

INFLUENCE OF HORMONES AND CAROTENOIDS ON SIGNALLING, IMMUNOCOMPETENCE AND PERFORMANCE IN A LIZARD

Helen Jane Place

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, in
fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2012

DECLARATION

I declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

A handwritten signature in black ink, appearing to read 'H. L. L. L.', with a long horizontal stroke extending from the bottom of the signature.

(Signature of candidate)

18th day of May, 2012 in Johannesburg

ABSTRACT

The animal kingdom contains a spectacular diversity in colour signals used to indicate quality. The challenge of understanding this diversity lies in identifying and interpreting constraints on signals that maintain signal honesty. I used an integrative approach to measure the effects of potential signal modulators on whole-animal performance, ornaments, condition and immunocompetence in the lizard *P. i. wilhelmi*. This approach attempts to remove some of the uncertainty surrounding the validity of existing handicap models. First, I investigated seasonal changes in testosterone, corticosterone and carotenoids and compared these to seasonal changes in endurance, immunocompetence and body condition male *P. i. wilhelmi*. I also determined which colour patches were predictors of male quality by relating them to morphology, endurance, body condition and immunocompetence. I found some support for the immunocompetence handicap hypothesis so I tested whether testosterone was modulating ornaments and constraining signals through its immunosuppressive properties. I tested the immunocompetence-handicap hypothesis in male *P. i. wilhelmi* while also conducting parallel studies with free-ranging and captive-housed lizards to assess whether there were differences between the groups that could indicate exogenous factors influencing signalling in their natural environment. While experimentally elevated testosterone did affect endurance and the properties of their colour patches, immunosuppression was only evident in free-ranging lizards. To measure the extent of organisational effects in males I also manipulated testosterone in females and found no evidence of immunosuppression although testosterone did affect some aspects of colouration and endurance. Next, I tested the stress-linked immunocompetence handicap hypothesis by experimentally elevating testosterone, corticosterone, or both at the same time. I found that corticosterone had an isolated effect on one colour patch, and testosterone and corticosterone had opposing effects on endurance that were negated when both were elevated. Different colour patches were affected by either testosterone or corticosterone with little overlap, and the combination of the two had a different action to either hormone elevated in isolation. Finally, I tested the oxidative stress hypothesis of carotenoids as a limiter of signal output due to their requirement in the oxidative stress response. I found that supplemented carotenoids vastly improved endurance, immune response and influenced different

aspects of colouration to that of testosterone and corticosterone. My study examined four different hypotheses of constraints on signalling involving testosterone, corticosterone, interactions between testosterone and corticosterone and carotenoids in a consistent manner measuring multiple indices of quality as well as multiple colour signals. This study provides a unique integrative perspective on the roles played by each factor as well as prompting us to re-examine our approach to understanding constraints on signalling.

In memory of W.H.A. Place
1916-2010

ACKNOWLEDGEMENTS

This work was funded by the National Research Foundation (grant awarded to M.J. Whiting), and carried out under a Mpumalanga Tourism and Parks Agency (MTPA) permit number MPB5211/2. All experimental work was approved by the Animal Ethics Screening Committee at the University of the Witwatersrand (clearance numbers AESC 2007/48/03; AESC 2007/64/03; AESC 2008/40/04; AESC 2009/36/3).

I would like to thank the APES department for allowing me to use Pullen Farm for my project. I'd also like to thank the managers of Pullen farm, Johan Rademan and Pontie Esterhuizen who not only kept an eye on me during floods, lighting strikes and fires, but also always remembered to make the their neighbours aware of my project. I would also like to thank the farmers who took a keen interest in my progress and often provided some much needed company and support. In particular I'd like to thank Joe and Enola for their hospitality and kindness.

This project would never have been possible without a great deal of interdisciplinary collaboration. In particular I would like to thank David Gray for teaching me to do testosterone assays, allowing me to work in his lab and use his equipment, and for being patient whenever I arrived at his office with long lists of questions. Diana Hews (Indiana State University) provided a great deal of advice and she and Maria Thaker took care of assay kit validation. Maria Thaker (UKZN) also performed the corticosterone ELISA assays. Thierry Regnier and Sandra Combrink at TUT not only made their lab and equipment available to me, they also worked with me to figure out a protocol to measure carotenoid concentrations in very small blood samples, which took a great deal of their time. I would also like to thank their post-graduate students, in particular Johan Linde who took time to teach me lab techniques when I was very out of my depth. Bruce MacKay built the lizard treadmill and Oliver Wood built the treadmill housing as well as troubleshooting and fixing the treadmill when the mechanism wore out.

I would also like to thank my supervisors. Martin Whiting encouraged me to try a PhD in the first place, and gave me the freedom and independence (and funding and equipment) to make my own mistakes, which have taught me far more than I learned when everything went right. He also continued to supervise me after moving to Australia. Neville Pillay 'adopted' me when Martin moved overseas and has been an indispensable part of this project, by providing advice on statistics, reviewing manuscripts promptly and providing a sounding board that allowed me to develop my own ideas.

Members of the Animal Behaviour lab provided me with friendship and support through the course of this project. In particular I'd like to thank Jenny Lancaster, Tasmin Rymer, Sneha Joshi, Megan Jones and Luke Duncan.

I am grateful to Grant Craig and Phillippa Wagner for keeping my job open, even when I was away for months at a time on fieldwork. They also provided valuable veterinary advice, as well as assisting in the paperwork required to send blood samples overseas. I also received advice and assistance from Katja Koepfel at Johannesburg Zoo.

My friends particularly Dudley Wood, Lara van Niekerk, Nicola Webb, Oliver Wood and Vanessa Brueton provided much appreciated support and encouragement. My family also gave me wholehearted love and support, even though nobody had a clue of what I was doing. Special thanks go to my mother and father who survived long periods of worry while I was out in the field and then welcomed me home as I filled their freezer with dead animals and blood samples (and bragged about it to their friends later).

CONTENTS

ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	vi
CONTENTS.....	viii
LIST OF FIGURES.....	ix
LIST OF TABLES.....	xi
INTRODUCTION.....	1
Seasonal variation in testosterone, corticosterone and carotenoids in flat lizard plasma and associated changes in condition-dependent signals.....	12
Introduction.....	12
Methods.....	18
Results.....	25
Discussion.....	34
CHAPTER 2.....	38
Testing the immunocompetence handicap hypothesis in free-living and captive lizards.....	38
Introduction.....	38
Methods.....	41
Results.....	45
Discussion.....	52
Chapter 3: Testing the effects of exogenous testosterone on female flat lizards.....	58
Introduction.....	58
Methods.....	62
Results.....	65
Discussion.....	70
CHAPTER 4.....	74
Short-term effects of elevated testosterone, corticosterone and the interaction between testosterone and corticosterone in a lizard.....	74
Introduction.....	74
Methods.....	77
Results.....	81
Discussion.....	91
CHAPTER 5.....	96
Testing the effects of carotenoids on condition-dependent signals in a lizard.....	96
Introduction.....	96
Methods.....	99
Results.....	103
Discussion.....	110
DISCUSSION.....	112
REFERENCES.....	126

LIST OF FIGURES

Chapter 1

Figure 1.1. A template for visual peak sensitivity in <i>Platysaurus broadleyi</i>	23
Figure 1.2. Plasma testosterone levels in male <i>Platysaurus i. Wilhelmi</i> in 3 different seasons.....	25
Figure 1.3. Plasma corticosterone levels in male <i>Platysaurus i. wilhelmi</i> in 3 different seasons	26
Figure 1.4. Plasma carotenoid levels in male <i>Platysaurus i. wilhelmi</i> in 3 different seasons	26
Figure 1.5. Endurance of male <i>P. i. wilhelmi</i> in 3 different seasons.....	27
Figure 1.6. Body condition of male <i>P. i. wilhelmi</i> in 3 different seasons.....	28
Figure 1.7. Immune response of male <i>P. i. wilhelmi</i> over 3 different seasons.....	29
Figure 1.8. Chromatic contrast of 5 different body regions of male <i>P. i. wilhelmi</i> over 3 seasons.....	30
Figure 1.9. Brightness of 5 different body regions of male <i>P. i. wilhelmi</i> over 3 seasons.....	32

Chapter 2

Figure 2.1. Plasma testosterone levels in male <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	45
Figure 2.2. Body condition of male <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	46
Figure 2.3. Cell-mediated immune response of male <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	47
Figure 2.4. Endurance of male <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	48
Figure 2.5. Chromatic contrast of colour patches of captive male <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	49
Figure 2.6. Chromatic contrast of colour patches of free-ranging male <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	50
Figure 2.7. Brightness of captive male <i>P. i. wilhelmi</i> colour patches before and two weeks after insertion of testosterone implants.....	51
Figure 2.8. Brightness of free-ranging male <i>P. i. wilhelmi</i> colour patches before and two weeks after insertion of testosterone implants.....	52

Chapter 3

Figure 3.1. Potential outcomes of experimentally elevating testosterone in females.....	61
Figure 3.2. Plasma testosterone levels in female <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	65
Figure 3.3. Body condition of female <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	66
Figure 3.4. Chromatic contrast of colour patches of female <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	67

Figure 3.5. Brightness of colour patches of female <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	68
Figure 3.6. Cell-mediated immune response of female <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	69
Figure 3.7. Endurance of female <i>P. i. wilhelmi</i> before and two weeks after insertion of testosterone implants.....	70

Chapter 4

Figure 4.1. Plasma testosterone levels in male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	82
Figure 4.2. Plasma corticosterone levels in male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	83
Figure 4.3. Body condition of male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	84
Figure 4.4. . Chromatic contrast of colour patches of male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	85
Figure 4.5. Brightness of colour patches of male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	87
Figure 4.6. Black throat patch area of male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	88
Figure 4.7. Cell-mediated immune response of male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	89
Figure 4.8. Endurance of male <i>P. i. wilhelmi</i> before and two weeks after insertion of hormone implants.....	90

Chapter 5

Figure 5.1. Plasma carotenoid levels in male <i>P. i. wilhelmi</i> before and two weeks after beginning supplementation.....	103
Figure 5.2. Body condition of male <i>P. i. wilhelmi</i> before and two weeks after beginning supplementation.....	104
Figure 5.3. Endurance of male <i>P. i. wilhelmi</i> before and two weeks after beginning supplementation.....	105
Figure 5.4. Cell-mediated immune response of male <i>P. i. wilhelmi</i> before and two weeks after beginning supplementation.....	106
Figure 5.5. Chromatic contrast of colour patches of male <i>P. i. wilhelmi</i> before and two weeks after beginning carotenoid supplementation.....	107
Figure 5.6. Brightness of colour patches of male <i>P. i. wilhelmi</i> before and two weeks after beginning carotenoid supplementation.....	109

LIST OF TABLES

Chapter 1

Table 1.1. GLZ analyses of the influence of season on chromatic contrast in male <i>P. i. wilhelmi</i>	29
Table 1.2. Seasonal changes of colour patch brightness in male <i>P. i. wilhelmi</i>	31
Table 1.3. Chromatic contrast predicts aspects of male quality.....	33

Chapter 4

Table 4.1. The effects of testosterone, corticosterone or testosterone and corticosterone on colour patch brightness.....	86
Table 4.2. Comparing predictions and outcomes of three hypotheses to results seen in male <i>P. i. wilhelmi</i>	91

Chapter 5

Table 5.1. Changes in brightness of 5 different body parts in <i>P. i. wilhelmi</i> following carotenoid treatment.....	108
---	-----

Discussion

Comparison of studies on signal constraints, my data, and original predictions based on the literature.....	118
---	-----

INTRODUCTION

Ornaments and armaments provide information about the quality of an animal, such as aspects of condition (reviewed in Andersson, 1994) including health, immunocompetence (e.g., McGraw & Ardia, 2003); aggression, fighting ability (Olsson, 1994) and dominance status (e.g., Thompson & Moore, 1991). If these signals are honest, they reduce the costs of agonistic encounters because individuals are able to assess rivals (Lemos-Espinal et al., 1996) and therefore predict the outcome of a fight. One kind of armament used to assess rivals is a badge of status (Shorey, 1976). Badges of status include colour patches that can be used in behavioural displays (LeBas & Marshall, 2000). Lizard species often use badges of status (reviewed in Whiting et al., 2003), and these signals are usually plastic, where they can reliably indicate dynamic aspects and correlate with life history events, such as social status or age (Cooper & Greenburg, 1992). Another kind of plasticity found in some species is nuptial colouration (Olsson, 1994) where colour signals are only evident during the breeding season (Rand, 1990; Martín & López, 2001), while others show some degree of signal intensity increasing in the breeding season but colour signals are still evident, to a lesser extent, throughout the year (Salvador & Veiga, 2000).

To maintain the honesty of signal systems, signals which indicate individual quality must be costly to produce (Zahavi, 1975) and so these signals are usually reliable (Zahavi, 1975; Andersson, 1994; Grafen, 1990). Variation in trait expression is thought to be condition-dependent in some cases, where males need to be healthy in order to show maximal trait expression (Andersson, 1994). For an individual to have maximal trait expression, it must be able to bear the associated costs. These factors are called constraints on signalling, as they stop animals from using signals to give false information about their quality. For example, male goldfinches (*Carduelis tristis*) under nutritional stress are unable to lay down as much pigment into developing feathers as male goldfinches with sufficient food (McGraw et al., 2005). As yet, several mechanisms have been identified that constrain signals, such as parasite resistance (Hamilton & Zuk, 1982; Folstad & Karter, 1992), predation risk (Cooper & Greenburg,

1992; Martín & López, 1999; Stuart-Fox et al., 2003; Husak et al., 2006c), energetic constraints (Buchanan et al., 2001), social control (Mateos & Carranza, 1997) and endocrine constraints (Buchanan et al., 2003). Costly signalling theory (Zahavi, 1975; Grafen, 1990) states that on average signals of quality need to be constrained by some form of cost. These costs can be direct, such as the energetic cost of developing and maintaining a trait (Andersson, 1994), or as a result of the trait – for instance animals with brightly coloured ornaments or armaments or showy behavioural displays may be at risk of predation (e.g., Candolin, 1999).

The handicap principle

Zahavi (1975) proposed the idea of the handicap principle, but this was largely discounted and ignored for over two decades after the seminal paper was published (Grose, 2011). Once the field of honest signalling theory began to take off, Folstad and Karter (1992) proposed the immunocompetence-handicap hypothesis (ICHH). They proposed that a major constraint on male traits would be the ‘double-edged sword’ of testosterone. The model states that male-specific traits are mediated by male-specific hormones (i.e. androgens) and that levels of these hormones would be limited by an associated negative effect on the immune system, mainly shown by an increase in parasite load (Folstad & Karter, 1992). This hypothesis gained in popularity very quickly, probably due to the logic of the idea – males have androgens while females do not, males have secondary sexual traits and females (usually) do not, females tend to have better immune systems than males (reviewed in McGraw & Ardia, 2005a) and tend to be at higher risk of autoimmune disease (Grossman, 1990), which adds to the body of evidence for the immunosuppressive effects of testosterone (reviewed in Roberts et al., 2004).

Although the idea found its way into established theory quite quickly, experimental evidence did not concur (Roberts et al., 2004). Some studies did indeed find support for the ICHH, but others found no link between testosterone and immune function, sometimes no link between traits and testosterone in the first place, and some studies found testosterone to have an immune-enhancing effect. Additionally, other related factors began to be considered as signal constraints. One is the stress-linked

immunocompetence handicap hypothesis (SL-ICHH) which proposes that because testosterone and corticosterone share receptors, co-varying levels of these hormones may be influencing signal production rather than either hormone functioning independently.

Testosterone as a signal constraint

Testosterone is recognised as an important influence in signal production, particularly in sexually dimorphic species (Saino & Møller, 1994; Buchanan et al., 2001) where it promotes development and maintenance of secondary sexual characters (e.g. Saino & Møller, 1994; Zuk, 1996; Wade, 1997; Lemos-Espinal et al., 1996; Deviche & Cortez, 2005). These are often correlated with aspects of male quality such as body condition (Galeotti et al., 1997) or activity levels (Olsson et al., 2000), and possibly reduced parasite load (Hamilton & Zuk, 1982; Saino & Møller, 1994; but see Seutin, 1994). Dominant males can have significantly higher androgen levels (Greenberg & Crews, 1990), with accompanying increased mating success (Olsson et al., 2000), better locomotor performance than subordinates (Zuk, 1996; Robson & Miles, 2000; Bales et al., 2006) high activity levels (Lovern et al., 2001) and aggression (Nelson, 2000; Whittingham & Schwabl, 2002; Dugatkin, 2004; but see McDonald et al., 1995; McDonald et al., 2001). Dominant males also show an incidence of male-specific behaviours (Wingfield et al., 1990; Lovern et al., 2001) such as display behaviour and mate-guarding (Saino & Møller, 1995) as well as more elaborate ornaments than subordinates (Deviche & Cortez, 2005).

According to the handicap principle (Zahavi, 1975), in order for testosterone to be a constraint on signalling, there must be associated costs to having high circulating concentrations of the hormone. In fact, there are several costs associated with high levels of testosterone. For instance, increased male-specific behaviour such as mate-guarding, aggression and behavioural displays are energetically expensive (Buchanan et al., 2001) and high aggression increases the frequency of agonistic encounters so there is a likelihood of being injured in a fight. Increased metabolic rate can also lead to decreased body condition (Olsson et al., 2000). There is also the cost of lost foraging time or mating

opportunities (Lynn et al., 2000). Conspicuous ornaments and behaviours also increase predation risk (Zuk, 1996; Husak et al., 2006a).

One of the most studied costs of testosterone is its effect on the immune system. There are well-established links between testosterone and immunosuppression (e.g. Deviche & Cortez, 2005) as well as a common pattern of differences between immune function in males and females of sexually dimorphic species (reviewed in Ahmed & Talal, 1990; Folstad & Karter, 1992). While most studies of the immunosuppressive effects of testosterone have been on avian species, there is some evidence of its occurrence in lizard species as well (e.g., Veiga et al., 1998; Olsson et al., 2000). Because immune and endocrine systems are linked (Boonekamp et al., 2008), it is logical to assume that hormonally-linked traits, such as secondary sexual characteristics, should be influenced by the immune system (Zuk, 1996; Folstad & Karter, 1992; Slater & Schreck, 1997). The immunocompetence handicap hypothesis (ICHH) (Folstad & Karter, 1992) suggests that the immunosuppressive effects of testosterone constrain signal expression in that only high quality males should be able to withstand the costs associated with high levels of circulating testosterone (Folstad & Karter, 1992). Although testosterone obviously affects the immune system and usually suppresses it (Zuk, 1996; Veiga et al., 1998), in some studies it seems to enhance it (Lindström et al., 2001). In Great Tits (*Parus major*) testosterone levels had a positive correlation with cell-mediated immunocompetence (van Oers et al., 2011). Only some studies provide support for the ICHH (reviewed in Roberts et al., 2004), and this hypothesis requires more testing, particularly in non-avian taxa.

Stress and signaling

While dominant males are often constrained by the costs of testosterone, subordinate males are often constrained by stress. Being subordinate can be costly, not only because dominant individuals monopolise resources such as food, refuges and potential mates (e.g., Keys & Rothstein, 1991; Clutton-Brock, 1998; Stahl et al., 2001; Lendvai et al., 2006; Croney et al., 2007) or because they show aggression to subordinates (Keys & Rothstein, 1991; Amorim & Almada, 2005; Poisbleau et al., 2005; Heitor et al., 2006; Croney et al., 2007), subordinates often experience chronic stress responses (Creel, 2001)

which can be detrimental to health (Nelson, 2000; Wingfield, 2003; Millspaugh & Washburn, 2004). Increased allostatic load - the physiological cost of stress - is reflected by an increase in glucocorticoids (Goymann & Wingfield, 2004). The main cause of the increased allostatic load experienced by subordinates is a result of social interactions. Dominant individuals harass subordinates which result in a sharp increase in stress hormones in those subordinates (reviewed in Summers et al., 2005; Creel, 2001) through the hypothalamic pituitary axis (HPA) within minutes of a stressor occurring (Wingfield, 2003). Stress hormones act mainly to stimulate energy mobilisation (Möstl & Palme, 2002) which leads to an increase in activity (reviewed in Lowry & Moore, 2006). The degree and duration of a stress response differs according to various factors, including individual factors (such as social status, contest history and social history) and environmental factors (such as season and food availability) (Summers et al., 2005). Stress responses are modulated by corticotrophin-releasing factor which not only stimulates the production of glucocorticoids through the HPA axis, but also modifies neurotransmitters which changes the animal's response to increased levels of corticosterone (Lowry & Moore, 2006). Individual responses to stress differ, and can be influenced by life-history stage (Crespi & Denver, 2005), sex, temperature, body condition (Moore & Jessop, 2003) and status (Abbott et al., 2003; Summers et al., 2005). For example, subordinate individuals generally lose more fights than dominant individuals, which leads to an increase in stress hormones in them, which in turn leads to a decrease in reproduction for that particular individual (Creel, 2001). Dominant and subordinate individuals have different responses to stress in terms of hormone secretion (Abbott et al., 2003). Generally, an increase in corticotrophin-releasing hormone results in an increase in glucocorticoids. Increased corticosterone levels can also result in increased stamina and reduced resting metabolic rate (Cote et al., 2006; Miles et al., 2007), although this must be coupled with increased food consumption to balance increased energy expenditure (Cote et al., 2006; Creel, 2001), but is generally associated with decreased reproduction (Dewsbury, 1982; Creel, 2001).

Stressful situations often influence aggressive behaviour (reviewed in Summers et al., 2005) independently of testosterone (Denardo & Licht, 1993). This means that agonistic

encounters, and consequently dominance, may be influenced by corticosterone. In an experiment with side blotted lizards (*Uta stansburiana*), males implanted with corticosterone had reduced home ranges whereas control males increased their home ranges (DeNardo & Sinervo, 1994a). Aggressive encounters are not the only area of behaviour that are typically considered to be controlled by testosterone but are influenced by corticosterone. For example, cichlid fish that had recently lost fights show less courtship behaviour when presented with a female (Amorim & Almada, 2005) than when they had not fought, regardless of their testosterone level. Alternative reproductive strategies of marine iguanas (*Amblyrhynchus cristatus*) can be predicted by corticosterone levels rather by testosterone levels (Berger et al., 2005). Because agonistic behaviour affects territory size and shape, corticosterone is vitally important to consider in territorial behaviour.

One hypothesis on constraints to signalling is that females are actually selecting males which can cope with stressful situations because this indicates a high quality male (Bortolotti et al., 2009a). The detrimental effects of long-term stress are far-reaching, leading to decreased reproductive output (Wingfield, 2003), body condition and immune response (Morici et al., 1997) and increased parasite load (e.g., Amo et al., 2006) and, eventually, damage to internal organs (Nelson, 2000). An animal that can withstand all of these and still produce an ornament in order to increase fitness must be of good quality (Bortolotti et al., 2009a).

Interactions between testosterone and corticosterone

Changes in corticosterone tend to cause an inverse response in testosterone levels (reviewed in DeNardo & Sinervo, 1994b; but see Roberts et al., 2006). The mechanism for this is in Leydig cells which produce testosterone. These cells have receptors for glucocorticoids and if stress levels become too high, enzymes blocking corticosterone from receptors cease to be effective and glucocorticoids curtail testosterone production (Nelson, 2000). The degree of suppression can vary according to individual genetic variation and may be an underlying cause for alternative reproductive strategies (Knapp & Moore, 1997) because territorial males may have different thresholds for

corticosterone, thereby limiting testosterone production. Territorial or dominant males also differ from subordinate males in terms of having a smaller degree of corticosterone increase following a fight, which could be beneficial if the animal has frequent agonistic encounters (Knapp & Moore, 1996). In side-blotched lizards (*Uta stansburiana*), testosterone supplementation increased territory size while lizards with corticosterone implants were unable to maintain territories. Lizards with both corticosterone and testosterone implants were no different to controls, suggesting that the hormonal effects directly counteract one another (DeNardo & Sinervo, 1994a). One possible explanation is that this is a result of an increase in glucocorticoids after energetically costly displaying behaviour which is mediated by testosterone (Emerson & Hess, 2001), but the relationship between these hormones is still relatively unexplored.

The stress-linked immunocompetence handicap-hypothesis (SL-ICHH) suggests that the apparent immunosuppressive effects of testosterone are actually due to co-varying corticosterone levels (Buchanan et al., 2003). This is supported by evidence that, in isolation and controlling for corticosterone levels, testosterone actually benefits the immune system, rather than suppressing it (Roberts et al., 2009). One study found that neither hormone had any effect on the immune system in isolation, but a combination of the two did (Roberts et al., 2006).

Carotenoids as a beneficial signal modulator

Factors maintaining signal honesty do not always have to be directly detrimental to the animal in order to have a cost to the signaller. In some cases, signal modulators are actually beneficial to the animal. For example, some signals are energetically expensive (reviewed in Hasson, 1997) while energy stores are not harmful they are limited and allocating them to signals means that antioxidants are shunted away from other parts of the animal's physiology, making limiting oxidative stress more difficult. One example of this is carotenoid-based signalling. Carotenoids are common red, orange or yellow pigments (Weedon, 1965; Goodwin, 1984) which play a role in modulating and enhancing the immune system (Alexander et al., 1985) usually through reducing oxidative stress by removing and blocking free radicals (Ames, 1983; Bendich & Olson,

1989; Krinsky, 1989; Prabhala et al., 1990; Saino et al., 1999; Hill, 1999; McGraw, 2005). While the pigments are relatively common, they cannot be synthesised by animals and so must be obtained from the diet (Hill et al., 2002), often from foods that are limited in the environment (Hill, 1999).

Carotenoid-based colouration is common in many taxa (e.g., Saino et al., 1999; Andersson et al., 2002; Saks et al., 2003; Pryke & Andersson, 2003) and provide much information about an individual's health. They can correlate with various indices of quality, ranging from sperm quality (Peters et al., 2004), nutritional state (McGraw, 2005), body size (Kodric-Brown & Brown, 1984), condition (von Schantz et al., 1999; Andersson et al., 2002), aggression (Kodric-Brown & Brown, 1984) and parasite load (Figuerola et al., 1999). The trade-off between the allocation of carotenoids to the immune system or to signals means that it is likely to be an honest signal (Gray, 1996), as an animal in poor condition would not be able to spare carotenoids to use in signals and would mobilise them from the skin to use in immune defences (Lozano, 1994; Tella et al., 2004). Immune constraints on colour have been demonstrated, mainly in bird species (e.g., Saino et al., 1999; Saino et al., 2000; McGraw & Ardia, 2003; McGraw & Ardia, 2005b), where carotenoid-based colouration is a reliable predictor of immune ability as well as past history of mounting an immune-response to infections (Lozano, 1994; Alonso-Alvarez et al., 2004). This leads to a 'beneficial but limited' relationship between general animal physiology and carotenoids (Lozano, 1994), which plays a part in the trade-off in animal signalling between the requirements of the immune system and development and maintenance of colour signals (von Schantz et al., 1999; Peters et al., 2004).

Study system

Platysaurus intermedius wilhelmi is a small (maximum snout-vent length 83 mm) cordylid lizard found on rocky outcrops in southern Africa. It breeds in spring and early summer (Branch, 1998). Males are territorial and highly aggressive towards one another. Male *Platysaurus* sp. signal to rivals using colour patches and if a contest escalates to a fight, damaging fights, incorporating chases and bites, ensue (Korner et al., 2000).

Platysaurus i. wilhelmi is sexually dimorphic, with males larger than females (Lailvaux et al., 2003b). Females and juveniles are drab with blueish-white venters, black and brown dorsal regions and straw-coloured tails. In contrast males are brightly coloured with blue and black venters, green flanks and blue-green legs and bright orange tails. This dichromatism is characteristic of the genus (Branch & Whiting, 1997). Other *Platysaurus* species are highly aggressive (Korner et al., 2000) and their brightly coloured badges have been found to correlate with various measures of male quality (e.g., Whiting et al., 2003; Whiting et al., 2006). Their dorsally compressed bodies allow them to use small crevices as refuges, particularly on smooth granite domes, which flake in flat sheets. While very little is currently known about their ecology and behaviour, the closely related *P. broadleyi* has been studied extensively and male colouration has been found here to be an important indicator of status (Whiting et al., 2003) and fighting ability (Whiting et al., 2006). The well-documented colour patches in other members of the genus which function as indicators of status as well as the highly aggressive nature of these male lizards suggest that signals are important in this system to minimize agonistic encounters. The red tail colouration suggests the presence of carotenoid-based colouration and the numerous *Ficus* (sp) trees in their habitat makes it likely that carotenoids are present in their diet.

Aims and predictions

The immunocompetence handicap hypothesis has been widely tested with mixed results (Hasselquist et al., 1999; reviewed in Buchanan et al., 2003; Zuk, 1996; Westneat et al., 2003). One issue is that the immunosuppressive effects of testosterone have not been extensively tested (Deviche & Cortez, 2005). The effect on the immune system is partly regulated genetically, through the major histocompatibility (MHC) complex (Ahmed et al., 1987), but the demands on the immune system are dynamic, depending on a variety of factors, such as season, previous exposure, and parasite load. There is a trade-off between signal intensity and immunocompetence – signal intensity is a plastic response, mediated by the demands of the immune system. As testosterone is self-regulated, this is a fascinating example of a trade-off which could be influenced by environmental factors, such as food availability, social stress and previous exposure to pathogens. Although

many studies have tested the immunocompetence handicap hypothesis, they have not taken into account other factors that could affect either colouration or immunity, either in conjunction with testosterone or as a result of increased levels of testosterone.

The aim of my study was to assess the immunocompetence-handicap hypothesis in conjunction with other hypotheses regarding signal constraints (the stress-linked immunocompetence-handicap hypothesis; the hypothesis that corticosterone acts as a constraint on signal production independently of testosterone, and the oxidative stress hypothesis, that the supply of carotenoids constrains signal expression due to the demands of the immune system) in *Platysaurus i. wilhelmi*. The integrative approach of studying factors that could prevent the relationship between testosterone and immunocompetence from being a simple case of increased testosterone increasing trait expression and decreasing immunocompetence allows us to investigate a whole-animal model of how testosterone influences signals and behaviour.

My study involved field and laboratory studies, which covered four objectives: 1. to examine seasonal fluctuations in testosterone, corticosterone and carotenoids and relate these to signal intensity, immunocompetence, locomotor performance and body condition (chapter 1). 2. to test the ICHH by determining the relationship between testosterone and signal intensity, immunocompetence, body condition and locomotor performance (chapter 2). This study was carried out concurrently in the field and in captivity with one group of lizards kept under controlled conditions while another group was released with testosterone implants and recaptured after 14 days. 3. To investigate the effects of elevated testosterone in females to understand the interplay between organisational and activational effects in males (chapter 3). 4. To test the stress-linked immunocompetence handicap hypothesis by investigating the effects of testosterone, corticosterone and a combination of testosterone and corticosterone on signals, immunocompetence, endurance and body condition. 4. to test the oxidative-stress hypothesis by investigating the relationship between carotenoids, colour and immunocompetence, and to investigate the effects of changing carotenoid levels on performance and body condition.

Thesis structure

This thesis consists of an introduction, five experimental chapters and an overall discussion. The experimental chapters are written for publication and to minimise repetition in this format, the abstracts have not been included in each experimental chapter. As my approach involved using consistent methodology to test different hypotheses there is a great deal of overlap in methodology and so only new aspects are included in detail in later chapters. As these chapters are intended for publication there will be some overlap in the introductions of each chapter and the main introduction. Figure numbering is sequential and separate for each chapter. A complete reference is included after the discussion and conclusion.

CHAPTER 1

Seasonal variation in testosterone, corticosterone and carotenoids in flat lizard plasma and associated changes in condition-dependent signals

Introduction

Many different animal species rely on signals to avoid the costs of agonistic encounters with rivals (Parker, 1974). Ornaments used in contests are called armaments (Berglund et al., 1996). Typical armaments are badges of status (Shorey, 1976), which are bright colour patches used in behavioural displays (LeBas & Marshall, 2000) that can indicate various aspects of quality, such as aggression, status and/or fighting ability (reviewed in Whiting et al., 2003). In order for these signals to be maintained in a system, they need to carry some cost as without cost dishonest signalling would be possible and therefore the signal system would become unreliable (Zahavi, 1975). These costs can be directly detrimental effects of modulating factors such as immunosuppression due to high levels of circulating testosterone (Folstad & Karter, 1992).

Traditional understanding of endocrine systems assumed that hormones affect behaviour or performance in a linear way (Arnold, 1983). More recent research has found that factors constraining signalling are complex, often interrelated, webs of multiple feedback loops which include endocrine effects (Husak et al., 2009; Ketterson et al., 2009) but also changes in sensitivity to hormones, morphology and energy uptake. These factors are not often considered as measures of performance but they do play a vital physiological role and highlight the importance of investigating multiple effects of individual factors (Husak & Irschick, 2009; Husak et al., 2009). In order to tease apart these relationships, we need to begin by examining two different relationships. First, we must investigate natural fluctuations in both signal-modulating fluctuations and in signals. Second, we need to determine which signals are actually indicative of male quality in the study system.

Testosterone levels influence signal production by promoting the development and maintenance of secondary sexual characters that have been selected to signal male quality (e.g. Saino & Møller, 1994; Zuk, 1996; Wade, 1997; Lemos-Espinal et al., 1996; Deviche & Cortez, 2005), particularly in sexually dimorphic species (Saino & Møller, 1994; Buchanan et al., 2001; e.g. Deviche & Cortez, 2005; Saraiva et al., 2010). These traits are often correlated with other measures of quality such as body condition (Galeotti et al., 1997) or activity levels (Olsson et al., 2000), and possibly reduced parasite load (Hamilton & Zuk, 1982; Saino & Møller, 1994; but see Seutin, 1994). Males with increased testosterone often have high mating success (Olsson et al., 2000), and dominance status (Greenberg & Crews, 1990), as well as improved locomotor performance (Zuk, 1996). Locomotor performance also has important implications in dominance status (Robson & Miles, 2000; Bales et al., 2006). Testosterone affects behaviour (Saino & Møller, 1995) in males and females (Lovern et al., 2001), increasing activity levels (Lovern et al., 2001), aggression (Nelson, 2000; Whittingham & Schwabl, 2002; Dugatkin, 2004; but see McDonald et al., 1995; McDonald et al., 2001) and male-specific behaviours (Wingfield et al., 1990; Lovern et al., 2001), such as display behaviour and mate-guarding (Saino & Møller, 1995). In many species, there are seasonal fluxes in testosterone, increasing shortly before, or during the breeding season, which coincides with changes in behaviour and/or social status. For example, in green anoles (*Anolis carolinensis*) testosterone increases significantly in spring and stays elevated during the breeding season (Jenssen et al., 2001) which coincides with an increase in dewlap area (Irschick et al., 2006).

The main hypothesis concerning constraints to testosterone-based signalling is the immunocompetence-handicap hypothesis (ICHH) which proposes that testosterone levels are constrained by the associated immunosuppressive effects of the hormone (Folstad & Karter, 1992). There is mixed support for this hypothesis (Roberts et al., 2004) and more recently there has been a call for a more integrative approach, considering other factors and feedback loops involved in testosterone-based signalling (e.g., Husak et al., 2009). Many traits affected by testosterone (for example receptor density, aggression, growth and development) may not be directly linked to performance although they have

important implications for physiology and condition. Other effects are neutral, part of suites of effects termed ‘hormonal pleiotropy’ (Husak & Irschick, 2009; Husak et al., 2009; Ketterson et al., 2009).

Testosterone is not the only hormone involved in signal expression and immunocompetence. Social interactions are stressful and result in the release of stress hormones (reviewed in Summers et al., 2005; Creel, 2001). Glucocorticoids (such as cortisol or corticosterone) are released within minutes of a stressful event (Wingfield, 2003) and they help the animal cope mainly by mobilizing energy (Möstl & Palme, 2002). Chronic elevation in stress hormones can have long-term detrimental effects on an individual’s health (Nelson, 2000; Wingfield, 2003; Millspaugh & Washburn, 2004). The extent of the stress response can be shaped by several factors, such as social status, social history (particularly history of contests) and environmental factors, such as season and food availability (Summers et al., 2005). Responses are modulated by corticotrophin-releasing factor which not only stimulates the production of glucocorticoids through the HPA axis, but also modifies neurotransmitters which changes the animal’s response to increased levels of corticosterone (Lowry & Moore, 2006). Generally, an increase in corticotrophin-releasing hormone, which results in an increase in glucocorticoids, leads to an increase in activity in a wide variety of taxa (reviewed in Lowry & Moore, 2006). Increased corticosterone levels can also result in increased stamina and reduced resting metabolic rate (Cote et al., 2006; Miles et al., 2007), although this must be coupled with increased food consumption to counter increased energy expenditure (Cote et al., 2006; Creel, 2001) and is generally associated with decreased reproduction (Dewsbury, 1982; Creel, 2001). Moderate increases in stress hormones can be associated with increased reproduction because they mobilise energy (Moore & Jessop, 2003). In marine iguanas (*Amblyrhynchus cristatus*), males adopting different reproductive strategies had similar levels of testosterone, but differed significantly in glucocorticoid levels, and territorial males had more elaborate signals and higher haematocrit (which indicated performance), but a reduced cell-mediated immune response and poorer body condition than non-territorial males (Berger et al., 2005).

In most species, testosterone and corticosterone concentrations follow a circadian rhythm (Romero & Remage-Healey, 2000), as well as changing seasonally (Möstl & Palme, 2002). There are seasonal patterns in glucocorticoid levels (Romero et al., 2006), possibly due to the stress of the cold and all the associated catabolic processes (Huber et al., 2003), as well as differences in social stress and energetic requirements inside and outside of the breeding season (Moore & Jessop, 2003). There seems to be a trade-off between immunocompetence and energetically costly behaviors, which changes with environmental conditions (such as cold weather), but this does not seem to be due to limitations on nutrients as the actual mounting of an immune response is not very energetically costly (Svensson et al., 1998). Stress is associated with a decrease in immune response (Svensson et al., 1998), which could be a way of preventing autoimmune diseases which may occur as a result of injury during a stressful event, leading to an increase in immune response (Raberg et al., 1998).

Carotenoids are generally considered to be beneficial but limited in that they are required in physiological processes and using them in signals requires a trade-off with physiology, mainly to the immune system (Lozano, 1994). Carotenoids are fat-soluble hydrocarbons that cannot be synthesised by animals and must be obtained from the diet (Goodwin, 1984; Hill et al., 2002). Carotenoids protect against cancer and tissue damage by removing and blocking free radicals (Ames, 1983; Bendich & Olson, 1989; Krinsky, 1989; Prabhala et al., 1990; Saino et al., 1999; Hill, 1999; McGraw, 2005) which is crucial to the health of an individual (McGraw, 2005). They are usually limited in the environment (Hill, 1999) and sometimes require foraging for unusual food items (Negro et al., 2002).

Carotenoid-based pigments are allocated to colour signals in a variety of taxa (e.g., Saino et al., 1999; Andersson et al., 2002; Saks et al., 2003; Pryke & Andersson, 2003; Dijkstra et al., 2007). These signals have been shown to provide key information about sperm quality (Peters et al., 2004), nutritional state (McGraw, 2005), body size (Kodric-Brown & Brown, 1984), condition (von Schantz et al., 1999; Andersson et al., 2002), aggression (Kodric-Brown & Brown, 1984) and parasite load (Figueroa et al., 1999). Carotenoid-

based colouration is likely to be an honest signal (Gray, 1996). If the animal is not in optimal condition, carotenoids that could have been used to produce colour pigmentation are mobilised from the skin and used in the immune system instead (Lozano, 1994; Tella et al., 2004), and so colour expression can be constrained by immunological processes (Hill, 1999). This has been shown in a variety of birds (e.g., Saino et al., 1999; Saino et al., 2000; McGraw & Ardia, 2003; McGraw & Ardia, 2005b). Colour can indicate both the ability of an individual to withstand infection and how well they have coped with past infections (Lozano, 1994; Alonso-Alvarez et al., 2004). This leads to a trade-off between using carotenoids in signals and in reducing oxidative stress, caused by build-up of free radicals (von Schantz et al., 1999; Peters et al., 2004).

Lizards are good model organisms for studies in behavioural endocrinology. They display carotenoid-based ornamentation which is unusual in mammals which are often the models used in carotenoid studies (Svensson & Wong, 2011). They are generally easier to observe and more abundant than more commonly used bird and mammal models and their ornaments and armaments are easy to quantify. Bird colours are particularly complex as there is an interplay between dynamic colouration in beak colour (e.g., McGraw et al., 2006c), and colour laid down in feathers which occurs over a short time period and then remains fixed (e.g., Andersson et al., 2002). Lizards are also relatively easy to house without disrupting a social system such as removing birds from their roles in parental care. Plasticity in colouration is relatively common in lizards (Husak et al., 2007), changing with age, social status and various other social factors (Lemos-Espinal et al., 1996). Some species only display colour signals during the breeding season (Cooper & Greenburg, 1992), which is known as nuptial colouration (Rand, 1990; Martín & López, 2001), while others have colouration which shows a lesser degree of plasticity in that the intensity of signals increases during the breeding season but does not vanish entirely during the rest of the year (Olsson, 1994).

