples (Calogero et al. 1998; Goymann et al. 2002a). Previous studies have shown that there are harmful effects of increased androgen and corticosterone levels in birds including increased basal metabolic rate (Buchanan et al. 2001) and immunosuppression (Folstad & Karter 1992; Sapolsky et al. 2000). In small birds, another disadvantage of using plasma samples to measure hormone levels is that the volume and frequency at which blood samples can be obtained is limited (Goymann 2005), making it difficult to take repeated samples from the same individual over a short time frame (Buchanan & Goldsmith 2004). Blood sampling can be especially limited in nestlings; blood samples cannot be taken until nestling body mass is sufficient to withstand sampling, which means that early developmental effects cannot be examined. For example, in a passerine species, the pied flycatcher, *Ficedula hypoleuca*, young are in the nest for about 14 - 16 days (Jarvinen 1990), but for purposes of hormone assay, nestlings are not big enough to be blood sampled until they are 6 - 7 days old (Goodship & Buchanan 2006). In addition, there are increasing restrictions on invasive work, due to ethical concerns about animal welfare. To overcome some of the problems involved with blood sampling, a growing number of studies have reported the use of non-invasive faecal sampling to measure androgen concentrations (Goymann et al. 2002a; Rettenbacher et al. 2004). Non-invasive faecal sampling offers an advantage over blood sampling in that hormones can be measured

without causing disturbance or stress to the animal, and faecal sampling may be a better method to use in long term studies (Rettenbacher et al. 2004).

A number of studies have used non-invasive faecal sampling to measure gonadal steroids and corticosteroids in the faeces of birds and mammals (e.g. Bishop & Hall 1991; Cavigelli 1999; Cavigelli & Pereira 2000; Hirschenhauser et al. 1999a; Hirschenhauser et al. 1999b; Hirschenhauser et al. 2000; Kirkpatrick et al. 1990; Kotrschal et al. 1998; Langmore et al. 2002; Wasser et al. 1997; White et al. 1995). However, the validity of non-invasive hormone measurements relies on the assumption that the concentration of hormone metabolites measured in faeces, proportionally reflects the level of circulating hormone in plasma (Buchanan & Goldsmith 2004; Goymann 2005). To validate non-invasive techniques, biological (Buchanan & Goldsmith 2004) and biochemical (e.g. Goymann 2002a) validation studies are required. Biological validation is necessary for all hormone work, but within recent years there has been a decline in the number of studies reporting assay validations. In order to be confident about a hormone assay technique, information to be gathered from biological validations should include: 1) quantifying the cross-reaction of substances other than the target hormone with the antibody in the assay medium, 2) demonstrating that the assay quantitatively detects the amount of hormone present, 3) demonstrating that there is a linear relationship between the concentration of the target hormone and assay standards and 4) demonstration of blanks, assay detection limit and precision (Buchanan & Goldsmith 2004). In order to measure testosterone (T) using radioimmunoassay throughout this project, the biological validations specified by Buchanan & Goldsmith (2004) have been addressed (see 2.7.4.2). Biochemical validations are necessary to validate non-invasive hormone assays, because metabolites of hormones, rather than the actual hormones are being measured (Goymann et al. 2006). It is important to be sure that excreted hormone levels relate to circulating levels and that excreted hormone levels are biologically meaningful.

In plasma, the target hormone, e.g. T or corticosterone, can be measured by hormone assay techniques. However, most steroids, including T and corticosterone are metabolized by the liver and gut (Palme 2005). Therefore, in excreted faeces, the actual hormone is either no longer present, or only present in very minor amounts, and

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syringe into 2 separate plastic tubes to later count for total radioactivity. Faecal samples were collected at 0, 1, 2, 3, 4, 6, 8, 12, 24, 48 and 70 h post [^{3}H]T injection on non-absorbent plastic sheets inserted underneath the perches of each cage. A plastic sheet was replaced in each cage 1 h before a collection time. Care was taken to collect the whole of each faecal sample (faeces and urine) by cutting around the sample on the plastic sheet and inserting both the sample and plastic sheet cutting into a plastic tube kept on ice. Within 1 h of collection, faecal samples were stored at -80 °C until further analysis.

7.2.3 Radiolabeled T extraction

Faecal samples collected from birds between 0 – 70 h post injection with [^{3}H]T were analysed at the University of Veterinary Medicine, Vienna. Each faecal sample was extracted with 1 ml of 60 % methanol by shaking for 20 min and centrifuging at 1500 rpm for 10 min. To measure radioactivity, a 50 µl aliquot of supernatant was removed from each faecal sample, mixed with 4 ml of scintillation fluid (Quicksafe A®, No 100800, Zinsser Analytic, Maidenhead, UK) and counted in a scintillation counter (Packard Tri-carb 2100TR). To determine total radioactivity administered to each bird, two 50 µl aliquots of the [^{3}H]T injection solution were pipetted into two scintillation vials, mixed with 4 ml of scintillation fluid and counted in a scintillation counter.

7.2.4 High performance liquid chromatography (HPLC)

To characterise the excreted metabolites in the faeces of each species, straight-phase high performance liquid chromatography (HPLC) separations were performed following previously published methodology (Palme & Möstl 1997). One methanol extracted faecal sample containing the highest amount of radioactivity from each [^{3}H]T injected male bird was used to characterise to metabolites. Metabolites were separated by straight phase HPLC (n-hexane/chloroform: 70/30; flow: 2 ml/min) on a Lichrosorb Si 60 column (10 µm, 25 x 0.4 cm; Forschungszentrum Seibersdorf, Austria) using a linear methanol gradient from 0 to 60 % in the first 30 min, 6 % from the 30th to 35th min and thereafter up to the 10 % until the 40th min. To determine which fractions contained metabolites of [^{3}H]T radioactivity, 50 µl sub-samples of each fraction were mixed with 200 µl of scintillation cocktail (Packard Microscint ^{TM} PS) and counted on a scintillation counter (Packard Top Count) in duplicate. Once aliquots had been

removed from each HPLC fraction for counting radioactivity, the rest of the fraction was dried down in a glass test tube overnight in an oven at 40 °C.

Dried fractions within the glass test-tubes were transported back to Cardiff University to test whether T-RIA cross-reacted with the androgen metabolites in each fraction. At Cardiff University, the dried fractions within the glass test tubes were stored at -20 °C until further analysis. To remove the dried fractions from the sides of the tubes, 300 µl of dichloromethane was added to each glass test tube, which was then vortexed on an orbital shaker (IKA ® KS 130 basic) at 720 rpm for 60 min. The dichloromethane in each glass test-tube was left to evaporate to dryness in a fume cupboard (2 - 3 h). Once dry, fractions were reconstituted in 50 µl of assay buffer, vortexed for 30 s (V400 Multitube vortexer, Alpha Laboratories) and stored at 4 °C overnight. The following day, each glass test-tube was vortexed on an orbital shaker at 800 rpm for 60 min before duplicate aliquots (20 µl) of each fraction were analysed by T-RIA.

7.2.5 GnRH-challenge of androgen production

To test whether an increase in androgen production and release induced by GnRH can be traced quantitatively in the faeces of pied flycatchers, great tits, canaries and zebra finches, six males of each species were injected i.p. with 1 µg chicken GnRH-I (Bachem, Merseyside). Each of these experimental males also served as a control and were injected i.p. with 50 µl PBS, 5 days before dosing with chicken GnRH-I. All injections were administered between 0900 – 1000 h. Each bird was blood sampled (approximately 100 µl) immediately before and 30 min after injecting with chicken GnRH-I. Faecal samples were collected at 0, 1, 2, 3, 4, 6, 8 and 12 h post injection on non-absorbent plastic sheets inserted underneath the perches of each cage. The plastic sheet was replaced in each cage 1 h before a collection time. Within 1 h of collection, faecal samples were stored at -80 °C until further analysis. As little disturbance as possible was caused to the birds during the 12 h collection period by removing the plastic sheet to another room to collect the faecal samples. Blood samples were centrifuged in a bench top centrifuge (Boeco) at 1300 rpm for 15 min and plasma was stored at -20 °C until analysed by T-RIA. Faecal samples were pooled for each individual at each collection time and androgens were extracted and analysed by T-RIA.

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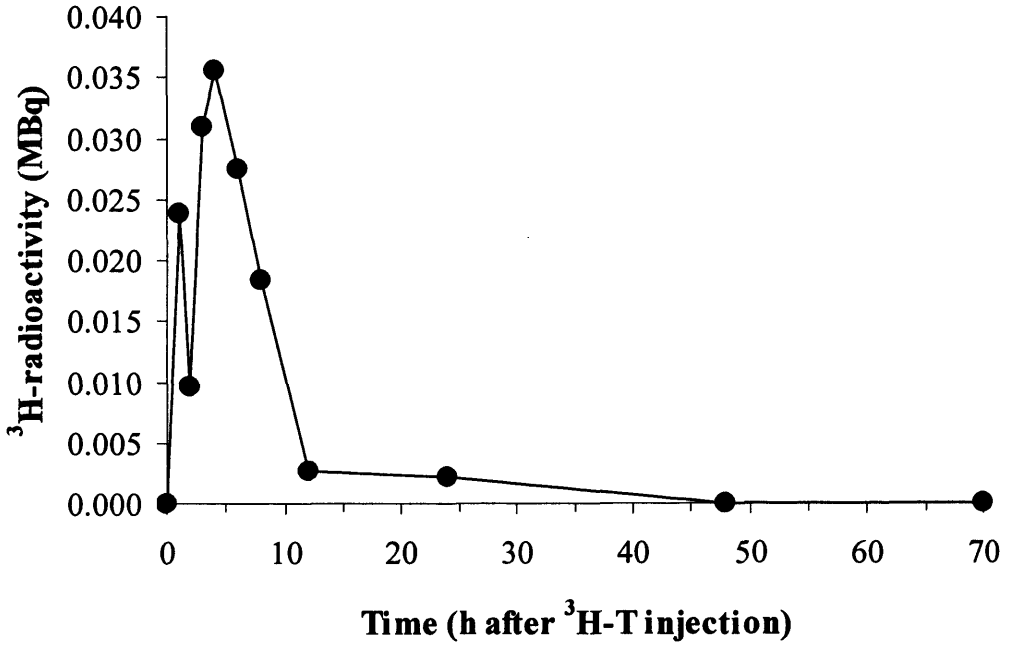

Figure 7.1 Time course (0 – 70 h) of [^{3}H] T excretion after injection in a male pied flycatcher.

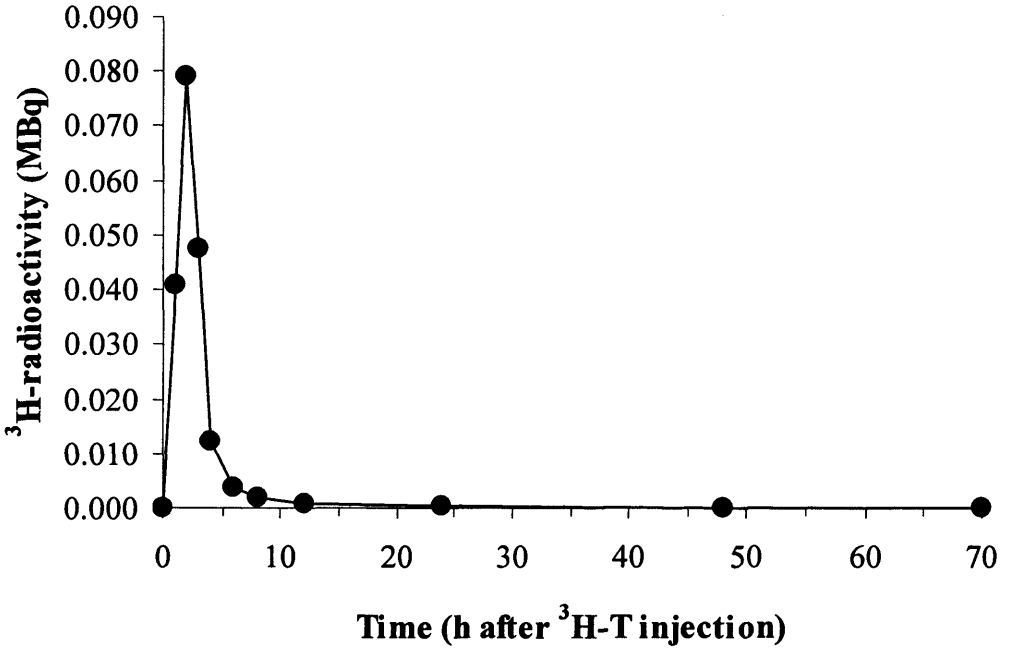

Figure 7.2: Time course (0 – 70 h) of [^{3}H] T excretion after injection in a male great tit.