Flat lizards (*Platysaurus* sp.) are brightly coloured and highly aggressive when defending territories and also in access to females (e.g., Whiting et al., 2006). The common flat lizard (*Platysaurus intermedius wilhelmi*) is an ideal system for addressing constraints to

signalling. They are strongly sexually dimorphic with brightly coloured males and drab females, as is characteristic of this genus (Branch & Whiting, 1997). The closely related *P. broadleyi* defends territories, adopts alternate reproductive tactics, and uses colour to signal fighting ability (Whiting et al., 2003; Whiting et al., 2006).

The first aim of this study was to test for seasonal fluctuations in testosterone, corticosterone and carotenoids (in this case β -carotene) in three seasons: winter, the early breeding season (in spring, before the rains) and summer. I attempted to relate these fluctuations with signal intensity, immunocompetence, locomotor performance and body condition. The second aim was to investigate correlations between colour and performance as well as gross morphology. Costly-signalling theory maintains that the production and maintenance of signals are too costly for low-quality males to express maximal trait expression (Zahavi, 1975; Grafen, 1990), and so by exploring the relationship between potential signals and condition-dependent measures of male quality (immune response, endurance and condition), we can begin to understand signals as condition-dependent predictors of male quality. Defining male quality is difficult and so measures of whole-animal performance (defined as the ability of an animal to perform a task) were used as a proxy of fighting ability (Husak et al., 2007).

I predicted that in summer, at the height of the breeding season, there would be an increase in testosterone, corticosterone and β -carotene. I predicted that there would be an associated increase in conspicuousness of colour patches and a decrease in immune function, as well as an increase in body condition due to the increased abundance of insects during the rainy months. I predicted that in winter, outside of the breeding season, there would be a decrease in testosterone, corticosterone and β -carotene. I also predicted that, in winter, there would be an increase in immune function, a decrease in signal conspicuousness and a decrease in body condition.

Methods

Study system

Platysaurus intermedius wilhelmi is a small (maximum snout-vent length 83 mm) cordylid lizard found on rocky outcrops in southern Africa. They breed in spring and early summer (Branch, 1998). These lizards are sexually dimorphic: males are colourful and significantly larger than females (Lailvaux et al., 2003b), which are drab (Branch, 1998). Males have green flanks and front legs, greenish-blue back legs and blue-black abdomens and orange tails. They have black throats with the black colouration, extending down the chest and occasionally onto the abdomen. Juvenile males resemble females, which are brown with straw coloured tails. Their dorsally compressed bodies allow them to use narrow crevices as refuges, particularly on smooth granite domes, which exfoliate to produce sheet rock.

I conducted field work primarily at Pullen Farm (S 25°34,358' ; E 031°10.879') and some neighbouring areas, all in the Crocodile River Nature Conservancy, a 30 000 ha area situated approximately 30 km east of Nelspruit, South Africa. The area is lowland mesic savannah, characterised by *Dichrostachys*, *Acacia* and *Rhus* trees, and dense grasses broken up by large, exposed granite bedrock. The weather is generally warm (average temperatures between 13° – 27° C) with the rainy season from September to March. I took all measurements at a field station within a few kilometres of the capture sites.

Handling and marking of lizards

Each season (winter, spring and summer) in 2008, I captured 45 lizards on lines of Catchmaster® 48R non-toxic glue traps (27 x 13 cm) and Trapper® glue boards (26 x 12 cm). Trapping involved observing a lizard until it entered a refuge and then surrounding the refuge with traps. I monitored traps continuously and removed lizards as soon as they became stuck, removing excess glue with cooking oil. This is a standard protocol for capturing lizards (Ellinger et al., 2001; Whiting & Alexander, 2001). I recorded each capture location using a GPS. I took blood samples in the field immediately upon capture,

all other measures were taken over the course of three days at the field station. Upon completion of measurements lizards were marked and released at the point of capture. I marked lizards permanently by writing a small number on the dorsum, just above the base of the tail with a battery-operated mini-cauteriser (Kyron Code Y2565). This technique has been used successfully with snakes where marks remained visible for at least two years (Winne et al., 2006).

Blood samples

Immediately upon capture, I took blood samples from the suborbital sinus, using a heparinised microhematocrit capillary tube to draw approximately 150 μ L of blood. This is a standard procedure for small lizards (e.g. Woodley & Moore, 1998; Cox et al., 2005a; Husak et al., 2007) which has been used successfully in previous studies on flat lizards (e.g. Whiting et al., 2003). In order to avoid confounding samples for corticosterone with handling stress, I measured the time from capture until the end of taking the sample. After three minutes I stopped sampling in all cases. To avoid circadian fluctuations in hormone levels from confounding my results, I took all blood samples within the first four hours of activity (ranging between 08h00 and 12h00 in winter and 05h00 to 09h00 in summer, depending on prevailing weather patterns) and recorded the time that the sample was taken. I placed samples on ice until they could be centrifuged at 3000 rpm for four minutes before separating and freezing the plasma (within hours of collection) at the field station where it was stored until it could be assayed. Due to the small volume of blood in samples I was unable to measure testosterone, corticosterone and carotenoids for each lizard. Instead, I used the plasma from 15 lizards in each assay as a subsample of the group of 45. Each sample was assayed individually.

Hormone assays

I assayed testosterone by using DPC Coat-A-Count Total Testosterone kits following a pilot assay with experimental dilutions of different samples in order to find the optimal dilution ratio. These kits have been validated with plasma from closely related lizards (Whiting et al., 2006).

Corticosterone was analysed using an ELISA kit (Biocom Biotech), with dilutions optimised using previously established protocols according to Wada et al (2007). Samples were diluted at 1:60 using a steroid replacement buffer and an external standard was used to calculate the intra- and interplate coefficient of variation (intra-plate coefficient of variation = 8.76% and inter-plate coefficient of variation = 11.15%).

Determining existing carotenoids in *P. i. wilhelmi* plasma

To determine which carotenoids were present, I followed the methods outlined by Lichtenthaler (1987). I pooled five plasma samples to reach a volume of 400 µl which I mixed with 500 µl of acetone and centrifuged. I removed the supernatant and washed the pellet with another 200 µl of acetone before combining the supernatants and dried them under nitrogen until the volume was reduced to 400 µl. I added 500 µl of deionised water and 500 µl diethyl ether and then removed the yellow layer on top of the solution. This was dried again under nitrogen to reduce to 500 µl.

I used a solvent system of petroleum hydrocarbon, dioxane and 2-propanol (70:30:10) and used both paper and precoated silica aluminium plates in thin-layer chromatography (Lichtenthaler, 1987). This showed that the extract matched β-carotene (reference sample from Kyron). I then took the paper that I had used for TLC and cut out the section where the extract had settled. I soaked it in diethyl ether and then ran it through a dual-beam spectrophotometer where it showed maximal absorbance to be 450 nm which is within the range of β-carotene which peaks between 420 - 500 nm (Lichtenthaler, 1987).

Measuring plasma concentrations of β-carotene

Following an established protocol for working with plasma carotenoids (e.g., Alonso-Alvarez et al., 2010), I performed a cold-extraction in a darkened room by mixing plasma with acetone stored in a -20° C freezer at a ratio of 1:10. This was centrifuged for 10 minutes at 11 000 rpm and then the supernatant was removed and used in analyses. I measured samples in duplicate in a SpectraMAX 190 ELISA plate reader (Molecular devices) running SoftMAX Pro 5.4.1 software. The small volume of ELISA plate wells allowed for duplicate sampling as well as reading all samples at the same time for added

precision. As the high acetone levels could damage the plastic of an ELISA plate, I mixed 20 μ l of distilled water with 150 μ l of the extract before it went into the plate.

These readings were compared to a standard curve made with a β -carotene reference standard (Sigma-aldrich) at ten different concentrations ranging from 0.1 mg/ml to 0.02 mg/ml. The standard curve had a $R^2 = 0.9$ and I used the equation of the curve to determine the concentrations of plasma samples.

Morphological measurements and spectroradiometry

I weighed lizards on an electronic balance to 1 decimal place. I measured their snout-to-vent length (SVL) as well as tail length with a plastic ruler to the nearest 1 mm while taking note of the condition of the tail (complete, broken, or regenerated). As a measure of general health, I calculated body condition by taking the residuals of $\ln$ SVL against mass (Fuxjager et al., 2010). I also measured head height (from the highest point of the head to below the lower jaw), head length (from the anterior edge of the tympanum to the tip of the snout), and head width (the distance between the widest points of the head) with digital calipers (to 0.01 mm). Head dimensions can be an indication of bite force and thus, fighting ability (e.g. Husak et al., 2006b).

I measured spectral reflectance of the throat, abdomen, flank, front and back legs and tail of each lizard, using a USB2000 optic spectrometer (Ocean Optics, Inc.), running on OOIBase32™ version 2.0.0.6 (Ocean Optics, 2002). Readings were taken using a fibre-optic cable linking the optic spectrometer to an AIS mini-DT light source (Analytical Instrument Systems inc.) which emits light over the entire visible spectrum (300-700 nm). The probe is mounted in a holder which ensures that the readings are taken from the same distance (5 mm) and angle (45°) each time. A hole in the probe holder enabled me to measure precise sites. And to avoid stray light from reaching the probe I covered the hole while taking readings. To maximise precision I used an average of two readings for each measurement in analyses and before measuring each lizard I took calibration readings of a black standard and a 99% diffuse white standard.

Immunocompetence

To measure immune response, I used a delayed-type hypersensitivity test using phytohaemagglutinin (PHA) (Smits et al., 1999). For each lizard, I injected one hind foot with 0.03 ml phosphate buffered saline (PBS) containing 50 mg of PHA (Sigma, L-8754) with a Hamilton syringe (1710 N, 22S ga/51 mm/pst2). The other hind foot received an injection of 0.03 ml PBS as a control. I alternated which side was the control every five lizards. I measured the thickness of each foot prior to injections and 24 hours later with a pressure-sensitive spessimeter (Teclock SM-112) to 0.01 mm. I measured each foot twice, before and after PHA injection and used the mean thickness as the measure of swelling. PHA injected tissues show a large infiltration of immune cell types, such as basophils, eosinophils, heterophils, lymphocytes, macrophages and thrombocytes (Martin Li et al., 2006), and a higher degree of swelling indicates a stronger immune response. This technique has been particularly successful with lizards (e.g. Saks et al., 2003; López & Martín, 2005; Belliure et al., 2004), and provides a way of challenging the immune system (Norris & Evans, 2000) which gives a more accurate result than looking at measures of immune defence without manipulation, as this would be affected by prior exposure or illness (Adamo, 2004). PHA cell-mediated response has a significant effect on survival and can be used as a proxy for large-scale immunity (Møller & Saino, 2004); it is also one of the more feasible approaches for field-based studies with small animals (reviewed in Demas et al., 2011).

Endurance

I measured endurance as the length of time a lizard walked on a belt (30 x 40 cm) (e.g. Sinervo et al., 2000; Garland et al., 1990) moving at 0.4 km.h^{-1} before it failed to maintain its position more than three times (Miles et al., 2007). I measured the endurance of each lizard twice, with trials 24 hours apart. Immediately prior to trials I raised the body temperature of the lizards by floating them in plastic bags over warm water of 35°C . Immediately before the test, I measured the lizard's body temperature with a cloacal thermometer. I took the longest time walking on the treadmill as the measure of maximal endurance (Irschick & Garland, 2001) which is a biologically relevant measure of male quality (Arnold, 1983; Irschick & Garland, 2001).

Visual modelling

I used microspectrophotometry data from a closely related lizard (*Platysaurus broadleyi*) (Fleishman et al., 2011) to model the visual system of flat lizards because visual systems are generally conserved (Osorio & Vorobyev, 2005), particularly in diurnal lizards (Loew et al., 2002; Fleishman et al., 2011). I created templates of the four main cone types using the methodology in Govardovskii et al (2000), including a correction for associated oil-droplets (Hart & Vorobyev, 2005). The four templates are for two short-wave cones (UVS and SWS), one medium wave (MWS) and one long wave (LWS) cone (figure 1.1).

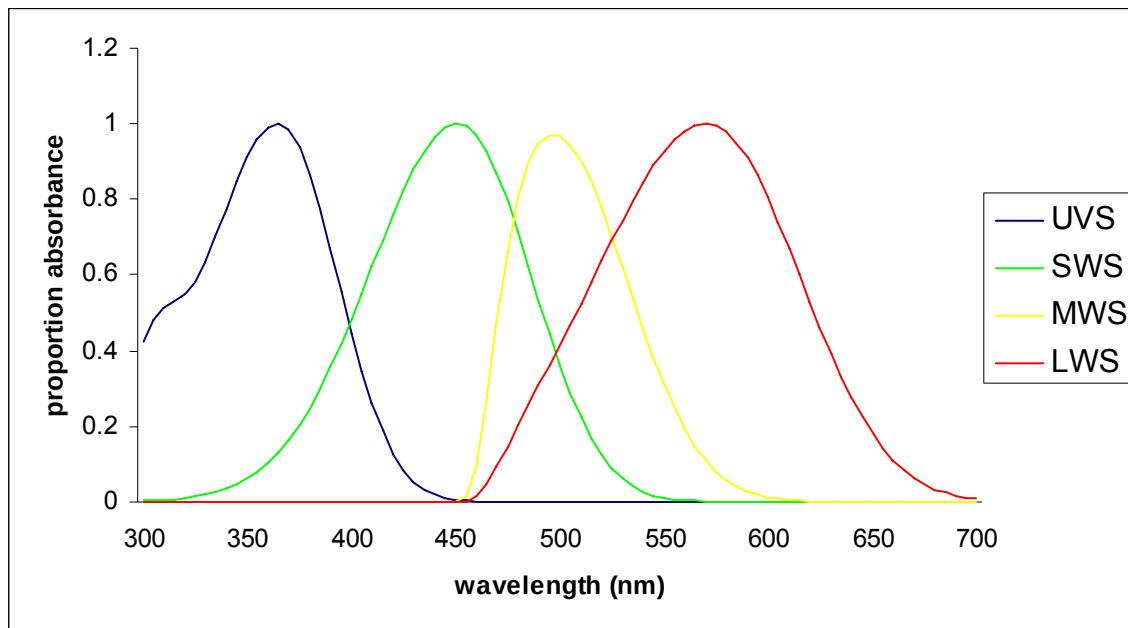


Figure 1.1. A template for visual pigment peak sensitivity in *Platyraurus broadleyi*

Each reflectance reading was then multiplied by the ambient irradiance. To measure irradiance I used an average of three readings taken on a rocky outcrop during usual lizard activity hours where lizards were found close to the ground and parallel to the rocks in order to measure the light available to a lizard (and therefore to model the light reaching the lizards' eyes). I then used the pigment templates and irradiance to calculate the position of each colour patch in colour-space using the protocol outlined by Endler & Mielke (2005). Once these readings were processed I calculated their chromatic contrast from the surrounding rocks using an average of three rock measurements.

Chromatic contrast is calculated as the Euclidean distance (Δ_T) between two colours in tetrahedral colour-space and can be calculated as follows:

$$\Delta_T = \sqrt{(SWS1_{lizard} - SWS1_{rock})^2 + (SWS2_{lizard} - SWS2_{rock})^2 + (RH2_{lizard} - R2_{rock})^2 + (LWS1_{lizard} - LWS1_{rock})^2}$$

To measure brightness I used the value of Q_T , the total amount of light reaching the eye (Endler & Mielke, 2005).

Statistical analyses

To compare concentrations of testosterone, corticosterone and β -carotene over different seasons, I used a MANOVA with the season as the categorical predictor. Although it would have been preferable to include these data in a larger model, constraints in the size of blood sample obtainable from a small lizard lead to these being subsamples of the complete group and so they are analysed separately. In the case of a significant result I used a Tukey-HSD post-hoc test to pinpoint pairwise differences. To compare endurance, immunocompetence, colour and body condition in different seasons, I used a Generalized Linear Model (GLZ) using a multinomial error structure and a Logit link function.

Season was the dependent variable and in each case I used the variable under investigation (i.e. hormone concentration, endurance, immunocompetence or colour measures) as the count variable. In analysing endurance I included body temperature as a covariate. I used β -estimate coefficients to assess differences between first order effects and descriptive statistics to determine the direction of change. Graphs were used to show patterns of change by comparing the medians and interquartile ranges for each season. I examined the role of colour patches as predictors of male quality by running a General Linear Model (GLM) with colour measures as covariates and the measure of male quality (endurance, immune response, body size and head dimensions) as the dependent variable. All statistics were performed in STATISTICA 6.0 (Statsoft).

Ethical note

This study was approved by the Animal Ethics and Screening Committee of the University of the Witwatersrand (AESC 2007/48/3), with a provincial permit from the Mpumalanga Parks Board (MPB5211).

Results

Seasonal variation in testosterone, corticosterone and β -carotene

Season was a significant predictor of testosterone levels ($F_{2,49} = 80.05$; $p < 0.001$), with levels in summer higher than winter and spring (Tukey-HSD post-hoc, figure 2). In contrast, season was not a significant predictor of corticosterone ($F_{2,46} = 1.05$; $p = 0.36$) or β -carotene ($F_{2,43} = 1.42$; $p = 0.251$; figures 1.2, 1.3 and 1.4).

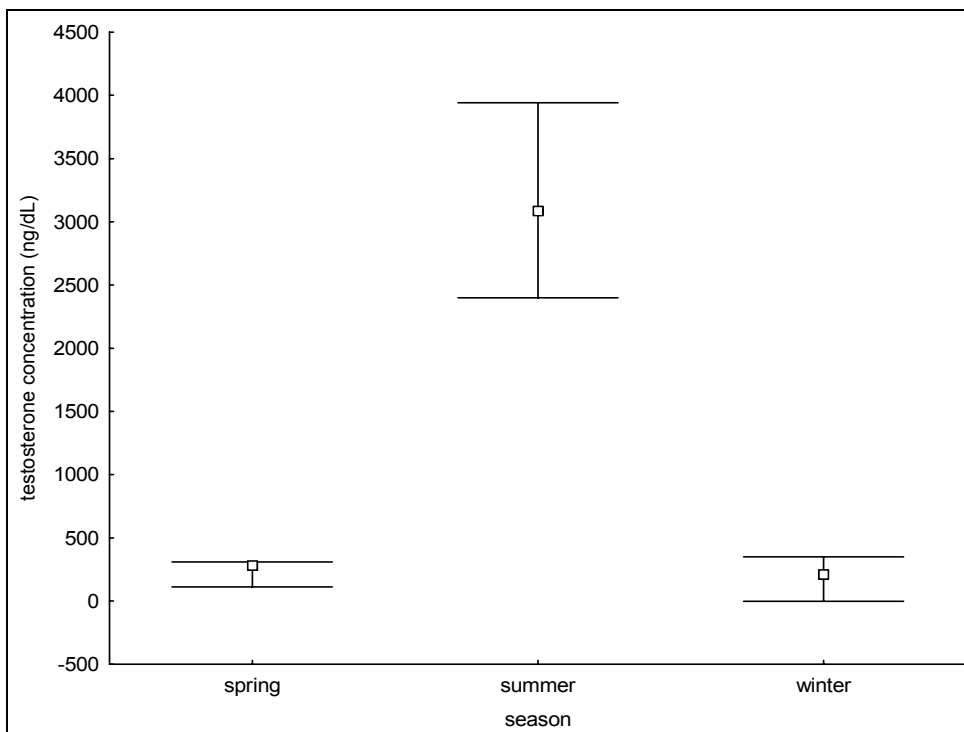


Figure 1.2. Testosterone was significantly higher in summer, measured in a subsample of 15 *P. i. wilhelmi* lizards per season. Values are shown as mean and range.

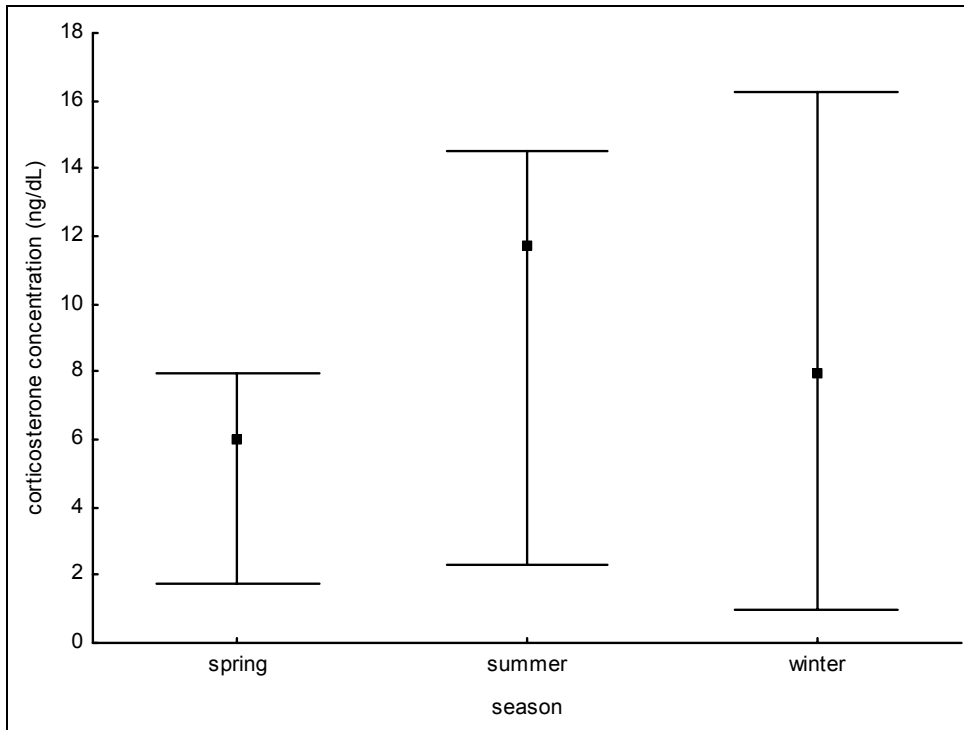


Figure 1.3. Season did not predict corticosterone levels across three seasons in a subsample of 15 *P. i. wilhelmi* lizards per season.

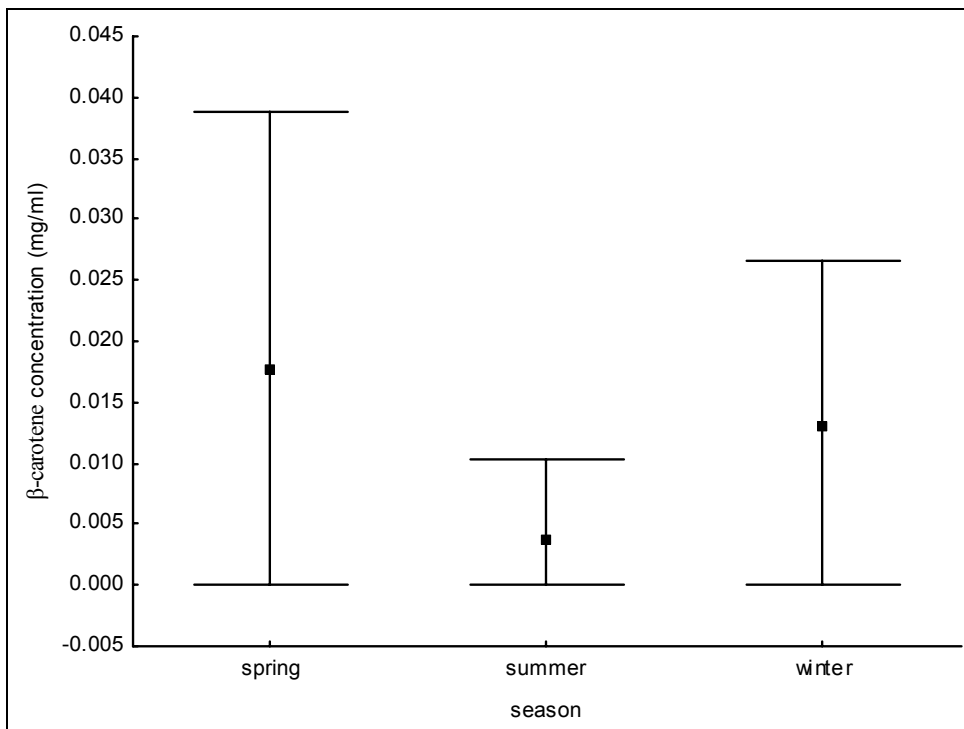


Figure 1.4. β -carotene levels were not predicted by season in three seasons in a subsample of 15 male *P. i. wilhelmi* lizards. Values are shown as means with whiskers indicating range.

Seasonal variation in endurance, body condition, immune response and spectral reflectance

Season was a significant predictor of endurance (Wald $\chi^2_2 = 2082.15$; $p < 0.001$) with *Platysaurus i. wilhelmi* lizards showing greatest endurance in winter, next greatest in summer ($\beta = 0.766$) and the shortest endurance in spring ($\beta = 0.240$ figure 1.5).

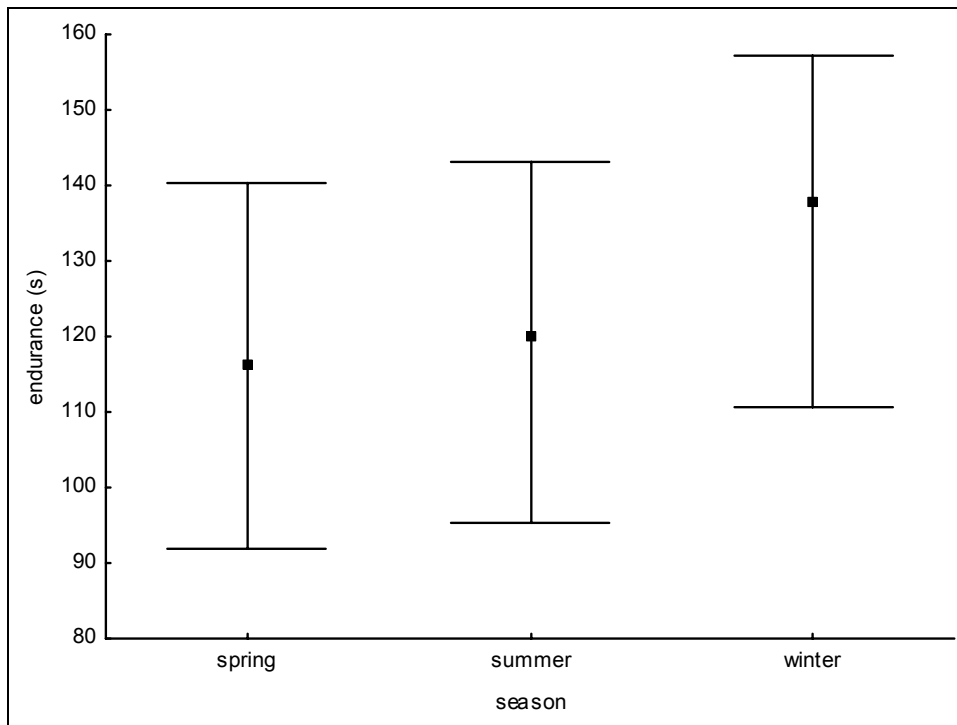


Figure 1.5. *Platysaurus i. wilhelmi* showed the greatest endurance in winter followed by lizards caught in summer. I measured 46 lizards in spring, 47 in summer and 52 in winter. Values are given as means with bars indicating range.

Body condition was not predicted by season (Wald $\chi^2_2 = 2.88$; $p = 0.24$) over the course of the year (see figure 1.6).

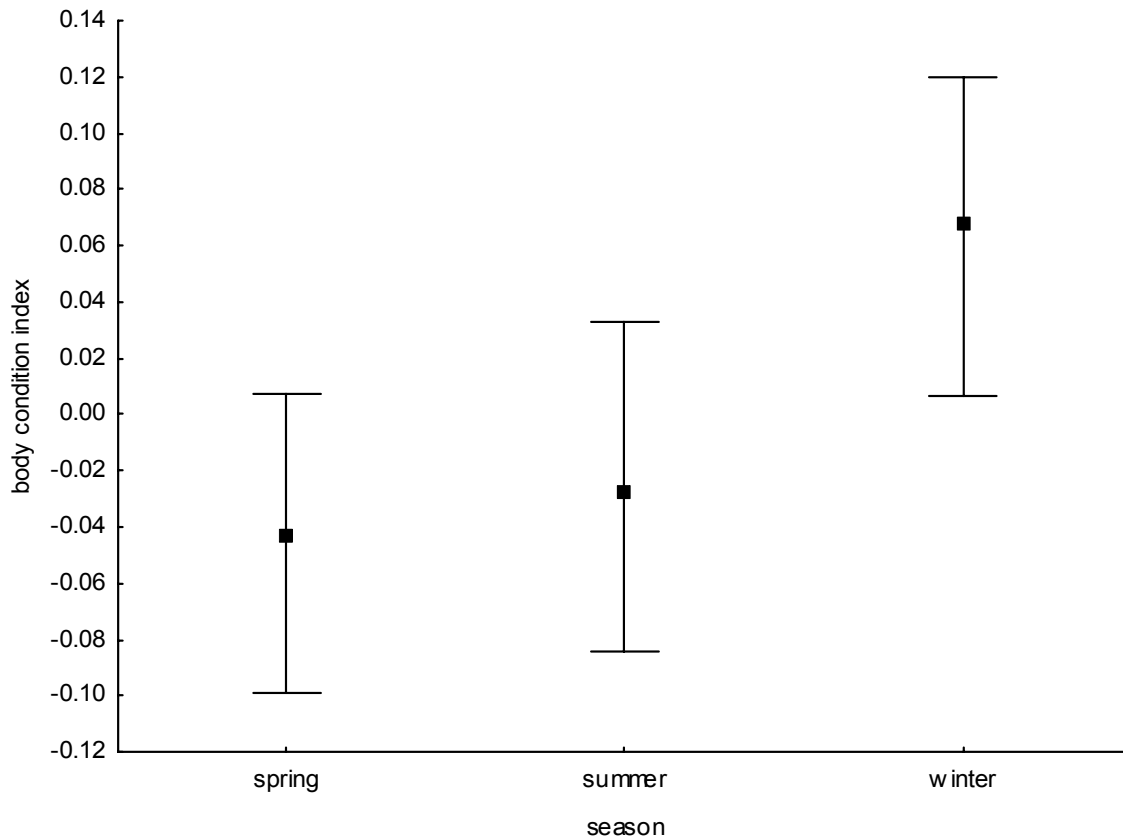


Figure 1.6. *Platysaurus i. wilhelmi* body condition did not change in three seasons, based on a sample of 46 lizards in spring, 47 in summer and 52 in winter. Values indicate means with bars showing range

Season was a significant predictor of immune response (Wald $\chi^2_2 = 146.37$; $p < 0.001$) with males showing a decrease in summer ($\beta = 0.550$) while there was no significant difference between winter and spring ($\beta = 0.002$, figure 1.7).

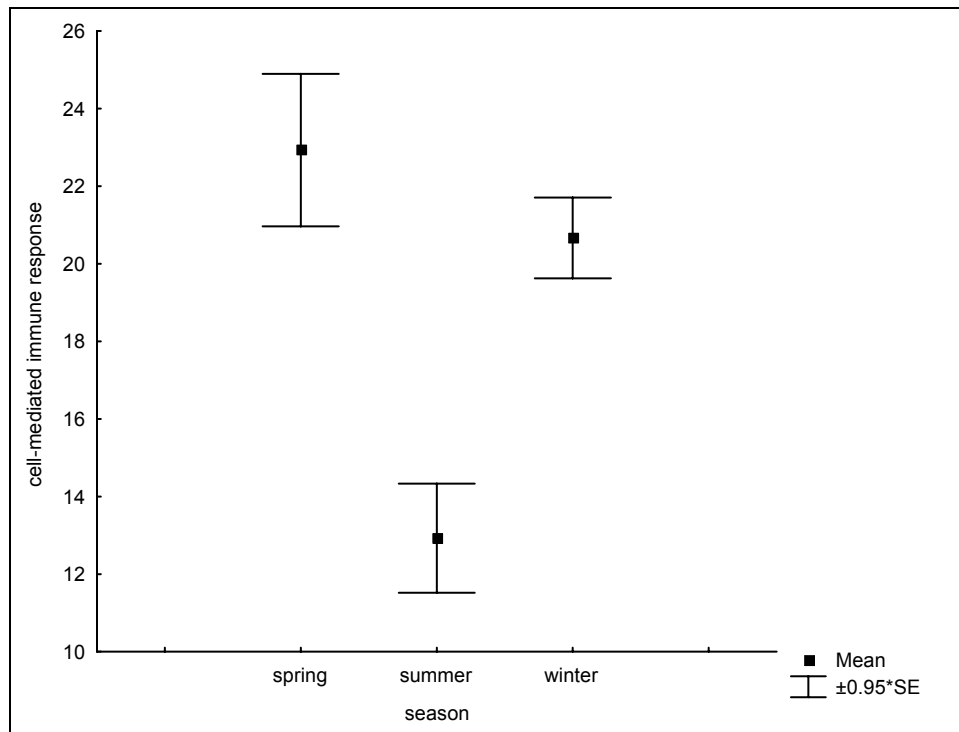


Figure 1.7. Immune response was lower in summer compared to winter and spring in *P. i. wilhelmi* (n = 46 lizards in spring, 47 in summer and 52 in winter). Values are shown as means with whiskers indicating standard error.

Season was not a predictor of chromatic contrast (figure 1.8 and table 1.1).

Table 1.1. GLZ analyses of the influence of season on chromatic contrast of colour patches

Body region	Wald χ^2	p
Flank	0.22	0.90
Front leg	0.20	0.90
Back leg	0.34	0.84
Abdomen	0.32	0.85
Tail	1.98	0.37

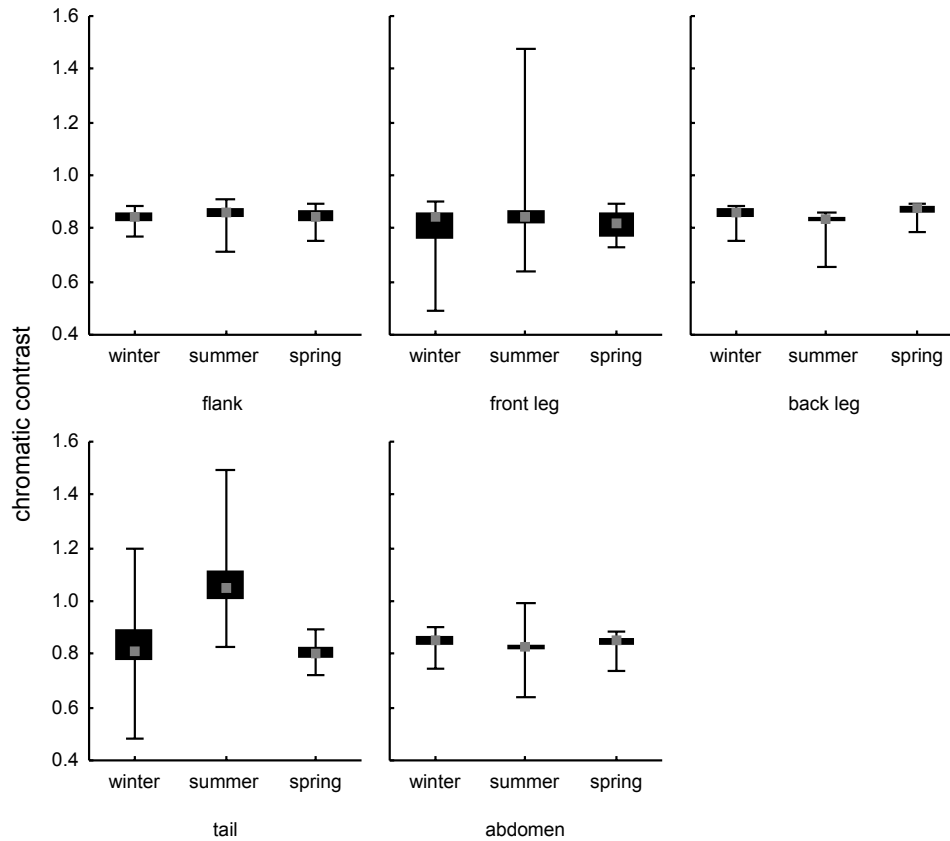


Figure 1.8. Chromatic contrast did not differ in any body region of *P. i. wilhelmi* between spring, summer and winter in *P. i. wilhelmi*. (n = 46 lizards in spring, 47 in summer and 52 in winter). Values indicate medians with boxes showing interquartile range and whiskers indicating total range.

Season was a significant predictor of brightness values in all body regions between different seasons (see table 1.2 and figure 1.9). All colour patches were brightest in winter (flank $\beta = 0.655$, front leg $\beta = 0.592$, back leg $\beta = 0.712$, abdomen $\beta = 0.515$, tail $\beta = 0.083$), and front leg ($\beta = 0.100$), abdomen ($\beta = 0.402$) and tail ($\beta = 0.202$) colour was the least bright during summer.

Table 1.2: all colour patches were brightest in winter, and several regions were the least bright during summer

Body region	Wald χ^2	p	Direction of change
Flank	17560.93	$p < 0.001$	Brighter in winter, no difference between spring and summer
Front leg	7067.87	$p < 0.001$	Brighter in winter and least bright in summer, spring intermediate
Back leg	19154.55	$p < 0.001$	Brighter in winter, no difference between spring and summer
Abdomen	10978.03	$p < 0.001$	Brightest in winter and least bright in summer, spring intermediate
Tail	937.29	$p < 0.001$	Brightest in winter and least bright in summer, spring intermediate.

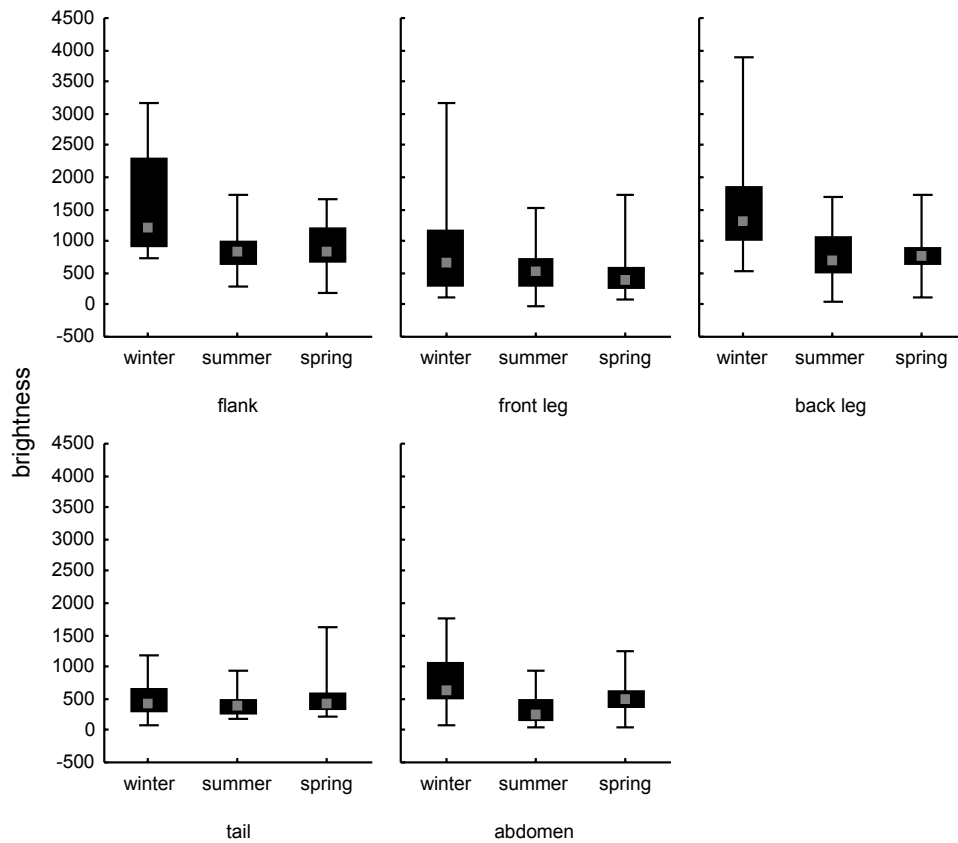


Figure 1.9. Season was a predictor of colour patch brightness in *P. i. wilhelmi*. (n = 46 lizards in spring, 47 in summer and 52 in winter) over three seasons. Values show medians with boxes showing interquartile range and whiskers showing total range.

Morphological predictors of male quality

Chromatic contrast of various body regions was a significant predictor for aspects of male quality (see table 1.3). The only aspect not predicted by any morphological characters was body condition, although weight (a component of body condition) was a predictor of head width.

Table 1.3. Chromatic contrast predicts aspects of male quality, and weight predicts head width

Indicator of quality	Predictors	$F_{1,147}$	p	Direction of influence
Endurance	SVL	4.81	0.030	positive
	Front leg chromatic contrast	8.48	0.004	negative
	Back leg chromatic contrast	9.81	0.002	positive
	Tail chromatic contrast	8.34	0.004	positive
Immune response	Tail chromatic contrast	7.11	0.009	negative
	Back leg chromatic contrast	10.26	0.002	positive
Size (snout-vent-length)	Flank brightness	4.61	0.033	positive
Head size (indicator of bite-force)	Head width predicted by weight	6.12	0.015	positive
	Head length:	11.36	0.001	positive
	Flank chromatic contrast	6.02	0.155	positive
	Front leg chromatic contrast	10.42	0.001	positive
	Back leg chromatic contrast			
Body condition	No predictors	-	-	

Discussion

The aim of my study was to test for seasonal fluctuations in testosterone, corticosterone and carotenoids and for associated effects on whole-animal performance and sexually selected traits, as well as to look for correlations between signals and indices of male quality.