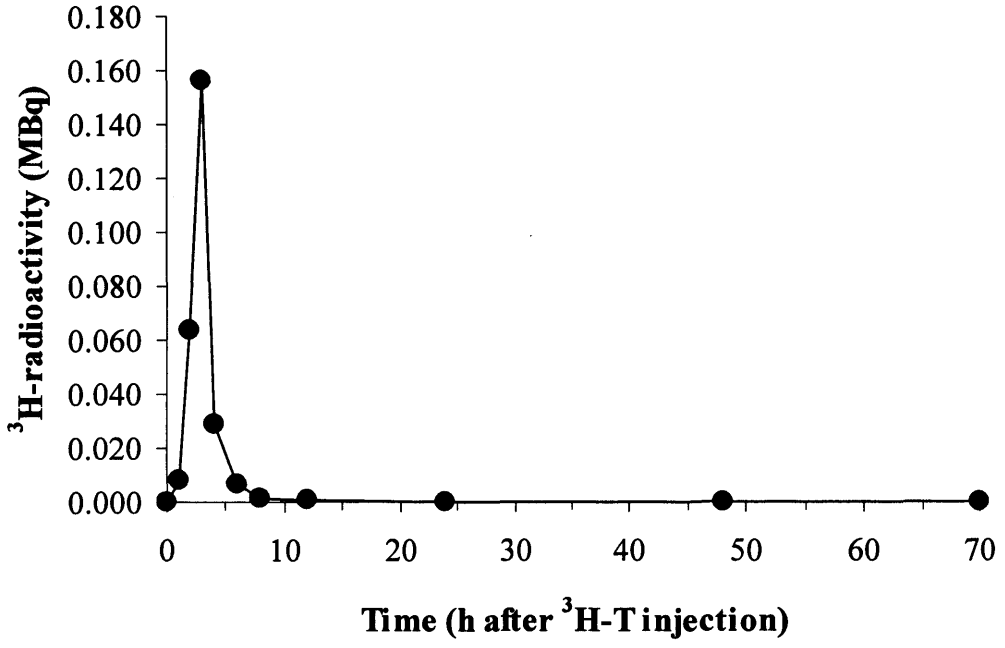

Figure 7.3: Time course (0 – 70 h) of [^{3}H] T excretion after injection in a male canary.

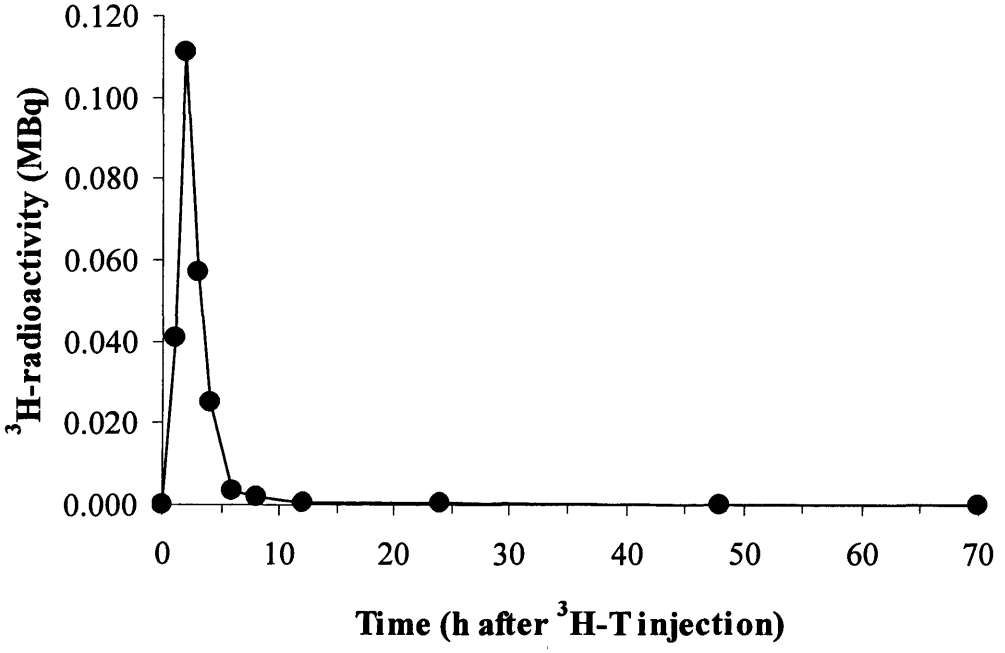

Figure 7.4: Time course (0 – 70 h) of [^{3}H] T excretion after injection in a male zebra finch.

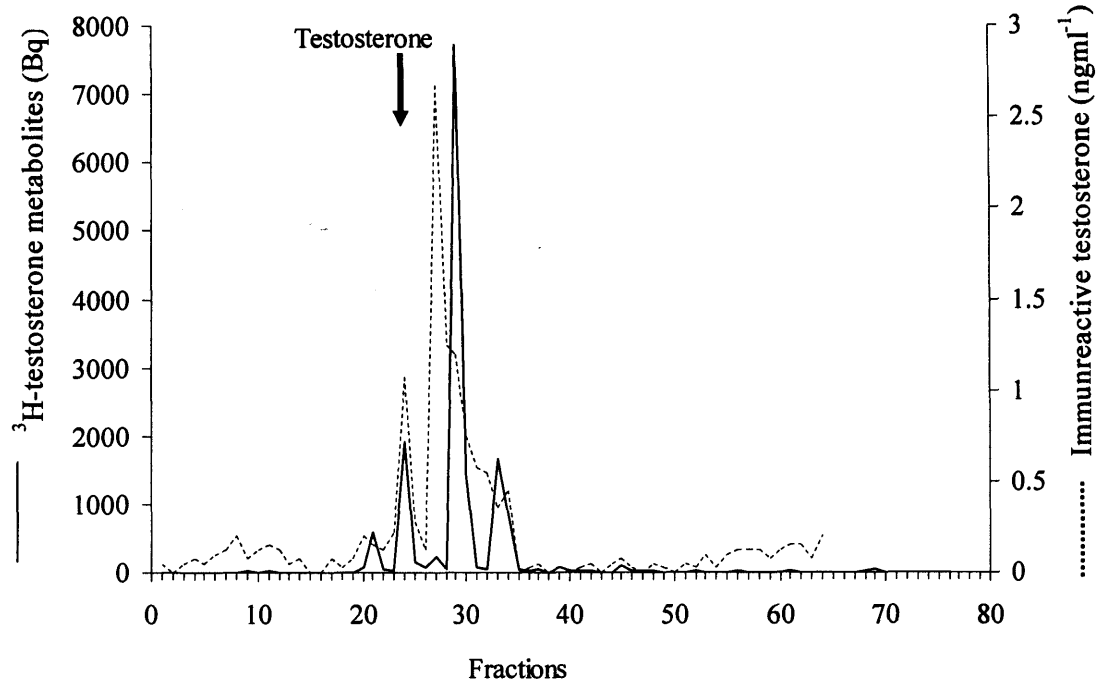

Figure 7.5: Pied flycatcher: HPLC profile of metabolites of i.p. injected [^{3}H] T (solid line) and immunoreactive substances measured with the T-RIA (dashed line). Data are for 1 male sampled at 4 h post injection.

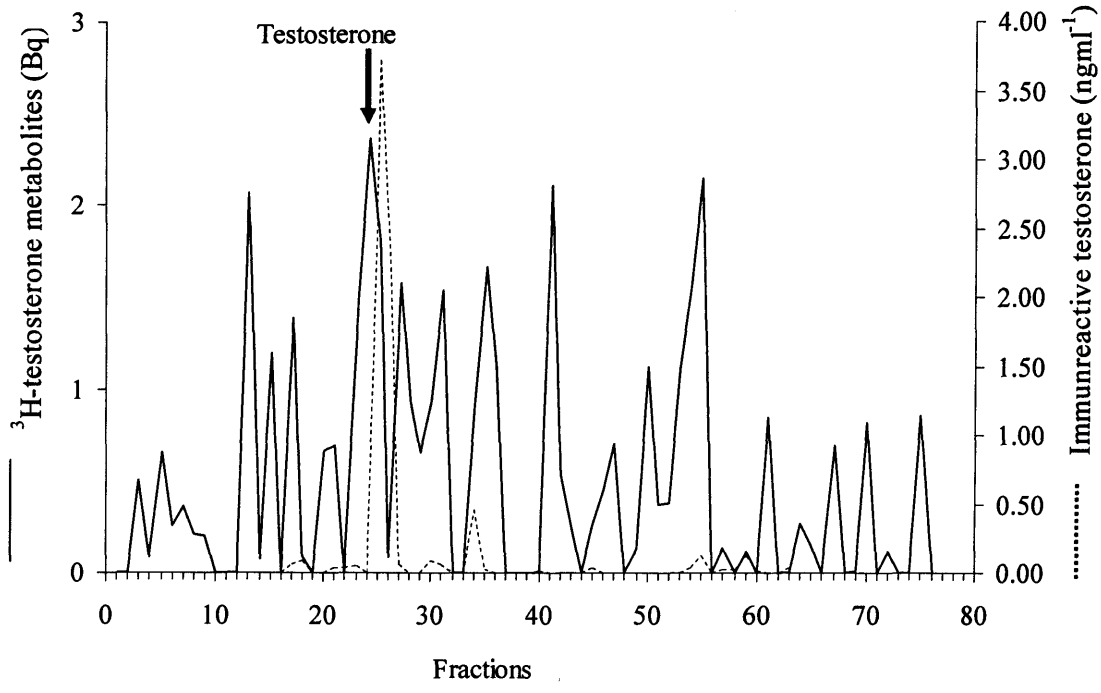

Figure 7.6: Great tit: HPLC profile of metabolites of i.p. injected [^{3}H] T (solid line) and immunoreactive substances measured with the T-RIA (dashed line). Data are for 1 male sampled at 2 h post injection.

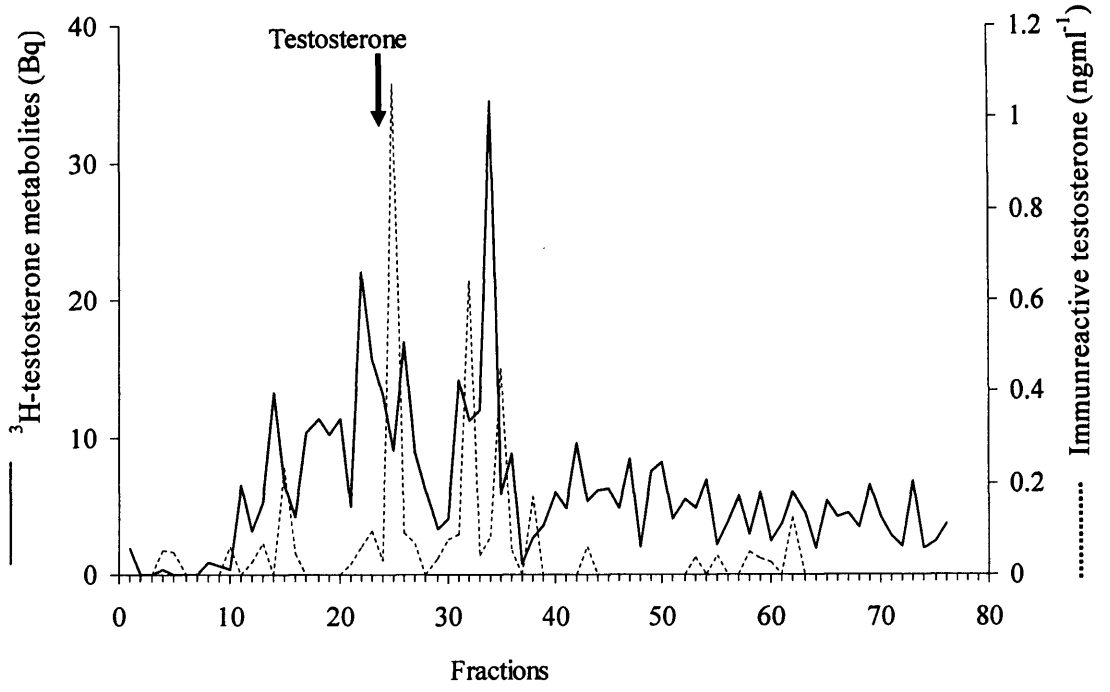

Figure 7.7: Canary: HPLC profile of metabolites of i.p. injected [^{3}H] T (solid line) and immunoreactive substances measured with the T-RIA (dashed line). Data are for 1 male sampled at 3 h post injection.

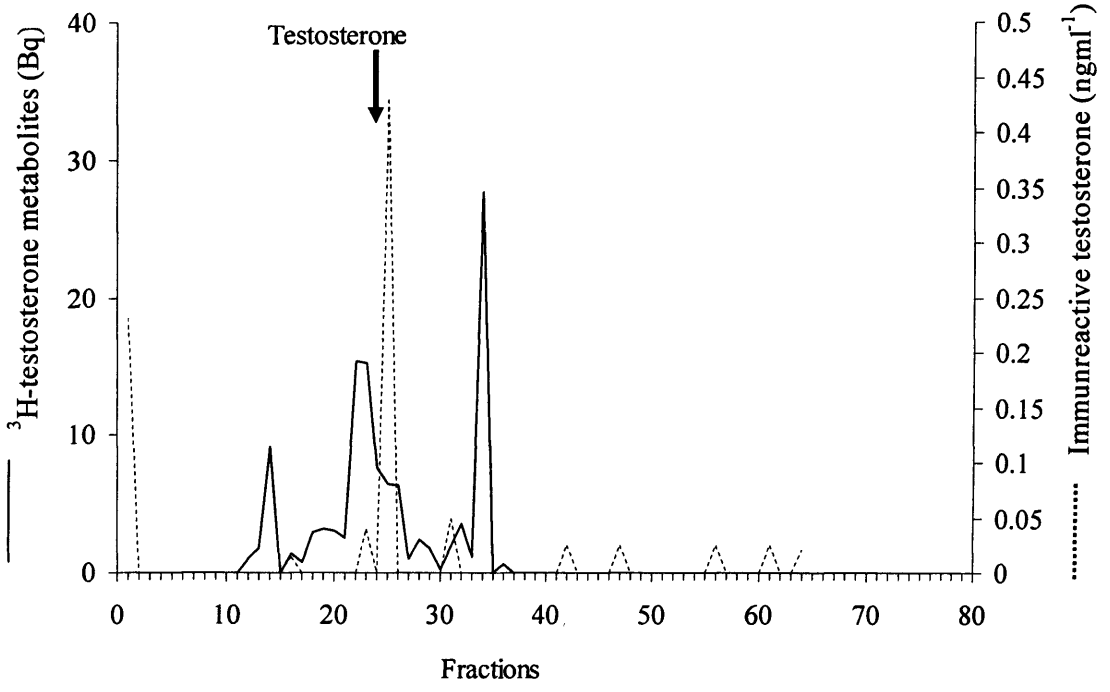

Figure 7.8: Zebra finch: HPLC profile of metabolites of i.p. injected [^{3}H] T (solid line) and immunoreactive substances measured with the T-RIA (dashed line). Data are for 1 male sampled at 2 h post injection.

7.3.2 GnRH-challenge of androgen production

The results of this study show that an injection of GnRH did not result in any apparent increase in excreted androgen metabolite levels in any of the four study species. Excreted androgen metabolite levels measured with T-RIA were not significantly different at any hour (0, 1, 2, 3, 4, 6, 8 and 12 h) post i.p. injection with GnRH in male pied flycatchers (one-way ANOVA $F_{7,38} = 0.45$, n = 6, p = 0.865; figure 7.9), male great tits (one-way ANOVA $F_{7,40} = 1.81$, n = 6, p = 0.112; figure 7.10) and male canaries (one-way ANOVA $F_{7,39} = 2.06$, n = 6, p = 0.072; figure 7.11). Androgen metabolites were not significantly higher in zebra finch faeces post GnRH injection, although excreted androgen metabolites at 6 and 8 h post injection were lower than pre-injection levels (one-way ANOVA $F_{7,40} = 5.31$, n = 6, p <0.001; figure 7.12).

Excreted androgen metabolite levels of control birds injected with PBS also did not change significantly in male pied flycatchers (one-way ANOVA $F_{7,40} = 0.48$, n = 6, p = 0.840), great tits (one-way ANOVA $F_{7,40} = 0.79$, n = 6, p = 0.603) and canaries (one-way ANOVA $F_{3,20} = 1.54$, n = 6, p = 0.235). Androgen metabolite levels of male zebra finches were significantly higher before injecting with PBS compared with androgen metabolite levels measured at 2 and 4 h post injection (one-way ANOVA $F_{3,20} = 3.17$, n = 6, p = 0.047).

Comparison of plasma T levels between 0 and 30 min post dosing with GnRH, showed that only plasma T levels in Zebra finches were significantly increased by GnRH injection (two sample t-test T = 2.92, n = 6, p = 0.019). Plasma T levels were not elevated at 30 min post dosing in pied flycatchers (two sample t-test T = 0.40, n = 6, p = 0.701), great tits (two sample t-test T = 1.14, n = 6, p = 0.287) and canaries (two sample t-test T = 1.21, n = 6, p = 0.262).

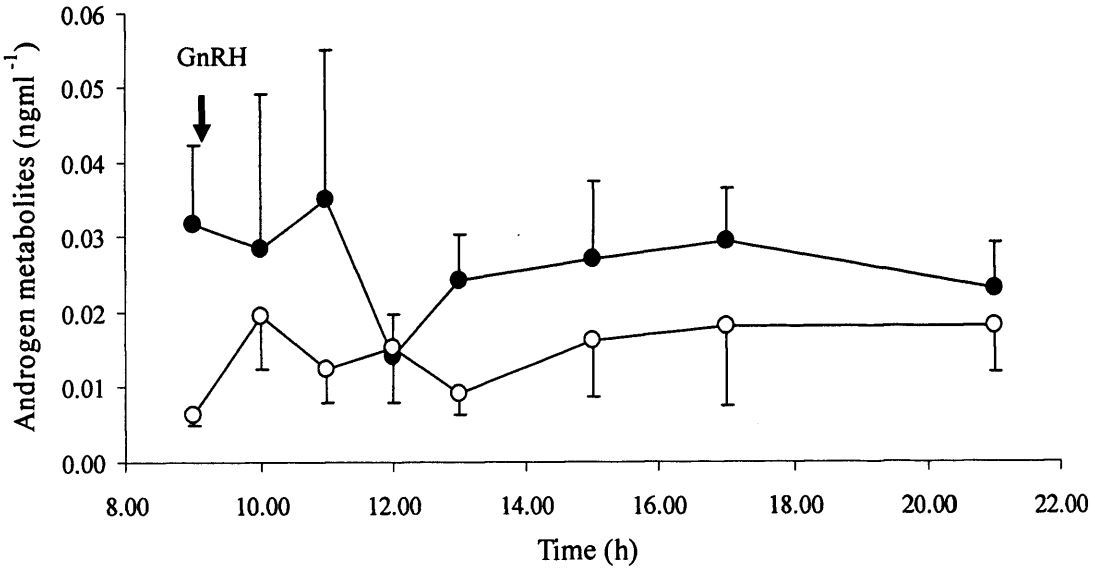

Figure 7.9: Pied flycatchers: Mean ± S.E. excreted androgen metabolite concentrations in six males injected at 09:00 h with GnRH (full circles) and six males injected with saline (open circles).

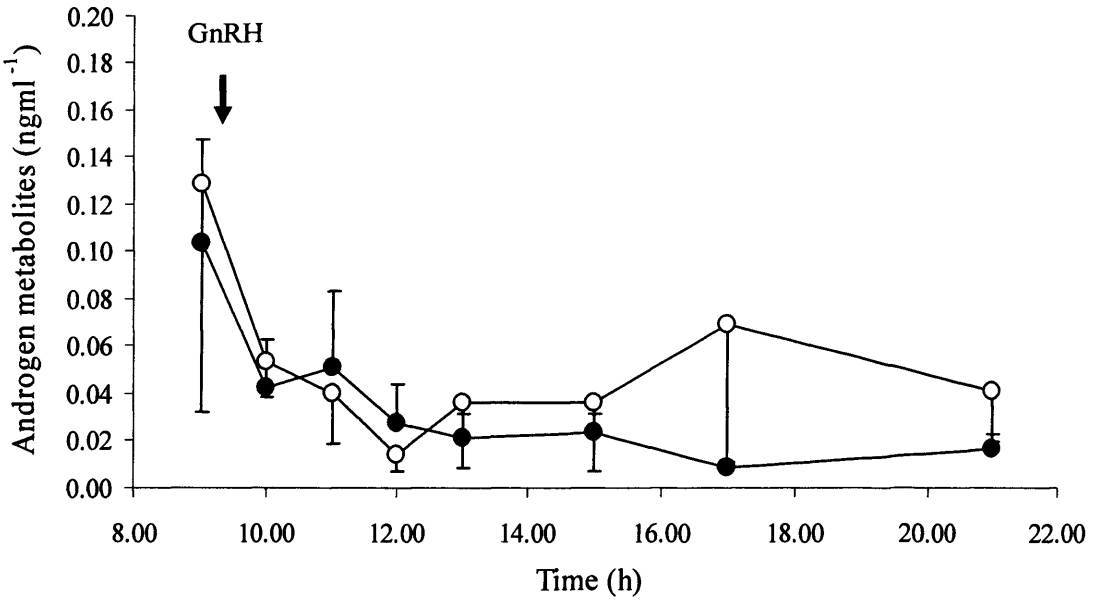

Figure 7.10: Great tits: Mean ± S.E. excreted androgen metabolite concentrations in six males injected at 09:00 h with GnRH (full circles) and six males injected with saline (open circles).