Testosterone increased significantly during the height of the breeding season. This increase corresponded to a decrease in cell-mediated immune response, but there were no changes in β -carotene or corticosterone in the different seasons sampled. *Platysaurus i. wilhelmi* showed greater endurance outside of the breeding season and while there was no change in body condition, all colour patches showed changes in brightness between the different seasons. Season was not a predictor of body condition, but there was a large degree of variation in condition which may be due to incidental environmental stress. This population of *P. i. wilhelmi* would have experienced significant environmental stress in spring in 2008, when the rains began much later than usual which lead to the deaths of a large number of animals in the conservancy. As insect activity began later than usual it is possible that this affected the seasonal patterns in body condition. This has been observed in great tits (*Parus major*) where environmental stress, particularly related to low arthropod availability affects body condition (Carrascal et al., 1998).

Elevation of testosterone with the correlated depression of immune response provides some support for the immunocompetence-handicap hypothesis. This hypothesis has had mixed support in the past with only about 50% support in one meta-analysis (Roberts et al., 2004). Testosterone peaking during the breeding season indicates its role in mediating sexually selected signals and behaviour, particularly in controlling nuptial colouration (Olsson, 1994).

Little data has been collected on the types and levels of carotenoids found in reptiles. Most studies have been on birds, where costs could be temporary because carotenoids

incorporated into the feathers may only be allocated to signals for a short period of time (after a moult) (Navara & Hill, 2003). However, it is unknown whether or not reptiles use more carotenoids in their signals during the breeding season than in other months of the year. Seasonal variation in carotenoid-mediated sexual signals and immunocompetence has not yet been investigated (Faivre et al., 2003). Understanding the types of carotenoids used is also important as which carotenoids are used and which are excreted also differs between taxa (Hill, 1999). The presence of β -carotene in lizard plasma suggests that there is a possibility of their having a role to play in constraints on signalling in *Platysaurus i. wilhelmi*. While there was no seasonal change in circulating plasma β -carotene concentrations, carotenoid concentrations in plasma do not always accurately reflect levels in the tissues (Pérez-Rodríguez, 2009). Blood samples are the most viable option to obtain carotenoid samples in small lizards compared to biopsy-type sampling used in larger animals but care must be taken in interpreting plasma carotenoid levels in future studies. In order to understand the role of carotenoids in signals, manipulations could provide a clearer picture than correlations to potentially unreliable plasma concentrations. Corticosterone concentrations showed a non-significant trend towards an increase during the breeding season compared to outside of the breeding season. Although this is not be a significant seasonal fluctuation, it is still a possibility that this does have a role to play in signalling and with manipulations and larger samples we should be able to see if this is the case.

As locomotor performance is associated with dominance (Perry et al., 2004) and endurance is an important aspect of patrolling territories, I expected an increase in endurance associated with increasing testosterone in the summer months. This was not the case as endurance was greater in lizards caught in winter. The decrease in endurance in the breeding season is not entirely unprecedented. In *Lacerta monticola* dominant males had larger heads but more asymmetrical femur length than subordinates and so exhibited a trade-off between locomotor performance and dominance (López & Martín, 2002), which could be based on conflicting pressures of sexual and natural selection by allocating energy to reproduction and to predator avoidance. Sexual signals or displays often increase conspicuousness to predators (Zuk & Kolluru, 1998) thereby increasing

predation risk in the breeding season when lizards are patrolling territories and signalling to conspecifics. Consequently, male *Platysaurus i. wilhelmi* would compete for territories and females in the breeding season. In winter, their energetic demands would not be as high, which could lead to greater energy allocation to performance. In winter I rarely saw *Platysaurus i. wilhelmi* in their usual places on rocky outcrops and along crevices and found them frequently in the vegetation along the edges of outcrops and often relatively near to other male *P. i. wilhelmi* males (pers. obs.), so there is also a possibility that they need increased endurance to forage over a larger area when food is not as abundant as during the breeding season.

In most cases here, predictors of male quality are chromatic aspects of colouration. Bearing in mind that testosterone levels changed over the seasons, it is surprising that the brightness of colour patches seems to be more plastic than the chromatic aspects where brightness has been standardised. In all cases, colours were brighter in winter, so as these lizards live on light-brown coloured granite outcrops, they would be more conspicuous when their colours were darker during the breeding season.

For endurance, immunocompetence, body size and head dimensions, there was a positive correlation between a trait and the chromatic contrast of one or several body regions. This is logical since a high chromatic contrast between the lizard and the rock would make a signal more conspicuous to conspecifics and therefore they are appropriate signals of male quality. The only exception to this would be the relationship between the chromatic contrast of the tail and immune response: males with better immune response had less conspicuous tails. This supports the hypothesis of oxidative stress hypothesis, where there is a trade-off between the allocation of carotenoids used in immune response and used in signals (Lozano, 1994).

Platysaurus i. wilhelmi males are highly aggressive to conspecific males. The fact that head dimensions, which are an important aspect of bite force, are uncoupled from indicators of body size suggests that this trait is linked to other aspects of quality than size. This is also true for *Anolis carolinensis* where testosterone predicted dewlap size

and bite force – both sexually selected traits – but this effect disappeared with correction for body size (Husak et al., 2007). Another study on *A. carolinensis* found bite force to be a plastic trait (Irschick et al., 2006), which makes it likely to be condition-dependent.

This study provides support for the ICHH in that seasonal fluctuations of testosterone correspond to a decrease in cell-mediated immunocompetence. This can be investigated further by performing experimental manipulations. The relationship between tail colour and immunocompetence also suggests that carotenoids are involved in a trade-off between health and signalling. Fluctuations over the course of the year also show that there is some plasticity of signals and so this is a good system to begin an in-depth study on integrative aspects of constraints to signalling, particularly since colour measures predict aspects of quality in these lizards.

CHAPTER 2

Testing the immunocompetence handicap hypothesis in free-living and captive lizards

Introduction

Folstad and Karter (1992) proposed the immunocompetence-handicap hypothesis (ICHH), which suggested that testosterone-mediated traits are limited by their effect on the immune system where testosterone acts as a 'double-edged sword'. Only high quality males should be able to withstand the immune costs associated with high levels of circulating testosterone. Links between androgens and immunocompetence have been found mainly in birds (Folstad & Karter, 1992). Moreover, in sexually dimorphic species, females are sometimes less susceptible to parasites than males (e.g. Deviche & Cortez, 2005) or have a better immune response (Folstad & Karter, 1992), lending credence to the immunosuppressive effects of testosterone. Because immune and endocrine systems are linked, it is logical to assume that hormonally-linked traits, such as secondary sexual characteristics, should be influenced by the immune system (reviewed in Ahmed & Talal, 1990). This leads to a trade-off between risk of infection and the potential of increased reproductive success (Folstad & Karter, 1992; Zuk, 1996; Slater & Schreck, 1997), and males of lower status may have less elaborate ornaments and lower androgen levels (Folstad & Karter, 1992).

Androgens play a significant role in modulating behavioural plasticity and the development of secondary sexual characters (Saraiva et al., 2010). Elevated levels of testosterone promote the development and maintenance of secondary sexual characters such as colouration, that have been selected to signal male quality, particularly in sexually dimorphic species (e.g. Saino & Møller, 1994; Lemos-Espinal et al., 1996; Zuk, 1996; Wade, 1997; Deviche & Cortez, 2005). These secondary sexual characters can include armaments such as badges of status, which are signals used to assess rivals during contests (Lemos-Espinal et al., 1996; Whiting et al., 2003). These badges are typically used in behavioural displays (LeBas & Marshall, 2000) and can provide information on

aspects of male condition and quality such as health, immunocompetence (e.g., McGraw & Ardia, 2003), aggression, fighting ability (Olsson, 1994) and dominance status (e.g., Thompson & Moore, 1991). These signals are often correlated with other measures of quality such as body condition (Saino & Møller, 1994; Buchanan et al., 2001) or activity levels (Galeotti et al., 1997), and possibly reduced parasite load (Olsson et al., 2000). Testosterone increases activity levels (Lynn et al., 2000; Lovern et al., 2001), aggression (Lovern et al., 2001), and male-specific behaviours (Nelson, 2000; Whittingham & Schwabl, 2002; Dugatkin, 2004; but see McDonald et al., 1995; McDonald et al., 2001) such as display behaviour and mate-guarding (Wingfield et al., 1990; Lovern et al., 2001). Behaviours indicative of illness – such as reduced activity and reduced food consumption, may limit the viability of an infection by minimising a pathogen's access to nutrients and keeping the body at an undesirable temperature (Ashley et al., 2009). For example, male songbirds with increased testosterone suppressed behaviour associated with illness compared to controls after an experimental immune challenge which may be another trade-off to increased testosterone (Ashley et al., 2009). Increased testosterone is also linked to high mating success (Hamilton & Zuk, 1982; Saino & Møller, 1994; but see Seutin, 1994) and improved locomotor performance (Olsson et al., 2000) which has important implications for dominance status (Zuk, 1996) with dominant males having significantly higher androgen levels than subordinates (Robson & Miles, 2000; Bales et al., 2006). Locomotor performance is related to dominance in nature (Perry et al., 2004) and is also related to the ability of male lizards to establish larger and/or better territories in the field (Garland et al., 1990). This may be due to testosterone increasing muscle size (Husak & Irschick, 2009) and although there is strong evidence of testosterone improving 'burst' speed in sprinting, there is very little unequivocal evidence of the effects of testosterone on endurance (Husak & Irschick, 2009).

A meta-analysis (reviewed in Roberts et al., 2004) found that approximately half of twenty-two studies testing Folstad and Karter's (1992) ICHH model have found support for it, which means that in order to study any constraints on testosterone in sexually dimorphic characters we need to test the benefits of testosterone empirically first.

Testosterone clearly plays multiple, complex roles in behaviour and physiology which

may explain the equivocal results in testing the ICHH. Recently, there has been a move to investigate the roles of hormones in a more integrative manner (Husak et al., 2009) by investigating multiple aspects of health, fitness and performance concurrently. One approach, which is currently lacking, is to attempt to tease apart exogenous (such as changes in behaviour or dominance) and endogenous (purely physiological such as direct responses of the immune system or metabolism with testosterone) factors by manipulating the influence that the external environment has on an animal. For example, if maintaining high levels of testosterone is predominantly costly in terms of energetic expenditure (Buchanan et al., 2001), social costs such as increased agonistic encounters (Eisenegger et al., 2011) and environmental hazards such as increased predation risk (Deviche & Cortez, 2005) might change other physiological factors which we may wrongfully attribute to testosterone or exposure to parasites and pathogens (Cox & John-Alder, 2007). In this case, free-ranging males with high testosterone levels should show a different response to increased testosterone levels than males kept in a controlled captive environment. If increased activity leads to logically increased access to high-quality nutrients due to increased time foraging or distance travelled, we could expect free-ranging animals to show less of a response to elevated testosterone than captive animals, particularly in light of recent evidence showing that signals are often controlled by fluctuating demands of the immune system (Greiner et al., 2010). These demands of the immune system may be ameliorated by food containing high quality antioxidant pigments (Lozano, 1994).

Using *Platysaurus intermedius wilhelmi* as a model, my study had two aims. First, I aimed to test the immunocompetence-handicap hypothesis. I predicted that with experimentally elevated testosterone, males would show an improvement in endurance and body condition (due to the building of muscle mass) and that their colour patches would become more conspicuous against their habitat than males with control implants. The second aim was to investigate the extent to which exogenous factors may be influencing the effects of testosterone on physiology by comparing the effects of testosterone manipulation on captive and free-ranging lizards. By comparing the effects of testosterone on males in a controlled environment with males exposed to other factors,

I investigated whether other factors, such as nutrient availability or social factors, might be influencing signals and immunocompetence. As the traditional theory states that testosterone is limiting merely in terms of immunocompetence, I predicted that males which are free-ranging for the duration of the experiment would show no difference in their response to elevated testosterone than males in a captive setting during this experiment as the modulating factors would be entirely endogenous and not affected by environmental factors.

Methods

Study system

Platysaurus intermedius wilhelmi is a small (maximum snout-vent length 83 mm) cordylid lizard found on rocky outcrops in southern Africa. They breed in spring and early summer (Branch, 1998). These lizards are sexually dimorphic with colourful males (Lailvaux et al., 2003b) with green flanks and front legs, greenish blue back legs and blue-black abdomens and bright orange tails. Females are drab brown and black with straw-coloured tails (Branch, 1998). As with all flat lizards they have dorsally-compressed bodies, allowing them to use narrow crevices as refuges. Their habitat is primarily on smooth granite domes and outcrops which exfoliate in large sheets.

I conducted field work primarily at Pullen Farm (S 25°34,358' ; E 031°10.879') in the Crocodile River Nature Conservancy, a 30 000 ha area situated approximately 30 km east of Nelspruit, South Africa. The area is a lowland mesic savannah, with large exposed regions of granite bedrock.

Handling and marking of lizards

I captured lizards on lines of Catchmaster® 48R non-toxic glue traps (27 x 13 cm) and Trapper® glue boards (26 x 12 cm). In addition to the trapping and marking protocol outlined in detail in chapter 1, to facilitate recapturing of free-living lizards I marked lizards temporarily by writing larger numbers on their dorsum with a Pilot® white opaque non-toxic xylene-free paint pen.

Testosterone

I assayed testosterone from blood collected from the suborbital sinus according to the protocol outlined in chapter 1. Briefly, I drew a blood sample of approximately 150 μL of blood upon capture which was kept on ice until centrifugation and separation within hours of capture. Plasma was stored frozen at the field station and then transported to the University of the Witwatersrand where it was stored until assayed. I assayed testosterone by using DPC Coat-A-Count Total Testosterone kits following a pilot assay with experimental dilutions of different samples in order to obtain the optimal dilution ratio. These kits have been validated with plasma from closely related lizards (Whiting et al., 2006).

I made implants by filling 5 mm lengths of silastic tubing with 3 μL of a solution of testosterone (100 μg testosterone μL^{-1}) in dimethyl sulfoxide (DMSO). I sealed the ends of each tube with silicon adhesive and the DMSO evaporated through the tubing, leaving the hormone in the lumen. Control implants were made in the same way but filled with pure DMSO (Cox et al., 2005a).

The effects of testosterone on free-ranging and captive lizards

I collected 40 males and measured morphological characters, locomotor performance and cell-mediated immune response (as described below). Once measurements were complete, I marked them and released them into the field, 20 with control implants and 20 with testosterone implants. I left the study site undisturbed for two weeks and then recaptured marked males. I had a recapture rate of 60 %, recapturing 12 testosterone-supplemented males and 12 control males. I collected blood samples immediately upon capture in order to validate the efficacy of the implants and then repeated all of the measurements that I had taken before their release.

To measure the effects of testosterone in isolation from variable environmental factors I also collected 26 male *P. i. wilhelmi* and housed them indoors at the field station in individual semi-opaque plastic boxes with lids (230 x 320 x 140 mm) and fed them on wet cat food three times a week, supplemented with Beefee powder (® Bayer) once a

week and water *ad libitum*. The field station was within kilometres of the trapping sites and so the environmental conditions of the room were similar to those of the natural environment. After five days for adjustment to captivity, I took blood samples and measured endurance, morphology and immunocompetence. After two weeks I took a second blood sample from each lizard and measured endurance, immunocompetence, body condition and colouration again, before removing their implants and releasing them.

Morphological measurements and spectroradiometry

All morphological measurements were taken using the protocol outlined in chapter 1. I measured weight and snout-to-vent length (SVL) in order to calculate a body condition index by taking the residuals of $\ln$ SVL against mass (Fuxjager et al., 2010).

I measured the spectral reflectance of the throat, abdomen, flank, front and back legs and tails of each lizard as outlined in chapter 1. As in the previous study I used an average of two readings for each measurement in analyses and, before measuring each lizard, I took calibration readings of a black standard and a 99% diffuse white standard. I processed the reflectance readings using the same visual modelling technique as in chapter 1. Using *Platysaurus* visual templates (chapter 1), I calculated both chromatic and achromatic aspects of colour. The chromatic aspect of colour that I used is termed chromatic contrast which is the Euclidean distance in tetrahedral colour space between the area measured and the background habitat. This technique standardises brightness and so isolates the chromatic aspect of colouration. The achromatic aspect is measured as brightness i.e. the total light reaching the eye when reflected from the surface measured.

Immunocompetence

To measure immune response I used a delayed-type hypersensitivity test using phytohaemagglutinin (PHA) (Smits et al., 1999) as outlined in chapter 1. Briefly, I compared swelling of a foot injected with 0.03 ml phosphate buffered saline (PBS) containing 50 mg of PHA (Sigma, L-8754) with the opposite foot injected with pure PBS. A higher degree of swelling indicates a stronger immune response.

Endurance

I measured endurance as the length of time a lizard walked on a belt (30 x 40 cm) (e.g. Sinervo et al., 2000; Garland et al., 1990) moving at 0.4 km.h^{-1} before it failed to maintain its position more than three times (Miles et al., 2007) in keeping with the protocol outlined in chapter 1.

Statistical analyses

For each measurement I ran a Generalized Linear Model (GLZ) using a Logit link function. I used the period of measurement (i.e. before or after manipulation) as the dependent variable using a binomial error structure as there were two measurements for each lizard. I used implant type (testosterone or control) and location (free-ranging or captive) as categorical predictors in all cases. I used β -estimate coefficients to assess differences between first order effects and descriptive statistics to determine the direction of change. All statistics were performed in STATISTICA 6.0 (Statsoft). As GLZ analyses count data rather than means, graphs indicate medians, interquartile and range.

Ethical note

This study was approved by the Animal Ethics and Screening Committee of the University of the Witwatersrand. Clearance numbers; carried out under 2007/64/03, with a provincial permit from the Mpumalanga Parks Board MPB5211.

Results

Testosterone levels

Implant type was a significant predictor of testosterone concentration (Wald $\chi^2_1 = 23588.3$, $p < 0.001$) in *P. i. wilhelmi* where lizards with testosterone implants had higher testosterone levels ($\beta = 1.164$) two weeks after the insertion of implants than lizards with control implants. Captive males had lower baseline testosterone levels ($\beta = 0.141$) than free-ranging males (figure 2.1).

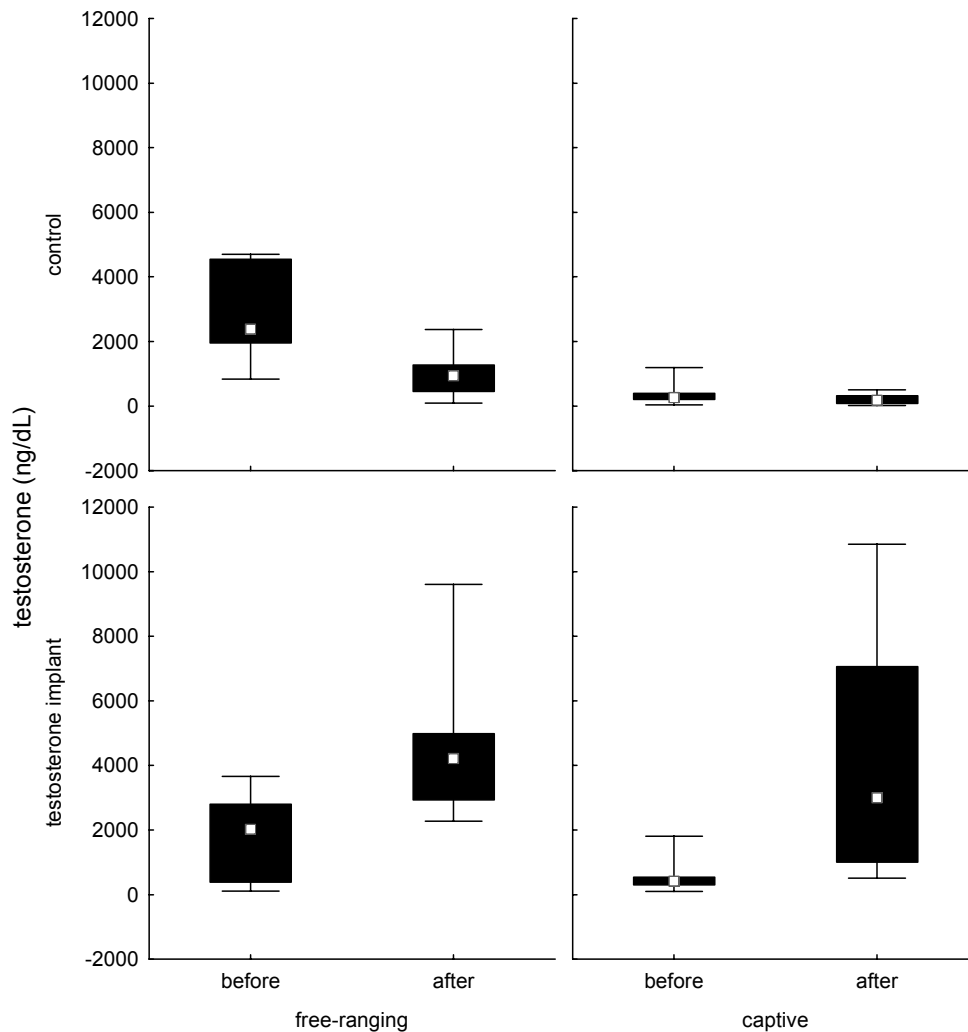


Figure 2.1. Testosterone levels before the insertion of implants and two weeks after insertion of implants in *P. i. wilhelmi*. In all cases testosterone implants increased testosterone significantly, while baseline levels of testosterone were lower in captive than in free-ranging lizards at the beginning of the study. Values indicated are medians with boxes showing interquartile range and whiskers showing total range.

Body condition

Neither experimentally elevated testosterone (Wald $\chi^2_1 = 0.2$; $p = 0.69$) nor housing condition predicted body condition in *P. i. wilhelmi* (Wald $\chi^2_1 = 0.2$; $p = 0.617$ figure 2.2).

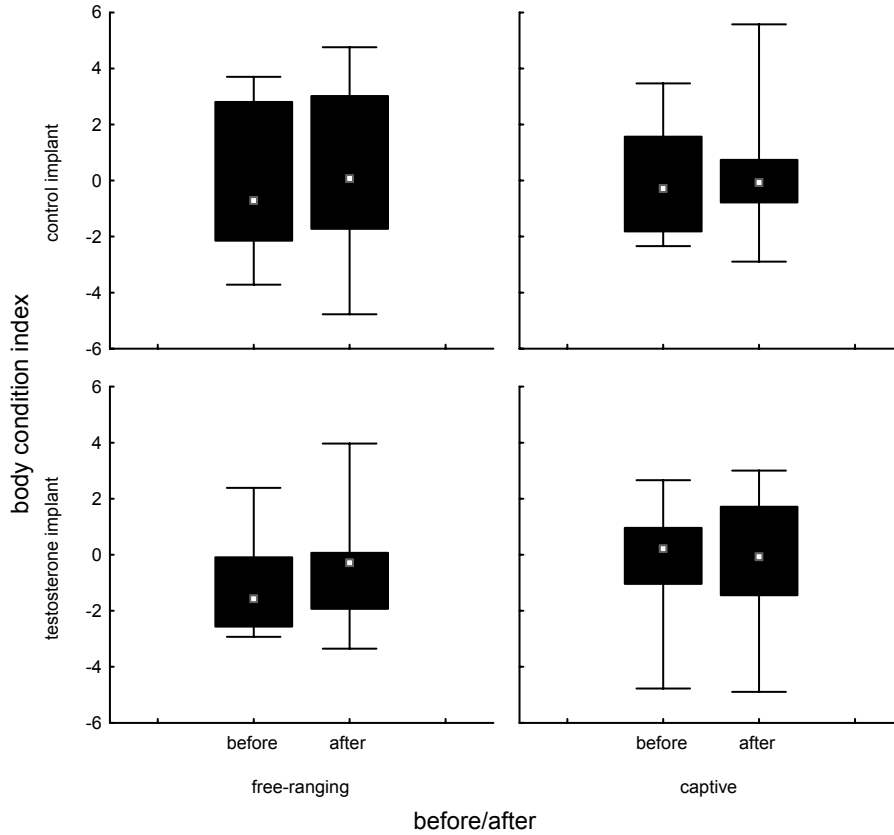


Figure 2.2. Experimentally elevated testosterone did not predict body condition in *P. i. wilhelmi*, which shows that body condition was not influenced by exogenous testosterone. Values indicated are medians with boxes showing interquartile range and whiskers showing range.

Immune response

Implant type and housing condition were predictors of immune response (Wald $\chi^2_1 = 5.3$, $p = 0.021$). Free-living *P.i wilhelmi* males with testosterone implants showed a decrease in immune response ($\beta = 0.134$, figure 2.3).

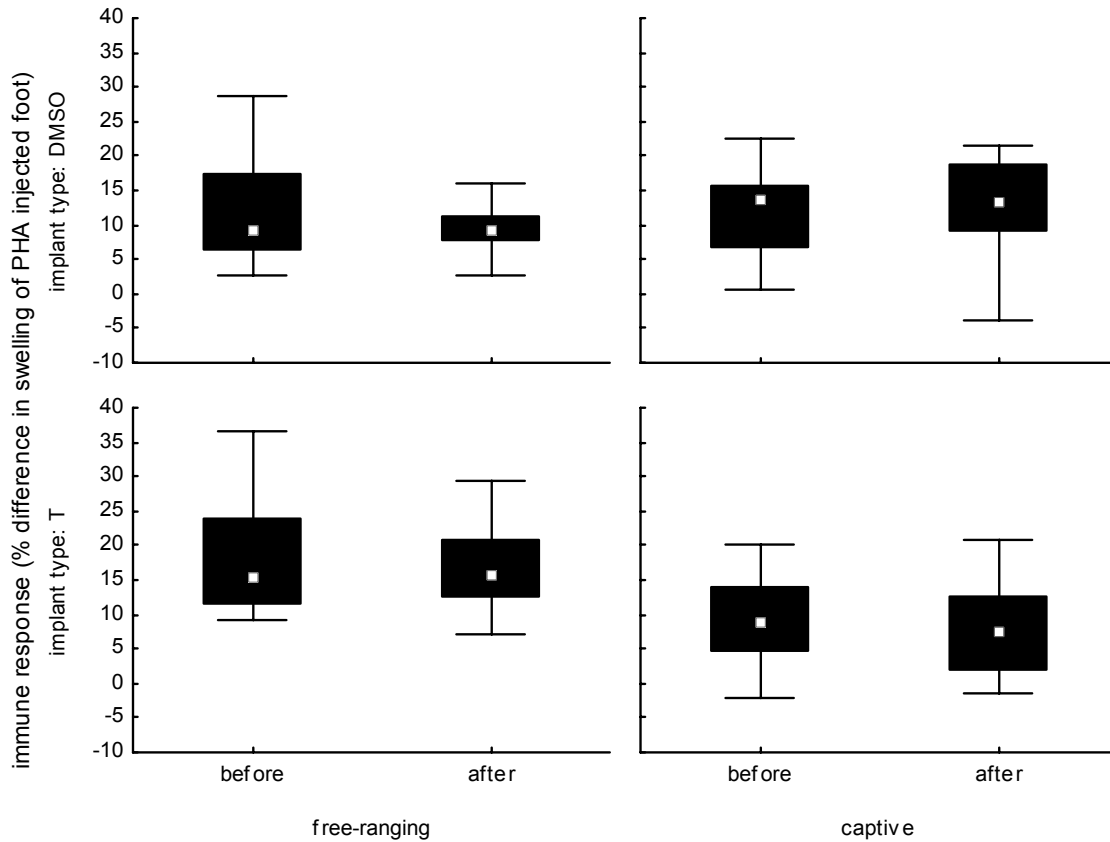


Figure 2.3. Free ranging *P. i. wilhelmi* males with testosterone implants showed a significant decrease in immune response two weeks after the insertion of implants. Values indicate medians with boxes indicating interquartile range and whiskers showing total range.

Endurance

Implant type was a significant predictor of endurance (Wald $\chi^2_1 = 55.3$, $p < 0.001$). Both free-living and captive lizards with testosterone implants ran for longer than their respective controls ($\beta = 0.187$), and free-living lizards ran for longer than captive lizards ($\beta = 0.753$). There was an interaction between housing condition and implant type, where free-living lizards with testosterone implants improved more than their captive counterparts over the course of the experiment ($\beta = 0.0439$, figure 2.4).

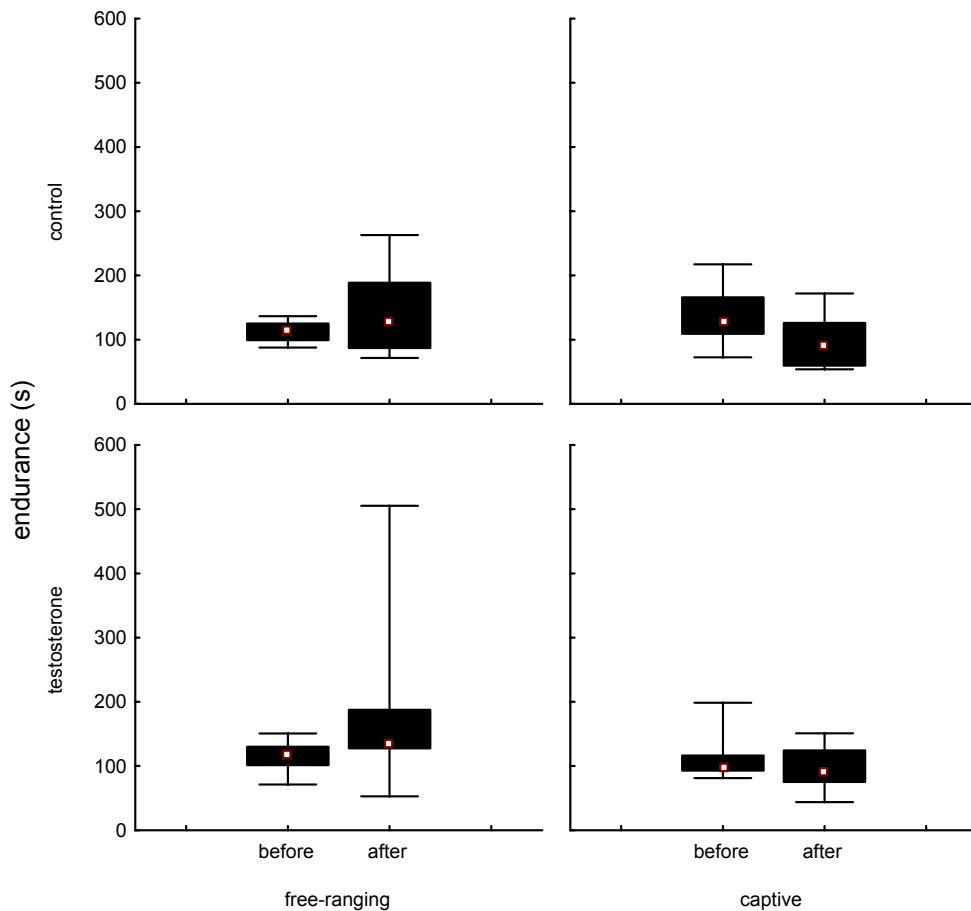


Figure 2.4. Implant type predicted endurance in *P. i. wilhelmi* with lizards with testosterone implants outperforming control individuals significantly and this effect was significantly more pronounced in free-ranging lizards. Values indicated medians with boxes showing interquartile range and whiskers indicating total range.

While lizard body temperature was a significant covariate in the model, a correlation showed only a weak link explaining very little of the variance between body temperature

and time on the treadmill ($R^2 = 0.0331$; $r = 0.18$, $p = 0.21$) which suggests that it is not of overriding significance in this case.

Chromatic contrast

Neither testosterone (Wald $\chi^2_1 = 0.4$; $p = 0.513$) nor captivity (Wald $\chi^2_1 = 0.6$; $p = 0.422$) were predictors of the chromatic aspect of conspicuousness between lizards and rocks (figures 2.5 and 2.6).

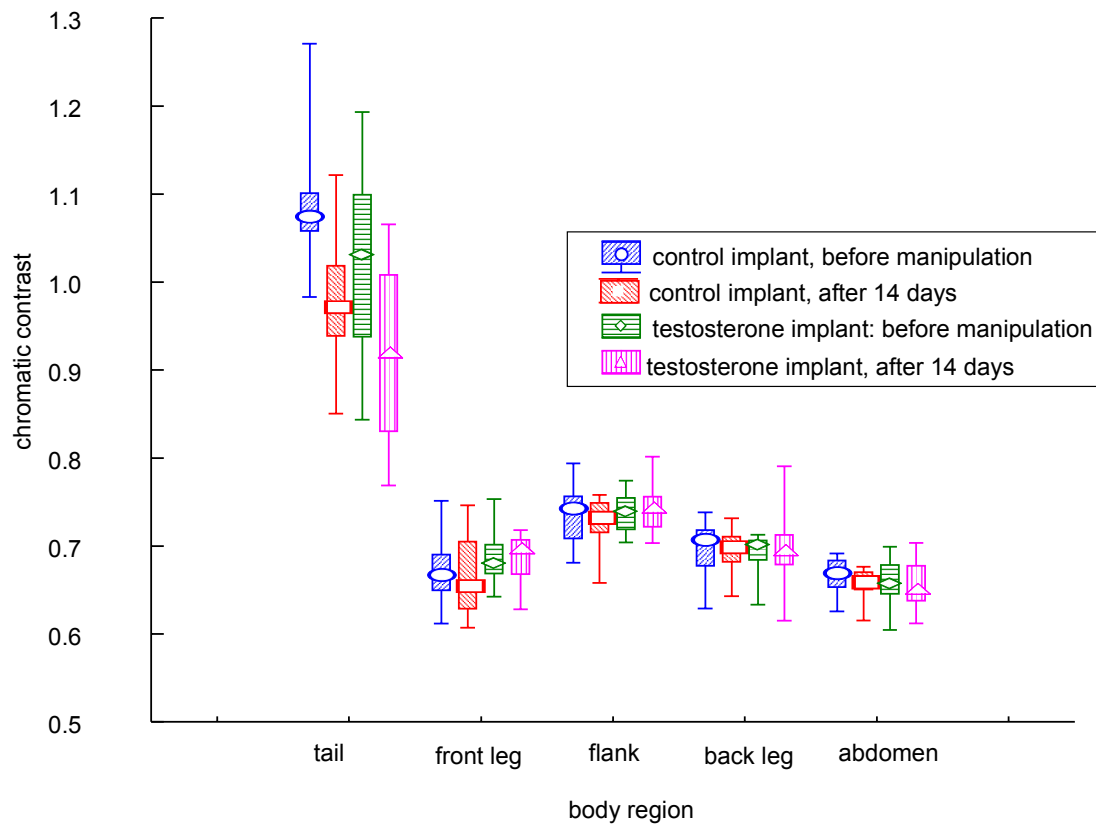


Figure 2.5. Testosterone did not predict chromatic contrast of captive *P. i. wilhelmi*. Boxes show inter-quartile range and whiskers indicate total range

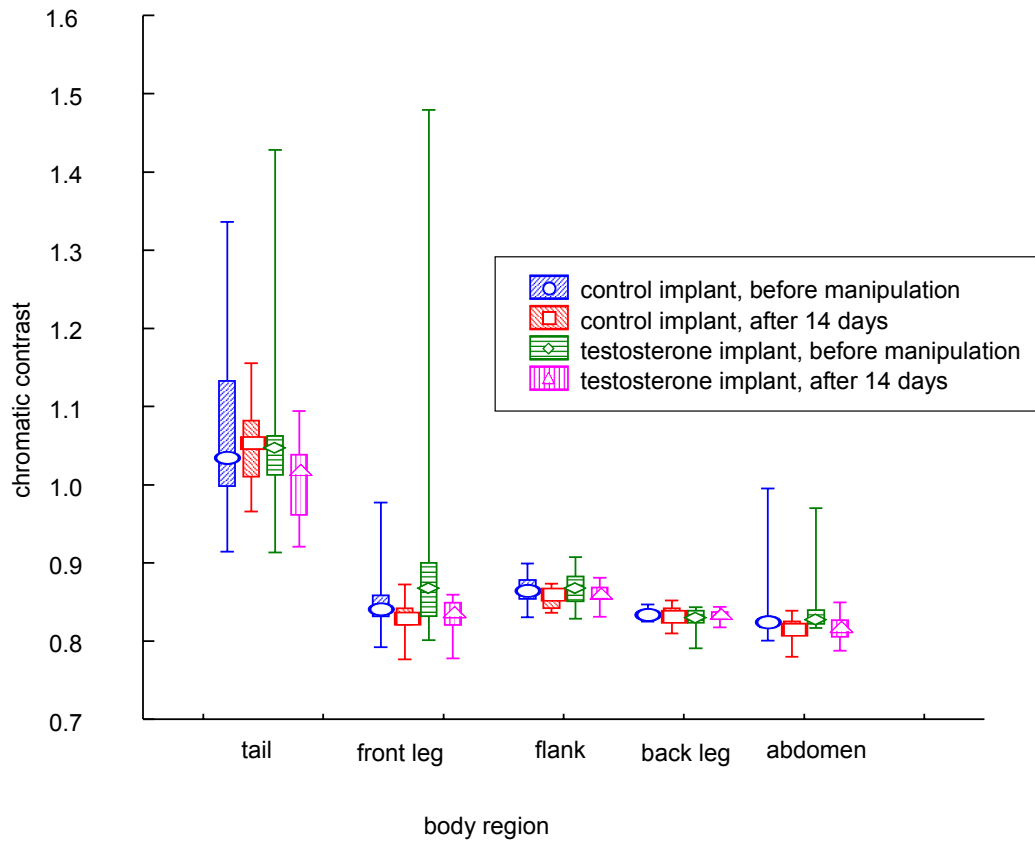


Figure 2.6. Chromatic contrast between colour patches and rock habitat was not predicted by testosterone after 14 days in free-ranging *P. i. wilhelmi*. Boxes indicate inter-quartile range and whiskers show total range.

Brightness

Testosterone implants predicted brightness in both captive and free-living *P. i. wilhelmi* (Wald $\chi^2_1 = 2382.4$; $p < 0.001$, Figure 2.7). Lizards with testosterone implants had darker back legs ($\beta = 0.070$) and abdomens ($\beta = 0.367$) and brighter tails ($\beta = 0.014$), flanks ($\beta = 0.05$) and front legs ($\beta = 0.165$) than lizards with control implants.

Housing condition (free-living or captive) had a significant effect on brightness ($\beta = 0.753$) where the changes were in the same direction but more pronounced in the free-living lizards than captive lizards in all cases except for back leg brightness which was the same in both groups ($\beta = 0.147$, figures 2.7 and 2.8).