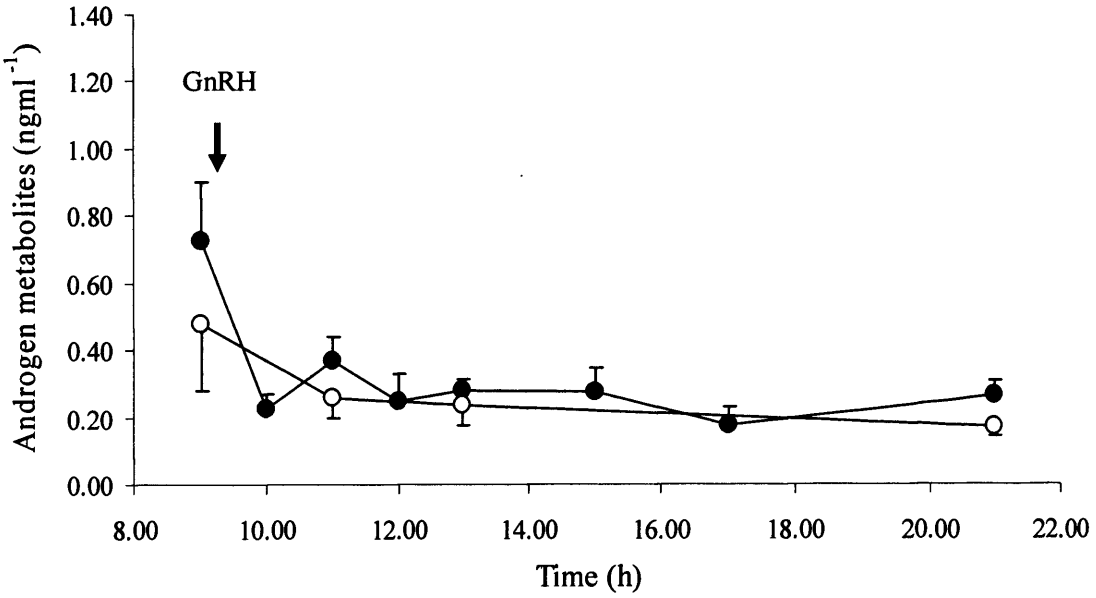

Figure 7.11: Canaries: Mean ± S.E. excreted androgen metabolite concentrations in six males injected at 09:00 h with GnRH (full circles) and six males injected with saline (open circles).

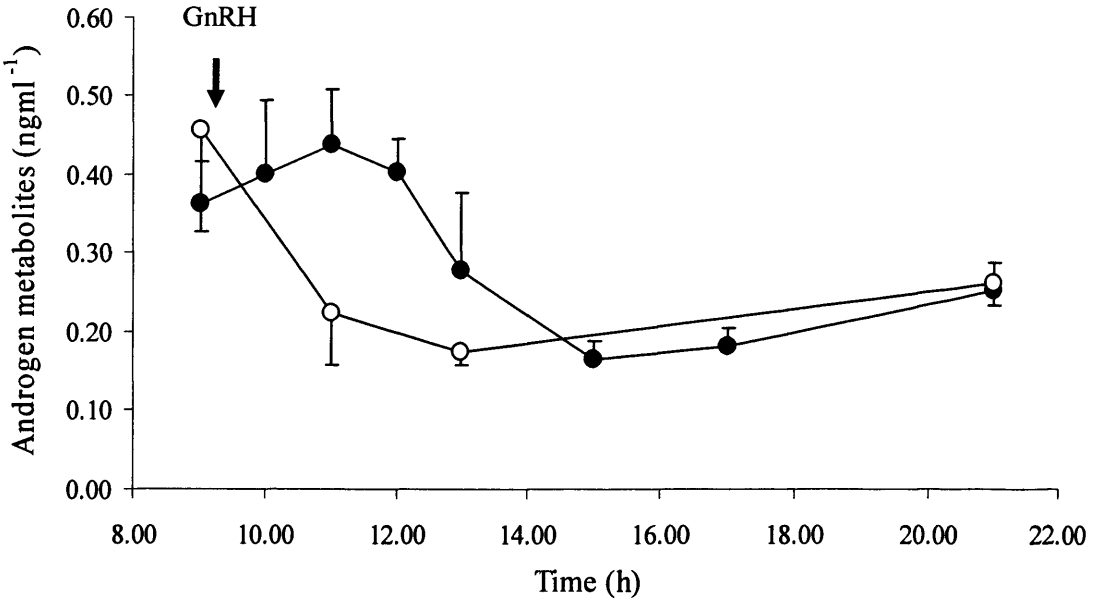

Figure 7.12: Zebra finches: Mean ± S.E. excreted androgen metabolite concentrations in six males injected at 09:00 h with GnRH (full circles) and six males injected with saline (open circles).

7.4 DISCUSSION

The results of the HPLC and subsequent T-RIA analysis of HPLC fractions showed that androgen metabolites were excreted in the faeces of a male pied flycatcher, great tit, canary and zebra finch in this study. Furthermore, T itself contributed to a peak of excreted hormones in each species, indicating that [^{3}H] T injected into the birds was not completely metabolized. The number of excreted androgen metabolites differed between species, thus highlighting the need for biochemical validation studies on non-invasive faecal sampling to be carried out at a species-specific level.

From a faecal sample of a male pied flycatcher, 4 radioactive peaks in the HPLC fractions were visible. A minor peak indicated the presence of T and 1 major and 2 minor peaks indicated metabolites of injected [^{3}H] T. These peaks were found in the same fractions that also showed immunoreactivity, indicating that the antibody used in the T-RIA in this study detected T as well as true metabolites of T. This confirms that the T-RIA used for the work detailed in this project (chapters 5 & 6) picks up the majority of the excreted androgen metabolites in pied flycatchers. In the male great tit, analysis of radioactivity in HPLC fractions showed that T as well as many other metabolites of injected [^{3}H] T were excreted. However, it is currently unclear why the radioactivity level in each HPLC fraction of the great tit was so low and further work is needed to investigate the profile of metabolite excretion in other faecal samples from great tits. T-RIA analysis of each HPLC fraction from the male great tit, indicated that the assay detected T and 1 other metabolite of [^{3}H] T. Analysis of the HPLC fractions from a male canary showed that T itself contributed to one of the main excreted hormones and was strongly detected with the T-RIA. One major and approximately 12 other minor metabolites of injected [^{3}H] T were detected with T-RIA in the male canary. In the faecal sample of a male zebra finch, analysis of radioactivity in the HPLC fractions showed that T as well as 2 major and a few minor metabolites were excreted. T-RIA detected T in the HPLC fraction of the zebra finch, although surprisingly, the major metabolite in fraction 34 was not detected with the T-RIA, although this metabolite had been detected with T-RIA in the other species. In addition, 4 minor peaks of the T-RIA did not correlate with metabolites of [^{3}H] T in the zebra finch. This may possibly have been because in comparison with the other species, the zebra finch excreted some different metabolites that did not cross-react

with the antibody used in the T-RIA in this study (Goymann 2005). This suggests that the T-RIA used throughout this project would not be appropriate for quantifying androgen metabolite excretion in zebra finches and that alternative antibodies should be tried for any such study. It is not possible to draw general conclusions about the differences between insectivorous and granivorous species concerning the identification of metabolites excreted and measured by the T-RIA, as there was much variation between each species. Therefore, this study shows the need for biochemical validation studies to be carried out at a species-specific level.

The commercial antibody used in the T-RIA in this study was different to that used in a similar study to measure faecal androgen metabolites in European stonechats (Goymann et al. 2002a). The antibody used in this study was chosen because it has been previously used to measure plasma T in pied flycatchers, great tits, canaries and zebra finches (Goodship & Buchanan 2006; unpublished data). Generally, the results of this study show that the different species excreted different metabolites of [^{3}H]T; some of these metabolites cross-reacted with the T-RIA antibody and other metabolites did not cross-react. This indicates that an antibody used in a T-RIA suitable for detecting steroid metabolites in one species does not necessarily work for others, which makes it difficult to draw general conclusions about what to expect (Goymann 2005; Palme 2005). Due to the many different types of metabolites that can be present in faeces, previous studies have recommended that group-specific antibodies are used in immunoassays when determining faecal steroids (Palme 2005; Thiel et al. 2005). For example, previous studies which have tried several different antibodies to find a suitable one to measure corticosterone metabolites in black grouse, *Tetrao tetrao* (Baltic et al. 2005) and capercallies, *T. urogallus* (Thiel et al. 2005) have found that the best antibody to use (one that gives high cross-reactivity with faecal corticosterone metabolites) is also used in chickens (Rettenbacher et al. 2004) and may represent the best antibody to detect corticosterone metabolites excreted in galliforms. Compared with these studies on galliforms, a study on a passerine species, the European stonechat, has found that another antibody gives a high cross-reactivity with faecal corticosterone metabolites (Goymann et al. 2002b).

When hormone metabolites are assayed in faecal samples, the concentration of metabolites in the sample represent the end-point measurement (Goymann et al. 2006). From the time when a hormone is produced to the time when metabolites of the hormone are measured in the faeces, many factors may influence the final concentration of excreted metabolites. For example, factors which can influence the final concentration of metabolites measured in faeces include: storage conditions of faecal samples (Khan et al. 2002; Möstl et al. 2005; Palme 2005) the length of time which elapses between metabolite excretion and measurement (Goymann 2005; Khan et al. 2002), animal metabolism rates and digestive processes (Goymann 2005; Goymann et al. 2002a) (Goymann et al. 2006). The way in which faecal samples are stored and the length of time taken until analysis are important issues, as it is known that faecal hormones are further metabolized by bacterial enzymes after excretion (Palme 2005). Therefore, freezing faecal samples immediately after excretion and storing samples in a freeze dried state has been previously recommended (Khan et al. 2002; Palme 2005). The results of this study showed that there were differences between species in the time taken for androgen metabolites to be excreted after [^{3}H]T injection. Peak excretion of androgen metabolites ranged from 2 h in the male great tit and zebra finch to 4 h in the pied flycatcher. As steroids are metabolized in the liver and excreted via the bile into the gut, there was a time lag between [^{3}H]T injection and the appearance of androgen metabolites in the faeces (Palme 2005). It is known that gut passage times, digestive processes and rates of liver metabolism vary between species (Goymann 2005; Goymann et al. 2002a), which would explain why the peak excretion of androgen metabolites differed between species.

In this study there was also a difference between species in the total [^{3}H] T radioactivity recovered in the faeces between 0 – 70 h post injection. Total recovery of radioactivity ranged from 46.3 % from a male pied flycatcher to 82.1 % from a male canary. These different recovery rates were unexpected results, especially as faecal androgen metabolites were mainly excreted within 12 h after [^{3}H] T injection in all species, and radioactivity in the faeces was down to background levels after 48 h. It is possible that a methodological error was made whilst injecting [^{3}H] T into the pied flycatcher, which may explain the low [^{3}H] T total recovery rate in this bird. If the needle of the injection

was made too deep, [^{3}H] T may have been injected into body tissue and could not pass into the circulatory system.

The results of this study also unexpectedly show that administration of GnRH did not lead to any apparent increase of excreted androgen metabolites in any species. Unfortunately, these results mean that it cannot be confirmed that the T-RIA in this study was able to pick up differences in androgen metabolites relating to biologically meaningful differences in circulating T levels, by each passerine species. Plasma T was found to be elevated after 30 min post GnRH injection in zebra finches, although there was no rise in plasma T after GnRH injection in the other species. A previous study on male European stonechats has shown that excreted androgen metabolites peaked in the faeces within 1.3 - 2.7 h after administration of GnRH (Goymann et al. 2002a). Compared with the study on European stonechats, a similar result was expected in this study. However, surprisingly, only a non-significant increase in faecal androgen levels were measured 1 – 2 h after GnRH injection in pied flycatchers and zebra finches, and faecal androgen levels actually decreased after GnRH injection in great tits and canaries. It is possible that these unexpected results can be explained by methodological errors. The lights in the animal house room were switched on at 0800 h which may have caused a surge in circulating hormone levels in the birds and could explain why faecal androgen levels were high before GnRH was injected at 0900 h. It is also possible that an error was made with the injections; GnRH may have been injected too deeply into each bird and did not pass into the blood stream.