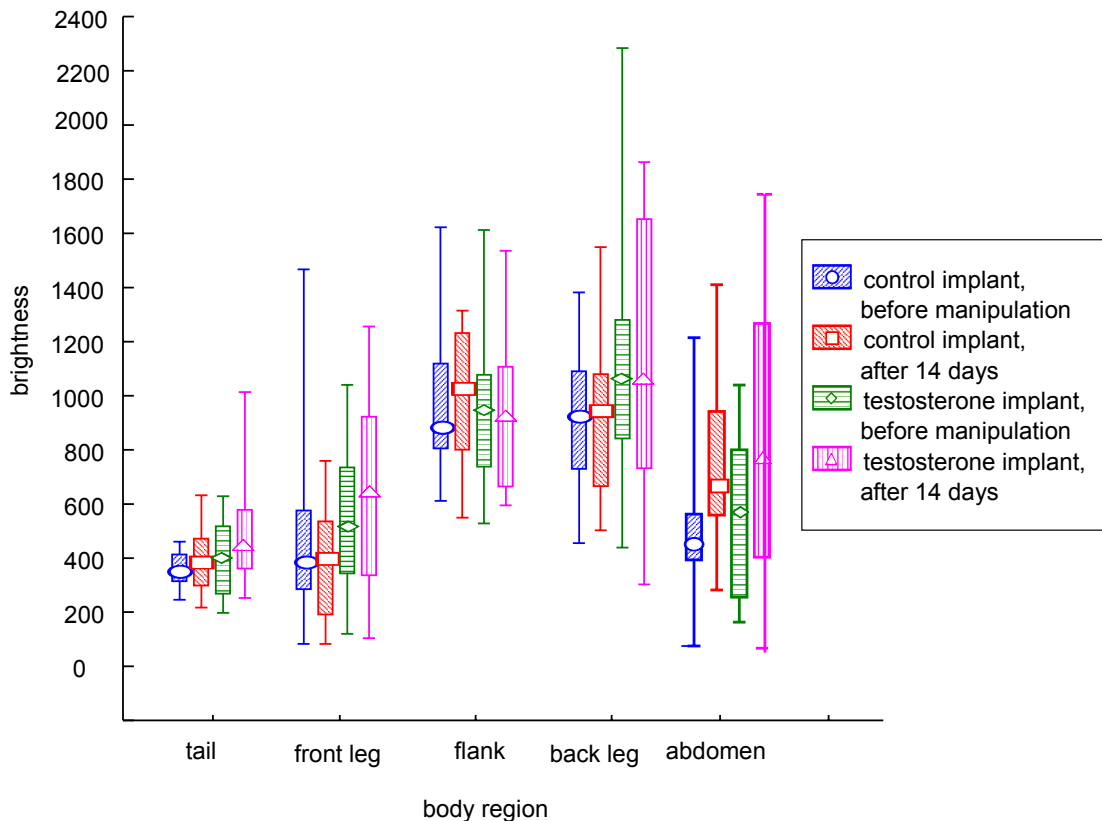


Figure 2.7. Experimentally increased testosterone lead to darker back legs and abdomens and brighter flank, tail and front leg regions in *P. i. wilhelmi*. This effect was visible in both groups (captive and free-ranging) but changes were of a smaller magnitude in captive lizards (shown here). Boxes indicate inter-quartile range and whiskers show total range

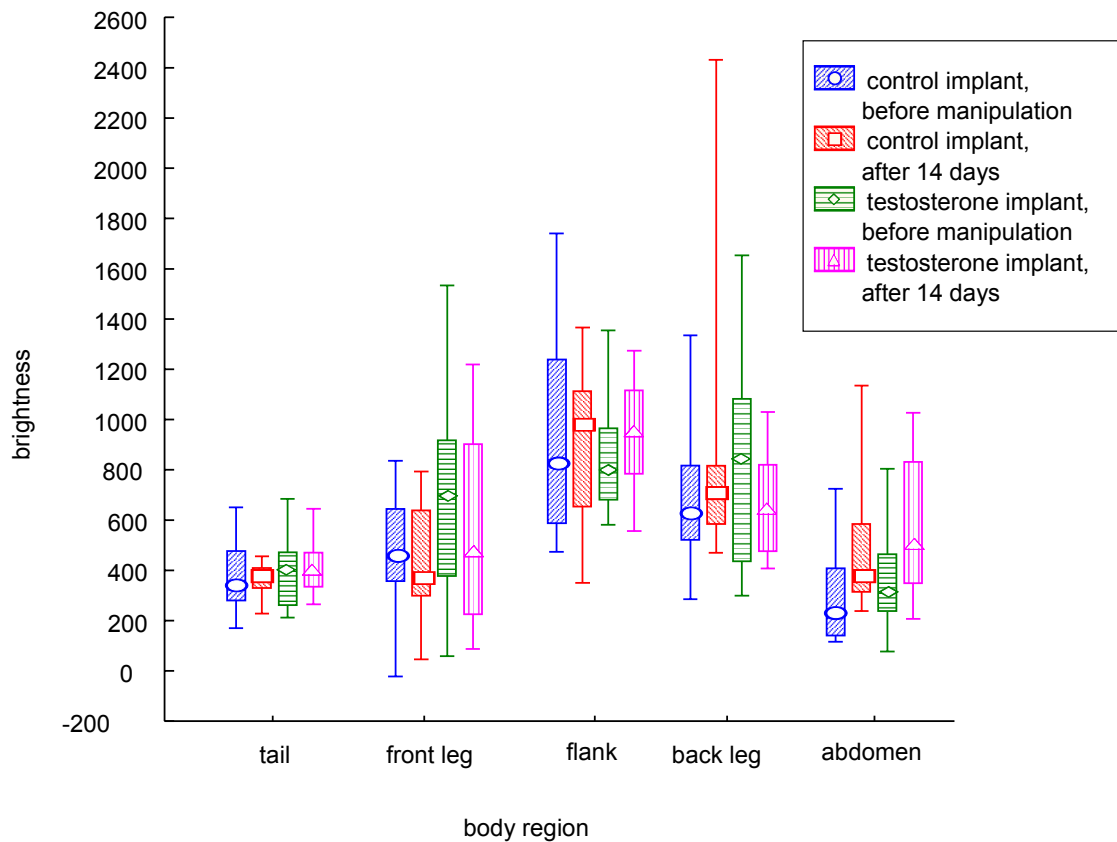


Figure 2.8. Changes in brightness showed a higher magnitude change in free-ranging (shown here) than in captive *P. i. wilhelmi*. In all cases, males with testosterone implants had darker back legs and abdomens and brighter flank, tail and front leg regions than control lizards. Boxes indicate inter-quartile range and whiskers show total range.

Discussion

The aim of my study was to test the immunocompetence-handicap hypothesis in *P. i. wilhelmi* and to compare the effects of experimentally elevated testosterone on free-living and captive lizards.

Both free-living and captive lizards with testosterone implants showed no change in body condition or chromatic contrast of colour patches. Testosterone-implanted free-living males showed a decrease in immune response compared to free-living control lizards while there was no difference between testosterone-implanted and control males in

captivity. Both free-living and lab-housed lizards (free-living and captive-housed) with elevated testosterone performed better in endurance trials than control males and also had brighter flanks and front leg colour and darker back legs, tails and abdomens than control males.

Testosterone implants raised circulating plasma testosterone levels by an average of 200%. While this is out of their natural population range, that is not unique to this study (e.g., Grear et al., 2009) and as there were effects of testosterone implants on phenotype it is unlikely that receptors were saturated before insertion of implants. Male *Platysaurus i. wilhelmi* with testosterone implants showed greater endurance than males with control implants as well as brighter flanks and front legs and darker back legs, abdomens and tails than males with control implants. Males confined for the course of the experiment showed less of an improvement in endurance following testosterone implantation than free-ranging males. Free-ranging males also showed a significant decrease in immunocompetence that was not apparent in captive males.

I predicted a decrease in body condition as the energetic costs of testosterone took effect (e.g. Ketola et al., 2009; Malo et al., 2009). This was not the case in either free-ranging or captive males. While males in captivity had no opportunity to increase their activity levels or territory patrols, the fact that there was no change in body condition for free ranging males is counter-intuitive. One possible explanation is that if the supplemented testosterone (with its concurrent increased endurance) lead to an increase in male home range size then the quality of food available might have outweighed the costs of moving in such a large area, particularly when insects tend to aggregate in small areas.

If the immunocompetence-handicap model was supported, I would have expected a decrease in delayed-type-hypersensitivity (as a measure of cell-mediated immunity) following the insertion of testosterone implants. This was the case in free-ranging males but not in captive males. This discrepancy between captive and free-ranging individuals has been reported in birds (Ketterson et al., 2001) (where it was considered to be an

artefact of stress in captivity) and is another indication that we need to investigate the costs and constraints of endocrine control of signalling in a more integrative manner.

The demands of the immune system are dynamic and affected by a variety of environmental factors (Greiner et al., 2010) and recent evidence shows that testosterone-mediated traits and immunocompetence work through a feedback loop rather than a linear relationship (Greiner et al., 2010). This difference between captive and free-ranging lizards may be explained by the different stressors to which captive and free-ranging animals were exposed. Captive animals in general are under chronic stress as a by-product of captivity and they are not affected by other more acute stressors (such as social interactions or the need to find food) which affect their free-ranging counterparts. One major difference between free-ranging and captive animals is that animals in a captive environment would experience more chronic stress than free-ranging males. This has been found in cheetahs (Terio et al., 2004) over a long time period as well as in wild chukars (*Alectoris chukar*) who mounted and maintained a chronic stress response for several days after capture (Dickens et al., 2009). As testosterone and corticosterone share receptors and are inversely related it is possible that differing levels of corticosterone may be affecting my results. The stress-linked immunocompetence-handicap hypothesis proposes that testosterone does not affect signals directly but, instead, it covaries with corticosterone. Therefore, corticosterone levels are actually used to modulate signals of male quality and changes in testosterone are merely coincidental (Bortolotti et al., 2009b). Other studies have found that only a combination of corticosterone and testosterone has an effect, rather than either hormone in isolation (Roberts et al., 2006). While my study does not investigate the effects of corticosterone directly, the role played by captivity on the effectiveness of testosterone implants suggests that the influence of stress on lizard physiology is considerable and needs to be explored.

Captive and free-ranging male flat lizards with testosterone implants showed a significant increase in endurance and ran for longer than control males over the course of the experiment. The improvement was more pronounced in free-ranging lizards compared to captive males. This effect of housing is logical - while muscle mass may increase, the

muscles are not necessarily ‘trained’ and so the benefits of larger muscles will not have as great an effect on the outcome of endurance trials (Husak & Irschick, 2009). As mentioned above, endurance is an important predictor of social status where it is established that males with high levels of testosterone are often dominant. This may be due to links between dominance and home-range size where dominant lizards have to cover large areas while patrolling their territories (Sinervo et al., 2000). While the territory size of *P. i. wilhelmi* is not known, they are highly active, particularly in the presence of other males, and some individuals do cover large areas (pers obs).

If testosterone does mediate male signals, I would predict that males with artificially elevated testosterone would show an increase in conspicuousness against their rocky habitat. As colour has two aspects – achromatic (i.e. brightness) and chromatic (location in colour-space), male *P. i. wilhelmi* with testosterone implants are expected to become less bright in order to contrast with their light-coloured granite habitat (achromatic aspect) as well as having a larger Euclidean distance between their colour-patches and rocks (chromatic aspect). Males with testosterone implants had darker back legs, abdomens and tails and brighter flanks and front legs. This is somewhat counter-intuitive because the posterior area (i.e. back legs, abdomens and tails) did become more conspicuous against the granite of their habitat than prior to manipulation, their flanks and front legs became brighter which would be less conspicuous against the rocks than at the beginning of the experiment.

As the brightness of the posterior region of male lizards (tail, hind leg and abdominal colours) responded to experimentally elevated testosterone by becoming darker, it is logical to assume that these regions are for signalling. This is most frequently found in predator-prey signalling where lizards direct predation to the tail which can be autotomised (Cooper, 2001) and changes in predation-pressure may very well be a limiting factor in testosterone-based colouration in this region. Tails that become colourful at maturity have also been linked to a decrease in ‘risky’ behaviour which compensates for the associated predation-pressure (Hawlana, 2009). Adult male flat lizards do have more colourful tails than the straw-coloured tails of females and juveniles

and I have observed males holding the centre portion of the tail up in the air when displaying to another male (pers obs).

Males tend to interact by standing side-on in an elevated position (pers. obs.) and it seems strange that their flank and front leg colouration would become brighter (i.e. more similar to the granite background) as these areas are the closest to opponent males during displays. There are two possible explanations for this: the front body regions contrast more strongly with the black throat region and therefore actually make displays more conspicuous; or the measurement of immune response could have increased the demands of the immune system and therefore have affected colouration. This effect being evident in males with testosterone implants rather than controls is in keeping with the idea of the immunocompetence-handicap hypothesis where the feedback loop between the immune system and signals affects lizard brightness.

Contrary to my prediction, experimentally elevated testosterone did not affect chromatic aspects of *P. i. wilhelmi* colour patches. This is surprising, considering that bright colours are male-specific and in sexually dimorphic species male traits are usually testosterone dependent. Some studies have found similar results. For example, mandrills (*Mandrillus sphinx*) lack sufficient variation in a healthy population to show a link between male facial colouration and condition or parasites (Setchell et al., 2009). We must consider the possibility that testosterone is actually not the mediator of the chromatic aspects of male colouration. In a previous study on the closely related *P. broadleyi*, testosterone levels were related to throat colour hue (i.e. a chromatic aspect of colour) but not with brightness (i.e. achromatic aspects) at all (Whiting et al., 2006).

There is also the possibility that as trials took place in the breeding season; males were already functioning with maximal testosterone levels for available receptors. Additional testosterone will not change traits if the animal has no free receptors and thus we would not observe a change. Receptor saturation can be a significant factor in controlling sexual dimorphism in lizards (Cox et al., 2005b). However, the fact that there were significant

changes in some factors (endurance, immunocompetence and brightness) makes this unlikely because if receptors were saturated there should be little change at all.

While the predictions that we can make and test with the ICHH are simple, the systems involved are so complex that there is seldom a clear relationship between testosterone and the animal under study. We must remember that there may be indirect effects, such as increased movement or extended activity time, leading to greater exposure to parasites and parasites that may compromise the individual's health and immune system (Cox & John-Alder, 2007). My study provides a new perspective on the traditional studies of the immunocompetence-handicap hypothesis in that I have investigated the effects of testosterone on multiple aspects of performance and phenotype as well as examining the effects on isolated animals and free-ranging animals which show that there are also exogenous factors affecting their responses. The results suggest that while testosterone plays an important role in signalling, locomotor performance and immunocompetence, it is not the only factor involved. While the ICHH is an important starting point in investigating these relationships, other, more complex models should also be investigated, particularly in light of the differences between endogenous and exogenous factors suggested here.

Chapter 3: Testing the effects of exogenous testosterone on female flat lizards

Introduction

The immunocompetence-handicap hypothesis (ICHH) predicts that testosterone functions as a ‘double-edged sword’ where its influence on male signalling and behaviour is hampered by associated immunosuppressive effects because increased testosterone leads to immunosuppression (e.g. Folstad & Karter, 1992). This requires two main assumptions: first, that testosterone is mediating male secondary sexual traits and second, that it has immunosuppressive properties. Testosterone is an important mediator of sex differences and influences morphological, physiological and behavioural phenotypes (reviewed in Ketterson et al., 2005; Møller et al., 2005). While the evidence for the ICHH is equivocal with only approximately half of the studies investigating it finding any support for the model (reviewed in Roberts et al., 2004), we cannot discount it without investigating the effects of testosterone completely.

One potentially confounding factor is that male signals may, to some extent, be under organisational rather than activational control. The action of a hormone on a trait is regarded as ‘activational’ if changing levels of that hormone affect trait expression (Hews et al., 1994). This means that trait expression should co-vary with testosterone levels (Hews et al., 1994). A hormone has an ‘organisational’ effect on a trait if it plays a role in the development of that trait, but the trait becomes fixed during early life and cannot be significantly altered with additional hormone exposure. This also applies to traits where organisational effects are required to lay the pathways for interactions with hormones at a later stage (Lovern et al., 2001). One way to test the relative influence of organisational and activational effects is to determine the effects of testosterone on females because they will have been exposed to significantly less testosterone than males over the course of their development. Hormones act as proximate factors regulating sex-specific phenotype expression, and suites of traits are mediated by the same hormones (Ketterson et al., 2005). For example, female ruffs that were given subcutaneous testosterone implants

caused the development of male-specific behaviour as well as male-specific plumage (Lank et al., 1999). These effects were reversible, indicating that these traits are activational and controlled by testosterone. In a study on tree lizards, alternative reproductive tactics are androgen-controlled and fixed within 60 days post-hatching, but testosterone continues to affect growth rates after this period (Hews & Moore, 1996). Females also developed male colouration only if administered androgens as juveniles, not when given testosterone as adults which suggests that these colours are under organisational control (Hews & Moore, 1995). There have also been studies on the organisational effects of testosterone: elevating testosterone in juvenile *Sceloporus undulatus* females led to the development of male-specific colouration whereas castrated males did not develop this colouration (Cox et al., 2005b). In adult tree lizards (*Urosaurus ornatus*), males with different throat colours did not differ either in testosterone or in corticosterone levels (Thompson & Moore, 1992). Increasing testosterone levels in lizards at an early age resulted in colour differences between males with testosterone implants and controls, even when using a relatively low dose of testosterone (Hews et al., 1994). Further manipulations of the hormone did not change trait expression and individuals with similar levels of trait expression can have vastly different hormone levels (Hews et al., 1994). For example, the face colour of *Sceloporus undulatus erythrocheilus* lizards increased in intensity when testosterone levels were experimentally increased, while ventral colour increased significantly more in males than females, suggesting that testosterone may have had an organisational effect in changing the limits of trait expression (Rand, 1992).

Testosterone is secreted by males and females (Nelson, 2000). While testosterone plays a role in several aspects of behaviour and physiology in males and females (reviewed in Staub & De Beer, 1997) the role of testosterone in females is still relatively unclear (Zysling et al., 2006). Besides the obvious roles of testosterone in influencing male-specific ornaments, behaviour, and sperm production, testosterone plays a pivotal role in the growth and development of juveniles, particularly in muscle and organ formation as well as neuron survival (Nelson, 2000; Staub & De Beer, 1997). Females also show seasonal fluctuations in testosterone, which peak during vitellogenesis, strongly

suggesting a role in female reproduction. There are not very many empirical studies on the effects of manipulations of testosterone in females, despite a wealth of information on the effects on males. To date, research (mostly on birds) showed that elevated testosterone in females leads to decreased cell-mediated immunity, increased aggression as well as increased corticosterone and HPA responsiveness (Zysling et al., 2006), decreased weight, delayed moulting and egg-laying (Edler et al., 2010; Clotfelter et al., 2004) and lack of brood patches for incubation (Clotfelter et al., 2004). Elevated testosterone in European starlings did change bill colour from black to yellow in females (De Ridder et al., 2002) but this is a rare example of elevating testosterone resulting in colour changes in females. While these studies do show us a great deal of the effects of testosterone on behaviour and activity levels, there is very little information on the effects of testosterone on colour ornaments in females. Much of the existing research on the effects of testosterone on females involves research on dark-eyed juncos (*Junco hyemalis*) which are weakly dimorphic, so there are not as many potential effects of testosterone on phenotype.

Manipulation of testosterone in females has two potential outcomes (fig 3.1). If elevated testosterone in females results in similar changes to behaviour and morphology as males, it can be assumed that these traits are purely activational. If they respond differently, then either the effects on males are organisational or else organisational effects have left females insensitive to testosterone levels (Zysling et al., 2006) which would allow the evolution of male traits to occur independently (Ketterson et al., 2005).

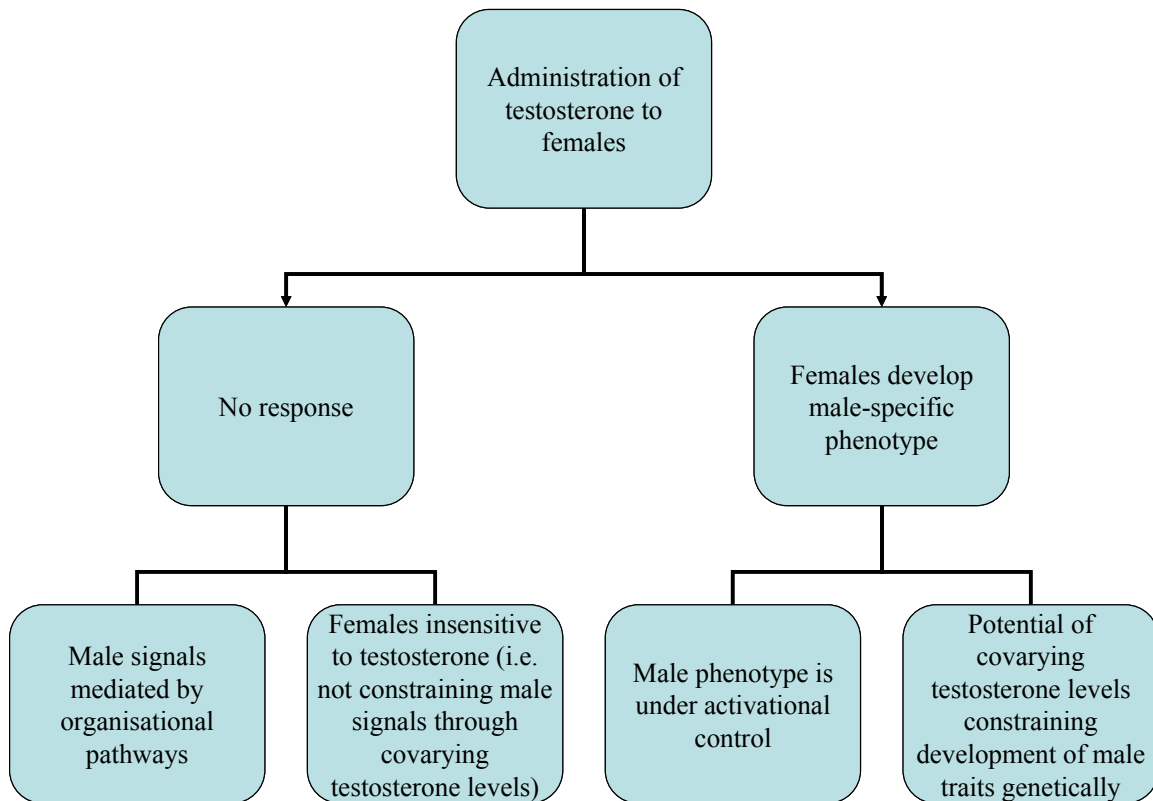


Figure 3.1. Potential outcomes of experimentally elevating testosterone in females

Lizards are a good model for studying condition-dependent colour signals. Plasticity in lizard colouration is relatively common (Husak et al., 2007), where signals are often plastic, changing with age, social status and various other social factors (Lemos-Espinal et al., 1996). Male *Platysaurus i. wilhelmi* with experimentally elevated testosterone showed improved endurance, brighter flanks and front legs, and darker back legs, abdomens and tails than control males (chapter 2). Also, the colour of these body regions predicts endurance, immunocompetence and body size including head dimensions (chapter 1). Males and females are similar genetically and so may have testosterone levels that co-vary with the testosterone levels in males. This may lead to constraints on male signalling based on the effects that high testosterone levels have on females (Ketterson et al., 2005). Testosterone is likely to be costly for both sexes, but females will reap fewer benefits as they will not be attempting to control territories or attract females. For example male and female anoles with testosterone implants showed increased activity levels (Lovern et al., 2001). While males may benefit from this increase in activity due to increased active time being used to defend territories or pursue females,

females would bear a high energetic cost at no reward. Another cost to females could be the effects of additional androgens on fitness, for example in spotless starlings increased testosterone reduced extra-pair offspring to females (García-Vigón et al., 2008). While a few studies have shown the effects of testosterone in females, very few have quantified the costs that these increased levels bring to the females (e.g., De Ridder et al., 2002).

Flat lizards (*Platysaurus*) are a good system to test relationships between hormones, colour signals and physiology. They are small, sexually dichromatic lizards with drab females and brightly-coloured males. Males are territorial and highly aggressive. The closely related *P. broadleyi* has been studied extensively and male colouration has been found to be an important indicator of status (Whiting et al., 2003) and fighting ability (Whiting et al., 2006). Their strong sexual dimorphism (characteristic of this genus (Branch & Whiting, 1997)) makes them an ideal candidate to explore the effects of testosterone on females which have very different colour phenotypes to males.

The aim of this study was to investigate the effects of testosterone on females in general with a view to understanding its role on constraints in male signalling. I predicted that with experimentally increased testosterone, females would show an increase in conspicuousness to resemble males and an increase in endurance but at the cost of reduced immune response and body condition.

Methods

Study system

Platysaurus intermedius wilhelmi is a small (maximum snout-vent length 83 mm) cordylid lizard found on rocky outcrops in Mpumalanga and Kwazulu-Natal provinces, South Africa as well as a limited distribution in Swaziland and Mozambique (Branch, 1998). In contrast to brightly coloured blue, green and orange males, females are drab and resemble juveniles with a brown and black dorsum, white and blue venter and straw coloured tail. I conducted field work at Pullen Farm (S 25.57°, E 031.18°) situated in the Crocodile River Nature Conservancy, a 30 000 ha area approximately 30 km east of Nelspruit, South Africa. For further details concerning the study species see chapter 1.

Collection and husbandry

I collected 38 females on lines of Catchmaster® 48R non-toxic glue traps (27 x 13 cm) and Trapper® glue boards (26 x 12 cm) according to protocols outlined in chapters 1 and 2. Lizards were housed indoors in individual semi-opaque plastic boxes (32 cm x 23 cm x 14 cm) in a room in the conservancy and fed on wet cat food three times a week, supplemented with Beefee® powder (Bayer) once a week and water *ad libitum*. All procedures were conducted on lizards while they were in captivity. Lizards were kept in captivity for five days prior to the first set of blood samples and corresponding measures. All of the measurements took place over three days, following which they were given implants, returned to their boxes and kept relatively undisturbed for 14 days at which point the blood samples and measurements were repeated.

Elevation of testosterone

I elevated testosterone levels in female flat lizards by following the protocol used on males in chapter 2. Nineteen lizards received testosterone implants and 19 received control implants.

Testosterone assay

I used blood collected from the suborbital sinus as according to the protocol outlined in chapter 1 in order to quantify plasma testosterone levels. I took all blood samples between 08h00-12h00, the period of peak activity for lizards, and noted the time taken to acquire a sample. I kept blood samples on ice until they could be centrifuged (usually within minutes of collection). After centrifuging samples at 3000 rpm for three minutes I separated the plasma and froze it immediately, until it could be assayed in the lab.

To validate the success of implants at raising testosterone levels I assayed testosterone by using DPC Coat-A-Count Total Testosterone kits using the protocol in chapter 1.

Morphological measurements and spectroradiometry

I measured the same morphological variables as the male lizards in chapter 2. Briefly I measured snout-to-vent length, and weight. I also measured spectral reflectance of the abdomen, front leg, flank, back leg and tail regions and used visual modelling techniques to compare differences in colour in a biologically significant way (For more detail see chapter 1).

Immune response

To measure immune response I used a delayed-type hypersensitivity test using phytohaemagglutinin (PHA) (Smits et al., 1999). I compared the swelling of one hind foot injected with a solution of phytohaemagglutinin in phosphate-buffered saline (PBS) with another foot injected with pure PBS. A higher degree of swelling indicates a higher cell-mediated immune response. For further details see chapter 1.

Endurance

I measured endurance as the length of time a lizard walked on a belt (30 x 40 cm) (e.g. Sinervo et al., 2000; Garland et al., 1990) moving at 0.4 km.h^{-1} before it failed to maintain its position more than three times (Miles et al., 2007). For more details on the methods of endurance measurements see chapter 1.

Statistical analysis

For each measurement I ran a Generalized Linear Model (GLZ) using a Logit link function. I used the time of the measurements as the dependent variable using a binomial error structure as there were two measurements for each lizard. This has been used successfully in endocrine studies in the past (e.g. Redpath et al., 2006). I used implant type (testosterone or empty) as a categorical predictor in all cases. I used β -estimate coefficients to assess differences between first order effects and descriptive statistics to determine the direction of change. All statistics were performed in STATISTICA 6.0 (Statsoft). Graphs show medians and interquartile range.

Ethical note

This study was approved by the Animal Ethics and Screening Committee of the University of the Witwatersrand. Clearance numbers; carried out under 2007/64/03, with a provincial permit from the Mpumalanga Parks Board MPB5211.

Results

Testosterone implants predicted circulating plasma levels significantly (figure 3.1; Wald $\chi^2_1 = 6358.9$ $p < 0.001$), with testosterone implants raising circulating plasma concentrations by up to 51 times the original level ($\beta = 2.344$). The range of testosterone concentrations is slightly greater than natural male breeding-season levels (chapter 1), with females averaging approximately 7000 ng.dL^{-1} while natural male levels during summer average at approximately 5087 ng.dL^{-1} .

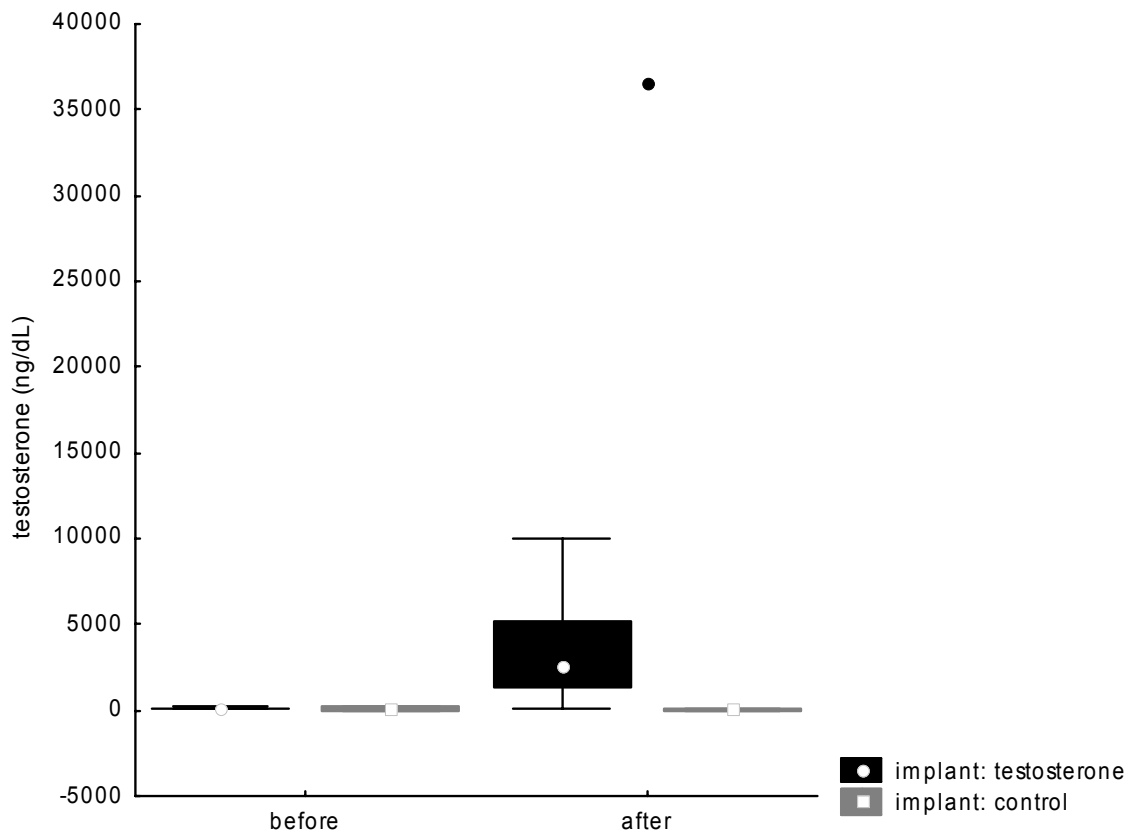


Figure 3.2. Testosterone levels in female *P. i. wilhelmi* are predicted by implant type. Lizards with testosterone implants had higher testosterone levels after 14 days than lizards with control implants. Values show medians, boxes show interquartile range and whiskers indicate range.

Implant type (testosterone or control) did not predict body condition in female *P. i. wilhelmi* 14 days after insertion of implants (figure 3.3; Wald $\chi^2 = 0.069$, $p = 0.79$)

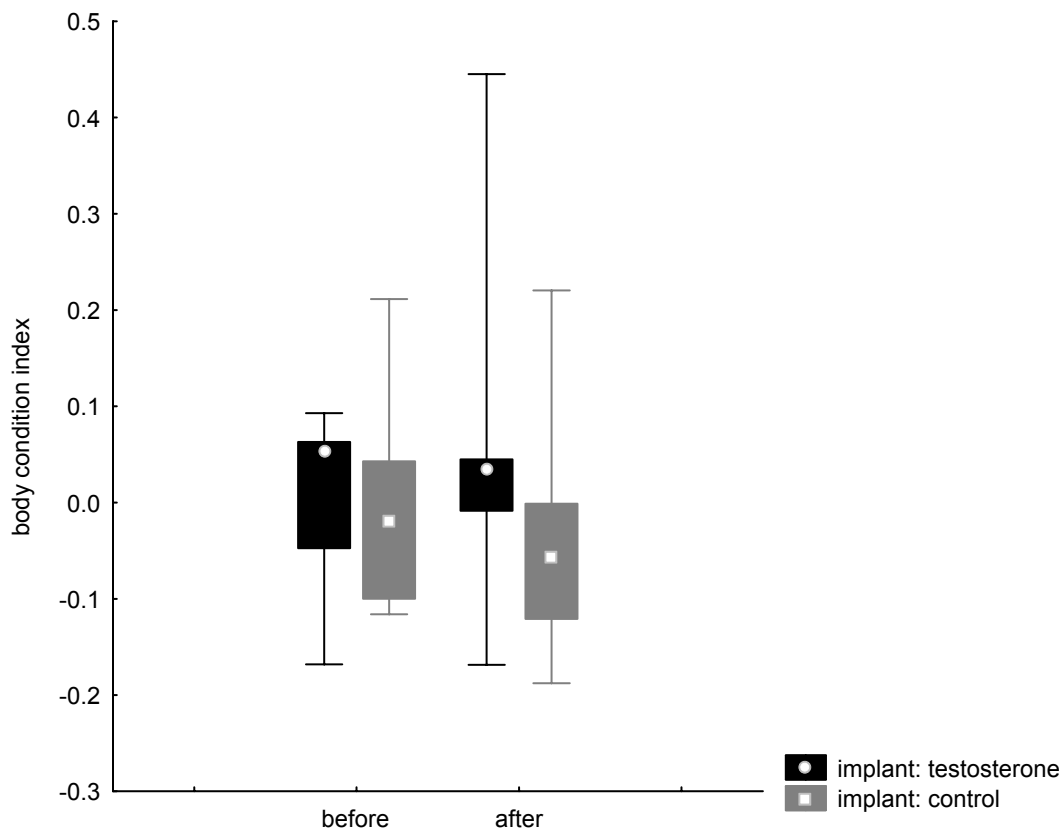


Figure 3.3. Body condition was not predicted by elevated testosterone in female *P. i. wilhelmi*. Values indicate medians, boxes show interquartile range and whiskers show total range.

Chromatic contrast was not predicted by implant type in female *P. i. wilhelmi* with testosterone implants and control implants (Wald $\chi^2_1 = 0.49$, $p = 0.48$, see figure 3.4.).

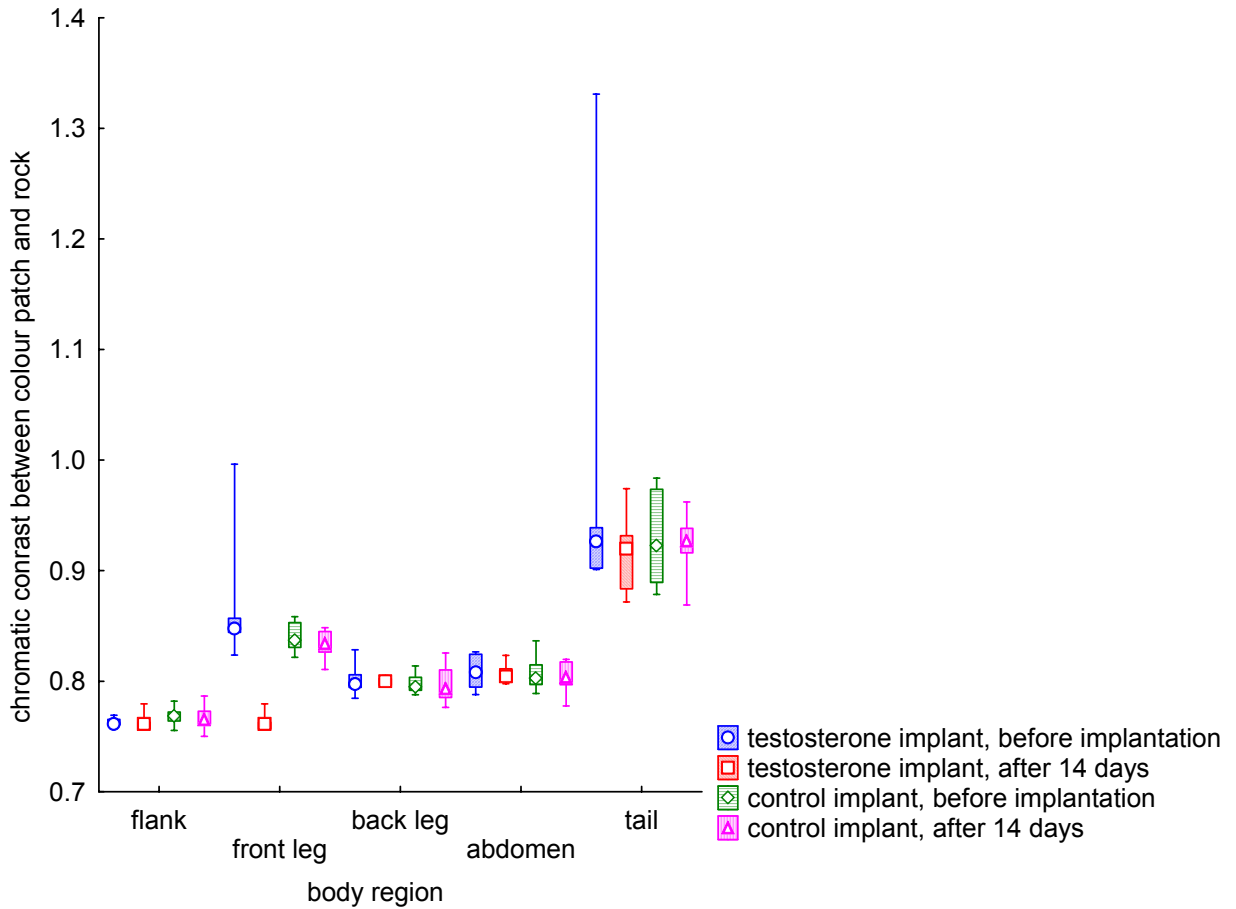


Figure 3.4. Testosterone implants did not predict chromatic contrast for any body region in female *P. i. wilhelmi*. Values indicate medians; boxes show interquartile range and whiskers show total range.

Implant type was a predictor of brightness (Wald $\chi^2_1 = 79.64$, $p < 0.001$) in that female *P. i. wilhelmi* with testosterone implants had less bright flanks ($\beta = 0.065$), front legs ($\beta = 0.214$), abdomens ($\beta = -0.134$) and tails ($\beta = -0.146$). There was no effect of elevated testosterone on back leg brightness ($\beta = -0.003$, figure 3.5.).

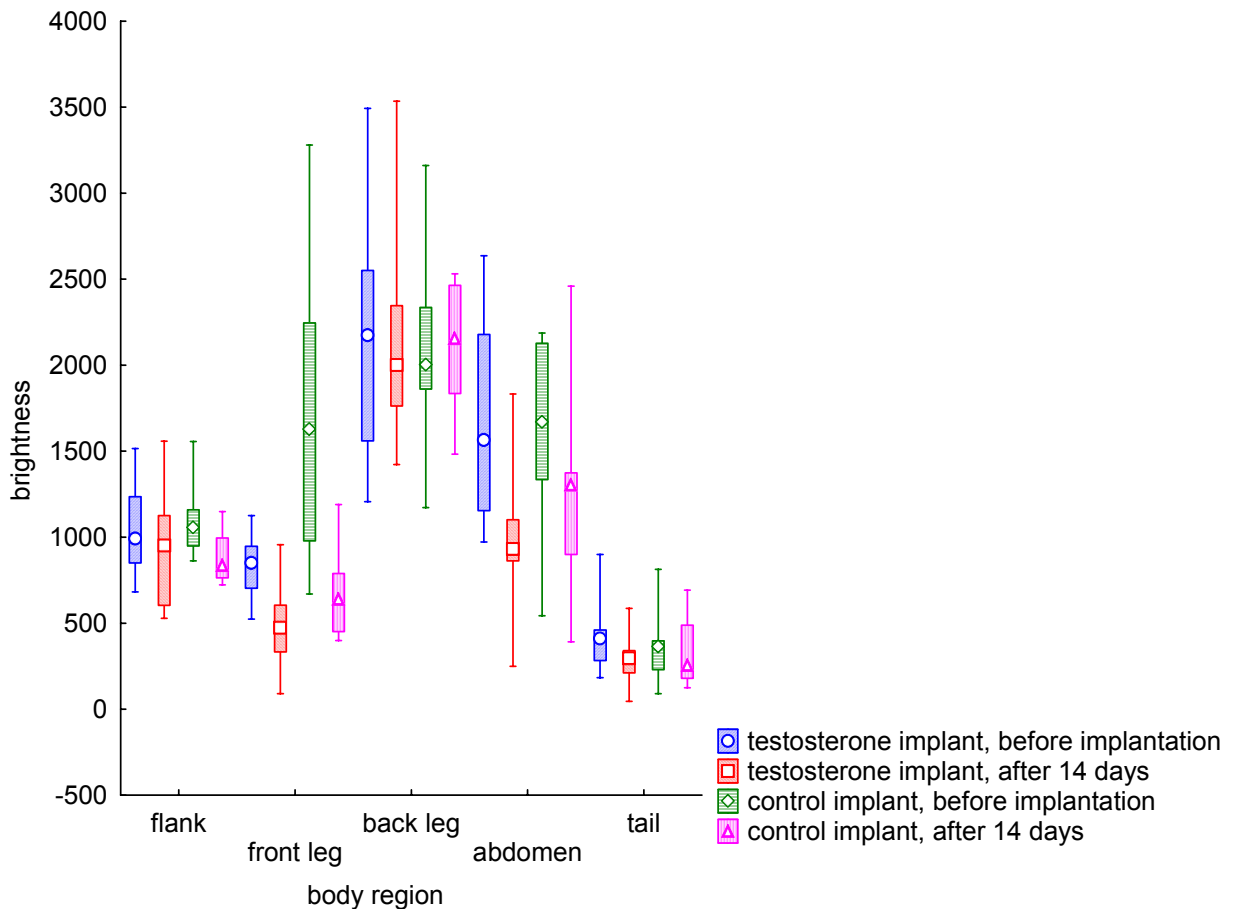


Figure 3.5. Implant type was a significant predictor of brightness in female *P. i. wilhelmi*, with females with testosterone implants having less bright flank, front leg, abdomen and tail regions two weeks after the insertion of implants than females with control implants. Values indicate medians with boxes showing interquartile range and whiskers showing total range.

Immune response was not predicted by experimentally elevated testosterone in female *P. i. wilhelmi* (Wald $\chi^2_1 = 0.362$, $p = 0.58$, figure 3.6).

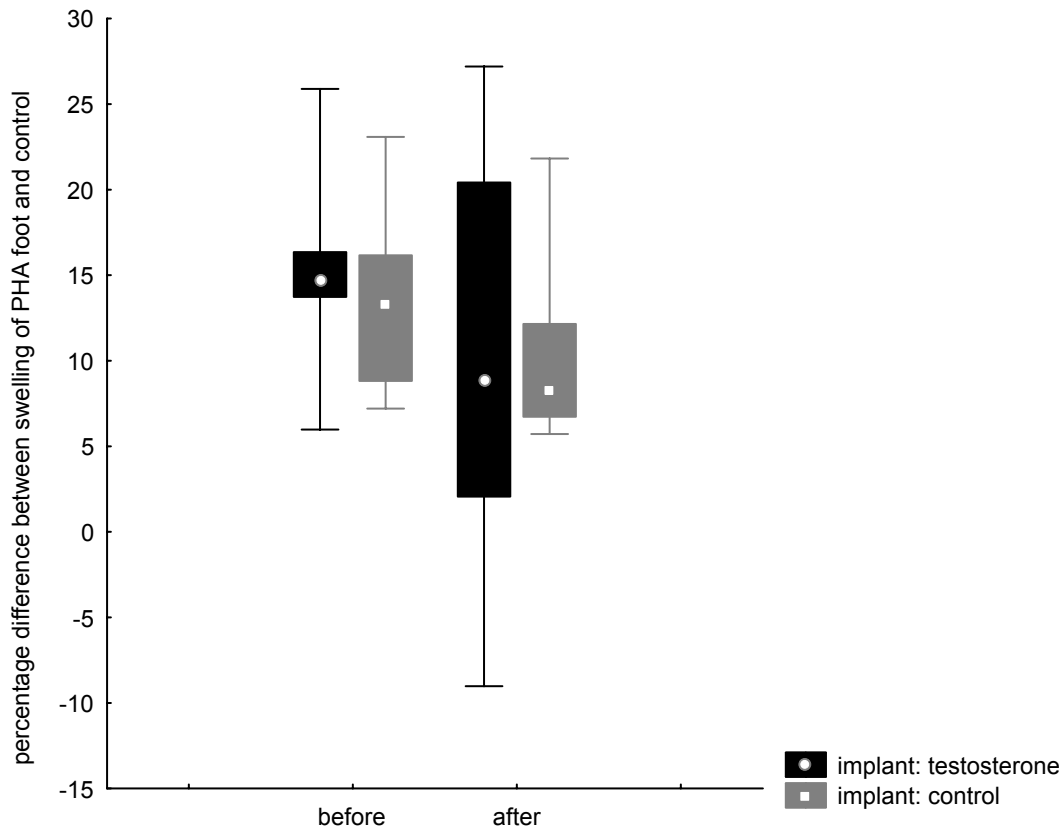


Figure 3.6. Experimentally elevated testosterone was not a significant predictor of cell-mediated immune response in female *P. i. wilhelmi*. Values indicate medians with boxes showing interquartile range and whiskers showing total range.

Experimentally elevated testosterone was a significant predictor of endurance (Wald $\chi^2_1 = 54.755$, $p < 0.001$). Female *P. i. wilhelmi* with testosterone implants outperformed control females implants 14 days after insertion of implants ($\beta = 0.283$, figure 3.7).

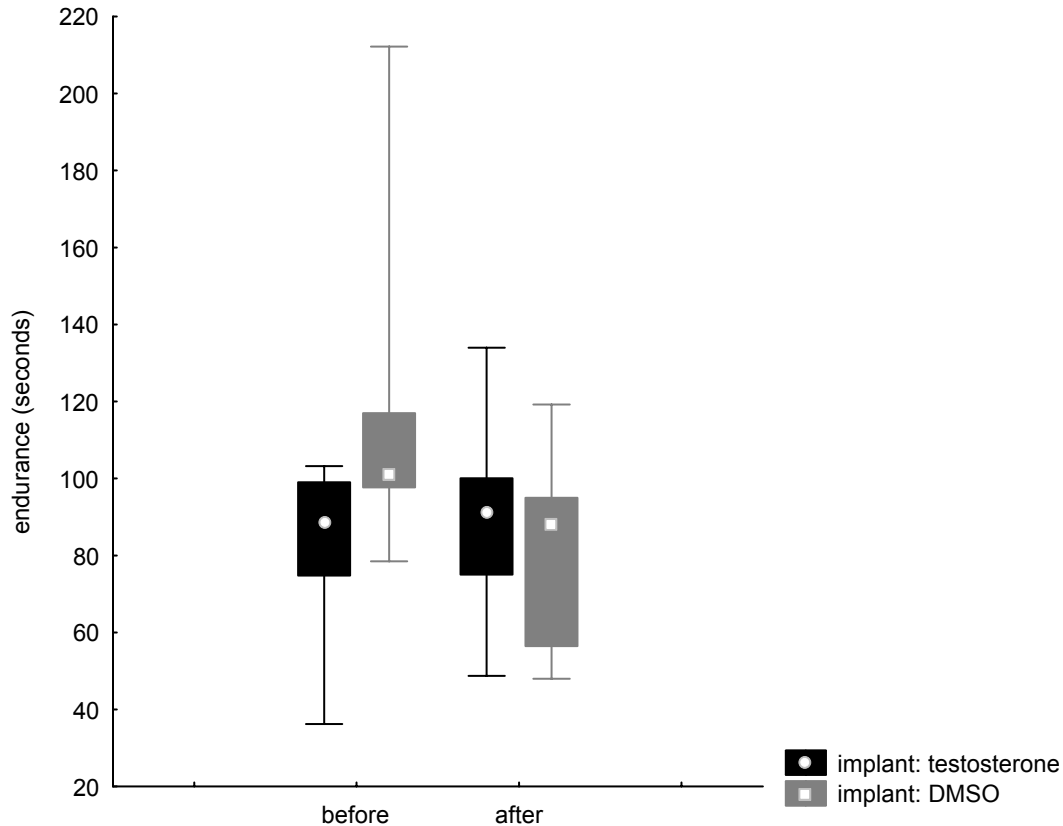


Figure 3.7. Female *P. i. wilhelmi* endurance was predicted by implant type. Lizards with testosterone implants outperformed control lizards 14 days after insertion of implants ($n = 18$ lizards per treatment). Values indicate medians and boxes show interquartile range. Whiskers show total range.

Discussion

The aim of my study was to determine whether testosterone affects females in the same way as it affects males in order to determine whether female sensitivity is a potential constraint to signalling or if there is some organisational effect at play that is worth investigating further.

Testosterone implants raised plasma testosterone in female *P. i. wilhelmi* to levels slightly higher (approximately 20% higher) than that of circulating testosterone in males during the breeding season (chapter 1). This was a 50-fold increase, but there was little

difference between females with testosterone implants and control females in immune response or body condition. Females with testosterone implants showed better endurance than control females as well as a decrease in brightness in the flank, abdomen, front leg and tail regions.

Male *P. i. wilhelmi* given testosterone under the same conditions (chapter 2) as in the current study showed improved endurance as well as brighter flanks and front legs and darker back legs, abdomens and tails than control males. The similarity in the effects of testosterone on endurance on males and females suggests that this is a condition-dependent trait that is controlled by testosterone. That this effect is activational rather than organisational makes sense since endurance is also related to the ability of male lizards to establish larger and/or better quality territories in the field (Garland et al., 1990). Endurance (and thus territory acquisition and defence) is a plastic response affected by testosterone levels. In a species which is highly aggressive and territorial it makes sense that their ability to defend their territories is governed by their state at that time rather than through developmental or genetic factors that are fixed during development. Consequently the costs of testosterone, endurance (and therefore territory size) should be an honest signal of a male's ability to withstand these costs.

The effects of experimentally elevated testosterone on female *P. i. wilhelmi* were to make the flank, abdomen, tail and front leg regions less bright than control females. While this response is similar in the effect on the abdomen and tail region as in males (chapter 2), the effect on the flank and front leg region is different. The darker colouration would make females more conspicuous against the light-coloured rock in their habitat than with their natural colouration. Darker colour would also make them more similar to males than their original pale blue-white ventral regions. In some species, females with experimentally elevated testosterone showed vast changes in a relatively short space of time (Lank et al., 1999). In other species females showed the development of some male traits, but not others, showing interplay between organisational and activational effects in phenotype expression. The fact that in this case females showed some change in

colouration suggests that some aspects of bright colours of the male flat lizards are under activational control.

Another possibility for the limited effects of testosterone on females is that the effects of testosterone in males are mediated through organisational pathways. As the results suggest that organisational factors might be constraining signal production, this suggests that a future study measuring the effects of testosterone in juveniles would be the final step in determining the extent to which this is happening.

Male *P. i. wilhelmi* in a similar experiment (chapter 2) showed changes in brightness in all body regions measured following the insertion of testosterone implants while chromatic contrast did not change at all. The similar response of females suggests that the chromatic aspects of colouration are fundamentally fixed in terms of sensitivity to testosterone through pathways that must have been laid down at some early stage in their development. Alternatively, since these colours do correlate to measures of male quality, there is a possibility that there are other factors mediating these ornaments such as different hormones or environmental factors, or else that they are genetically determined and therefore a fixed signal of genetic quality.

While the change in endurance is similar to that of males it is surprising that there were no measurable costs to the added testosterone. There is the possibility that females maintain significantly lower testosterone levels as it affects their reproductive cycle where it plays a role in vitellogenesis (Rhen et al., 1999). Another factor to consider in future studies would be the effect of testosterone on oestrogen levels, as oestrogen and testosterone can have directly opposing effects on physiology (e.g., Lund et al., 2004). Alternatively the increase in conspicuousness associated with bright colouration influenced by testosterone might be associated with increased predation pressure which may be too high for maintenance of high testosterone levels. Alternatively, Bateman's principle (Bateman, 1948) states that females and males differ in immune capability not because males have higher testosterone levels but rather due to differing life histories leading to different priorities in energy allocation. Because testosterone influences

muscle growth it is also possible that the energy required to maintain additional muscle may be too high for females to bear, particularly considering that they do not need to patrol territories or fight off intruders, and would be more likely to allocate extra energy to reproduction.

Female *P. i. wilhelmi* with testosterone implants showed improved endurance and overall darker colouration than females with control implants. This is a similar effect to that seen in males (chapter 2) and shows that these traits are under activational control, independent of sex. What is uncertain are the costs associated with high levels of testosterone as females showed no change in body condition or immune response associated with increased testosterone. There was also no effect of exogenous testosterone on the chromatic aspect of colour, which we know to be indicative of male quality (chapter 1). What remains to be investigated are alternative constraints on signalling that may be associated with testosterone. For example testosterone is known to affect the bioavailability of carotenoids (Blas et al., 2006) and in some cases leads to increased circulating carotenoids, which have immune-enhancing properties (McGraw et al., 2006a). As chromatic aspects of colour did not respond to exogenous testosterone in adults what remains is to test for organisational effects of testosterone by manipulating testosterone in juveniles. This would be impractical without the knowledge of the effects of testosterone on adult females, and it adds to a neglected aspect of behavioural endocrinology.

CHAPTER 4

Short-term effects of elevated testosterone, corticosterone and the interaction between testosterone and corticosterone in a lizard

Introduction

Animals often use signals to avoid the costs of aggressive encounters (Parker, 1974). These signals are common in males of sexually dimorphic species and are often attributed to high testosterone levels (e.g. Deviche & Cortez, 2005; Saraiva et al., 2010). In order for these signals which can indicate many aspects of male quality such as status, aggression or fighting ability, (reviewed in Whiting et al., 2003) to be maintained they must be honest and therefore carry some cost which inferior males are unable to bear (Zahavi, 1975). The immunocompetence-handicap hypothesis (ICHH) predicts that testosterone-mediated signals are constrained by the immunosuppressive effects of high circulating levels of testosterone (Folstad & Karter, 1992). However, a recent meta-analysis found that only about half of the studies that tested the ICHH showed any support for the model (Roberts et al., 2004).

Few studies empirically test either the direct immunosuppressive effects of testosterone or the effect of testosterone on signal expression. Many studies assume that since a species shows sexual dimorphism, any differences must be attributed to testosterone levels. Several studies have found that changing levels of testosterone does not affect signals (e.g. Husak et al., 2007), particularly in systems where the effects of testosterone are organisational, where costs are only incurred temporarily during development, or where changes are only evident at the life-history level (Ros et al., 2006). Similarly, the evidence for the immunosuppressive effects of testosterone is limited: while testosterone usually suppresses the immune system (Rantala et al., 2007), it has also been found to improve immunocompetence (Zuk, 1996; Veiga et al., 1998). Other studies have found that when controlling for corticosterone, testosterone actually benefits the immune

system, rather than suppressing it (Lindström et al., 2001). Recent studies have called for a more integrative approach to behavioural endocrinology since cause and effect are often confounded, such as in the case of parasite load, in which parasite load depends on many other factors such as exposure rates, risky behaviour, and home range size (Husak et al., 2009; Irschick & Garland, 2001). Signal expression and immunocompetence should therefore be examined in the context of whole animal performance, behaviour and physiology.

There are many other factors involved in the production of secondary sexual signals. Corticosterone has significant effects on signals, general health (usually measured using body condition as a proxy), and immunocompetence (e.g. Berger et al., 2005). Also, glucocorticoids (such as cortisol or corticosterone) are released within minutes of a stressful event (Wingfield, 2003) which allows the animal to cope mainly by mobilizing energy (Möstl & Palme, 2002) (reviewed in Lowry & Moore, 2006). Increased corticosterone levels can also result in increased stamina and reduced resting metabolic rate (Cote et al., 2006; Miles et al., 2007), and is generally associated with decreased reproduction (Dewsbury, 1982; Creel, 2001).

Levels of testosterone and corticosterone tend to co-vary suggesting the possibility of a dual influence on signal modulation. For example, in lizards with chronic stress, testosterone and corticosterone are inversely proportional, and corticosterone administered during an aggressive encounter, decreases aggression (DeNardo & Sinervo, 1994b). In a number of taxa, increased corticosterone levels result in a decrease in testosterone levels (reviewed in DeNardo & Sinervo, 1994b). The response of testosterone to changing corticosterone levels can differ greatly between individuals. In tree lizards (*Urosaurus ornatus*), experimentally restrained males had an increase in corticosterone, but floater males had a much larger concurrent decrease in testosterone than territorial males when restrained (Knapp & Moore, 1997). Territorial males can also have less of a stress response than floater males which may be linked to the higher frequency of agonistic encounters that they experience while defending a territory (Knapp & Moore, 1996). The stress-linked immunocompetence handicap hypothesis (SL-

ICHH) proposes that rather than testosterone levels constraining maximal signal output, co-varying corticosterone levels act as a constraint through an increase in energy requirements due to energy mobilisation and increased activity levels in animals with increased stress hormones (Owen-Ashley et al., 2004; Møller, 1995; Evans et al., 2000; Buchanan, 2000). This increase in energy expenditure is adaptive in terms of chronic, short-term stress, as it allows animals to remove themselves from stressful situations. In more chronic situations (such as maintaining territories during a breeding season), the costs of maintaining high testosterone and corticosterone levels is significantly greater than in acute stressful situations. One study found that neither hormone had any effect on the immune system in isolation, but a combination of the two did (Roberts et al., 2006).

Bartolotti (2009) proposed that corticosterone by itself acts as a signal constraint since males need to be of a significantly high quality to withstand the costs of elevated corticosterone while maintaining signals and territories. For example, comb area of male red grouse with elevated corticosterone responded less to testosterone implants than those of a control group with lower levels of glucocorticoids (Bortolotti et al., 2009a). Considering that these traits are often sexually selected, if corticosterone constrains signals, this would mean that females choose mates based on corticosterone-mediated than testosterone levels. In a population with the same/similar stressors, a male which can maintain ornaments will have demonstrated superior quality (indicative of underlying genetic quality) in coping with stressors (Bortolotti et al., 2009a; Moore et al., 2011). Female choice in zebra finches (*Taeniopygia guttata*) is linked to corticosterone levels in males (Roberts et al., 2007a). There is also a feedback loop where, rather than hormone combinations affecting immunocompetence, a challenge to the immune system can result in signals being downgraded. For example, where animals may increase territory size (where patrolling it requires greater energy sacrifice) and therefore face a combination of high energy requirements and increased pathogen exposure, the ability to maintain signals could be an accurate measure of male quality (Bortolotti et al., 2009a).

Male flat lizards are territorial and highly aggressive. They maintain territories on rocky outcrops where they have frequent encounters between rivals, which makes

corticosterone a likely factor to be involved in their behavioural physiology. They are sexually dichromatic, as is characteristic of this genus (Branch & Whiting, 1997) with males displaying striking green, blue, black and orange colouration. These colour patches have been found to correspond to various aspects of health and performance (chapter 1) and respond to testosterone manipulation to some degree (chapters 2 and 3).

The aim of my study was to examine the effects of testosterone, corticosterone and a combination of the two on body condition, signal (colour) expression, whole-animal performance, and immunocompetence. I predicted that if testosterone is the overriding factor in constraining signals due to immunosuppression (ICHH), males with experimentally elevated testosterone will show an increase in signal conspicuousness, a decrease in immune response, and an increase in locomotor performance while the converse would be true for males with elevated corticosterone. If the constraint on signalling is the interaction between testosterone and corticosterone, I predicted that males implanted with testosterone and corticosterone will show a greater decrease in immune response than control males or males implanted with only testosterone or only corticosterone. For males with both corticosterone and testosterone implants, I also tested the prediction that there would be no change in colouration or performance because their combined effects should counter-act each other.

Methods

As the methods used in this study are similar to previous studies in this thesis (chapters 1-3) detailed protocols have been omitted. Only novel methods have been included here.

Study system

Platysaurus intermedius wilhelmi is a small, sexually dimorphic lizard found on rocky outcrops in southern Africa. Males are both larger than females (Lailvaux et al., 2003b) and more colourful (Branch, 1998). Females are black and brown with straw-coloured tails while males have green flanks and front legs, greenish-blue back legs and blue abdomens. They have black throats with the black colouration continuing down the chest and occasionally onto the abdomen. Their throats are black and they have bright orange

tails. They have dorsally-compressed bodies, and they live on rocky outcrops where they are particularly suited to sleep in small crevices mainly made by granite domes which exfoliate in sheets. They breed in spring and early summer (Branch, 1998).

I conducted field work primarily at Pullen Farm (S 25.57°, E 031.18°) and some neighbouring farms, all in the Crocodile River Nature Conservancy, a 30 000 ha area situated approximately 30 km east of Nelspruit, South Africa.

Capture of lizards

I captured adult male *P. i. wilhelmi* on lines of glue traps (Catchmaster® 48R non-toxic glue traps: 27 x 13 cm; and Trapper® glue boards: 26 x 12 cm). I recorded each capture site with a GPS. For detailed handling protocol see chapter 1.

Elevation of testosterone and corticosterone

I made implants by filling 5 mm lengths of silastic tubing with 3 μL of a solution of either testosterone (100 μg testosterone μL^{-1}) or corticosterone (100 μg corticosterone μL^{-1}) in dimethyl sulfoxide (DMSO). I sealed the ends of each tube with silicon adhesive and the DMSO evaporated through the tubing, depositing the hormone in the lumen of the tube. Control implants were made in the same way but filled with pure DMSO (Cox et al., 2005a). Implants were inserted subcutaneously on the lizard's venter (for further details see chapter 2). This is an established method for elevating steroid concentrations in small lizards, and can elevate hormone levels for at least three months following implantation (Miles et al., 2007).

Husbandry

Lizards were housed individually according to protocols outlined in chapter 2. After a five day acclimatisation period, I measured performance, morphology and immunocompetence (described below) and then allocated lizards to one of four treatments. They were assigned to four treatment groups ($n = 20$ in each implant group) based on the type of implant they received. Lizards were given: (1.) one testosterone implant and a control (empty) implant; (2.) one corticosterone implant and one empty implant; (3.) one testosterone implant and one corticosterone implant; and (4.) two

control implants. After two weeks I took another blood sample from each lizard and measured the lizards again, before removing the implants and releasing lizards at the point of capture.

Morphological measurements

I measured snout-to-vent length (SVL) and weighed each lizard in order to calculate a body condition index as the residual on a simple regression of mass on $\ln$ SVL. For more details see chapter 1. I measured the size of black ventral patches by scanning the ventral surface of each lizard on a flat-bed scanner (Canon 2000U) using Omnipage SE (© 2002 Scansoft, Inc) software. I calculated the area with Simple PCI graphics software (Compix incorporated Sewickley U.S.A.) which I calibrated against known lizard SVL.

Visual modelling

I measured spectral reflectance of the tail, front leg, back leg, flank and abdomen of each lizard. Each measurement is an average of two readings and the spectrometer was calibrated between each lizard. I used microspectrophotometry data on a closely related lizard (*Platysaurus broadleyi*) (Fleishman et al., 2011) to create pigment templates for 4 cone type (2 short-wave cones, 1 medium wave and 1 long wave cone). From this, I calculated achromatic and chromatic measures of colour. Achromatic is concerned with brightness, which is the total reflectance of the colour. For chromatic aspects of colour I calculated the Euclidean distance between the colour measured and a measure of the rocky habitat. This method standardises brightness so that the measures are independent of each other. For further details see chapter 1.

Measure of testosterone and corticosterone

I measured corticosterone and testosterone in plasma that I obtained from blood samples taken from the suborbital sinus. I took blood samples five days after capture once lizards had begun to habituate to captivity and then took another sample from each lizard 14 days after insertion of implants (for protocol see chapters 1 and 2). In all cases, I recorded the time from touching the box (in which they were housed) to remove the lid until the end of sampling in order to control for confounding effects of handling stress. No sample took

longer than three minutes to obtain, and a regression of sampling time and corticosterone was not significant (Spearman $r = 0.33$, $p > 0.05$).

I assayed testosterone by using DPC Coat-A-Count Total Testosterone kits following a pilot assay with experimental dilutions of different samples in order to find the optimal dilution ratio. These kits have been validated with plasma from closely related lizards (Whiting et al., 2006). Corticosterone was analysed using an ELISA kit (Biocom Biotech), with dilutions optimised using previously established protocols according to Wada et al (2007) For more detail see chapter 1.

Measures of immunocompetence

I measured a delayed-type hypersensitivity to phytohaemagglutinin (PHA) in order to measure immunocompetence. I compared swelling of a foot injected with 0.03 ml phosphate buffered saline (PBS) containing 50 mg of PHA (Sigma, L-8754) to swelling of a foot injected with pure PBS. The degree of swelling indicates cell-mediated immune response. For details see chapter 1.

Endurance

I measured endurance as the time a lizard walked on a belt (30 x 40 cm) moving at 0.4 km.h⁻¹ until it failed to maintain its position three times. Each lizard was measured twice, with trials 24 hours apart. There was a significant effect of body temperature on endurance (Wald $\chi^2_1 = 164.598$; $p < 0.001$) and was therefore included as a covariate in the analyses (below).

Statistical analyses

I used a Generalized Linear Model (GLZ) with a logit link function and binomial error structure to analyse the data from lizards at the beginning of the experiment and two weeks after the insertion of implants. I used the time of the measurement as the dependent variable, the implant type as a categorical predictor and the measurement in question as a count variable. I used β -estimates to determine the precise location of differences and in the event of a significant β -estimate I used descriptive statistics to

gauge the direction of change. In all cases, α was set at 5%, with p-values of 0.05 and smaller considered significant. All graphs show medians with interquartile ranges.

Ethical Note

This study was approved by the Animal Ethics and Screening Committee of the University of the Witwatersrand. Clearance numbers; carried out under 2008/40/04, with a provincial permit from the Mpumalanga Parks Board MPB5211.

Results

Efficacy of implants

Testosterone implants significantly predicted circulating testosterone levels in lizards (Wald $\chi^2_2 = 29226.63$; $p < 0.001$, figure 4.1). Males with one testosterone implant and one empty implant showed an increase of approximately 8-fold in circulating testosterone ($\beta = 1.882$). The highest recorded testosterone concentration was approximately three times the average natural peak summer testosterone (chapter 1). Males with one testosterone implant and one corticosterone implant showed an 80% increase in testosterone ($\beta = 0.226$) while males with only empty implants showed a significant decrease in testosterone levels ($\beta = 2.108$).

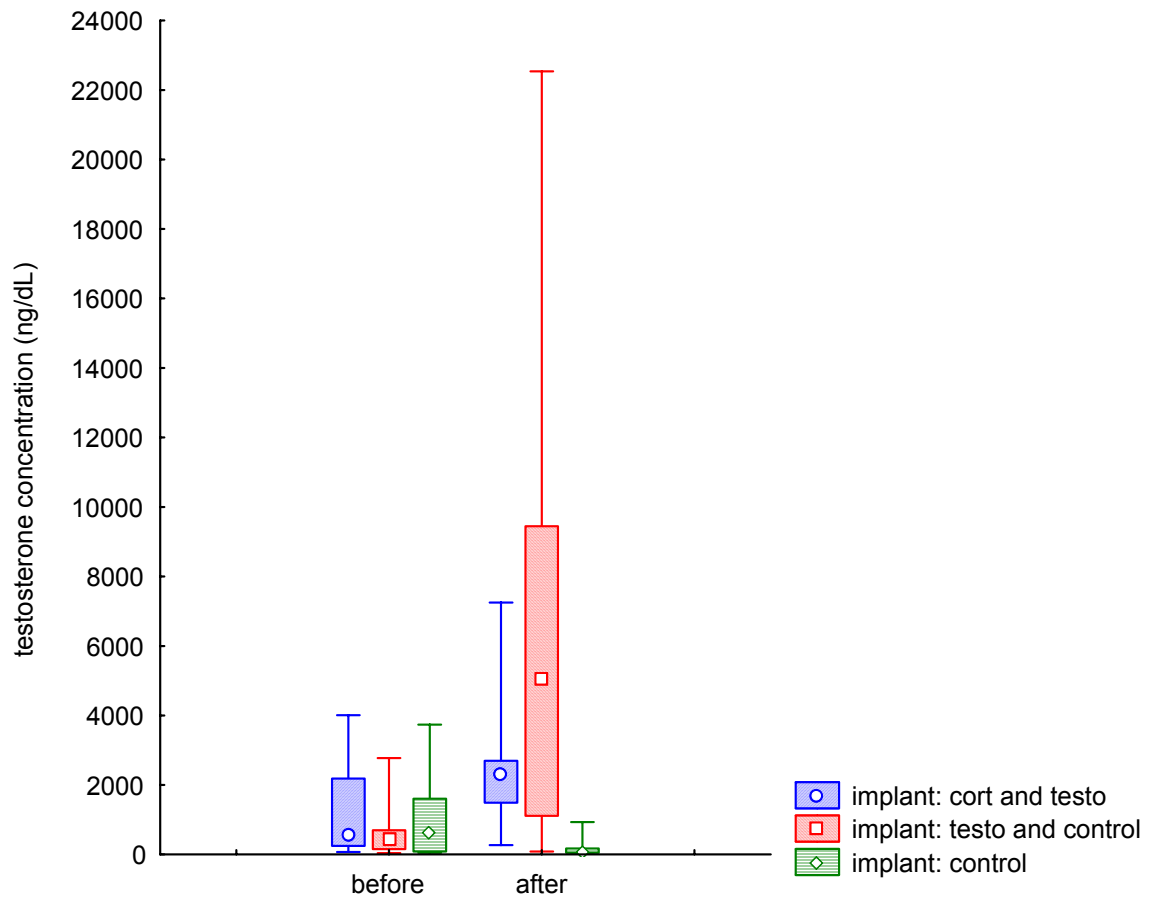


Figure 4.1. Testosterone levels in male *P. i. wilhelmi* before and two weeks after insertion of hormone implants. Lizards with testosterone only showed an eight-fold increase in circulating testosterone two weeks after insertion of implants, and lizards with one testosterone and one corticosterone implant increased circulating testosterone by approximately 80%. Values shown are medians with boxes indicating interquartile range and whiskers showing total range

Corticosterone levels were also predicted by the combination of implants (Wald $\chi^2_2 = 81.89$; $p < 0.001$). *Platysaurus i. wilhelmi* males with one corticosterone implant and one empty implant showed the largest increase in circulating concentrations of approximately 250% ($\beta = -0.671$) and lizards with one testosterone implant and one corticosterone implant showed an increase of about 150% ($\beta = -0.362$). Control males, with two empty implants showed a slight decrease in corticosterone levels over the course of the experiment ($\beta = 1.033$, figure 4.2).

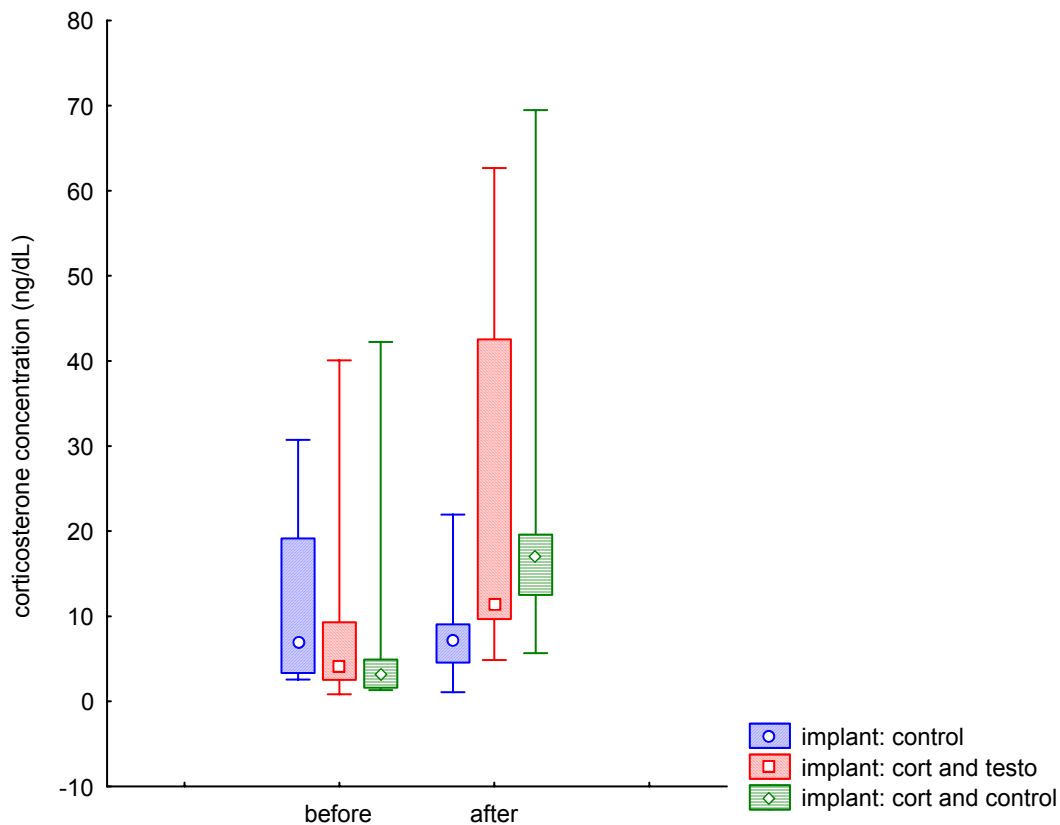


Figure 4.2. Corticosterone levels in male *P. i. wilhelmi* before and two weeks after insertion of implants. Lizards with corticosterone implants showed the greatest increase in corticosterone levels, while lizards with one testosterone implant and one corticosterone implant showed a smaller increase. Values indicate medians, boxes show interquartile range, and whiskers show total range.

Body condition

Implant type was not a predictor of body condition (Wald $\chi^2_3 = 0.223$; $p = 0.97$, figure 4.3), indicating that elevated testosterone and/or corticosterone did not affect it.

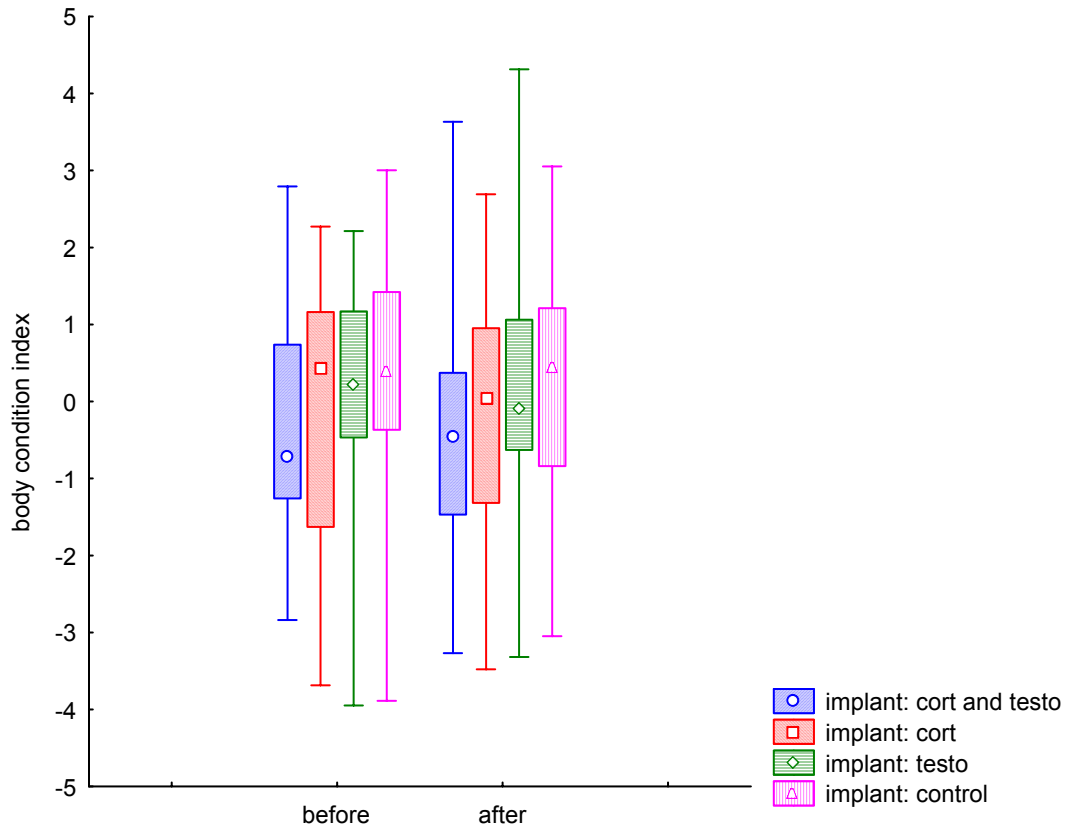


Figure 4.3. Implant combination was not a predictor of body condition in male *P. i. wilhelmi*. Values represent medians with boxes showing interquartile range and whiskers showing total range.

Effects of manipulation on signal intensity

Implant type did not predict chromatic contrast of any of the body regions (flank Wald $\chi^2_3 = 0.004$; $p = 0.99$; front leg Wald $\chi^2_3 = 0.002$; $p = 0.99$; back leg Wald $\chi^2_3 = 0.003$; $p = 0.99$; abdomen Wald $\chi^2_3 = 0.0009$; $p = 0.99$; tail Wald $\chi^2_3 = 0.006$; $p = 0.99$, figure 4.4)

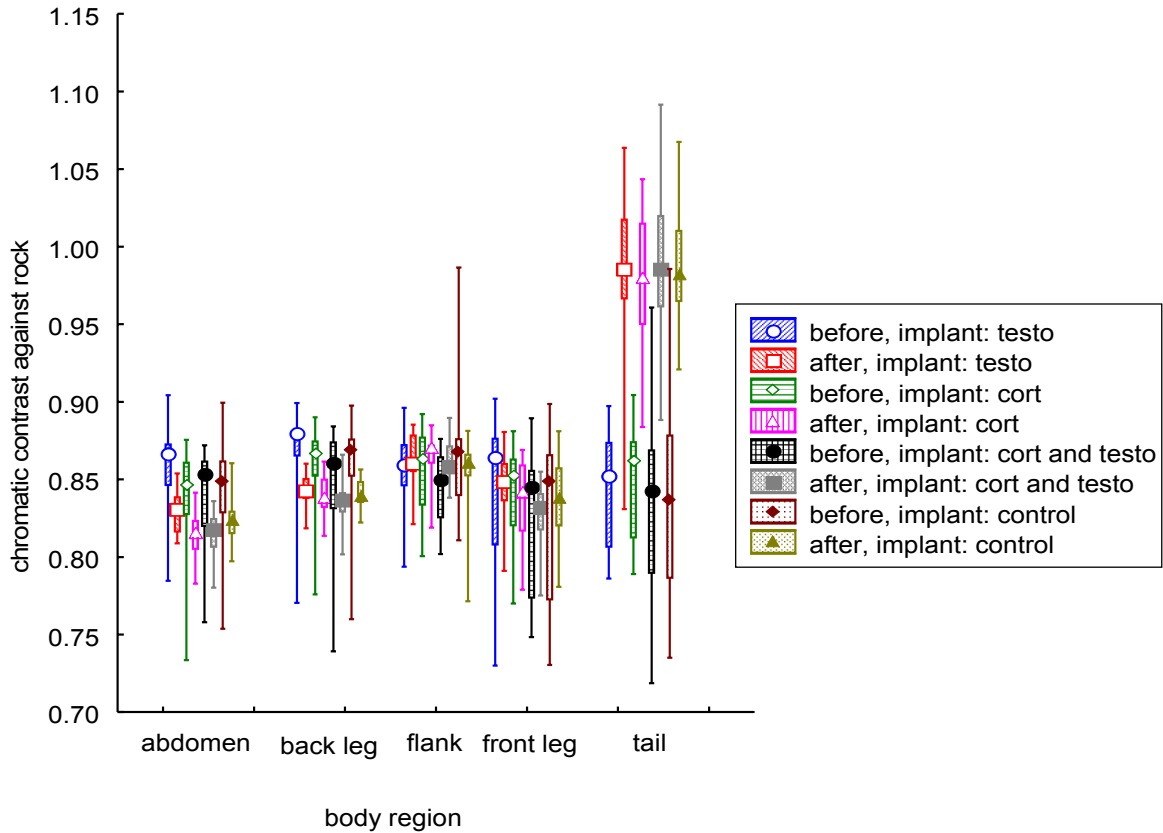


Figure 4. 4. There was no effect of implant type on chromatic contrast in male *P. i. wilhelmi*. Values represent medians, with boxes showing interquartile range and whiskers showing total range.

There were several significant effects of different implant combinations on colour patch brightness (see table 4.1, figure 4.5): Males with testosterone implants showed a significant increase in brightness of the abdomen and back leg, and a decrease in flank brightness. Males with corticosterone implants showed an increase in back leg, front leg and tail brightness and a decrease in abdomen brightness. Males with one testosterone and one corticosterone implant showed a decrease in abdomen brightness and a slight decrease in tail brightness.

Table 4.1: Statistical outputs from GLZ models comparing effects on brightness of different implant combinations. Implant combinations are given as: Cort (corticosterone), Tcort (testosterone and corticosterone), T (testosterone) and control.

Body region	Wald χ^2	p	β	Direction of change
Flank	119.82	< 0.001	0.031	Cort: no change
			0.081	Tcort: brighter
			0.063	T: darker
			0.049	Control: darker
Front leg	37.33	< 0.001	0.009	Cort only: no change
			0.061	Tcort: less bright
			0.003	T: no change
			0.068	Control: no change
Back leg	108.26	< 0.001	0.023	Cort: brighter
			0.080	Tcort: much brighter
			0.082	T: no change
			0.025	Control: much brighter
Abdomen	49.70	< 0.001	0.065	Cort: less bright
			0.074	Tcort: least bright
			0.020	T: no change
			0.029	Control: brighter
Tail	27.17	< 0.001	0.022	Cort: no change
			0.009	Tcort: no change
			0.057	T: much darker
			0.045	Control: brighter

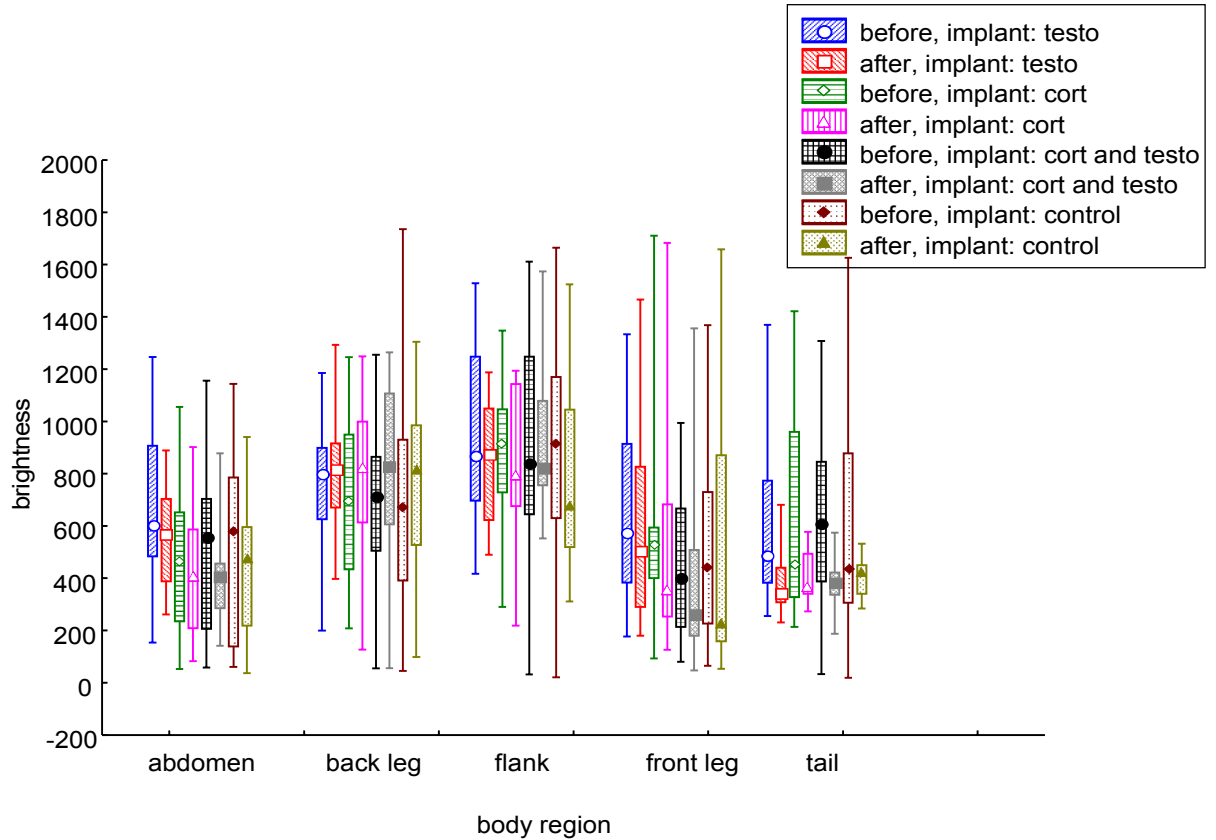


Figure 4.5. Colour patch brightness before and two weeks after the insertion of hormone implants in male *P. i. wilhelmi*. Lizards with only testosterone implants had brighter abdomen and back leg patches and darker flanks. Colour patches in lizards with corticosterone implants increased in brightness of the back leg, front leg and tail colour, and had darker abdomens following manipulation. Lizards with elevated testosterone and corticosterone had darker abdomens and slightly darker tail colour two weeks after receiving implants. Values indicate medians with boxes showing interquartile range and whiskers showing total range.