In conclusion, the results of the radioinfusion study showed that T and androgen metabolites were excreted in the faeces of a male pied flycatcher, great tit, canary and zebra finch after an injection with [^{3}H] T. Furthermore, the antibody used in the T-RIA in this study, was able to pick up T and androgen metabolites in the faeces of these species. The number and type of excreted androgen metabolites varied considerably between species, indicating that validation studies should be undertaken for individual species. The GnRH study was unsuccessful; it is recommended that this study be repeated under natural light conditions after further practice with injection techniques.

Chapter 8

Relationships between testosterone, nestling development, sex and adult quality

8.1 INTRODUCTION

It has been discussed in previous chapters of this thesis that testosterone (T) influences begging behaviour in nestling birds (Schwabl 1996a; Eising & Groothuis 2003). The results of this project have found that enhanced nestling T levels can increase begging behaviour in pied flycatchers, *Ficedula hypoleuca* (see chapter 4), and a field experiment showed that pied flycatcher parents preferentially feed nestlings displaying the greatest begging intensity (chapter 5). Previous studies have found that nestling age and sex may influence T levels and begging behaviour (Adkins-Regan et al. 1990; Kilner 2002; Leonard et al. 2005; Ottinger et al. 2001; Price & Ydenberg 1995), and begging signals may remain honest due to the immunosuppressive effects involved with T production (Folstad & Karter 1992). In addition, adult T levels are also related to some measures of adult quality and may indicate parental ability (e.g. Sætre et al. 1995; Stoehr & Hill 2000; Sanz 2001; Garamszegi et al. 2004). The aim of this chapter was to draw together some studies on pied flycatchers investigating relationships between nestling age, sex, immune response, adult quality and circulating T levels.

8.1.1 Nestling age and sex effect on T

Steroid hormone levels of nestling birds may be influenced by nestling age and sex (Adkins-Regan et al. 1990; Ottinger & Abdelnabi 1997; Silverin & Sharp 1996), which in turn may affect begging signals (Fargallo et al. 2003; Kilner 2002; Leonard et al. 2005; Price et al. 1996; Saino et al. 2003; Teather 1992). Steroid hormone levels have been compared between different ages and sexes, mostly in young galliformes (Ottinger & Abdelnabi 1997; Tanabe et al. 1979; Woods & Podczaski 1974; Woods et al. 1975). In young chickens, *Gallus domesticus* and quail, *Coturnix japonica*, androgen levels are highest during the embryonic period and they decline to lower levels after hatching (Ottinger et al. 2001; Tanabe et al. 1979). The gonads develop early on during the embryonic period in galliformes and males have higher circulating androgen levels than females (Ottinger & Abdelnabi 1997; Ottinger et al. 2001; Woods & Podczaski 1974; Woods et al. 1975). In passerines, a study on great tits, *Parus major* has found that nestling T levels of both sexes are significantly higher in the first 2 days after hatching compared with other times during the nestling period (Silverin & Sharp 1996). In the same study on great tits, male T levels were significantly higher than female nestlings

only in the first 2 days after hatching (Silverin & Sharp 1996). A study on zebra finches, *Taeniopygia guttata* has shown that T levels of male nestlings are highest during the first week after hatching compared with T levels sampled at other times during the nestling and juvenile period (Adkins-Regan et al. 1990). In the study by Adkins-Regan et al. (1990) it was found that female nestling zebra finches tended to have higher T levels than males throughout development. Another study on canaries *Serinus canaria*, has also found that nestling females have higher T levels than nestling males between 35 and 55 days of age (Weichel et al. 1986).

8.1.2 Cell-mediated immune response and T

Although signals can sometimes carry deceptive information (Maynard Smith & Harper 2003; Searcy & Nowicki 2005), signals must be reliable often enough if they are to be maintained through natural selection (Searcy & Nowicki 2005). Theoretical models of signalling systems show that the honesty of signals can be maintained by the costs involved with signal production (Godfray 1995a; Kilner 1997; Zahavi 1975). Several sources of physiological costs have been identified with the production of T including: increased basal metabolic rate (Buchanan et al. 2001), immune suppression (Folstad & Karter 1992) and increased levels of corticosterone (Evans et al. 2000). The immunocompetence handicap hypothesis (ICHH) suggests that signals may depend on T for development, so high levels of T are needed for the development of a large signal and that only high quality individuals can withstand the immunosuppressive costs associated with high T levels (Folstad & Karter 1992). In birds, cell-mediated immune response can be tested using an injection of phytohaemagglutinin (PHA) into the wing web (Buchanan et al. 2003). PHA is a plant lectin which promotes a hypersensitivity reaction and has been extensively used to investigate immune function in birds (Lochmiller et al. 1993).

8.1.3 Breeding state, adult quality and T

In breeding birds, adults of both sexes can show pronounced seasonal variations in plasma T levels (e.g. Johnsen 1998; Silverin 1991; Silverin & Wingfield 1982; Van Duyse et al. 2003; Wingfield & Silverin 2002). At the start of the breeding season when male plasma T levels are high, females may use several signals that indicate male quality when choosing a mate. For example, male song quality and male plumage

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8.2.3 Cell-mediate

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from the branchial vein on the right wing to assay circulating plasma T levels. Into the left wing web, 50 µl of a suspension of PHA (Sigma L-8754) (24 mg of PHA in 4.8 ml of 1 x PBS) was injected, and the response (swelling on the left wing web) was measured 24 h post-injection. Phosphate buffered saline (PBS) was not injected into the right wing web as a control measure in this study, as a previous study has shown that there is no significant effect of the injection itself on the size of the swelling on the wing web (Smits et al. 1999). Three repeated measurements were taken with the pressure sensitive spessimeter on the left wing web on each occasion. Immune response was determined as the thickness increase (mm) of the left wing web (thickness post-injection minus thickness pre-injection).

8.2.4 Breeding state, adult quality and T

Between 1st April – 30th June in 2003 and 2004, adult males (n = 61) and females (n = 94) were weighed (accuracy 0.25 g) and their blood sampled (approximately 100 µl) once to assay circulating plasma T levels. To investigate plasma T levels in different states of breeding, adults were categorised into 3 groups: 1) pre-laying, 2) egg incubation and 3) feeding young.

Forehead patch size and plasma T levels were measured in a sample of males in 2003 (n = 24) and 2004 (n = 11). In 2003, males were caught and measured when feeding their young. In 2004, males were caught during the pre-laying state of the breeding season. Blood samples were taken from the males (approximately 100 µl) to measure plasma T and the white forehead patch of each individual was photographed in front of a piece of graph paper using a digital camera (Sony DCR-TRV25E). The digital images of forehead patches were downloaded into a PC, and the area of white was calculated using the software package, ImageJ 1.30 (http://rsb.info.nih.gov/ij/index.html).

The relationship between parent T levels and nestling hatching and fledging success in 2003 and 2004 was investigated. The majority of adult females were sampled for plasma T during egg incubation. Therefore, adult females which were sampled during egg incubation were used to investigate nestling hatching and fledging success in this study. The majority of adult males in 2003 were sampled for plasma T whilst feeding

young and were used to investigate nestling hatching and fledging success. As most adult males sampled for plasma T in 2004 were mostly caught early in the season, it was not known which nestbox these males occupied, and therefore males caught in 2004 were not used to investigate nestling hatching and fledging success in this study.

8.2.6 Statistical analysis

All analyses were performed in MINITAB 14.0. One way ANOVA was used to compare mean brood plasma T levels between different broods, and was also used to compare adult plasma T levels between different breeding states. GLM ANOVA models were used to investigate the effect of brood age or nestling sex (sex factor nested within brood) on nestling plasma T levels. GLM ANOVA was also used to test the effects of nestling plasma T, mass, age, sex and hatching date on variation in PHA response. Finally, GLM ANOVA was used to test the effects of adult male plasma T, male mass, date and year on the size of the male forehead patch. A Wilcoxon matched-pairs signed-ranks test was used to determine any differences between the number of male and female nestlings within broods. Spearman rank-order correlations were used to investigate the relationship between parent T levels and nestling hatching and fledging success. When parametric tests were used, data were checked for normality using the Kolmogorov-Smirnov test, and where necessary data were transformed to meet the assumptions of the distribution of residuals.

Figure 8.1: Mean
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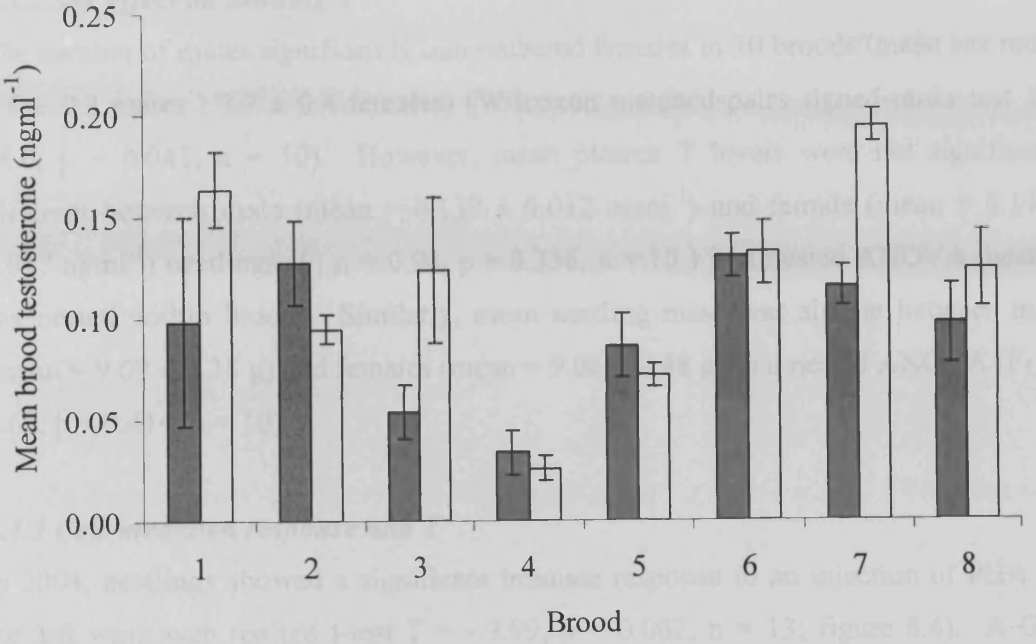

Figure 8.2: Mean ± S.E. brood plasma testosterone in 8 broods aged 7 days old (grey bars) and in the same broods aged 11 days old (open bars).

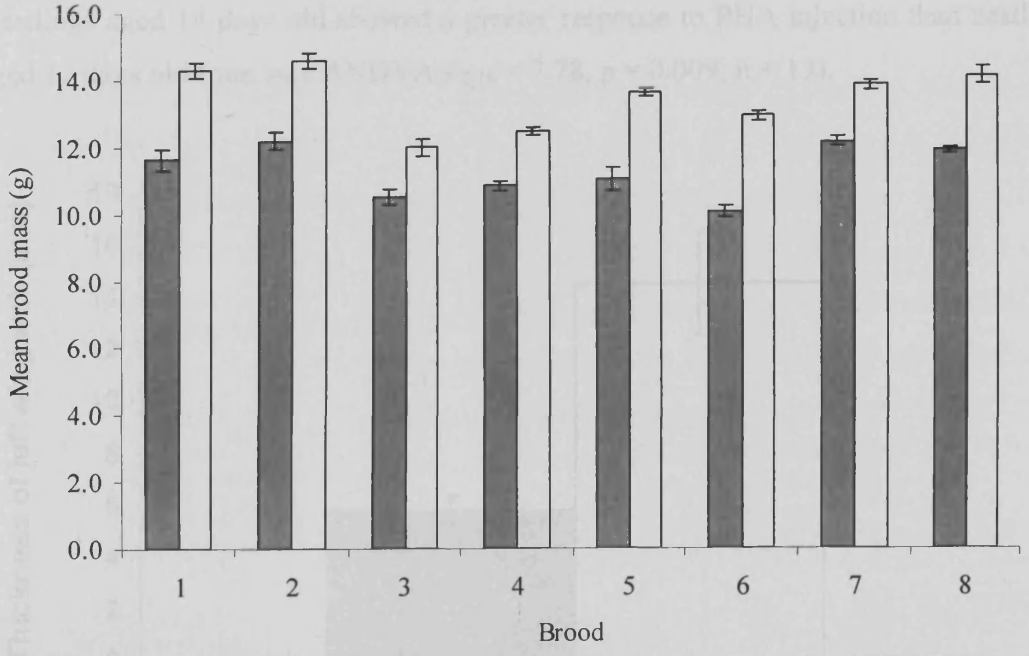

Figure 8.3: Mean ± S.E. brood mass in 8 broods aged 7 days old (grey bars) and in the same broods aged 11 days old (open bars).