Snout-vent length was significantly related to the area of the black throat patch (Wald $\chi^2_1 = 53.903$; $p < 0.001$), so I used it as a continuous predictor in the model. Black throat patch area was predicted by implant combination (Wald $\chi^2_3 = 63.962$; $p < 0.001$, figure 4.6). Males with only corticosterone implants showed a marked decrease in area of the throat patch ($\beta = 0.075$) and males with one testosterone implant and one corticosterone implant showed a slight decrease in patch area ($\beta = 0.061$). Males with testosterone ($\beta = 0.029$) or control ($\beta = 0.166$) implants did not show a significant change in area.

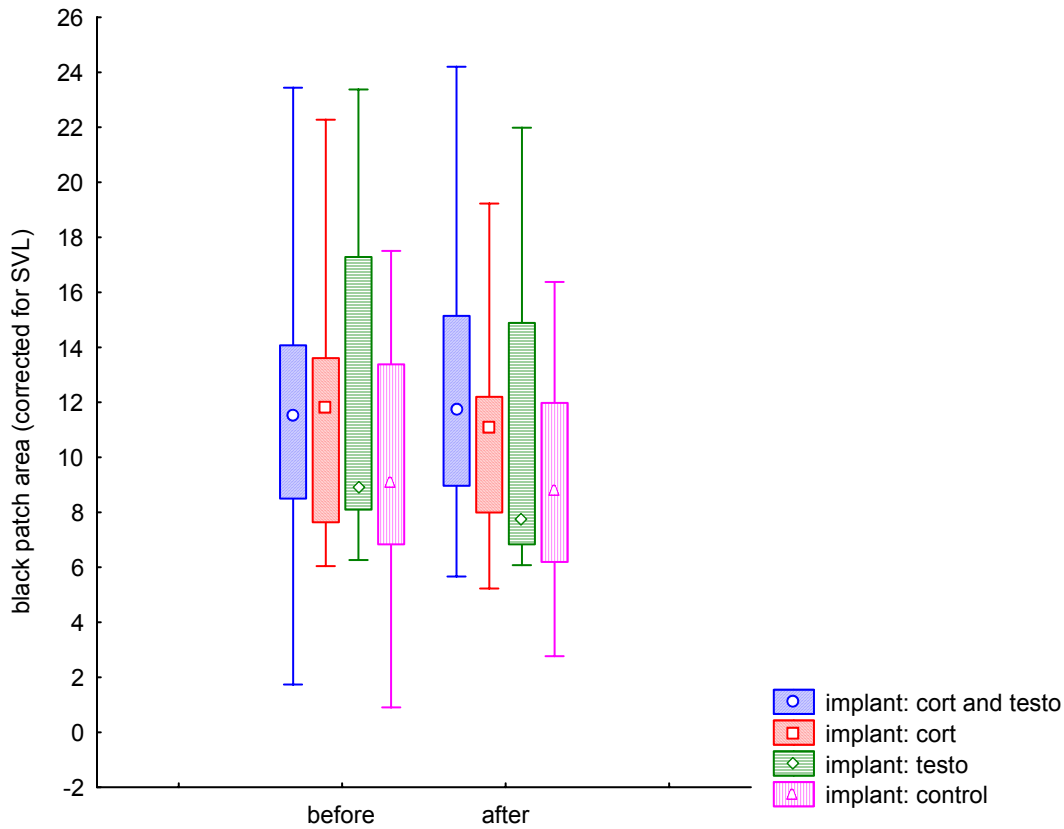


Figure 4.6. Black throat patch are in *P. i. wilhelmi* before and two weeks after insertion of corticosterone (cort), testosterone (testo), a combination (cort and testo) or control implants. Values shown here are medians, wiith boxes indicating interquartile range and whiskers showing total range.

Immune response

Implant type did not predict delayed-type sensitivity response to phytohaemagglutinin (Wald $\chi^2 = 1.115$; $p = 0.77$, figure 4.7).

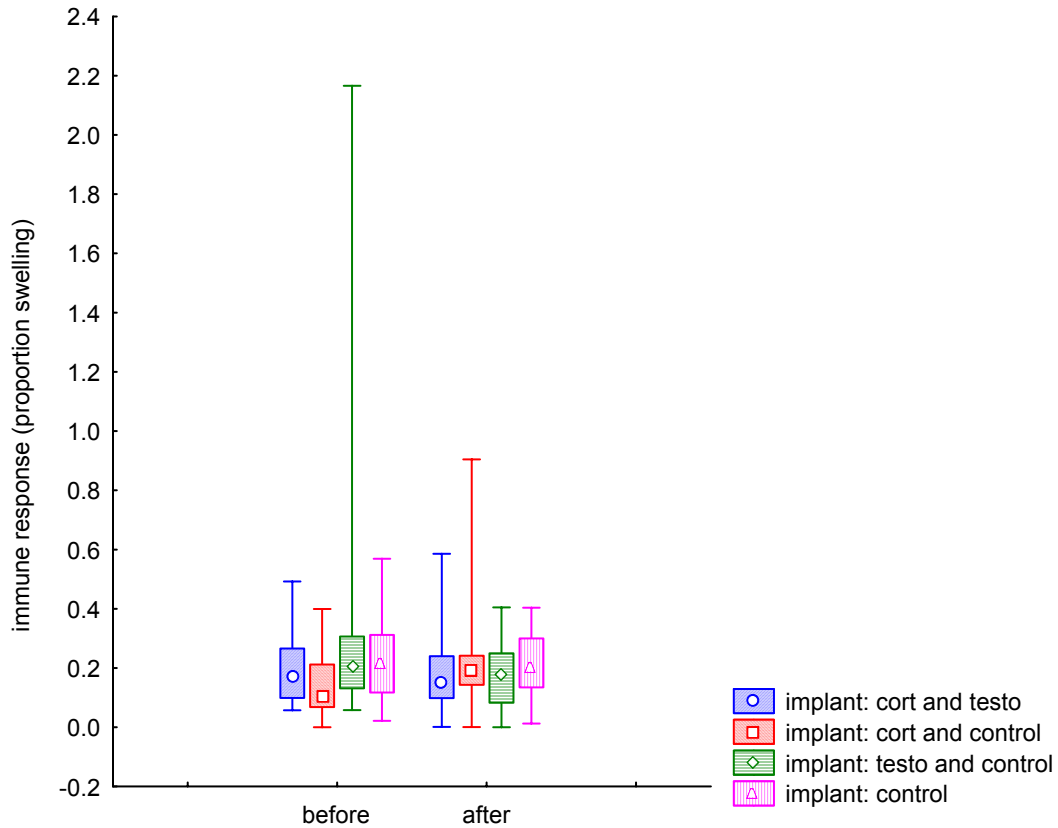


Figure 4.7. Cell-mediated immune response to phytohaemagglutinin in male *P. i. wilhelmi* before and two weeks after insertion of implants. Values indicated are medians with boxes showing interquartile range and whiskers showing total range.

Implant type was a significant predictor of endurance (Wald $\chi^2_3 = 20.434$; $p < 0.001$, figure 4.8). The only group in this study that did not show a significant change in endurance was the group of *P. i. wilhelmi* with one testosterone and one control implant ($\beta = 0.035$). The groups of lizards with control implants showed the smallest decrease in endurance ($\beta = 0.104$) and the group with corticosterone only, showed the largest decrease ($\beta = 0.082$). The group with one testosterone and one corticosterone implant was intermediate to the control group and the corticosterone only group ($\beta = 0.012$).

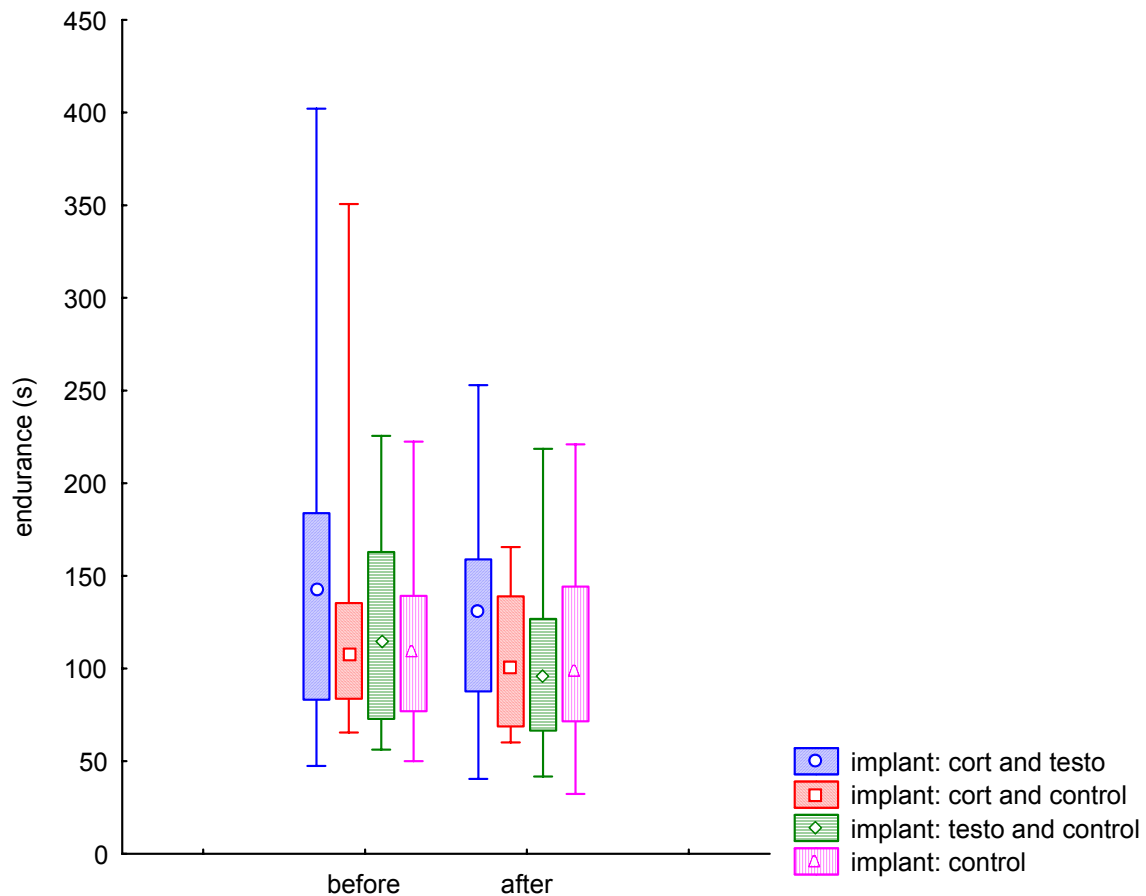


Figure 4.8. Endurance of male *P. i. wilhelmi* before and two weeks after the insertion of hormone implants. Values represent medians while boxes show interquartile range and whiskers show total range

As the purpose of this study was to test the predictions laid out by the ICHH, SL-ICHH and the corticosterone-only models of constraints on signalling, I have compared the results from manipulations on *P. i. wilhelmi* with the predictions made from current

understanding of the associated hypotheses. While none of the existing models fit the results perfectly, there is some overlap between predictions and study outcomes (see table 2).

Table 4.2: Comparing predictions and outcomes of the three hypotheses: the ICHH, SL-ICHH and corticosterone-only, to results seen in *P. i. wilhelmi*

Overriding factor	Testosterone only		Testosterone x Corticosterone		Corticosterone only	
	Prediction	Result	Prediction	Result	Prediction	Result
Body condition	↑	-	-	-	↓	-
Immune response	↓	-	↓↓	-	↓	-
endurance	↑	↑	-	↑	↓	↓
colour	↑	↑↓	-	↑↓	↓	↑↓

Discussion

The aim of my study was to test predictions of the immunocompetence handicap hypothesis, the stress-linked immunocompetence handicap hypothesis and the corticosterone-only hypothesis. *Platysaurus i.wilhelmi* males with testosterone implants showed improved endurance and a brighter abdomen and back legs and darker flanks two weeks after insertion of implants compared to before implants. Lizards with only corticosterone implants showed a dramatic decrease in endurance and their back legs, front legs and tails became brighter following insertion of implants than at the start of the experiment. Their throat patches also became smaller and their abdomens became less bright. There was no effect of any implant combination on immune response or body condition. Lizards with testosterone and corticosterone implants had darker abdomens and tails. Lizards with testosterone implants ran for longer than all other treatment groups, while lizards with only corticosterone implants performed the worst and lizards

with both testosterone and corticosterone showed endurance levels intermediate to the testosterone and corticosterone groups.

Lizards with testosterone implants showed a much greater increase in circulating testosterone levels than lizards with a combination of testosterone and corticosterone implants. The increase in testosterone was also much greater than the relative increase in corticosterone even though the implants were made in exactly the same way, with equal concentrations of testosterone and corticosterone. This suggests an *in vitro* effect, possibly through suppression of their endogenous hormone production through feedback loops. There is evidence of high corticosterone levels suppressing the production of testosterone (Nelson, 2000; Moore et al., 1991) as well as effects of gonadal steroids on the HPA axis and associated stress response (Viau, 2002) and this may explain the discrepancy in testosterone levels between the testosterone group and the testosterone and corticosterone groups.

One of the key predictions of the stress-linked immunocompetence handicap hypothesis is that a major cost of elevated corticosterone is in energy mobilisation (Lowry & Moore, 2006). I did not detect any significant change in body condition in any treatment. If a stress response requires a costly energy mobilisation, then a change in body condition, as energy reserves are accessed, would have been apparent. I also expected a change in body condition with the administration of testosterone, as it increases metabolic rate (Husak et al., 2006c) which would suggest similar effects of testosterone and corticosterone on body condition. Because there was no difference between hormone treatments and control animals, therefore the costs of testosterone and corticosterone are not likely to be directly due to energy requirements. Another study on free-ranging males of this species found that body condition did not change with experimentally elevated testosterone (chapter 2), so it is unlikely that effects were ameliorated by the provision of food to lizards in captivity.

Neither testosterone nor corticosterone seems to play a role in plasticity of chromatic contrast, which means that either this component of colouration is static after

development (i.e. an organisational effect) or else there are other factors controlling chromatic aspects of colouration. Endocrine effects on brightness are quite complex: testosterone and corticosterone both have an effect on brightness but they seem to influence different body regions. To add further complexity, lizards with both testosterone and corticosterone implants did not show the combined effects of both other treatments. For example in zebra finches (*T. guttata*) a combination of the two hormones has a different effect than the sum of the effect of each hormone individually (e.g. Roberts et al., 2007c). This suggests that the interaction between the two hormones acts differently to the hormones individually.

Corticosterone implants resulted in a decrease in black throat patch area. *Platysaurus i. wilhelmi* live on light-coloured granite outcrops and often display by holding a pushup pose and displaying their throats against the rocky backdrop (pers obs). The black contrasts very strongly against the rock, which is light-coloured granite, and therefore it might be an indicator of male quality. If so, this fits the model of corticosterone being the sole limiter of a signal, as testosterone did not affect patch area at all.

I found no evidence of immunosuppression either due to testosterone or corticosterone. The immunocompetence handicap hypothesis predicts that the major constraint to testosterone-based signalling is in its suppression of the immune system (Folstad & Karter, 1992). Also, I previously demonstrated that testosterone was linked to immunosuppression only in free-ranging *P. i. wilhelmi* (chapter 2). The prediction that this pattern may be stress-mediated through linked fluctuations in corticosterone does not hold in these manipulations, which suggests that there are other factors involved here which are not apparent in a captive situation. While testosterone had an effect on the brightness of some body regions as well as improving locomotor performance, it had no effect on immunocompetence. While immunosuppression is often predicted to be the mechanism of signal constraint, there are other costs to maintaining high levels of corticosterone and testosterone (such as energetic costs) and so while the predictions of the immunocompetence-handicap hypothesis do not hold in terms of the mechanism

controlling signal honesty, they clearly play a role in signal mediation even if the mechanism is not entirely clear yet.

In terms of locomotor performance, all of my predictions were supported: testosterone had a positive effect and corticosterone had a negative effect on lizard endurance. The combination of the two showing an intermediate effect does fit in with the existing theory of the effects of corticosterone and testosterone counteracting each other. This has been found before particularly with regard to aggressive behaviour (e.g., Denardo & Licht, 1993) Endurance has important implications in male dominance (Perry et al., 2004) as well as territory acquisition and maintenance and so animals with low testosterone or under high stress may not be able to maintain their status.

The purpose of this study was to test three different constraints on signalling based on three existing models of: the ICHH where testosterone-modulated signals of male quality are constrained by testosterone-linked immunosuppression; the stress-linked immunocompetence-handicap hypothesis (SL-ICHH) where co-varying corticosterone levels respond to elevated testosterone levels and carry an energetic cost to mounting a stress-response; and the hypothesis that costs of maintaining high corticosterone levels act as a constraint on signals of male quality. All three hypotheses have mixed support in the literature, but they are not usually all tested at the same time. This multi-model approach allows us to examine multiple effects of hormones that may be acting as signal mediators and to investigate their interactions, which provide a new perspective in animal signalling theory. I show that none of these models (the ICHH, SL-ICHH and corticosterone-only models) are able to explain all of the results completely, because no single factor regulates signal expression and therefore all of these factors should be taken into account rather than choosing a single model. While there was no effect of testosterone or corticosterone on immune response, testosterone, corticosterone and a combination of the two had different effects on colouration, particularly the change in black throat patch area with elevated corticosterone. The field of behavioural endocrinology has recently started considering the links between performance, morphology and the endocrine system as multifaceted and multidirectional feedback

loops with many complex interactions with dynamic costs taking place to regulate sexually selected traits (Rubenstein & Hauber, 2008). While the traditional hypotheses such as the immunocompetence-handicap hypothesis and the stress-linked immunocompetence handicap hypothesis are useful models for generating testable predictions, it is becoming more conventional to consider that honest-signalling models involve feedback loops rather than simple cause and effect (Safran et al., 2008). It is increasingly apparent that these systems are much more complex than originally thought.

CHAPTER 5

Testing the effects of carotenoids on condition-dependent signals in a lizard

Introduction

The immune system is often modulated and enhanced by carotenoids (Alexander et al., 1985), which are common yellow, orange or red pigments (Weedon, 1965; Goodwin, 1984). Carotenoids are fat-soluble hydrocarbons that can only be synthesised by photosynthetic organisms, some bacteria and fungi (Goodwin, 1984), and animals must obtain them from their diet (Hill et al., 2002). Carotenoids protect against cancer and tissue damage by removing and blocking free radicals (Ames, 1983; Bendich & Olson, 1989; Krinsky, 1989; Prabhala et al., 1990; Saino et al., 1999; Hill, 1999; McGraw, 2005) which is crucial to the health of an individual (McGraw, 2005). Carotenoids form the precursors to vitamin A (Hill, 1999) which is associated with decreased cancer risk, increased lung function and improved immune response in multiple animal species (reviewed in Bendich, 2004). They are usually limited in the environment (Hill, 1999) and sometimes require foraging for unusual food items. For example, the Egyptian vulture (*Neophron percnopterus*) obtains carotenoids from eating ungulate faeces (Negro et al., 2002). Dietary intake can be used as a predictor of carotenoid concentration (Negro et al., 2000; Hill et al., 2002), although some species have more efficient absorption mechanisms than others (Tella et al., 2004). Carotenoids are not only responsible for yellow, orange and red colouration but they can also form chemical complexes with proteins which can lead to purple, blue or green colours (reviewed in Svensson & Wong, 2011)

Animals use signals to communicate aspects of their condition and quality to individuals around them. Signals are usually used in behavioural displays (LeBas & Marshall, 2000) and can provide information on aspects of male condition and quality such as health, immunocompetence (e.g., McGraw & Ardia, 2003), aggression, fighting ability (Olsson, 1994) and dominance status (e.g., Thompson & Moore, 1991). In order for these signalling systems to be effective there must be some cost associated with them (Zahavi,

1975). Until recently, most of the factors in models associated with quantifying costs of signals have involved signal-constraining factors that are detrimental to an individual's health and survival. For instance, the immunocompetence-handicap hypothesis posits that testosterone-mediated traits are modulated by the immunosuppressive action of testosterone (Folstad & Karter, 1992). The stress-linked immunocompetence-handicap hypotheses states that these traits are modulated by changes in corticosterone which can have detrimental effects on health, particularly when at high concentrations over an extended period of time (Møller, 1995; Evans et al., 2000).

In some cases the factor modulating a signal is actually beneficial to the animal, but limitations in supply mean that allocating this resource to a signal makes it unavailable to other physiological roles. This constraint on allocation of the modulating factor serves to limit its use in signals (Olson & Owens, 1998). An example of this kind of trade-off is in signals that require active broadcast, such as vocalising or display behaviour. These active signals require energy allocated to signalling that has to be diverted from other physiological processes (Andersson, 1994). An extreme example is the electric discharges used by gymnotiform electric fish (*Brachyhypopomus gauderio*), which require the reduction of energy allocated to other physiological functions in order to maintain signal output reducing energy used in other metabolic functions such as growth or reproduction (Stoddard & Salazar, 2011). The oxidation-handicap model suggests that there is a trade-off in the allocation of carotenoids to immune function (largely as antioxidants) and locking them away in ornaments (Lozano, 1994). There are other costs to signalling than directly harmful effects of signal-modulators. Most studies concerning the role of carotenoids on condition-dependent signalling have been on birds, where costs could be temporary because carotenoids used in feathers may only be allocated to signals for a short period of time (after a moult) (Navara & Hill, 2003). Some studies on birds do concern ornaments that are not in feathers, such as bill colour in which carotenoid-based colouration is likely to be an honest signal (Gray, 1996), because if the animal is not in optimal condition, carotenoids that could have been used in colour are mobilised from the skin and used in the immune system instead (Lozano, 1994; Tella et al., 2004), and so colour expression can be constrained by immunological processes (Hill, 1999).

There is a complex relationship between oxidative stress and carotenoids (Bendich, 2004; Svensson & Wong, 2011; Bendich & Olson, 1989). Carotenoids increase antioxidant defenses (cell-mediated response is correlated with plasma carotenoid levels) (Alonso-Alvarez et al., 2004) which reduce oxidative stress. Carotenoids also boost immune response (e.g., Blount et al., 2003) which causes an increase in free radical production (Costantini, 2008). Taxa may differ in their carotenoid use, ability to absorb or manipulate carotenoids as well as the types of circulating carotenoids. As such, the importance as well as the specific roles of carotenoids are likely to differ vastly among species (Pérez-Rodríguez, 2009). Recent studies have even suggested that carotenoids are insignificant as antioxidants in certain animal taxa (e.g., Perez-Rodriguez et al., 2008; Losdat et al., 2010). The handful of species used in most carotenoid studies (largely bird species) means that there is a need for studies on more, different, taxa in order to understand the relationships between immune response, carotenoids and signals clearly (Svensson & Wong, 2011) as well as to understand the phylogenetic signal of carotenoids in different systems.

Flat lizards (*Platysaurus*) are a good system to test for the influence of carotenoids in signalling because they are strongly sexually dimorphic with brightly coloured males and drab females, as is characteristic of this genus (Branch & Whiting, 1997). The closely related *P. broadleyi* defends territories, adopts alternate reproductive tactics, and uses colour to signal fighting ability (Whiting et al., 2003; Whiting et al., 2006). They have dynamic signals (compared to fixed colouration in bird feathers) as well as bright and easily measurable colouration. They also have access to carotenoids from figs (*Ficus* sp) which are found scattered along the edge of the rocky outcrops, which they inhabit.

The aim of my study was to investigate the relationship between carotenoids, signal expression, and immunocompetence, and to investigate the effects of changing carotenoid levels on performance and body condition in adult male *P. i. wilhelmi* lizards. If carotenoids are a limiting resource mobilized for immunocompetence and signal production and maintenance, then there are two testable hypotheses: (1.) carotenoids

should be in limited supply and so experimental supplementation of these pigments should produce a visible change in male *P. i. wilhelmi* phenotype; (2.) As the oxidative stress hypothesis involves a trade-off between two positive outcomes, it is expected that carotenoids can have advantages that are not related to signaling (such as endurance and immunocompetence) (adapted from Svensson & Wong, 2011).

I predicted that following carotenoid supplementation, male *P. i. wilhelmi* would show better condition and an improvement in endurance and immunocompetence. As more carotenoids would be available to allocate to ornaments I predicted that male colour would become more conspicuous against its background.

Methods

Study system

Platysaurus intermedius wilhelmi is a small (maximum snout-vent length 83 mm) cordylid lizard found on rocky outcrops in southern Africa (Branch, 1998). They have dorsally compressed bodies which allows them to fit into narrow crevices which are common in their habitat of smooth exfoliating granite. Fig trees (*Ficus sp.*) are found along the edges of some areas of the outcrops and lizards tend to gather near them and forage for insects. *P. i. wilhelmi* show sexual dimorphism both in size, with males larger than females (Lailvaux et al., 2003b), and also in colouration. Males have bright green flanks and front legs, blue and black venters and back legs and orange tails whereas females and juveniles are drab brown and black with blue-white venters and straw coloured tails.

I conducted field work primarily at Pullen Farm (S 25°34,358', E 031°10.879') and some neighbouring areas, all in the Crocodile River Nature Conservancy, a 30 000 ha area situated approximately 30 km east of Nelspruit, South Africa. I worked at the beginning of the rainy season, which begins in September and ends in March. In order to maintain consistency in studies on constraints on signalling in this study, species the methodology in this study is very similar to the protocol employed in previous studies (chapters 1-3). For this reason detailed methodology has been omitted unless it deviates from previous chapters.

Handling and marking of lizards

I captured lizards on lines of glue traps (Catchmaster® 48R non-toxic glue traps (27 x 13 cm) and Trapper® glue boards (26 x 12 cm)) using standard protocol for capturing lizards (e.g., Ellinger et al., 2001; Whiting & Alexander, 2001). I recorded each capture location using a GPS. Since lizards were kept in solitary conditions I did not mark them during the experiment but I did mark them permanently prior to release with a mini-cauteriser (Kyron Code Y2565) to avoid recapturing individuals. For more detail on the capture and handling see chapters 1-3.

Comparing the effects of β -carotene on captive lizards

I collected 56 males and housed them individually indoors in semi-opaque plastic boxes with lids (230 x 320 x 140 mm) and fed them on wet cat food three times a week, supplemented with Beefe powder (® Bayer) once a week and water *ad libitum*. Five days after adjustment to captivity, I took blood samples and measured endurance, morphology and immunocompetence. After 14 days I took a second blood sample from each lizard and measured endurance, immunocompetence, body condition and colouration again. Half of these lizards (28) were in the β -carotene treatment group and received supplementation during the course of the experiment and the remaining 28 were control males given no supplementation. Carotenoids were supplemented using a technique traditionally used in non-invasive hormone supplementation in reptiles (e.g. Belliure et al., 2004). β -carotene dissolved in a carrier oil (denatured sunflower oil) was deposited using a dropper on each lizard's dorsal surface every second day. Lizard skin contains a high concentration of lipids which allows lipophilic molecules to pass through the scales and enter the bloodstream (Mason, 1992). This technique ensures that all males receive the same volume of carotenoids independently of how much they eat since lizards in captivity for short time periods often do not eat sufficient food to control levels of supplementation. Males in the supplementation group were treated with 1 ml of oil containing 20 μ g/ml β -carotene (adapted from McGraw & Ardia, 2003) and control males were treated with pure carrier oil.

Measurement of plasma carotenoid concentration

I assayed β -carotene from blood collected from the suborbital sinus. Immediately upon capture, I used a heparinised microhaematocrit capillary tube to draw approximately 150 μ L of blood. This is a standard procedure for small lizards (e.g. Woodley & Moore, 1998; Cox et al., 2005a; Husak et al., 2007) which has been used successfully in previous studies on flat lizards (e.g. Whiting et al., 2003). I noted the time of capture as well as the start and end bleeding time for each sample so that I could exclude samples involving long handling times. I took all blood samples between 08:h00-12:h00, the period of peak activity for lizards. After centrifuging samples at 3000 rpm for three minutes I separated the plasma and froze it immediately, until it could be assayed in the lab.

Measuring plasma concentrations of β -carotene

After five days to habituate to captivity I collected approximately 150 μ L of blood from the suborbital sinus using a heparinised microhaematocrit capillary tube. Following an established protocol for working with plasma carotenoids (e.g., Alonso-Alvarez et al., 2010) I performed a cold-extraction of β -carotene (see chapter 1 for details on extraction) and then measured the supernatant in a SpectraMAX 190 ELISA plate reader. All spectral absorbance levels were compared to a 10-point standard curve measured at the same time. I measured all samples in duplicate and used the average of the readings in analysis.

Morphological measurements and spectroradiometry

I measured weight and snout-to-vent length (SVL) as per the protocol outlined in previous chapters. The residuals of $\ln$ SVL against mass provided an index of body condition (Fuxjager et al., 2010) which I used as a proxy to measure overall condition of individuals.

I measured spectral reflectance of the throat, abdomen, flank, front and back legs and tail regions of each lizard, using a USB2000 optic spectrometer (Ocean Optics, Inc.), running on OOIBase32™ version 2.0.0.6 (Ocean Optics, 2002) using the protocol from chapter 1. All measurements used are the average of two readings, with the machine calibrated

between measurements for each lizard. I used existing data on the visual system of the closely related *Platysaurus broadleyi* (Fleishman et al., 2011) to model colour according to the visual system of the receiver (competing male lizards). This was processed into separate chromatic and achromatic aspects of colour: achromatic being brightness or the sum of reflectance reaching the eye and chromatic being the chromatic contrast between the colour and the rock making up their habitat, once brightness is standardised.

Endurance

I measured endurance as the length of time a lizard walked on a belt (30 x 40 cm) (e.g. Sinervo et al., 2000; Garland et al., 1990) moving at 0.4 km.h⁻¹ before it failed to maintain its position more than three times (Miles et al., 2007). Each lizard was tested twice with trials 24 hours apart and the best time was taken as maximal endurance.

Immunocompetence

To measure immune response I used a delayed-type hypersensitivity test using phytohaemagglutinin (PHA) (Smits et al., 1999). I compared the swelling produced in a lizard's foot when injected with 0.03 ml phosphate buffered saline (PBS) containing 50 mg of PHA (Sigma, L-8754) with a control foot injected with pure PBS. The degree of welling indicates the stress of the animal's cell-mediated immune response (e.g. Saks et al., 2003; López & Martín, 2005; Belliure et al., 2004). For further details see chapter 1.

Statistical analyses

For each measurement, I ran a Generalized Linear Model (GLZ) using a logit link function. I used the period of measurement (before or after manipulation) as the dependent variable and so I used a binomial error structure. I used treatment type (β -carotene or control) as categorical predictor in all cases. I used β -estimate coefficients from the GLZ to assess differences between first order effects and descriptive statistics to determine the direction of change. All statistics were performed in STATISTICA 6.0 (Statsoft). Graphs show medians with inter-quartile ranges.

Ethical note

This study was approved by the Animal Ethics and Screening Committee of the University of the Witwatersrand. Clearance numbers; carried out under 2009/36/03, with a provincial permit from the Mpumalanga Parks Board MPB5211/2.

Results

β-carotene levels

β-carotene supplementation did not significantly predict circulating plasma β-carotene levels (Wald $\chi^2_1 = 0.14$; $p = 0.71$; figure 5.1).

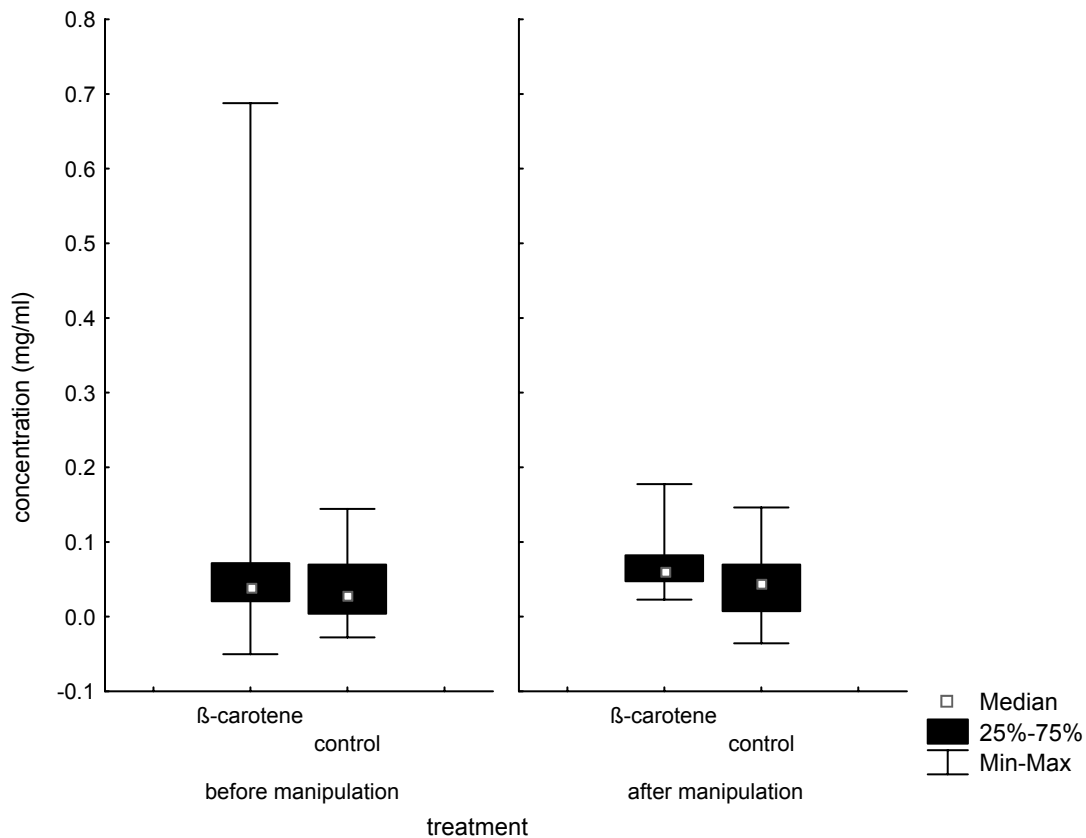


Figure 5.1. Supplementation of carotenoids did not predict circulating plasma carotenoid levels in *P. i. wilhelmi* measured after 14 days of supplementation. Values are medians with boxes showing interquartile range and whiskers showing total range

Body condition

β -carotene did not predict body condition in supplemented compared to control males (Wald $\chi^2_1 = 0.03$; $p = 0.95$, figure 5.2).

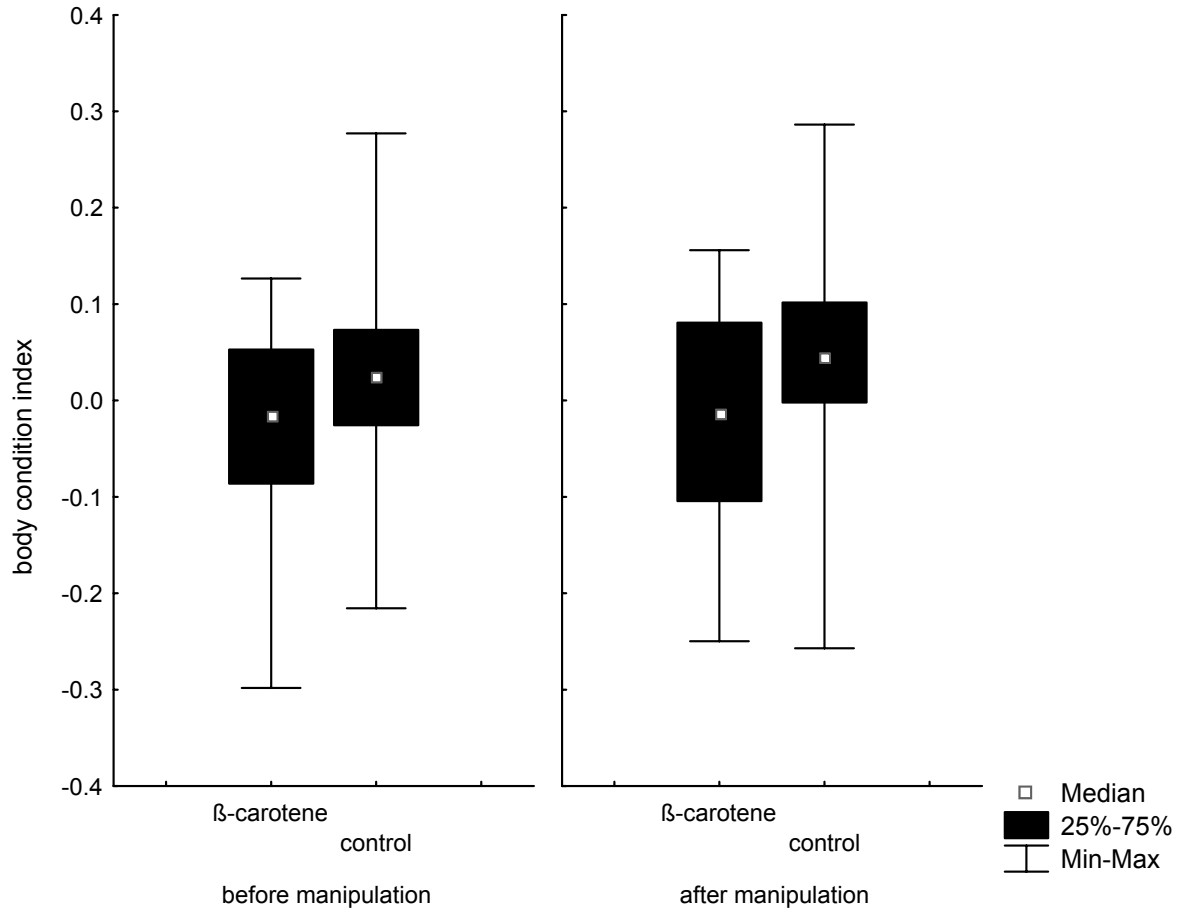


Figure 5.2. Supplemented β -carotene did not predict body condition in *P. i. wilhelmi* after 14 days.

Values shown are medians with boxes indicating interquartile range and whiskers showing total range

Endurance

Supplemented β -carotene was a predictor of endurance (Wald $\chi^2_1 = 1480.17$; $p < 0.001$) with supplemented *P. i. wilhelmi* males running for longer than the control group ($\beta = -0.688$) and improving by approximately 60% after supplementation (figure 5.3).

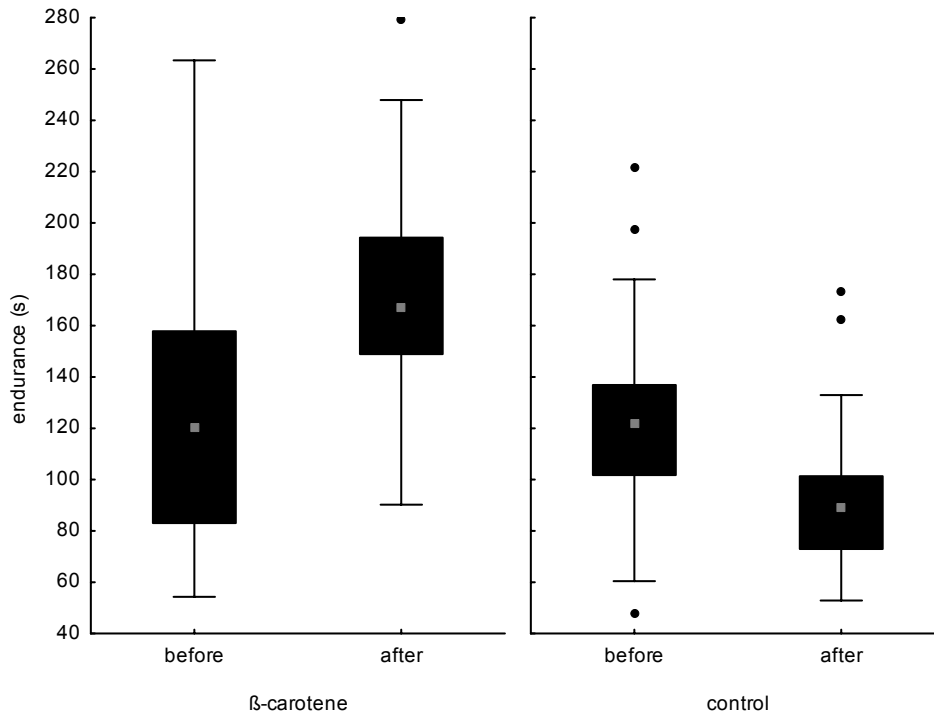


Figure 5.3. Supplemented β -carotene predicted endurance in male *P. i. wilhelmi*. Values shown are medians with boxes indicating interquartile range and whiskers show range

Lizard body temperature was a significant covariate (Wald $\chi^2_1 = 1612.45$; $p < 0.001$). However, body temperature and endurance were not significantly correlated ($R^2 = 0.000274$; $r = -0.017$, $p = 0.86$) and explained very little of the variance of endurance which suggests that it influenced outcome of the results in an unpredictable way.

Immune response

Cell-mediated immune response was predicted by β -carotene supplementation (Wald $\chi^2_1 = 81.56$; $p < 0.001$, figure 5.4). *Platysaurus i. wilhelmi* lizards receiving β -carotene showed a better immune response than control lizards which showed a decrease in immune response ($\beta = 0.369$).

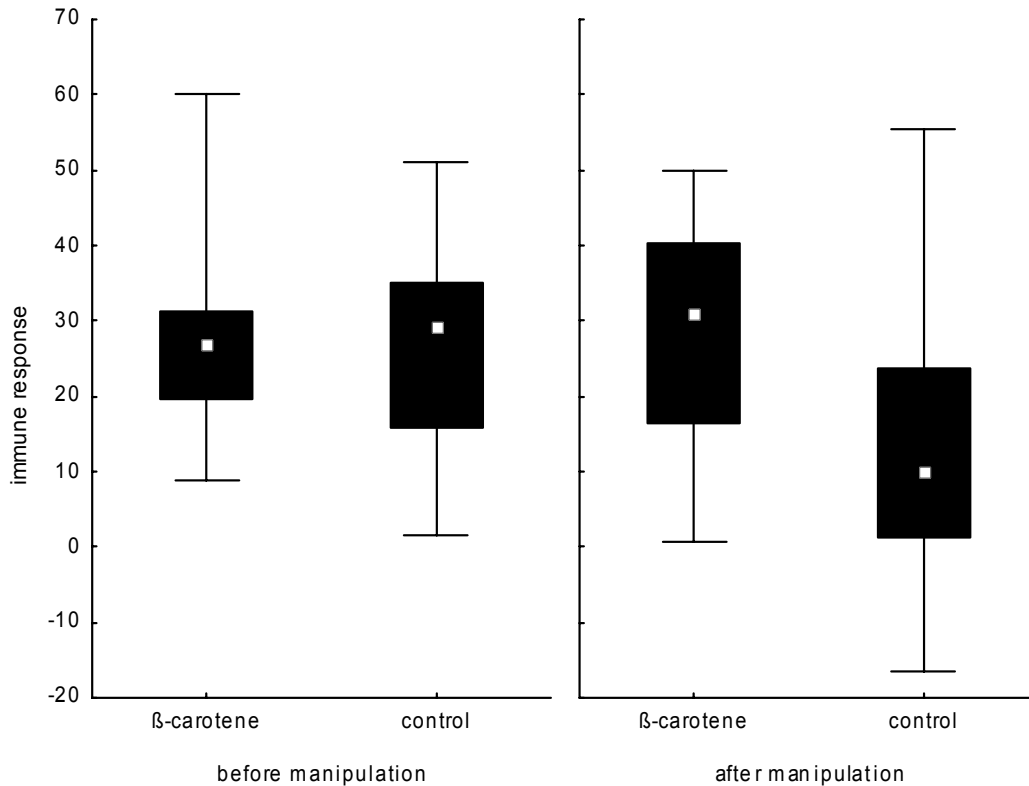


Figure 5.4. Cell-mediated immune response was predicted by β -carotene supplementation in *P. i. wilhelmi*. Values show medians while boxes indicate interquartile range and whiskers show total range.

Chromatic contrast

β -carotene supplementation was not a predictor of chromatic contrast of lizard colour patches against their rock habitat (flank Wald $\chi^2_3 = 0.0007$; $p = 0.97$; front leg Wald $\chi^2_3 = 0.0000$; $p = 0.99$; back leg Wald $\chi^2_3 = 0.00006$; $p = 0.99$; abdomen Wald $\chi^2_3 = 0.0003$; $p = 0.98$; tail Wald $\chi^2_3 = 0.0007$; $p = 0.97$, figure 5.5). While there were changes over the course of the experiment they were independent of treatment so carotenoid supplementation did not affect colouration in this case.

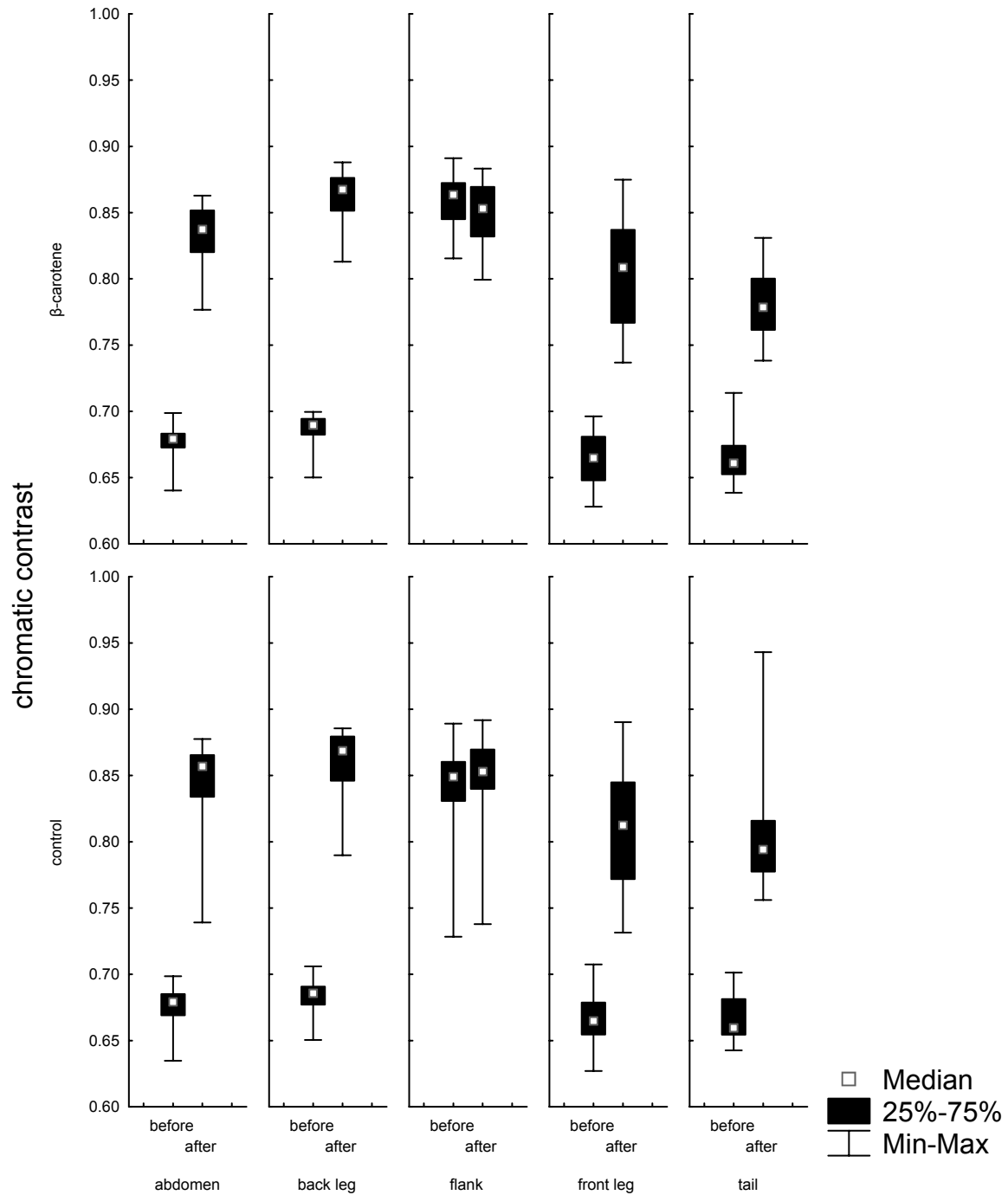


Figure 5.5. Treatment with β -carotene did not predict chromatic contrast of colour patches in *P. i. wilhelmi* as control and β -carotene supplemented lizards responded the same way, independently of treatment. Values shown are medians with boxes showing interquartile range and whiskers showing total range.

Brightness

Receiving β -carotene predicted brightness of colour patches in *P. i. wilhelmi*. (figure 5.6), mostly decreasing the brightness of patches on the body parts mentioned, except for the tail which increased in brightness (Table 5.1).

Table 5.1: Changes in brightness in 5 body parts following β -carotene treatment in male *P. i. wilhelmi*

Body region	Wald χ^2	p	β	Direction of change
Flank	89.03	< 0.001	0.063	Decreased brightness
Front leg	13.34	< 0.001	0.030	Decreased brightness
Back leg	113.01	< 0.001	0.074	Decreased brightness
Abdomen	27.62	< 0.001	0.045	Decreased brightness
Tail	70.38	< 0.001	0.080	Increase in brightness

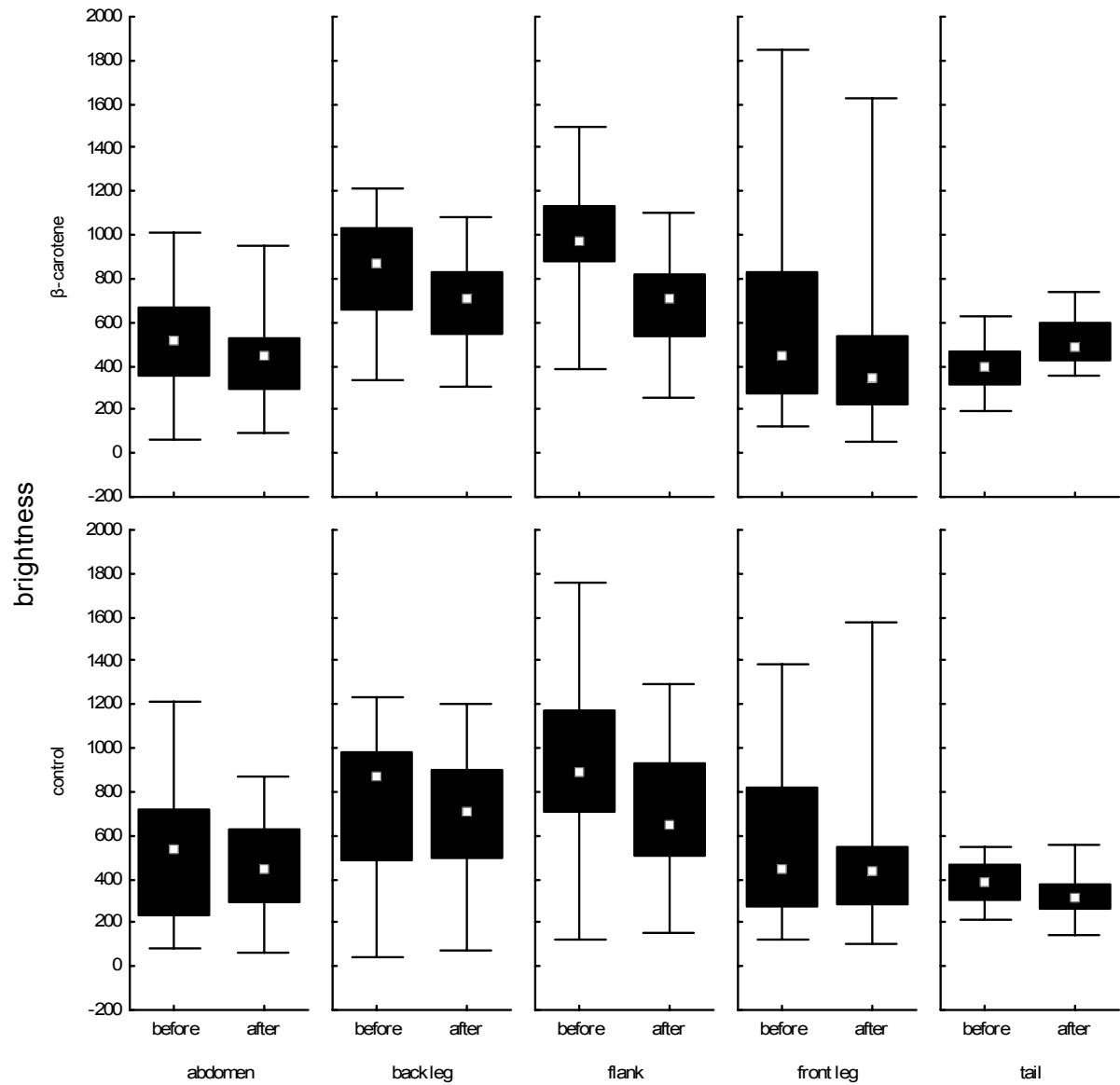


Figure 5.6. β -carotene supplementation predicted brightness of colour patches in *P. i. wilhelmi*. Values show medians with boxes showing interquartile range and whiskers showing total range.

Discussion

Lizards with supplemented β -carotene outperformed control lizards in endurance and immune response. They also showed a decrease in brightness in all body regions except for the tail which became brighter. Only body condition was not affected by carotenoid supplementation.

Supplementation of β -carotene did not significantly change circulating carotenoid levels in adult male *Platysaurus intermedius wilhelmi*. Given the effect in response variables, it is likely that carotenoids are quickly mobilised following uptake. Besides the obvious effect of the β -carotene treatment it should be remembered that β -carotene (as with all carotenoids) is fat soluble (Goodwin, 1984) and so excretion would be limited or occur at low levels. These compounds interact in complex ways with other antioxidant molecules which can also affect their circulating levels (Morales et al., 2009). Moreover, carotenoids are stored in a variety of different tissues and organs in a non-uniform distribution (reviewed in Svensson & Wong, 2011; Surai et al., 2001). Lizards show vast differences in carotenoid allocation to different organs which varies across species (typically with highest concentrations in the liver and lowest concentrations in muscles) (Czeczuga, 1980) and these differences also extend across populations (Costantini et al., 2005). Taken together, these results indicate that plasma concentrations are not always an accurate measure of overall carotenoids (Pérez-Rodríguez, 2009). In future studies I recommend sacrificing a subsample of lizards in order to obtain carotenoid measures for multiple tissues. Taking multiple measures in blood samples from live animals does constrain the degree of measurement available in studies of small animals due to the volume of plasma required, but with the considerable effects seen here it is quite clear that the added carotenoids had an effect on lizard physiology regardless of the results of the assays used to measure circulating carotenoids.

If the oxidative-stress hypothesis (Lozano, 1994) of carotenoids as a beneficial but limited constraining factor holds then I expected two outcomes. Firstly, if carotenoids are limited, supplementation should show a visible change in phenotype of sexually selected

traits. In this case, colour patches all changed in brightness. As most colour patches became darker (decreased brightness), they would be more conspicuous against the light-coloured granite of their habitat and so would be more effective as signals. Secondly, carotenoid supplementation should benefit the animal in ways other than enhancing signals, which was the case in my study, since carotenoid supplementation prevented the decline in immune response associated with temporary captivity seen here in control lizards as well as in previous studies on this population (chapter 1, chapter 2).

Carotenoids cannot be manufactured by animals *de novo* (Goodwin, 1984) and tend to be limited in supply (Hill, 1999). This means that with the associated increase in endurance, which is linked to dominance (Perry et al., 2004), it is logical to assume that males with larger or high quality territories should have more access to carotenoids than subordinate counterparts which makes it both a signal and a positive feedback loop. This could also be affected by testosterone levels which are linked to endurance (chapter 2) as increased testosterone promotes the uptake (McGraw et al., 2006b) and bioavailability of carotenoids (Blas et al., 2006). Testosterone levels may even have more of an effect on carotenoid allocation than a challenge to the immune system (Martínez-Padilla et al., 2010), and the next step should be to investigate the interactions between carotenoids and testosterone.

An additional aspect that requires investigation is the relationship between corticosterone and carotenoid-based colouration. In the common lizard (*Lacerta vivipara*), carotenoid-based colouration was affected by elevation of corticosterone, but not affected by supplementing dietary carotenoids. The lack of evidence of a trade-off between carotenoids and physiological stress suggests an interplay between the organisational and activational aspects in the modulation of carotenoid-based signals (Fitze et al., 2009).

Our current understanding of constraints on signalling is based on the trade-offs between signal intensity and physiological effects of signal modulators. The addition of a beneficial signal modulator (i.e. β -carotene) confirms the current thinking that signals form only a small part in a big network of tradeoffs and feedback loops (e.g. Irschick &

Garland, 2001). There is a possibility that carotenoids play a role in ameliorating the detrimental effects of high hormone concentrations

Carotenoids are not synthesised by animals and so must be obtained exogenously through selected foods (Hill, 1999). Supplementing β -carotene did not alter plasma concentrations of the carotenoid, suggesting that it is mobilised rapidly for use in signals and physiological processes. Exogenous β -carotene resulted in increased immunocompetence and endurance as well as significant effects of brightness of colour patches which signal male condition and quality. While this supports the oxidative stress hypothesis (Lozano, 1994), there is a possible interaction between carotenoids and testosterone that should be investigated further. Testosterone which also increases endurance (chapter 2), and so may allow individuals to maintain larger territories and therefore have access to carotenoids which boost endurance, thereby showing an amplifying effect. While lines of research on testosterone and carotenoids as signal modulators are still generally independent (Blas et al., 2006) the similar effects on colouration and potential counteractive effects on the immune system suggest that these effects could be confounding and require manipulations in order to tease the effects of hormones and nutrients apart.

DISCUSSION

The aim of my project was to investigate constraints on signalling in *Platysaurus intermedius wilhelmi* by using an integrative approach, involving testing the multiple effects of hormones and carotenoids on both signal output and animal performance, which are proxies for fitness. This approach gives a unique perspective to understanding costly signalling theory and signal constraints. This discussion reviews the key findings of this approach in relation to four hypotheses in behavioural ecology, and attempts to relate them to the biology of the study species. This is followed by a conceptual framework which integrates my findings with predictions from the four hypotheses and compares this to information available in the literature of species commonly studied in the field of costs of signalling.

Key findings

In chapter 1, I investigated the relationships between signals and mediators (hormones and carotenoids). I found that testosterone was the only factor that changed seasonally and this was mirrored by changes in endurance and immunocompetence. I also found that colour predicted endurance, immunocompetence and size - and so could be considered to be an indicator of male quality.

The appearance of correlated changes in immunocompetence and testosterone prompted me to investigate whether the immunocompetence-handicap hypothesis was supported in *P.i.wilhelmi*. The immunocompetence handicap hypothesis (ICHH) proposes that testosterone plays a vital role in the production and maintenance of male secondary selected signals, but also constrains these signals through associated immunosuppressive properties of testosterone (Folstad & Karter, 1992). As this hypothesis has yielded equivocal results in the literature (Roberts et al., 2004), I was also interested as to whether other factors could be playing a role in constraining signalling. Thus, in chapter 2, I investigated the immunocompetence-handicap hypothesis concurrently in a free-living and captive (under controlled conditions) group of *P. i. wilhelmi*. I found that there was a link between testosterone and immunosuppression, but only in free-ranging lizards. Other effects of testosterone (on both free-living and captive lizards) included benefits to endurance and changes to brightness of certain body regions.

While the results from chapter 2 showed that testosterone does play a role in performance and in signal intensity, the difference in immune response between the groups led me to investigate, in chapter 4, the first logical factor that would be different between the groups – stress. While free-ranging males are exposed to all kinds of acute stressors such as agonistic encounters, predation, limited food availability or unpredictable environmental conditions (Vleck et al., 2000), lizards in captivity additionally experience chronic stress (Terio et al., 2004; Dickens et al., 2009) but other stressors such as lack of food availability, agonistic encounters or predation are eliminated. There are also two major hypotheses concerning the role of corticosterone in constraints to signalling: the stress-linked immunocompetence handicap hypothesis (SL-ICHH) which states that the

apparent effects of testosterone on immunocompetence are actually due to covarying corticosterone levels (Buchanan, 2000); the hypothesis that testosterone levels are actually coincidental to signal output and signals (and even testosterone levels) are in fact mediated by corticosterone concentrations (Bortolotti et al., 2009a).

In chapter 4, I compared the effects of corticosterone, testosterone and a combination of the two on male flat-lizards. I found that all three hypotheses (the ICHH, the SL-ICHH and corticosterone-only) were supported to some degree. Testosterone influenced signal production and endurance; corticosterone influenced the area of throat colour badges, and a combination of the two often had an intermediate effect. There was still absolutely no effect on immunocompetence even with manipulations of stress-hormones.

While testosterone did have some influence on colour intensity it did not affect chromatic contrast and therefore, does not fully explain sexual dichromatism. In chapter 3, I investigated the degree to which organisational pathways are set up for signal production and maintenance in males by measuring the effects of elevated testosterone on females. I also measured endurance, immune response, and body condition in order to assess whether there were any obvious costs to females of having elevated testosterone levels. Measuring the effects on females is a useful technique to understanding signals in males as it allows us to examine the effects of testosterone on lizard physiology without the confounding factor of organisational effects that are likely to be present in males. By testing the response of females to testosterone, I was able to compare the effects of testosterone on females to those found in males. Thus, I could explain the effects on males in light of what responses are plastic in females and therefore are activational rather than using fixed organisational pathways. Females did respond to the testosterone implants by showing an improvement in endurance and changes in colour brightness which might make them more conspicuous. This suggests that while some pathways are mediated through organisational pathways – specifically chromatic aspects of colour - males' signals are still plastic and mediated by testosterone, independent of sex and so manipulation of potential constraining factors is a valid method of attempting to understand signal constraints in this taxon. All that remains to tease apart the

organisational and activational effects is to investigate the effects of exogenous testosterone on juvenile *P. i. wilhelmi*. This has been done successfully with other species, particularly tree lizards (*Urosaurus ornatus*) where testosterone implants influenced growth and dewlap type (Hews & Moore, 1995).

Since there were obvious effects of testosterone and corticosterone on signals and measures of performance, it is clear that there are many mediators of signal quality, but they do not show any particular cost to the individual. While testosterone and corticosterone both have an effect on signals there was no apparent associated detrimental effect on the animal, such as a loss of condition or reduced immune response. Neither testosterone nor corticosterone nor a combination of the two affected body condition (used as a proxy for overall health). Immunocompetence was only affected in free-living lizards with testosterone implants, not by any other manipulation. The lack of obvious cost to endocrine signal mediators suggested that there was some kind of exogenous factor maintaining signal honesty. I tested the oxidative stress hypothesis (Lozano, 1994) which states that carotenoids that are used in signals are not available for their other physiological role as antioxidants and immune boosters. The trade-off here is that carotenoids are limited in the environment and cannot be synthesised by animals (Hill, 1999) so their allocation to signals is removing them from their role as antioxidants in the immune system. There is some evidence that *P. i. wilhelmi* is omnivorous (reviewed in Lailvaux et al., 2003a) and substantial data that the closely related *Platysaurus broadleyi* eats figs (Whiting & Greef, 1997). While I never observed *P. i. wilhelmi* eating figs, *Ficus* sp. trees were common at my field site, usually around the edges of the rocky outcrops inhabited by my study subjects. In addition, these trees attracted many insects and I often saw lizards eating insects that had been feeding on fallen figs. Carnivorous animals do obtain carotenoids from their food, albeit in lower quantities than herbivores (Olson & Owens, 1998), so whether they eat figs or eat fig-eating insects it is likely that they are obtaining carotenoid-rich food in these areas. In consequence, chapter 5 investigated the actions of supplemented carotenoids in captive *P.i.wilhelmi*. These had a marked effect on endurance, colour and immunocompetence, and it appeared to

counteract the immunosuppressive effect of captivity seen in control lizards here and as a general pattern in other studies with captive *P.i. wilhelmi* (chapters 2-4).

Implication of my findings

Overall, my study considered four hypotheses to explain signal constraints in sexually-selected trait development and maintenance in male *P. i. wilhelmi*. The immunocompetence handicap hypothesis, the stress-linked immunocompetence handicap hypothesis, the idea that corticosterone modulates signals independently, or that nutrient quality and availability constrain signals (the oxidative stress hypothesis) are all supported to some degree. Thus, I cannot unequivocally state that one of the hypotheses is supported. All four factors (testosterone, corticosterone, corticosterone and testosterone, carotenoids) play a role in the constraint of signals in *P.i.wilhelmi*. Logically, each possible modulator of sexually selected traits fits with their biology: *P. i. wilhelmi* is highly aggressive and territorial, both of which are generally controlled by testosterone (Nelson, 2000), and therefore having testosterone as a signal mediator is logical because this is indicative of fighting ability and aggression (Whiting et al., 2006) and male-specific behaviours such as mate-guarding (Saino & Møller, 1995). Frequent agonistic encounters between rivals would involve changes in corticosterone levels (Summers et al., 2005), particularly in subordinate males which generally show a dramatic increase in stress following losing a fight (Creel, 2001). If corticosterone acts as a signal mediator it could indicate male quality as they cope with stressors and still maintain signals (Bortolotti et al., 2009a). Since dominant males often have different levels of corticosterone in response to agonistic encounters, this could also signal social status (Creel, 2001) which fits with the handicap hypothesis which predicts that costs to signalling are different for high and low quality individuals (Grafen, 1990). Such encounters are also shaped by dominance and territorial behaviour which means that it is likely that corticosterone and testosterone interact in the acquisition and defence of territories. Fruit availability is patchy in the landscape (pers obs) and so food quality would be variable and consequently lizards that were able to maintain high-quality territories would have access to high-nutrient food and therefore display more intense ornaments, based on available carotenoids. To discount all of the models in favour of one

would eliminate a large part of the actual influence and interplay between signal modulators.

To my knowledge, this work is one of the most integrative studies of the effects of corticosterone, testosterone and carotenoids on signal constraint, and their interaction. The hypotheses that I tested are well-established in the literature, and there are several points which overlap, such as known effects on immunocompetence. Nonetheless, the study of constraints on signalling follows separate lines of investigation and there is very little research on interactive effects of signal modulators (Blas et al., 2006). This is despite a call for a more integrative approach in studies in behavioural ecology for over a decade (e.g., Irschick & Garland, 2001).

Conceptual framework

Table 1 compares the currently available evidence in the literature of the effects of corticosterone, testosterone and carotenoids on signals, performance, immunocompetence and body condition. This is not an exhaustive representation of the available literature, because I have chosen to represent the available information for species in which most aspects of signal constraints have been studied. While some species have been quite thoroughly tested, notably zebra finches (*Taenopygia guttata*) (e.g., Alonso-Alvarez et al., 2007a; Cynx et al., 2005; McGraw & Ardia, 2003) and barn swallows (*Hirundo rustica*) (e.g., Saino et al., 1997; Saino et al., 2002; Saino et al., 1995), my study is the most comprehensive investigation into these signal constraints to date.

Table 1. A comparison of studies on signal constraints, my results, and original predictions based on the literature. Data were obtained from 36 selected studies on various species. Arrows show the direction of effect and numbers correspond to references. Orange blocks indicate agreement with the predictions; grey blocks indicate partial disagreement and dark grey blocks show total disagreement. White blocks indicate no data. At the bottom there is a summary of the matching of predictions to results with a tick indicating agreement, a cross indicating disagreement and a question mark when the information is equivocal.

Study species	Testosterone				Corticosterone				testosterone x corticosterone				carotenoids				References
	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	
Predictions	↑	↓	↑	↓	↓	↓	↓	↓	-	↓ ↓	-	↓ ↓	↑	↑	↑	↑	
<i>Platysaurus intermedius wilhelmi</i> (common flat lizard)	↑	↓ in free-living males	↑	-	↑ ↓	-	↓ ↓	-	↑ ↓ -	-	↓	-	↑	↑	↑	-	Chapters 2-5
<i>Urosaurus ornatus</i> (tree lizard)	↑ (1,2)		↑ (4)			↓ (3)											(1. Weiss & Moore, 2004; 2. Hews & Moore, 1995; 3. French et al., 2006; 4. Robson & Miles, 2001)
<i>Norops sagrei</i> (brown anole)	↑ (2, 4)		- (5)	↑ (growth rate) (2)	↓ (3)			↑ (5)					- (1)				(1. Steffen et al., 2010; 2 Cox et al., 2009; 3. Tokarz, 1987; 4. Tokarz et al., 2002; 5. Tokarz et al., 1998)

Study species	Testosterone				Corticosterone				testosterone x corticosterone				carotenoids				References
	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	
Predictions	↑	↓	↑	↓	↓	↓	↓	↓	-	↓↓	-	↓↓↓	↑	↑	↑	↑	
<i>Taenopygia guttata</i> (zebra finch)	↑ (1)	↓ (3) - (7)		- (1) ↓ (5)		↑↓ (6)		↑ (6)		↑ (5)			↑ (2)	↑ (4)			(1. Cynx et al., 2005; 2. McGraw et al., 2003; 3. Alonso-Alvarez et al., 2007b; 4. McGraw & Ardia, 2007; 5. Roberts et al., 2007c; 6. Roberts et al., 2007b; 7. Birkhead et al., 1998)
<i>Carduelis tristis</i> (American Goldfinch)	↑ (2)	↑ (2)											↑	- (1)			(1. Navara & Hill, 2003; 2. McGraw et al., 2006a)
<i>Euplectes ardens</i> (Red-collared widowbird)	↑ (2)												↑ (1)	↑ (1)			(1. Pryke et al., 2001; 2. Pryke & Andersson, 2003)
<i>Hirundo rustica</i> (barn swallow)	↑ (4)	↓ (3)	- (5)			- (2) ↓ (6)							↑ (1)				(1. Saino et al., 1999; 2. Saino et al., 2002; 3. Saino et al., 1995; 4. Saino & Møller, 1994; 5. Saino et al., 1997; 6. Saino et al., 2003)
<i>Gasterosteus aculeatus</i> (three-spined stickleback)	↑ (2)	↓ (2)											↑ (1)	↑ (1)			(1. Folstad et al., 1994; 2. Kurtz et al., 2007)

Study species	Testosterone				Corticosterone				testosterone x corticosterone				carotenoids				References
	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	signals	Immuno-competence	performance	Body condition	
Predictions	↑	↓	↑	↓	↓	↓	↓	↓	-	↓↓	-	↓↓	↑	↑	↑	↑	
<i>Panorpa vulgaris</i> (scorpionfly)	↑	↓ (only old animals)															(Kurtz, 2007)
<i>Madrillus sphinx</i> (Mandrill baboon)	-	↓			-	↓				↓							(Setchell et al., 2010)
<i>Podopus sungorus</i> (Siberian hamster)		↑ (1)				↓ (3) - (2)											(1. Bilbo & Nelson, 2001; 2. Cl et al., 2010; 3. Zysling & Dema 2007)
pattern matching predictions?	✓	✓	✓	X	?	?	✓	X	X	?	X	X	✓	✓	✓	X	

In general, testosterone enhances signals and reduces immune response, as well as improving performance (Table 1). This concurs with my predictions and my data, although while there was an effect of testosterone on immune response, this was only evident in free-ranging male *P.i. wilhelmi*. In other species there are some exceptions to this pattern. For example, in Mandrill baboons, testosterone did not affect signals (Setchell et al., 2009). Another exception is that testosterone enhanced immune response in American goldfinches (McGraw et al., 2006a) through its effect on upregulating lipoproteins. Increased testosterone also enhanced immunocompetence in Siberian hamsters although the mechanism is possibly a direct result of testosterone acting on immune cells (Bilbo & Nelson, 2001).

My data matched the predictions for the effects of corticosterone in that colour patches became less conspicuous in their habitat and there was also a significant decrease in throat patch area. *Platysaurus i. wilhelmi* lizards with corticosterone implants also showed a decrease in endurance which matches my predictions. There were no effects of corticosterone on body condition or immune response, contrary to the prediction of a loss of condition and corticosterone-induced immunosuppression. The literature provides a more ambiguous pattern. As Table 1 shows, there are mixed results for the effects of corticosterone on signals and immunocompetence. For example, elevated corticosterone decreases signalling behaviour in brown anoles (Tokarz, 1987) which matches the predictions, but elevated corticosterone had no effect on signals in Mandrill baboons (Setchell et al., 2009). Similarly, corticosterone decreased immune response in tree lizards (Weiss & Moore, 2004), which matched the prediction of corticosterone having an immunosuppressive effect, but in other species the results were ambiguous, often with conflicting results in the same study species. For example, corticosterone was linked to immunosuppression in barn swallows (Saino et al., 2003), but another study on the same species found no relationship between corticosterone and immunocompetence (Saino et al., 2002). Despite the prediction that elevated corticosterone would decrease body condition through energy mobilisation (Lowry & Moore, 2006), I found no support for this, since my data showed no effect of corticosterone on body condition and in Table 1

the main pattern is for corticosterone to improve body condition, which is the case with zebra finches (Roberts et al., 2007c) and brown anoles (Tokarz et al., 1998).

Interactions between corticosterone and testosterone are not as well tested as the effects of either hormone independently (Table 1). I found that the general pattern was a disagreement between the predictions and the literature. My data supported the prediction that performance in *P.i.wilhelmi* individuals with testosterone and corticosterone implants – measured as endurance – would be intermediate to that of lizards with either testosterone or corticosterone implants. The literature does not provide sufficient data on the effects of the interaction of testosterone and corticosterone and it is clear that this area needs more study, particularly since the most studied aspect - immunocompetence - has produced directly conflicting results. A combination of corticosterone and testosterone resulted in improved immune response in zebra finches (Roberts et al., 2007c) and immunosuppression in Mandrills (Setchell et al., 2009). The notable pattern in Table 1 is that this combination of hormones has not been studied thoroughly and so generalisations on the effects of testosterone and corticosterone need to be made with caution.

Carotenoids generally enhance signals, immunocompetence, and performance (Table 1). One exception is in American goldfinches where carotenoid supplementation did not affect immune response despite the extensive carotenoid-based plumage seen in these birds (Navara & Hill, 2003). My data matched the predictions exactly: supplemented β -carotene *P. i. wilhelmi* males showed changes in signals to make them more conspicuous in their habitat, and improved their endurance and immunocompetence. The only point in which my data departed from the predictions of this theory was when there was no change in body condition with supplemented β -carotene.

Overall, it is also quite clear that some relationships are well understood – for example in most cases testosterone does increase signal output and often suppresses the immune system, and carotenoids are often associated with increased signals and immunocompetence (Table 1). On the other hand, corticosterone and the interaction between corticosterone and testosterone are less well researched and the results often

contradict the predictions (Table 1). It should also be noted that the studies shown here (and in general) were conducted over many years and with experimental protocols that changed through time as technology and protocols developed. The value of an exhaustive approach on a single model allowed me to also trouble-shoot and develop methodology in order to use a consistent approach.

Conclusion

While the conflicting effects of testosterone, corticosterone, a combination of corticosterone and testosterone, and carotenoids on signals, condition, immunocompetence and endurance suggest that the development of a new model is necessary, I do not think that it would be practical. Due to the variation already evident in the literature, it is unlikely that a new model would explain any more variation than is already included in existing hypotheses. A model combining existing hypotheses would be unwieldy and probably only predict signal constraints for a small group of animals that shared similar behavioural traits and physiology. There is also a possibility that other factors may be involved. For example, exogenous testosterone in zebra finches has an immunosuppressive effect, but this is countered by an associated increase in leptin (Alonso-Alvarez et al., 2007b). Generally, some aspect of male signals are fixed organisationally, such as dewlap type in tree lizards (*Urosaurus ornatus*) (Hews & Moore, 1995) or ventral colouration in *Sceloporus undulatus* (Cox et al., 2005a) but other signals are under activational control, such as femoral pore secretions and throat colour in tree lizards (Hews & Moore, 1995). Until such time as when we can understand and predict which aspects are organisationally and activationally controlled, we will not understand which signals are condition-dependent and which indicate genetic quality.

Rather than a new, all-encompassing (and probably also unreliable) model, a new research approach is needed. Instead of assessing the overriding signal modulator in every case, we should be taking a multifaceted approach to studying constraints on signalling by testing whether a factor (such as testosterone or corticosterone) plays a role in modulating signals and if so investigate the extent of its influence as well as the associated physiological and behavioural repercussions, as I have done here. There are

variable responses to manipulations in different hormones and nutrients. For example, in American goldfinches experimentally elevated testosterone improved immune response (McGraw et al., 2006a), while in several other species such as wall lizards (*Podarcis muralis*) exogenous testosterone suppressed immunocompetence (Martín et al., 2008). This variability means that a global integrative model for constraints on sexually selected traits is unlikely to work for many species.

We should also be focussing on the biological significance of what we can learn from signal constraints. By investigating the relative importance of different signal modulators, we can begin to generate functional hypotheses and explanations for the selective pressures that have influenced signalling through their constraints. For example, zebra finches (*Taenopygia guttata*) use carotenoids to counteract the immunosuppressive effects of testosterone (McGraw & Ardia, 2007). It would be interesting to establish whether this relationship is evident in other species, particularly since there is evidence for a positive link between testosterone and carotenoid bioavailability (Blas et al., 2006). Furthermore, we could investigate the relative role of carotenoids on the immune system of animals that do not display carotenoid-based coloration, such as mammals to ascertain whether their sexually selected or condition dependent traits change as a result of supplementation.

Another point of interest would be to investigate the differences between signals which are generated over a short time period and become fixed, such as horns, or bird feathers, and plastic signals such as colours in bird beaks, and skin in fish and lizards which can change over time and require maintenance of circulating hormone or carotenoid levels. There is also the possible effect of social control, for example in *Psammmodromus algirus* lizards, development of nuptial colouration in sexually maturing lizards is delayed by the presence of large males. Small male *P. algirus* with experimentally manipulated colouration to resemble large males were attacked significantly more often than large males with the same colour or small males who had not yet developed the colouration (Martín & Forsman, 1999). Another interesting topic would be to compare the predominance of corticosterone-based signals in systems with solitary social systems

when compared to large groups with a multitude of stressful social interactions. For example, in Mandrill baboons (*Mandrillus sphinx*) high levels of social interaction and aggression result in increased corticosterone levels which correlate with gut parasite load. Despite this classic relationship between social stress, corticosterone and parasite load, there was no effect of these changes in corticosterone on ornamentation (Setchell et al., 2009).

The integration of understanding by using a multidisciplinary approach involving collaboration between the ultimate approach of behavioural ecologists and proximate approaches of physiologists may provide a level of understanding that either side is unable to produce independently. While there is definitely a drive towards integrating factors such as behavioural ecology, physiology and performance, in general the field is very much broken into camps. There are very few studies which investigate more than one effect of a hormone on an animal's physiology, and behavioural ecologists often test many different aspects of fitness and signal quality without appropriately investigating the physiology behind it. Partnerships between chemists, biologists, immunologists and endocrinologists are required if we are going to achieve a better understanding of signal costs or male quality might provide.

In conclusion, my study is the most comprehensive and consistent study of its kind on constraints in signalling to date, and provides some support for four major hypotheses. An integrative approach to studying constraints on signalling provides a much information, and further investigation into interactions, for example between testosterone and carotenoids, might explain inconsistencies in the literature.