8.3.2 Sex effect on nestling T

The number of males significantly outnumbered females in 10 broods (mean sex ratio = 4.4 ± 0.3 males : 2.7 ± 0.4 females) (Wilcoxon matched-pairs signed-ranks test W = 48.0, p = 0.041, n = 10). However, mean plasma T levels were not significantly different between male (mean = 0.132 ± 0.012 $ngml^{-1}$) and female (mean = 0.144 ± 0.017 $ngml^{-1}$) nestlings ($F_{1,51}$ = 0.94, p = 0.336, n = 10) in a nested ANOVA (nestling sex nested within brood). Similarly, mean nestling mass was similar between males (mean = 9.09 ± 0.35 g) and females (mean = 9.06 ± 0.48 g) in a nested ANOVA ($F_{1,37}$ = 0.68, p = 0.414, n = 10).

8.3.3 Cell-mediated response and T

In 2004, nestlings showed a significant immune response to an injection of PHA into the left wing web (paired t-test T = - 3.99, p = 0.002, n = 13; figure 8.4). A GLM model with nestling response to PHA injection as the dependent variable showed that there was no significant effect of nestling age, sex, plasma T, mass and date on nestling PHA response (table 8.1). However, after removing non-significant factors from the GLM model (nestling sex, plasma T, mass and date), the final model showed that nestling age influenced response to PHA injection ($F_{2,10}$ = 7.78, p = 0.009, n = 13). Nestlings aged 14 days old showed a greater response to PHA injection than nestlings aged 12 days old (one way ANOVA $F_{2,10}$ = 7.78, p = 0.009, n = 13).

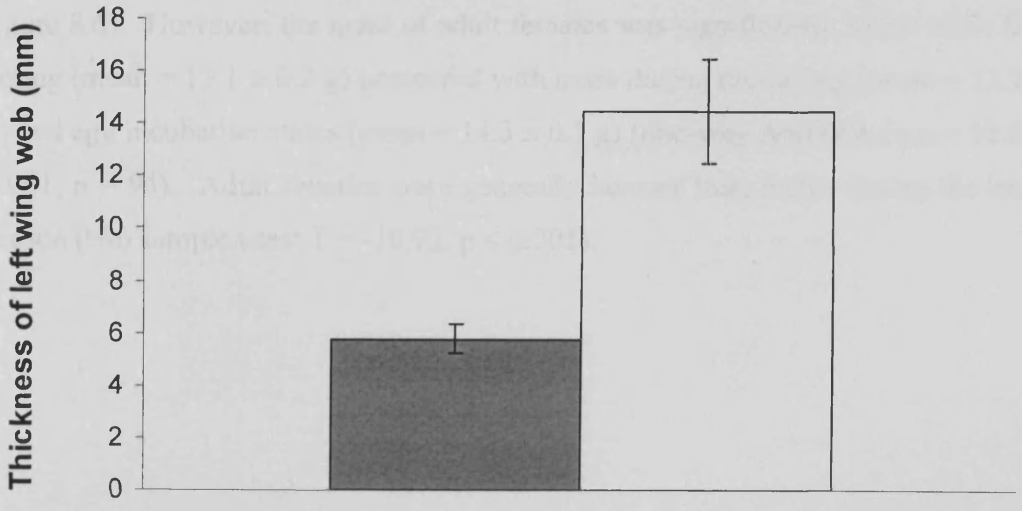

Figure 8.4: Mean ± S.E. thickness of nestling (n = 13) left wing web ± S.E. (mm) measured before PHA injection (grey bar) and 24 h after PHA injection (open bar).

Source	d.f.	F	P
Age	2	1.65	0.269
Sex	1	0.01	0.916
Plasma T	1	0.88	0.384
Mass	1	0.13	0.732
Date	1	0.75	0.419

Table 8.1: Nestling variables in a GLM model used to investigate variation in nestling response to PHA injection.

8.3.4 Breeding state, adult quality and T

Adult breeding states were not completely synchronised between April – June and plasma T levels of adult males were significantly different in each of 3 breeding states (figure 8.5). Male plasma T levels were highest during pre-laying (mean = 2.031 ± 0.358 $ngml^{-1}$), they decreased significantly during egg incubation (mean = 0.694 ± 0.338 $ngml^{-1}$) and they decreased significantly again when feeding young (mean = 0.099 ± 0.019 $ngml^{-1}$) (one way ANOVA $F_{2,58} = 63.61$, $p < 0.001$, $n = 61$). By the time adult males were feeding young, plasma T levels had fallen to 5 % of pre-laying state values and were similar to adult female T levels. Plasma T levels of adult females (mean = 0.144 ± 0.016 $ngml^{-1}$) did not vary significantly between breeding states (one-way ANOVA $F_{2,91} = 1.36$, $p = 0.261$, $n = 94$).

Adult male mass (mean = 12.6 ± 0.1 g) was not significantly different in each of the 3 breeding states between April – June (one-way ANOVA $F_{2,57} = 0.35$, $p = 0.703$, $n = 61$; figure 8.6). However, the mass of adult females was significantly lower when feeding young (mean = 13.1 ± 0.2 g) compared with mass during pre-laying (mean = 13.9 ± 0.9 g) and egg incubation states (mean = 14.3 ± 0.1 g) (one-way ANOVA $F_{2,90} = 11.02$, $p < 0.001$, $n = 94$). Adult females were generally heavier than males during the breeding season (two sample t-test $T = -10.92$, $p < 0.001$).

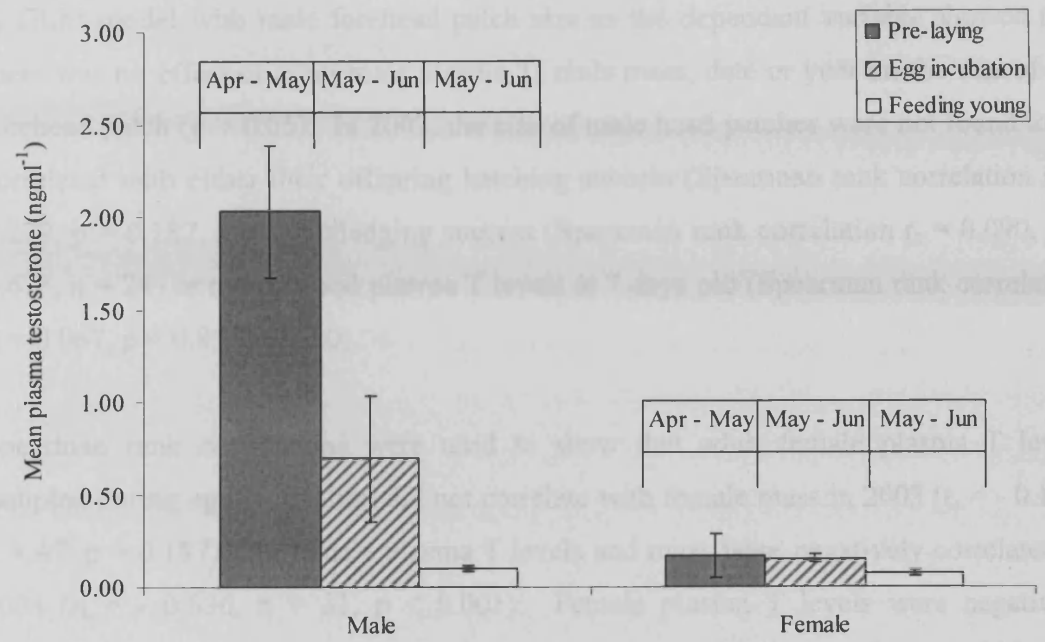

Figure 8.5: Mean ± S.E. plasma testosterone levels of adult males sampled during: pre-laying (n = 22), egg incubation (n = 6) and feeding young (n = 33), and adult females sampled in during: pre-laying (n = 2), egg incubation (n = 79) and feeding young (n = 13). Data were obtained between April – June in 2003 and 2004.

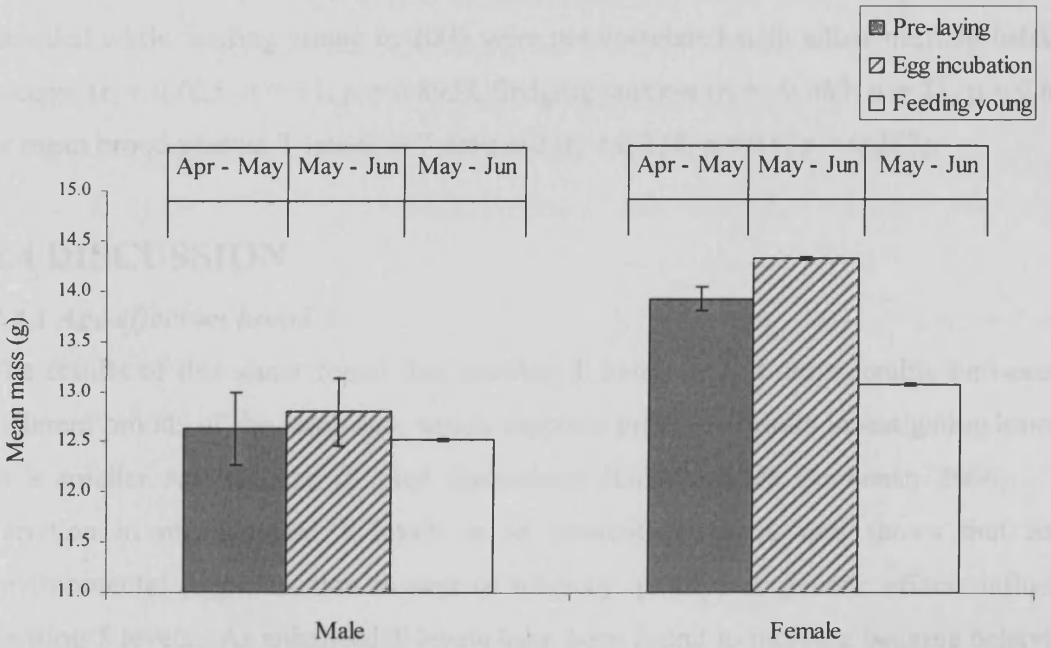

Figure 8.6: Mean ± S.E. mass of adult males sampled during: pre-laying (n = 22), egg incubation (n = 6) and feeding young (n = 33), and adult females sampled in during: pre-laying (n = 2), egg incubation (n = 79) and feeding young (n = 13). Data were obtained between April – June in 2003 and 2004.

A GLM model with male forehead patch size as the dependent variable showed that there was no effect of adult male plasma T, male mass, date or year on the size of the forehead patch ($p > 0.05$). In 2003, the size of male head patches were not found to be correlated with either their offspring hatching success (Spearman rank correlation r_s = 0.279, p = 0.187, n = 24), fledging success (Spearman rank correlation r_s = 0.090, p = 0.675, n = 24) or mean brood plasma T levels at 7 days old (Spearman rank correlation r_s = 0.067, p = 0.855, n = 10).

Spearman rank correlations were used to show that adult female plasma T levels sampled during egg incubation did not correlate with female mass in 2003 (r_s = - 0.196, n = 47, p = 0.187), but female plasma T levels and mass were negatively correlated in 2004 (r_s = - 0.636, n = 31, $p < 0.001$). Female plasma T levels were negatively correlated with nestling hatching success in 2003 (r_s = - 0.344, n = 47, p = 0.018), but not in 2004 (r_s = 0.214, n = 31, p = 0.249). Similarly, adult female plasma T levels were negatively correlated with nestling fledging success in 2003 (r_s = - 0.295, n = 47, p = 0.047), but not in 2004 (r_s = 0.143, n = 31, p = 0.451). Adult female plasma T levels were not correlated with mean brood T levels in 2003 (r_s = -0.460, n = 13, p = 0.114) (brood plasma T levels were not measured in 2004). Adult male plasma T levels sampled while feeding young in 2003 were not correlated with either nestling hatching success (r_s = 0.025, n = 31, p = 0.895), fledging success (r_s = -0.083, n = 31, p = 0.656) or mean brood plasma T levels at 7 days old (r_s = 0.318, n = 14, p = 0.267).

8.4 DISCUSSION

8.4.1 Age effect on brood T

The results of this study found that nestling T levels varied considerably between 20 different broods of the same age, which supports previous results investigating brood T in a smaller sample size of pied flycatchers (Goodship & Buchanan 2006). The variation in mean brood T levels is an interesting result, and shows that either environmental (e.g. variation in nest or territory quality) or genetic effects influence nestling T levels. As enhanced T levels have been found to increase begging behaviour in nestling birds, including pied flycatchers (Eising & Groothuis 2003; Schwabl 1996a; chapter 4), an environmental or genetic factor affecting plasma T levels may partially explain why nestling begging intensity and survival rates differ between broods (Price

et al. 1996; Price & Ydenberg 1995). Further studies using cross-fostering experiments are needed to differentiate between the effects of environmental or genetic factors on nestling T levels and begging behaviour.

In this study, plasma T levels did not differ between nestlings aged 7 and 11 days old. However, as studies on great tits and zebra finches have previously found that nestling T levels are highest within the first week of hatching compared with other times during the nestling period (Adkins-Regan et al. 1990; Silverin & Sharp 1996), it may be possible that had pied flycatcher T levels been sampled when nestlings were younger than 7 days old, an age effect on nestling T levels may have been recorded. Pied flycatcher nestlings were blood sampled to assay T levels at 7 days old throughout this project, as it was believed that nestlings younger than this would not have been able to withstand sampling procedures. However, as it was found in chapter 7 of this thesis that androgen metabolites can be measured in the faeces of pied flycatchers (figure 7.5), future studies may be able to measure androgen levels in nestlings too young to withstand invasive sampling. Previous studies have found that nestling quality or need may change with age (Kilner 2002; Rosivall et al. 2005), and begging signals may change as nestlings get older (Kilner 2002; Leonard et al. 2005; Price & Ydenberg 1995). For example, a study by Leonard et al. (2005) on tree swallows, *Tachycineta bicolour* found that nestling begging rate in response to playback sounds of a parent arriving at the nest was greater for older (7 – 9 days) than for younger nestlings (5 – 6 days). Another study on yellow-headed blackbirds, *Xanthocephalus xanthocephalus* showed that begging loudness increased with nestling aged between 5 – 10 days old (Price et al. 1996). It has been suggested that older nestlings might beg more readily because they need more resources and a number of studies on a range of species have shown that begging behaviour correlates with offspring need (Kilner 1995; Kilner 1997; Mondloch 1995; Price & Ydenberg 1995; Redondo & Castro 1992; Smith & Montgomerie 1991). However, in addition, older nestlings may also be able to exaggerate their begging postures more than younger nestlings, because the costs of begging have been found to decrease with age (Kilner 2002; Kilner 2001). For example, a study on nestling canaries has shown that postural begging is costly due to metabolic expenditure and reduces growth rate (Kilner 2001). In a study in which pairs of sibling canaries were forced to beg for different durations, Kilner (2001) showed that

excessive nestling begging slowed growth and that the energetic costs of begging had least effect on older nestlings that had completed most growth. As plasma T levels may be lower in older nestlings closer to fledging compared with nestling T levels immediately post hatching (Adkins-Regan et al. 1990; Silverin & Sharp 1996), older nestlings may also experience fewer costs involved with the production of T (Folstad & Karter 1992). In addition, older, larger nestlings may be able to afford a greater begging effort for the same level of hunger than younger smaller nestlings (Godfray 1995a; Johnstone & Grafen 1993; Kilner & Johnstone 1997). It can be suggested that the relationship between nestling T levels and begging behaviour may change with age and older nestlings may be able to beg more than younger nestlings with the same level of circulating T. As theory shows that the reliability of nestling begging signals are maintained by the costs involved with the display (Godfray 1991; Godfray 1995a), a decrease in the cost of signalling with age may mean that begging signals become less reliable as nestlings get older (Kilner 2002). Parents may compensate for less reliable begging signals in older nestlings by changing their provisioning rules (Kilner 2002; Lessells 2002; Rosivall et al. 2005).

8.4.2 Sex effect on nestling T

A sex difference in nestling plasma T levels and mass was not apparent from this study. Furthermore, the results of another study reported in this thesis in which begging behaviour was measured after manipulating nestling T levels (chapter 4), did not show any differences in begging behaviour between male and female nestlings. Differences in morphology and behaviour are a prominent feature of reproducing adult birds, but the time when sexual dimorphism is established in young birds can vary between species (Adkins-Regan et al. 1990; Ottinger & Abdelnabi 1997; Saino et al. 2003). Previous studies on passerine species have shown that male and female nestlings can have different plasma T levels (Adkins-Regan et al. 1990; Silverin & Sharp 1996; Weichel et al. 1986). In great tits, male nestlings have higher T levels than females for only the first 2 days after hatching (Silverin & Sharp 1996), whereas in zebra finches and canaries, females have been found to have higher plasma T levels than males (Adkins-Regan et al. 1990; Weichel et al. 1986). However, a recent study has shown for the first time that male zebra finches beg for longer than females in the first few days after hatching, and that begging duration was not due to a size difference between

sexes, as males were a similar size to females at hatching (Engelhardt et al. 2006). Furthermore, in the same study it was shown that T injected into eggs increased begging of female nestlings to similar levels as in male nestlings of the control group, but T treatment did not affect begging of males (Engelhardt et al. 2006). This recent study on zebra finches may suggest that male nestlings had higher plasma T levels than females, although this was not measured directly. Studies linking yolk T and begging behaviour provide useful information on maternal effects on nestling behaviour, but further studies are required to investigate relationships between maternal T, nestling plasma T and begging behaviour. One reason why sex differences in nestling begging behaviour occurs may be due to size differences between male and female nestlings. For example, in red-winged blackbirds, *Agelaius phoeniceus* (Teather 1992) and yellow-headed blackbirds (Price et al. 1996; Price & Ydenberg 1995), males are larger than females, and male nestlings beg more intensely, especially when hungry, which suggests that begging reflects higher nutritional requirements. In Eurasian kestrels, *Falco tinnunculus* female nestlings are larger than males, and as females gain more food from their parents, females nestlings may have a competitive superiority over males in this species (Fargallo et al. 2003). Parents of some species are also able to bias their provisioning to nestlings of one sex or another (Lessells 2002) which may be an advantage when the fitness returns of sons and daughters differ (Clutton-Brock 1991; Trivers & Willard 1973). Parents may be able to use morphological or behavioural traits of nestlings to identify offspring sex (Lessells 2002).

8.4.3 Cell-mediated immune response and T

The results of this study found that there was no significant effect of nestling plasma T levels on cell-mediated immune response to PHA injection. However, the ICHH predicts that each individual should have its own optimum level of T allowing maximum trait expression, while minimizing immunosuppression (Folstad & Karter 1992). Therefore, the variation in optimum T levels between individuals in the small sample size used in this study may explain why no significant relationship between nestling T levels and immune response was found. Nestling T levels may correlate either positively or negatively with immune response. Individuals with high T levels may suffer from immunosuppressive costs of T, but also, good quality individuals with high T levels may be able to withstand the immunosuppressive costs involved with T

production. Previous manipulative studies have provided support for the ICHH by showing that cell-mediated and / or humoral immune responses are suppressed by elevated T levels in several adult male passerine species including: European starlings, *Sturnus vulgaris* (Duffy et al. 2000) house sparrows (Evans et al. 2000), song sparrows, *Melospiza melodia* (Owen-Ashley et al. 2004) and dark-eyed juncos, *Junco hyemalis* (Casto et al. 2001). Corticosterone also has known immunosuppressive effects in birds (Evans et al. 2000; Marsh & Scanes 1994; Owen-Ashley et al. 2004). However, other studies have also found that males with elevated T levels do not always show a reduced immune response (reviewed by Roberts et al. 2004).

Previous studies have found that androgens of maternal origin may suppress nestling immunity. For example, yolk androgen concentrations increase with laying order in gulls (Eising et al. 2001; Groothuis & Schwabl 2002; Royle et al. 2001) and it has been found in black-headed gulls, *Larus ridibundus* that cell-mediated immunity decreases with hatching order (Muller et al. 2003), although elevated yolk T in the eggs of yellow-legged gulls, *Larus michahellis* did not affect cell-mediated immune response (Rubolini et al. 2005). A different study on eastern bluebirds, *Sialia sialis* has found that nestlings hatching from T-treated eggs show suppressed cell-mediated immune response (Navara et al. 2005). As yolk T has been shown to enhance nestling growth, it may be suggested that nestlings are subjected to a trade off between the positive effects of androgens on growth with the negative effects of androgens on reduced immunity (Groothuis & Ros 2005; Navara et al. 2005).

8.4.4 Breeding state, adult quality and T

The results of this study show that adult male plasma T levels differed significantly between breeding states. Male plasma T levels were highest during pre egg laying and had declined to basal levels by the time they were feeding young. A previous study by Silverin (1991) found a similar pattern in the changes of plasma T levels of adult male pied flycatchers over the breeding season. Plasma T levels were found to peak at the start of the breeding season during the early part of nest building and they rapidly decreased around the time eggs hatched, and remained at moderate levels during the parental phase (Silverin 1991). The functional significance of this hormonal peak was

suggested to increase mate guarding behaviour in order to avoid cuckoldry, and / or to increase courtship behaviour (Silverin 1991).

During mate choice, adult females of many passerine species may use several signals that indicate male quality, parental ability and the quality of resources held by the male in his territory (Gottlander 1987b; Part & Qvarnstrom 1997; Sætre et al. 1995; Sanz 2001; Siitari & Huhta 2002). Male 'badges of status' can be used by females when choosing a mate, and in collared flycatchers, the size of the white forehead patch is positively related to a male's lifetime reproductive success (Gustafsson et al. 1995). Furthermore, in collared flycatchers (Garamszegi et al. 2004) and house sparrows (Buchanan et al. 2001; Evans et al. 2000), plumage patch size is related to male plasma T levels. Increased costs of immunosuppression (Folstad & Karter 1992), and / or energetic costs (Buchanan et al. 2001) associated with large badge sizes, may show that T-dependent badge sizes act as honest indicators of male quality. However, this study did not find a significant relationship between male plasma T levels and forehead patch size in pied flycatchers; although this result may be explained by the timing that T levels were sampled at in relation to badge production. Adult male pied flycatchers undergo a partial moult each year between January and February before the start of the spring migration (Lundberg & Alatalo 1992). During the partial moult, adult males develop their conspicuous black and white plumage, including the patch of white feathers of the forehead (Lundberg & Alatalo 1992). In this study, adult male T levels were not sampled until April and May. Therefore, it may be suggested that as T levels were not measured at the time of badge production, a correlation between adult male T levels and forehead patch size should not be expected. As seen from the results of this study, circulating T levels can vary considerably over a season. Therefore, it may be very important to sample T levels at the time of signal production. In addition, the timing of T sampling may also explain why male head patch size and T levels did not correlate with brood T at 7 days old. As nestling T levels may change through development, the relationship between parent and offspring T levels may alter through the season. Previous studies have shown that the size and shape of the forehead patch in male pied flycatchers is highly variable between different individuals and populations (Dale et al. 1999). In a Spanish population of pied flycatchers, males have a large white forehead patch which may be an important signal of male quality to

females during mate finding (Potti & Montalvo 1991). However, in a Norwegian population, males have smaller forehead patches than the Spanish population and an observational study has found that females do not use the size of the forehead patch when choosing a mate (Dale et al. 1999). Male forehead patches in this study where found to vary considerably in size (see figure 1.3), and as males with very small head patches mated and raised broods successfully, patch size may not be an important indicator of male quality in this population.

This study found that female plasma T levels did not change significantly between breeding states. If female pied flycatchers allocate T into their egg yolks, this study shows that females don't have raised plasma T levels before egg laying. Since the discovery of maternally derived androgens in avian yolk (Schwabl 1993), a number of studies in a range of species have found that androgens are present in egg yolks and may naturally increase (Eising et al. 2001; French et al. 2001; Groothuis & Schwabl 2002; Lipar et al. 1999; Pilz et al. 2003; Royle et al. 2001; Schwabl 1993; Sockman & Schwabl 2000), or decrease (Gil et al. 1999; Schwabl et al. 1997) with laying order. There has not yet been a study to measure yolk T in the eggs of pied flycatchers. This study also found that female plasma T levels were negatively correlated with nestling hatching and fledging success in 2003, but not in 2004. Furthermore, there was no significant correlation between female plasma T levels and female mass in 2003, but female T levels were negatively correlated with female mass in 2004. This difference between years suggests that different environmental conditions may influence how female T levels relate to female condition, which in turn could affect nestling survival. Female T levels may correlate with territory quality or with other aspects of female condition that may affect hatching and fledging success of their young.

If plasma T levels of females are in some way related to yolk T levels, female pied flycatchers may influence the survival of their offspring through T levels deposited into their egg yolks. A range of studies have shown that yolk androgens can influence the growth and development of young birds in different ways in different species, some of these effects of androgens are positive, others negative (Navara et al. 2005). For example, studies which have manipulated T levels of egg yolks have shown that T increases nestling growth and begging in black-headed gulls and canaries (Eising &

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Chapter 9
Conclusions

The main results of this project have been discussed in detail within each chapter and this thesis will now conclude by drawing together the main findings. As many new questions have been generated by the work of this project, suggestions for further research will be made. Finally, a summary of the main results will be presented.

9.1 Nestling T and begging behaviour

The overall aim of this project was to investigate the role of testosterone (T) in controlling begging signals in nestling pied flycatchers, *Ficedula hypoleuca.* The aim of this project was addressed through interdisciplinary studies, integrating physiology, behavioural and evolutionary biology, using a combination of laboratory and field techniques. To date, there have been few studies to investigate endocrine mechanisms controlling begging behaviour in young passerines. Previous manipulation studies on passerine species including: canaries, *Serinus canaria* (Schwabl 1996a) and zebra finches, *Taeniopygia guttata* (Engelhardt et al. 2006) have shown that nestlings beg more intensively when hatching from T-treated eggs, compared with control eggs. Observational studies on non-passerine species including: thin-billed prions, *Pachyptila belcherie* (Quillfeldt et al. 2006) and white storks, *Ciconia ciconia* (Sasvári et al. 1999) have found that nestling endogenous T is correlated with begging behaviour. Observation and manipulation studies on gulls, *Laridae* have related nestling begging behaviour with steroid hormones including: endogenously-produced T (Groothuis & Meeuwissen 1992; Groothuis & Ros 2005), androgens of maternal origin (Eising & Groothuis 2003) and endogenously-produced corticosterone (Kitaysky et al. 2003; Kitaysky et al. 2001b). The work described in this thesis is the first to directly relate nestling endogenous T with begging behaviour in a passerine species.

The results of this project on pied flycatchers found that nestling endogenous plasma T levels were positively correlated with two measures of begging behaviour including: 1) the duration of the begging display and 2) the maximum height of the begging stretch (chapter 3). These results suggested that nestling T levels may be causally involved in

controlling begging signals in this species. In a manipulation study, the casual nature of T was confirmed, as it was found that the duration of begging displays by T-dosed nestlings were longer than controls (chapter 4). There was no effect of the T-dose on the maximum height of begging stretches, and from this finding it was suggested that the height of begging is not as T-dependent as the duration of begging displays.

The results of chapter 4 also showed that nestling circulating T levels were elevated through an oral dose of T. This finding may be useful for future studies wishing to increase plasma hormone levels in small animals, without using invasive methods (e.g. hormone implants). In this project, a pilot study was performed before the main study to investigate the level of dosing and length of time required to increase plasma T levels within physiological range in nestling pied flycatchers. Future studies to use oral-dosing techniques must also first quantify the level of dose suitable for specific species. The results of the manipulation study described in this project contrasted with previous manipulation studies on black-headed gulls, *Larus ridibundus,* which enhanced nestling endogenous T levels using T pellets implanted subcutaneously into the neck (Groothuis & Meeuwissen 1992; Groothuis & Ros 2005). In the study on black-headed gulls, it was found that T-implants did not increase nestling begging behaviour, but they did increase nestling territorial defence and aggressive pecking behaviour compared with control nestlings. Compared with the work on gulls, the results of this project suggest that T may have a different functional role on behaviour in altricial birds compared with semi-precocial species. This inter-species difference in the role of T on nestling begging behaviour should be further investigated. New studies are now required to manipulate nestling T levels in different species to fully explore the causal relationship between nestling T and behaviour. The results obtained from the oral-dosing technique used in this study may encourage further manipulation studies on small passerine nestlings.

9.2 Maternal T

Female birds of many species deposit androgens into their egg yolks at some point before laying (e.g. Schwabl 1993). So far, most of the previous studies which have investigated relationships between hormone levels and nestling begging behaviour, have measured maternally derived hormones. For example, it has been found that the

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hormone levels relate to circulating levels and that excreted hormones are biologically meaningful. From the biochemical validation studies carried out in this project, it was confirmed that the androgen metabolites excreted in the faeces by pied flycatchers (chapters 5 & 6), were quantitatively detected, as excreted androgens cross-reacted with the antibody used in T-RIA (chapter 7). From the biochemical validation studies, a lot of variation in the type of excreted metabolites was found between 4 different passerine species. The ability of the antibody used in the T-RIA to cross-react with the different excreted androgens also varied between species, and it was shown that the antibody used in this project was unsuitable to quantify androgen excretion in zebra finches. The results of this project therefore highlight the need for biochemical validation studies on non-invasive sampling to be carried out at a species-specific level. Unfortunately, the GnRH study was unsuccessful and this project cannot confirm that the T-RIA was able to pick up differences in androgen metabolites relating to biologically meaningful differences in circulating T levels by each passerine species. The possible methodological error made in the GnRH study may have been due to the lighting conditions in the animal room or injection techniques. It is recommended that, after correction of these errors, a future study repeats this GnRH study. Throughout this project when samples were collected for hormone assay, every care was taken to minimize the extent that hormones were metabolized by bacterial enzymes (Palme 2005), as this can alter the final level of hormones measured in a sample (Khan et al. 2002; Möstl et al. 2005; Palme 2005). In the field, blood and faecal samples were always transported at around 0 °C and were frozen within 5 h of collection. After each field season, plasma samples were stored at -20 °C and faecal samples were stored in a freeze-dried state until analysis. Future field studies measuring hormone levels in animals should be aware of the potential errors involved with collecting and storing samples for analysis, and it is recommended that studies report the methods that they use to minimise errors so that other studies can learn from them.

In summary, the results of this project have shown for the first time the covariation between nestling begging behaviour and circulating plasma T levels in passerines. Furthermore, a manipulation study confirmed that nestling T is responsible in part for the control of begging intensity, suggesting that nestling T and may be a mechanism that controls begging behaviour in nestling birds. Nestling T levels may have direct

fitness effects, as provisioning adults predominantly allocated food based on begging signals and brood T levels were positively related to fledging success. This project has also confirmed that T and the majority of androgen metabolites excreted by pied flycatchers were detected in non-invasive faecal samples, which may encourage new studies to investigate hormone levels and behaviour in young nestlings too small to sampled invasively. Future studies investigating the costs associated with begging behaviour may indicate the role of T on the evolution of begging behaviour in nestling pied flycatchers.

Summary of results

The main results of this project have been summarised below:

Chapter 3

1) Pied flycatcher nestling begging intensity increased over the duration of a short-term food deprivation study.

2) Nestling begging behaviour defined as: 1) the duration of the begging display and 2) the maximum height of the begging stretch, were positively correlated with nestling plasma T levels.

3) Mean brood T was positively associated with fledging success.

Chapter 4

4) Nestling plasma T levels were elevated through an oral dose of T.

5) The causal nature of the positive relationship between nestling circulating T levels and nestling begging behaviour was confirmed; the duration of begging displays were longer by T-dosed nestlings compared with controls.

6) There was no effect of T-dosing on the maximum height of nestling begging stretches, suggesting that the height of begging is not as T-dependent as begging display duration.

Chapter 5

7) Parents responded to nestling begging signals when allocating food resources. Parents preferentially fed nestlings that extended their neck highest in the nest, held their beak closest to the provisioning parent or were first to beg.

8) Parents did not respond to cues of nestling sex, mass or androgen levels at 7 days old when making feeding decisions.

9) Adult females reduced their provisioning rates in response to a short-term reduction in brood size, but adult males did not decrease their provisioning rates significantly.

10) During brood reduction, parents allocated food based more strongly on nestling behaviour.

11) Brood reduction did not alter nestling faecal androgen levels, although the brood may not have been reduced for long enough to see an effect of the manipulation.

Chapter 6

12) In a cross-fostering experiment, brood begging duration was longer by broods with reduced relatedness compared with control broods.

13) Adults did not appear to respond to reduced relatedness within a brood.

14) Mean faecal androgen levels of fostered siblings were higher than siblings remaining in their natal brood, although further studies are needed to test the robustness of the effect of cross-fostering on androgen levels.

Chapter 7

15) A radioinfusion study showed that T and androgen metabolites were excreted in the faeces of 4 different passerine species including: a male pied flycatcher, great tit, canary and zebra finch.

16) It was confirmed that the antibody used in the T-RIA throughout this project was able to detect T and androgen metabolites in pied flycatcher faeces.

17) The number of excreted androgen metabolites differed between species, thus highlighting the need for biochemical validation studies on non-invasive sampling to be carried out at a species-specific level.

18) The GnRH study was unsuccessful and did not lead to any apparent increase of excreted androgen metabolites in any species. It is recommended that the GnRH study should be repeated.

Chapter 8

19) Nestling plasma T levels were found to significantly vary between broods at 7 days old.

20) Plasma T levels of male and female nestlings were similar at 7 days old.

21) Plasma T levels were similar between nestlings aged 7 and 11 days old.

22) Cell-mediated immune response was not found to be influenced by nestling plasma T levels.

23) Adult male T levels differed significantly between breeding states and were highest at the start of the breeding season pre egg laying.

24) The size of the white forehead patch of adult males was not correlated with male plasma T levels.

25) Adult female plasma T levels did not differ significantly between different breeding states.

26) Adult female plasma T levels were negatively correlated with nestling hatching and fledging success.

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