REFERENCES

- Abbott, D. H., Keverne, E. B., Bercovitch, F. B., Shively, C. A., Mendoza, S. P., Saltzman, W., Snowdon, C. T., Ziegler, T. E. & Banjevic, M. 2003. Are subordinates always stressed? a comparative analysis of rank differences in cortisol levels among primates. *Hormones and Behavior*, **43**, 67-82.
- Adamo, S. A. 2004. How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour*, **68**, 1443-1449.
- Ahmed, S. A. & Talal, N. 1990. Sex hormones and the immune system--part 2. Animal data. *Bailliere's Clinical Rheumatology*, **4**, 13-31.
- Ahmed, S. A., Talal, N. & Christadoss, P. 1987. Genetic regulation of testosterone-induced immune suppression. *Cellular Immunology*, **104**, 91-98.
- Alexander, M., Newmark, H. & Miller, R. G. 1985. Oral beta-carotene can increase the number of OKT4+ cells in human blood. *Immunology Letters*, **9**, 221-224.
- Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. & Sorci, G. 2004. An Experimental Test of the Dose-Dependent Effect of Carotenoids and Immune Activation on Sexual Signals and Antioxidant Activity. *American Naturalist*, **164**, 651-659.
- Alonso-Alvarez, C., Bertrand, S., Faivre, B., Chastel, O. & Sorci, G. 2007a. Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 819 - 825.
- Alonso-Alvarez, C., Bertrand, S. & Sorci, G. 2007b. Energetic reserves, leptin and testosterone: a refinement of the immunocompetence handicap hypothesis. *Biology Letters*, **3**, 271-274.
- Alonso-Alvarez, C., Pérez-Rodríguez, L., Garcia, J. T. & Viñuela, J. 2010. Testosterone-mediated trade-offs in the old age: a new approach to the immunocompetence handicap and carotenoid-based sexual signalling. *Proceedings of the Royal Society B: Biological Sciences*, **in press**, 1 - 9.
- Ames, B. N. 1983. Dietary Carcinogens and Anticarcinogens. *Science*, **221**, 1256-1264.
- Amo, L., López, P. & Martín, J. 2006. Nature-based tourism as a form of predation risk affects body condition and health state of *Podarcis muralis* lizards. *Biological Conservation*, **131**, 402-409.
- Amorim, M. C. P. & Almada, V. C. 2005. The outcome of male-male encounters affects subsequent sound production during courtship in the cichlid fish *Oreochromis mossambicus*. *Animal Behaviour*, **69**, 595-601.
- Andersson, M. B. 1994. *Sexual Selection*. New Jersey: Princeton University Press.
- Andersson, S., Pryke, S. R., Örnborg, J., Lawes, M. J. & Andersson, M. 2002. Multiple Receivers, Multiple Ornaments, and a Trade-off between Agonistic and Epigamic Signaling in a Widowbird. *American Naturalist*, **160**, 683-691.
- Arnold, S. J. 1983. Morphology, Performance and Fitness. *American Zoologist*, **23**, 347-361.
- Ashley, N. T., Hays, Q. R., Bentley, G. E. & Wingfield, J. C. 2009. Testosterone treatment diminishes sickness behavior in male songbirds. *Hormones and Behavior*, **56**, 169-176.
- Bales, K. L., French, J. A., McWilliams, J., Lake, R. A. & Dietz, J. M. 2006. Effects of social status, age, and season on androgen and cortisol levels in wild male golden lion tamarins (*Leontopithecus rosalia*). *Hormones and Behavior*, **49**, 88-95.
- Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. *Heredity*, **2**, 349-368.
- Belliure, J., Smith, L. & Sorci, G. 2004. Effect of Testosterone on T Cell-Mediated Immunity in Two Species of Mediterranean Lacertid Lizards. *Journal of Experimental Zoology*, **301A**, 411-418.
- Bendich, A. 2004. From 1989 - 2001: what have we learned about the 'biological actions of beta-carotene'? *The Journal of Nutrition*, 2255 - 2305.
- Bendich, A. & Olson, J. A. 1989. Biological actions of carotenoids. *The FASEB journal*, 1927 - 1932.
- Berger, S., Martin Li, L. B., Wikelski, M., Romero, L. M., Kalko, E. K. V., Vitousek, M. N. & Rodl, T. 2005. Corticosterone suppresses immune activity in territorial Galapagos marine iguanas during reproduction. *Hormones and Behavior*, **47**, 419-429.

- Berglund, A., Bisazza, A. & Pilastro, A. 1996. Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biological Journal of the Linnean Society*, **58**, 385 - 399.
- Bilbo, S. D. & Nelson, R. J. 2001. Sex steroid hormones enhance immune function in male and female Siberian hamsters. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, **280**, R207-R213.
- Birkhead, T. R., Fletcher, F. & Pellatt, E. J. 1998. Sexual selection in the zebra finch *Taeniopygia guttata*, : condition, sex traits and immune capacity. *Behavioral Ecology and Sociobiology*, **44**, 179-191.
- Blas, J., Pérez-Rodríguez, Bortolotti, G. R., Viñuela, J. & Marchant, T. A. 2006. Testosterone increases bioavailability of carotenoids: Insights into the honesty of sexual signaling. *Proceedings of the National Academy of Sciences*, **103**, 18633-18637.
- Blount, J. D., Metcalfe, N. B., Birkhead, T. R. & Surai, P. F. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science*, 125 - 127.
- Boonekamp, J. J., Ros, A. F. H. & Verhulst, S. 2008. Immune activation suppresses plasma testosterone level: a meta-analysis. *Biology letters*, **4**, 741 - 744.
- Bortolotti, G. R., Mougeot, F., Martinez-Padilla, J., Webster, L. M. & Piertney, S. B. 2009a. Physiological Stress Mediates the Honesty of Social Signals. *PLoS ONE*, **4**, e4983.
- Bortolotti, G. R., Mougeot, F., Martinez-Padilla, J., Webster, L. M. I. & Piertney, S. B. 2009b. Physiological Stress Mediates the Honesty of Social Signals. *PLoS ONE*, **4**, e4983.
- Branch, W. R. 1998. *Field guide to the snakes and other reptiles of southern africa*. Cape Town: Struik Publishers.
- Branch, W. R. & Whiting, M. J. 1997. A new *Platysaurus* (Squamata: Cordylidae) from the Northern Cape Province, South Africa. *African Journal of Herpetology*, **46**, 124-136.
- Buchanan, K. L. 2000. Stress and the evolution of condition-dependent signals. *Trends in Ecology & Evolution*, **15**, 156-160.
- Buchanan, K. L., Evans, M. R. & Goldsmith, A. R. 2003. Testosterone, dominance signalling and immunosuppression in the house sparrow *Passer domesticus*. *Behavioral Ecology and Sociobiology*, **55**, 50 - 59.
- Buchanan, K. L., Evans, M. R., Goldsmith, A. R., Bryant, D. M. & Rowe, L. V. 2001. Testosterone influences basal metabolic rate in male house sparrows: a new cost of dominance signalling? *Proceedings of the Royal Society B: Biological Sciences*, **268**, 1337 - 1344.
- Candolin, U. 1999. The relationship between signal quality and physical condition: is sexual signalling honest in the three-spined stickleback? *Animal Behaviour*, **58**, 1261-1267.
- Carrascal, L. M., Senar, J. C., Mozetich, I., Uribe, F. & Domench, j. 1998. Interactions between environmental stress, body condition, nutritional status and dominance in great tits. *The Auk*, **115**, 727 - 738.
- Chester, E. M., Bonu, T. & Demas, G. E. 2010. Social defeat differentially affects immune responses in Siberian hamsters (*Phodopus sungorus*). *Physiology & Behavior*, **101**, 53-58.
- Clotfelter, E. D., O'Neal, D. M., Gaudioso, J. M., Casto, J. M., Parker-Renga, I. M., Snajdr, E. A., Duffy, D. L., Nolan, V. & Ketterson, E. D. 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? *Hormones and Behavior*, **46**, 171-178.
- Clutton-Brock, T. H. 1998. Reproductive skew, concessions and limited control. *Trends in Ecology & Evolution*, **13**, 288-292.
- Cooper, W. E. 2001. Multiple Roles of Tail Display by the Curly-Tailed Lizard *Leiocephalus carinatus*: Pursuit Deterrent and Deflective Roles of a Social Signal. *Ethology*, **107**, 1137-1149.
- Cooper, W. E. & Greenburg, N. 1992. Reptile coloration and behaviour. In: *Biology of the reptilia 18, Physiology E, Hormones, Brain and Behaviour* (Ed. by Gans, C. & Crews, D.), pp. 115-228. Chicago: The University of Chicago Press.
- Costantini, D. 2008. Oxidative stress in ecology and evolution: lessons from avian studies. *Ecology Letters*, **11**, 1238-1251
- Costantini, D., Dell'Omo, G., Casagrande, S., Fabiani, A., Carosi, M., Bertacche, V., Marquez, C., Snell, H., Snell, H., Tapia, W. & Gentile, G. 2005. Inter-population variation of carotenoids

- in Galápagos land iguanas (*Conolophus subcristatus*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **142**, 239-244.
- Cote, J., Clobert, J., Meylan, S. & Fitze, P. S. 2006. Experimental enhancement of corticosterone levels positively affects subsequent male survival. *Hormones and Behavior*, **49**, 320-327.
- Cox, R. M. & John-Alder, H. B. 2007. Increased mite parasitism as a cost of testosterone in male striped plateau lizards *Sceloporus virgatus*. *Functional Ecology*, **21**, 327-334.
- Cox, R. M., Skelly, S. L. & John-Alder, H. B. 2005a. Testosterone Inhibits Growth in Juvenile Male Eastern Fence Lizards (*Sceloporus undulatus*): Implications for Energy Allocation and Sexual Size Dimorphism. *Physiological and Biochemical Zoology*, **78**, 531 - 545.
- Cox, R. M., Skelly, S. L., Leo, A. & John-alder, H. B. 2005b. Testosterone Regulates Sexually Dimorphic Coloration in the Eastern Fence Lizard, *Sceloporus undulatus*. *Copeia*, 597-608.
- Cox, R. M., Stenquist, Derek S., Henningsen, Justin P. & Calsbeek, R. 2009. Manipulating Testosterone to Assess Links between Behavior, Morphology, and Performance in the Brown Anole *Anolis sagrei*. *Physiological and Biochemical Zoology*, **82**, 686-698.
- Creel, S. 2001. Social dominance and stress hormones. *Trends in Ecology & Evolution*, **16**, 491-497.
- Crespi, E. J. & Denver, R. J. 2005. Roles of stress hormones in food intake regulation in anuran amphibians throughout the life cycle. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, **141**, 381-390.
- Croney, C. C., Prince-Kelly, N. & Meller, C. L. 2007. A note on social dominance and learning ability in the domestic chicken (*Gallus gallus*). *Applied Animal Behaviour Science*, **105**, 254 - 258
- Cynx, J., Bean, N. J. & Rossman, I. 2005. Testosterone implants alter the frequency range of zebra finch songs. *Hormones and Behavior*, **47**, 446-451.
- Czeczuga, B. 1980. Carotenoids in some parts of certain species of lizards. *Comparative Biochemistry and Physiology*, **64B**, 755 - 757.
- De Ridder, E., Pinxten, R., Mees, V. & Eens, M. 2002. Short- and long-term effects of male-like concentrations of testosterone on female european starlings (*Sturnus vulgaris*). *The Auk*, **119**, 487 - 297.
- Demas, G. E., Zysling, D. A., Beechler, B. R., Muehlenbein, M. P. & French, S. S. 2011. Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. *Journal of Animal Ecology*, in press.
- Denardo, D. F. & Licht, P. 1993. Effects of Corticosterone on Social Behavior of Male Lizards. *Hormones and Behavior*, **27**, 184-199.
- DeNardo, D. F. & Sinervo, B. 1994a. Effects of Corticosterone on Activity and Home-Range Size of Free-Ranging Male Lizards. *Hormones and Behavior*, **28**, 53-65.
- DeNardo, D. F. & Sinervo, B. 1994b. Effects of Steroid Hormone Interaction on Activity and Home-Range Size of Male Lizards. *Hormones and Behavior*, **28**, 273-287.
- Deviche, P. & Cortez, L. 2005. Androgen control of immunocompetence in the male house finch *Carpodacus mexicanus* Müller. *The Journal of Experimental Biology*, 1287 - 1295.
- Dewsbury, D. A. 1982. Dominance Rank, Copulatory Behavior, and Differential Reproduction. *The Quarterly Review of Biology*, **57**, 135-159.
- Dickens, M. J., Earle, K. A. & Romero, L. M. 2009. Initial transference of wild birds to captivity alters stress physiology. *General and Comparative Endocrinology*, **160**, 76 - 83.
- Dijkstra, P. D., Hekman, R., Schulz, R. W. & Groothuis, T. G. G. 2007. Social stimulation, nuptial colouration, androgens and immunocompetence in a sexual dimorphic cichlid fish *Behavioral Ecology and Sociobiology*, **61**, 599 - 609.
- Dugatkin, L. A. 2004. *Principles of animal behaviour*. New York: W. W. Norton & Company, Inc.
- Edler, R., Goymann, W., Schwabl, I. & Friedl, T. W. P. 2010. Experimentally elevated testosterone levels enhance courtship behaviour and territoriality but depress acquired immune response in Red Bishops *Euplectes orix*. *Ibis*, no-no.
- Eisenegger, C., Haushofer, J. & Fehr, E. 2011. The role of testosterone in social interaction. *Trends in Cognitive Sciences*, **15**, 263-271.

- Ellinger, N., Schlatte, G., Jerome, N. & Hodl, W. 2001. Habitat Use and Activity Patterns of the Neotropical Arboreal Lizard *Tropidurus* (= *Uracentron*) *azureus weneri* (Tropiduridae). *Journal of Herpetology*, **35**, 395-402.
- Emerson, S. B. & Hess, D. L. 2001. Glucocorticoids, Androgens, Testis Mass, and the Energetics of Vocalization in Breeding Male Frogs. *Hormones and Behavior*, **39**, 59-69.
- Endler, J. A. & Mielke, P. W. 2005. Comparing entire colour patterns as birds see them. *Biological Journal of the Linnean Society*, **86**, 405-431.
- Evans, M. R., Goldsmith, A. R. & Norris, S. R. A. 2000. The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). *Behavioral Ecology and Sociobiology*, **47**, 156-163.
- Faivre, B., Preault, M., Salvadori, F., Thery, M., Gaillard, M. & Cezilly, F. 2003. Bill colour and immunocompetence in the European blackbird. *Animal Behaviour*, **65**, 1125-1131.
- Figuerola, J., Munoz, E., Gutierrez, R. & Ferrer, D. 1999. Blood Parasites, Leucocytes and Plumage Brightness in the Cirl Bunting, *Emberiza cirlus*. *Functional Ecology*, **13**, 594-601.
- Fitze, P. S., Cote, J., San-Jose, L. M., Meylan, S., Isaksson, C., Andersson, S., Rossi, J. & Clobert, J. 2009. Carotenoid-Based Colours Reflect the Stress Response in the Common Lizard. *PLoS ONE*, **4**, e5111.
- Fleishman, L. J., Loew, E. R. & Whiting, M. J. 2011. High sensitivity to short wavelengths in a lizard and implications for understanding the evolution of visual systems in lizards. *Proceedings of the Royal Society B: Biological Sciences*.
- Folstad, I., Hope, A. M., Karter, A. & Skorping, A. 1994. Sexually Selected Color in Male Sticklebacks: A Signal of Both Parasite Exposure and Parasite Resistance? *Oikos*, **69**, 511-515.
- Folstad, I. & Karter, A. J. 1992. Parasites, Bright Males, and the Immunocompetence Handicap. *The American Naturalist*, **139**, 603-622.
- French, S. S., Matt, K. S. & Moore, M. C. 2006. The effects of stress on wound healing in male tree lizards (*Urosaurus ornatus*). *General and Comparative Endocrinology*, **145**, 128-132.
- Fuxjager, M. J., Foutopoulos, J., Diaz-Uriarte, R. & Marler, C. A. 2010. Functionally opposing effects of testosterone on two different types of parasite: implications for the immunocompetence handicap hypothesis. *Functional Ecology*, in press.
- Galeotti, P., Saino, N., Sacchi, R. & Moller, A. P. 1997. Song correlates with social context, testosterone and body condition in male barn swallows. *Animal Behaviour*, **53**, 687-700.
- García-Vigón, E., Cordero, P. J. & Veiga, J. P. 2008. Exogenous testosterone in female spotless starlings reduces their rate of extrapair offspring. *Animal Behaviour*, **76**, 345-353.
- Garland, T., Jr., Hankins, E. & Huey, R. B. 1990. Locomotor Capacity and Social Dominance in Male Lizards. *Functional Ecology*, **4**, 243-250.
- Goodwin, T. W. 1984. *The biochemistry of the carotenoids*. London: Chapman & Hall.
- Govardovskii, V. I., Fyhrquist, N., Reuter, T. O. M., Kuzmin, D. G. & Donner, K. 2000. In search of the visual pigment template. *Visual Neuroscience*, **17**, 509-528.
- Goymann, W. & Wingfield, J. C. 2004. Allostatic load, social status and stress hormones: the costs of social status matter. *Animal Behaviour*, **67**, 591-602.
- Grafen, A. 1990. Biological signals as handicaps. *Journal of Theoretical Biology*, **144**, 517-546.
- Gray, D. A. 1996. Carotenoids and Sexual Dichromatism in North American Passerine Birds. *The American Naturalist*, **148**, 453-480.
- Grear, D. A., Perkins, S. E. & Hudson, P. J. 2009. Does elevated testosterone result in increased exposure and transmission of parasites? *Ecology Letters*, **12**, 528-537.
- Greenberg, N. & Crews, D. 1990. Endocrine and behavioral responses to aggression and social dominance in the green anole lizard, *Anolis carolinensis*. *General and Comparative Endocrinology*, **77**, 246-255.
- Greiner, S., Stefanski, V., Dehnhard, M. & Voigt, C. C. 2010. Plasma testosterone levels decrease after activation of skin immune system in a free-ranging mammal. *General and Comparative Endocrinology*, **168**, 466-473.
- Grose, J. 2011. Modelling and the fall and rise of the handicap principle. *Biological Philosophy*, **26**, 677 - 696.

- Grossman, C. J. 1990. Are there underlying immune-neuroendocrine interactions responsible for immunological sexual dimorphism? *Progress in NeuroEndocrinImmunology*, **3**, 75 - 82.
- Hamilton, W. D. & Zuk, M. 1982. Heritable True Fitness and Bright Birds: A Role for Parasites? *Science*, **218**, 384-387.
- Hart, N. S. & Vorobyev, M. 2005. Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, **191**, 381-392.
- Hasselquist, D., Marsh, J. A., Sherman, P. W. & Wingfield, J. C. 1999. Is avian humoral immunocompetence suppressed by testosterone? . *Behavioral Ecology and Sociobiology*, **45**, 167 - 175.
- Hasson, O. 1997. Towards a General Theory of Biological Signaling. *Journal of Theoretical Biology*, **185**, 139-156.
- Hawlena, D. 2009. Colorful tails fade when lizards adopt less risky behaviors. *Behavioral Ecology and Sociobiology*, **64**, 205 - 213.
- Heitor, F., Oom, M. d. M. & Vicente, L. 2006. Social relationships in a herd of Sorraia horses: Part I. Correlates of social dominance and contexts of aggression. *Behavioural Processes*, **73**, 170-177.
- Hews, D. K., Knapp, R. & Moore, M. C. 1994. Early Exposure to Androgens Affects Adult Expression of Alternative Male Types in Tree Lizards. *Hormones and Behavior*, **28**, 96-115.
- Hews, D. K. & Moore, M. C. 1995. Influence of androgens on differentiation of secondary sex characters in tree lizards, *Urosaurus ornatus*. *General and Comparative Endocrinology*, **97**, 86 - 102.
- Hews, D. K. & Moore, M. C. 1996. A critical period for the organization of alternative male phenotypes of tree lizards by exogenous testosterone? *Physiology & Behavior*, **60**, 425-429.
- Hill, G. E. 1999. Is There an Immunological Cost to Carotenoid-Based Ornamental Coloration? *The American Naturalist*, **154**, 589-595.
- Hill, G. E., Inouye, C. Y. & Montgomerie, R. 2002. Dietary Carotenoids Predict Plumage Coloration in Wild House Finches. *Proceedings: Biological Sciences*, **269**, 1119-1124.
- Huber, S., Palme, R. & Arnold, W. 2003. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). *General and Comparative Endocrinology*, **130**, 48-54.
- Husak, J. F., Fox, S. F., Lovern, M. B. & van den Bussche, R. A. 2006a. Faster lizards sire more offspring: sexual selection on whole-animal performance. *Evolution*, **60**, 2122-2130.
- Husak, J. F. & Irschick, D. J. 2009. Steroid use and human performance: Lessons for integrative biologists. *Integrative and Comparative Biology*, **49**, 354-364.
- Husak, J. F., Irschick, D. J., McCormick, S. D. & Moore, I. T. 2009. Hormonal regulation of whole-animal performance: Implications for selection. *Integrative and Comparative Biology*, **49**, 349-353.
- Husak, J. F., Irschick, D. J., Meyers, J. J., Lailvaux, S. P. & Moore, I. T. 2007. Hormones, sexual signals, and performance of green anole lizards (*Anolis carolinensis*). *Hormones and Behavior*, **52**, 360-367.
- Husak, J. F., Lappin, A. K., Fox, S. F. & Lemos-Espinal, J. A. 2006b. Bite-Force Performance Predicts Dominance in Male Venerable Collared Lizards (*Crotaphytus antiquus*). *Copeia*, **2**, 301 - 306.
- Husak, J. F., Macedonia, J. M., Fox, S. F. & Saucedo, R. C. 2006c. Predation Cost of Conspicuous Male Coloration in Collared Lizards (*Crotaphytus collaris*): An Experimental Test Using Clay-Covered Model Lizards. *Ethology*, **112**.
- Irschick, D. J. & Garland, T. 2001. Integrating function and ecology in studies of adaptation: Investigations of Locomotor Capacity as a Model System. *Annual Review of Ecology and Systematics*, **32**, 367-396.
- Irschick, D. J., Ramos, M., Buckley, C., Elstorr, J., Carlisle, E., Lailvaux, S. P., Bloch, N., Herrel, A. & Vanhooydonck, B. 2006. Are morphology - performance relationships invariant across different seasons? A test with the green anole lizard (*Anolis carolinensis*). *Oikos*, **114**, 49 - 59.

- Jenssen, T. A., Lovern, M. B. & Congdon, J. D. 2001. Field-testing the protandry-based mating system for the lizard, *Anolis carolinensis*: does the model organism have the right model? *Behavioral Ecology and Sociobiology*, **50**, 162 - 172.
- Ketola, T., Kortet, R. & Kotiaho, J. S. 2009. Endurance in exercise is associated with courtship call rate in decorated crickets, *Gryllobates sigillatus*. *Evolutionary Ecology Research*, **11**, 1131 - 1139.
- Ketterson, E. D., Atwell, J. W. & McGlothlin, J. W. 2009. Phenotypic integration and independence: Hormones, performance, and response to environmental change. *Integrative and Comparative Biology*, **49**, 365-379.
- Ketterson, E. D., Nolan, V. J., Casto, J. M., Buerkle, C. A., Clotfelter, E. D., Grindstaff, J. L., Jones, K. J., Lipar, J. L., McNabb, F. M. A., Neudorf, D. L., Parker-Renga, I. M., Schoech, S. J. & Snajdr, E. A. 2001. Testosterone, phenotype and fitness: a research program in evolutionary behavioral endocrinology. In: *Avian Endocrinology* (Ed. by Dawson, A. & Chaturvedi, C. M.), pp. 19 - 40. New Delhi: Narosa Publishing House.
- Ketterson, E. D., Nolan, V. J. & Sandell, M. 2005. Testosterone in Females: Mediator of Adaptive Traits, Constraint on Sexual Dimorphism, or Both? *The American Naturalist*, **166**, S85 - S98.
- Keys, G. C. & Rothstein, S. I. 1991. Benefits and costs of dominance and subordination in white-crowned sparrows and the paradox of status signalling. *Animal Behaviour*, **42**, 899-912.
- Knapp, R. & Moore, M. C. 1996. Male morphs in tree lizards, *Urosaurus ornatus*, have different delayed hormonal responses to aggressive encounters. *Animal Behaviour*, **52**, 1045-1055.
- Knapp, R. & Moore, M. C. 1997. Male morphs in tree lizards have different testosterone responses to elevated levels of corticosterone. *General and Comparative Endocrinology*, **273** - 279.
- Kodric-Brown, A. & Brown, J. H. 1984. Truth in Advertising: The Kinds of Traits Favored by Sexual Selection. *The American Naturalist*, **124**, 309-323.
- Korner, P., Whiting, M. J. & Ferguson, J. W. H. 2000. Interspecific aggression in flat lizards suggests poor species recognition. *African Journal of Herpetology*, **49**, 139-146.
- Krinsky, N. I. 1989. Antioxidant functions of carotenoids. *Free Radical Biology and Medicine*, **7**, 617-635.
- Kurtz, J. 2007. The correlation between immunocompetence and an ornament trait changes over lifetime in *Panorpa vulgaris* scorpionflies. *Zoology*, **110**, 336-343.
- Kurtz, J., Kalbe, M., Langefors, Å., Mayer, I., Milinski, M. & Hasselquist, D. 2007. An Experimental Test of the Immunocompetence Handicap Hypothesis in a Teleost Fish: 11-Ketotestosterone Suppresses Innate Immunity in Three-Spined Sticklebacks. *The American Naturalist*, **170**, 509 - 519.
- Lailvaux, S. P., Alexander, G. J. & Whiting, M. J. 2003a. Sex-based differences and similarities in locomotor performance, thermal preferences and escape behaviour in the lizard *Platysaurus intermedius wilhelmi*. *Physiological and Biochemical Zoology*, **76**, 511 - 521.
- Lailvaux, S. P., Alexander, G. J. & Whiting, M. J. 2003b. Sex-Based Differences and Similarities in Locomotor Performance, Thermal Preferences, and Escape Behaviour in the Lizard *Platysaurus intermedius wilhelmi*. *Physiological and Biochemical Zoology*, **76**, 511 - 521.
- Lank, D. B., Coupe, M. & Wynne-Edwards, K. 1999. Testosterone-induced male traits in female ruffs (*Philomachus pugnax*): autosomal inheritance and gender differentiation. *Proceedings of the Royal Society B: Biological Sciences*, 2323 - 2330.
- LeBas, N. R. & Marshall, N. J. 2000. The Role of Colour in Signalling and Male Choice in the Agamid Lizard *Ctenophorus ornatus*. *Proceedings: Biological Sciences*, **267**, 445-452.
- Lemos-Espinal, J. A., Smith, G. R. & Ballinger, R. E. 1996. Ventral Blue Coloration and Sexual Maturation in Male *Sceloporus gadoviae* Lizards. *Journal of Herpetology*, **30**, 546-548.
- Lendvai, A. Z., Liker, A. & Barta, Z. 2006. The effects of energy reserves and dominance on the use of social-foraging strategies in the house sparrow. *Animal Behaviour*, **72**, 747-752.
- Lichtenthaler, H. K. 1987. [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In: *Methods in Enzymology*, pp. 350-382: Academic Press.
- Lindström, K. M., Krakower, D., Lundstrom, J. O. & Silverin, B. 2001. The Effects of Testosterone on a Viral Infection in Greenfinches (*Carduelis chloris*): An Experimental Test of the

- Immunocompetence-Handicap Hypothesis. *Proceedings of the Royal Society B: Biological Sciences*, **268**, 207-211.
- Loew, E. R., Fleishman, L. J., Foster, R. G. & Provencio, I. 2002. Visual pigments and oil droplets in diurnal lizards: a comparative study of Caribbean anoles. *Journal of Experimental Biology*, **205**, 927-938.
- López, P. & Martín, J. 2002. Locomotor capacity and dominance in male lizards *Lacerta monticola*: a trade-off between survival and reproductive success? *Biological Journal of the Linnean Society*, **77**, 201 - 209.
- López, P. & Martín, J. 2005. Female Iberian wall lizards prefer scents that signal a better cell-mediated immune response. *Biology Letters*, **1**, 404 - 406.
- Losdat, S., Helfenstein, F., Gaude, B. & Richner, H. 2010. Effect of sibling competition and male carotenoid supply on offspring condition and oxidative stress. *Behavioral Ecology*, **21**, 1271-1277.
- Lovern, M. B., McNabb, F. M. A. & Jenssen, T. A. 2001. Developmental Effects of Testosterone on Behavior in Male and Female Green Anoles (*Anolis carolinensis*). *Hormones and Behavior*, **39**, 131-143.
- Lowry, C. A. & Moore, F. L. 2006. Regulation of behavioral responses by corticotropin-releasing factor. *General and Comparative Endocrinology*, **146**, 19-27.
- Lozano, G. A. 1994. Carotenoids, parasites and sexual selection. *Oikos*, **2**, 309 - 311.
- Lund, T. D., Munson, D. J., Haldy, M. E. & Handa, R. J. 2004. Androgen Inhibits, While Oestrogen Enhances, Restraint-Induced Activation of Neuropeptide Neurons in the Paraventricular Nucleus of the Hypothalamus. *Journal of Neuroendocrinology*, **16**, 272-278.
- Lynn, S. E., Houtman, A. M., Weathers, W. W., Ketterson, E. D. & Nolan, V. 2000. Testosterone increases activity but not daily energy expenditure in captive male dark-eyed juncos, *Junco hyemalis*. *Animal Behaviour*, **60**, 581-587.
- Malo, A. F., Roldan, E. R. S., Garde, J. J., Soler, A. J., Vicente, J., Gortazar, C. & Gomendio, M. 2009. What does testosterone do for red deer males? *Proceedings of the Royal Society B: Biological Sciences*, **276**, 971 - 980.
- Martin Li, L. B., Han, P., Lewittes, J., Kuhlman, J. R., Klasings, K. C. & Wikelski, M. 2006. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. *Functional Ecology*, **20**, 290 - 299.
- Martín, J., Amo, L. & López, P. 2008. Parasites and health affect multiple sexual signals in male common wall lizards, *Podarcis muralis*. *Naturwissenschaften*, **95**, 293-300.
- Martín, J. & Forsman, A. 1999. Social costs and development of nuptial coloration in male *Psammotromus algirus* lizards: an experiment. *Behavioral Ecology*, **10**, 396-400.
- Martín, J. & López, P. 1999. Nuptial coloration and mate-guarding affect escape decisions of male lizards *Psammotromus algirus*. *Ethology*, **105**, 439-447.
- Martín, J. & López, P. 2001. Risk of predation may explain the absence of nuptial coloration in the wall lizard, *Podarcis muralis*. *Evolutionary Ecology Research*, 889-898.
- Martínez-Padilla, J., Mougeot, F., Webster, L. M. I., Pérez-Rodríguez, L. & Pieltney, S. B. 2010. Testing the interactive effects of testosterone and parasites on carotenoid-based ornamentation in a wild bird. *Journal of Evolutionary Biology*, **23**, 902-913.
- Mason, R. T. 1992. Reptilian pheromones. In: *Biology of the Reptilia 18: Physiology E, Hormones, Brain and Behaviour* (Ed. by Gans, C. & Crews, D.), pp. 115-228. Chicago: The University of Chicago Press.
- Mateos, C. & Carranza, J. 1997. The role of bright plumage in male-male interactions in the ring-necked pheasant. *Animal Behaviour*, **54**, 1205-1214.
- McDonald, C. G., Reimchen, T. E. & Hawryshyn, C. W. 1995. Nuptial colour loss and signal masking in *Gasterosteus*: An analysis using video imaging. *Behaviour*, **132**, 963-977.
- McDonald, P. G., A., B. W. & Astheimer, L. B. 2001. The Influence of Testosterone on Territorial Defence and Parental Behavior in Male Free-Living Rufous Whistlers, *Pachycephala rufiventris*. *Hormones and Behavior*, **39**, 185-194.
- McGraw, K., Correa, S. & Adkins-Regan, E. 2006a. Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. *Behavioral Ecology and Sociobiology*, **60**, 117-122.

- McGraw, K. J. 2005. The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? *Animal Behaviour*, **69**, 757-764.
- McGraw, K. J. & Ardia, D. R. 2003. Carotenoids, Immunocompetence, and the Information Content of Sexual Colors: An Experimental Test. *The American Naturalist*, **162**, 704-712.
- McGraw, K. J. & Ardia, D. R. 2005a. Sex differences in carotenoid status and immune performance in zebra finches. *Evolutionary Ecology Research*, **7**, 251 - 262.
- McGraw, K. J. & Ardia, D. R. 2005b. Sex differences in carotenoid status and immune performance in zebra finches. *Evolutionary Ecology Research*, **7**, 251-262.
- McGraw, K. J. & Ardia, D. R. 2007. Do carotenoids buffer testosterone-induced immunosuppression? An experimental test in a colourful songbird. *Biology letters*, **3**, 375 - 378.
- McGraw, K. J., Correa, S. M. & Adkins-Regan, E. 2006b. Testosterone upregulates lipoprotein status to control sexual attractiveness in a colorful songbird. *Behavioral Ecology and Sociobiology*, **60**, 117 - 122.
- McGraw, K. J., Crino, O. L., Medina-Jerez, W. & Nolan, P. M. 2006c. Effect of Dietary Carotenoid Supplementation on Food Intake and Immune Function in a Songbird with no Carotenoid Coloration. *Ethology*, **112**, 1209-1216.
- McGraw, K. J., Gregory, A. J., Parker, R. S., Adkins-Regan, E. & Prum, R. 2003. Diet, plasma carotenoids, and sexual coloration in the zebra finch (*Taeniopygia guttata*). *The Auk*, **120**, 400-410.
- McGraw, K. J., Hill, G. E. & Parker, R. S. 2005. The physiological costs of being colourful: nutritional control of carotenoid utilization in the American goldfinch, *Carduelis tristis*. *Animal Behaviour*, **69**, 653-660.
- Miles, D. B., Calsbeek, R. & Sinervo, B. 2007. Corticosterone, locomotor performance, and metabolism in side-blotched lizards (*Uta stansburiana*). *Hormones and Behavior*, **51**, 548-554.
- Millsaugh, J. J. & Washburn, B. E. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. *General and Comparative Endocrinology*, **138**, 189-199.
- Møller, A. P. 1995. Hormones, handicaps and bright birds. *Trends in Ecology & Evolution*, **10**, 121-121.
- Møller, A. P., Garamszegi, L. Z., Gil, D., Hurtrez-Boussès, S. & Eens, M. 2005. Correlated Evolution of Male and Female Testosterone Profiles in Birds and Its Consequences. *Behavioral Ecology and Sociobiology*, **58**, 534-544.
- Møller, A. P. & Saino, N. 2004. Immune response and survival. *Oikos*, **104**, 299 - 304.
- Moore, F. R., Cornwell, R. E., Law Smith, M. J., Al Dujaili, E. A. S., Sharp, M. & Perrett, D. I. 2011. Evidence for the stress-linked immunocompetence handicap hypothesis in human male faces. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 774-780.
- Moore, I. T. & Jessop, T. S. 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. *Hormones and Behavior*, **43**, 37 - 47.
- Moore, M. C., Thompson, C. W. & Marler, C. A. 1991. Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. *General and Comparative Endocrinology*, **81**, 217-226.
- Morales, J., Velando, A. & Torresa, R. 2009. Fecundity compromises attractiveness when pigments are scarce. *Behavioral Ecology*, **20**, 117 - 123.
- Morici, L. A., Elsey, R. M. & Lance, V. A. 1997. Effects of long-term corticosterone implants on growth and immune function in juvenile alligators, *Alligator mississippiensis*. *The Journal of Experimental Zoology*, **279**, 156 - 162.
- Möstl, E. & Palme, R. 2002. Hormones as indicators of stress. *Domestic Animal Endocrinology*, **23**, 67-74.
- Navara, K. J. & Hill, G. E. 2003. Dietary carotenoid pigments and immune function in a songbird with extensive carotenoid-based plumage coloration. *Behavioral Ecology*, **6**, 909 - 916.
- Negro, J. J., Grande, J. M., Tella, J. L., Garrido, J., Hornero, D., Donázar, J. A., Sanchez-Zapata, J. A., Benítez, J. R. & Barcell, M. 2002. An unusual source of essential carotenoids. *Nature*, **416**, 807 - 808.

- Negro, J. J., Tella, J. L., Blanco, G., Forero, M. G. & Garrido-Fernández, J. 2000. Diet explains interpopulation variation of plasma carotenoids and skin pigmentation in nestling white storks. *Physiological and biochemical zoology*, **1**, 97 - 101.
- Nelson, R. J. 2000. *An introduction to Behavioural Endocrinology (2nd edition)*. Sunderland: Sinauer & Associates.
- Norris, K. & Evans, M. R. 2000. Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology*, **11**, 19 - 26.
- Olson, V. A. & Owens, I. P. F. 1998. Costly sexual signals: are carotenoids rare, risky or required? *Trends in Ecology & Evolution*, **13**, 510-514.
- Olsson, M. 1994. Nuptial coloration in the sand lizard, *Lacerta agilis*: an intra-sexually selected cue to lighting ability. *Animal Behaviour*, **48**, 607-613.
- Olsson, M., Wapstra, E., Madsen, T. & Silverin, B. 2000. Testosterone, ticks and travels: a test of the immunocompetence-handicap hypothesis in free-ranging male sand lizards. *Proceedings of the Royal Society B: Biological Sciences*, 2339 - 2343.
- Osorio, D. & Vorobyev, M. 2005. Photoreceptor Spectral Sensitivities in Terrestrial Animals: Adaptations for Luminance and Colour Vision. *Proceedings: Biological Sciences*, **272**, 1745-1752.
- Owen-Ashley, N. T., Hasselquist, D. & Wingfield, J. C. 2004. Androgens and the Immunocompetence Handicap Hypothesis: Unraveling Direct and Indirect Pathways of Immunosuppression in Song Sparrows. *The American Naturalist*, **164**, 490-505.
- Parker, G. A. 1974. Assessment Strategy and the Evolution of Fighting Behaviour. *Journal of Theoretical Biology*, **47**, 223 - 243.
- Pérez-Rodríguez, L. 2009. Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. *BioEssays*, **31**, 1116-1126.
- Perez-Rodriguez, L., Mougeot, F., Alonso-Alvarez, C., Blas, J., Viñuela, J. & Bortolotti, G. R. 2008. Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). *Journal of Experimental Biology*, **211**, 2155-2161.
- Perry, G., Levering, K., Girard, I. & Garland, T., Jr. 2004. Locomotor performance and social dominance in male *Anolis cristatellus*. *Animal Behaviour*, **67**, 37 - 47.
- Peters, A., Denk, A. G., Delhey, K. & Kempenaers, B. 2004. Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. *Journal of Evolutionary Biology*, **17**, 1111 - 1120.
- Poisbleau, M., Fritz, H., Guillon, N. & Chastel, O. 2005. Linear social dominance hierarchy and corticosterone responses in male mallards and pintails. *Hormones and Behavior*, **47**, 485-492.
- Prabhala, R. H., Garewal, H. S., Meyskens, J. F. L. & Watson, R. R. 1990. Immunomodulation in humans caused by beta-carotene and vitamin A. *Nutrition Research*, **10**, 1473-1486.
- Pryke, S. R. & Andersson, S. 2003. Carotenoid-based status signalling in red-shouldered widowbirds (*Euplectes axillaris*): epaulet size and redness affect captive and territorial competition. *Behavioral Ecology and Sociobiology*, **53**, 393-401.
- Pryke, S. R., Andersson, S. & Lawes, M. J. 2001. Sexual Selection of Multiple Handicaps in the Red-Collared Widowbird: Female Choice of Tail Length But Not Carotenoid Display. *Evolution*, **55**, 1452-1463.
- Raberg, L., Grahn, M., Hasselquist, D. & Svensson, E. 1998. On the Adaptive Significance of Stress-Induced Immunosuppression. *Proceedings: Biological Sciences*, **265**, 1637-1641.
- Rand, M. S. 1990. Polymorphic Sexual Coloration in the Lizard *Sceloporus undulatus erythrocheilus*. *American Midland Naturalist*, **124**, 352-359.
- Rand, M. S. 1992. Hormonal control of polymorphic and sexually dimorphic coloration in the lizard *Sceloporus undulatus erythrocheilus*. *General and Comparative Endocrinology*, **88**, 461-468.
- Rantala, M. J., Roff, D. A. & Rantala, L. M. 2007. Forceps size and immune function in the earwig *Forficula auricularia* L. *Biological Journal of the Linnean Society*, **90**, 509-516.
- Redpath, S. M., Mougeot, F., Leckie, F. M. & Evans, S. A. 2006. The effects of autumn testosterone on survival and productivity in red grouse, *Lagopus lagopus scoticus*. *Animal Behaviour*, **71**, 1297-1305.

- Rhen, T., Ros, J. & Crews, D. 1999. Effects of testosterone on sexual behavior and morphology in adult female leopard geckos, *Eublepharis macularius*. *Hormones and Behavior*, **36**, 119 - 128.
- Roberts, M. L., Buchanan, K. L., Bennett, A. T. D. & Evans, M. R. 2007a. Mate choice in zebra finches: does corticosterone play a role? *Animal Behaviour*, **74**, 921 - 929.
- Roberts, M. L., Buchanan, K. L. & Evans, M. R. 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. *Animal Behaviour*, **68**, 227-239.
- Roberts, M. L., Buchanan, K. L., Evans, M. R., Marin, R. H. & Satterlee, D. G. 2009. The effects of testosterone on immune function in quail selected for divergent plasma corticosterone response. *J Exp Biol*, **212**, 3125-3131.
- Roberts, M. L., Buchanan, K. L., Hasselquist, D., Bennett, A. T. D. & Evans, M. R. 2007b. Physiological, morphological and behavioural effects of selecting zebra finches for divergent levels of corticosterone. *Journal of Experimental Biology*, **210**, 4368-4378.
- Roberts, M. L., Buchanan, K. L., Hasselquist, D. & Evans, M. R. 2006. Effects of testosterone and corticosterone on immunocompetence in the zebra finch. *Hormones and Behavior*, **In Press, Corrected Proof**.
- Roberts, M. L., Buchanan, K. L., Hasselquist, D. & Evans, M. R. 2007c. Effects of testosterone and corticosterone on immunocompetence in the zebra finch. *Hormones and Behavior*, **51**.
- Robson, M. A. & Miles, D. B. 2000. Locomotor Performance and Dominance in Male Tree Lizards, *Urosaurus ornatus*. *Functional Ecology*, **14**, 338-344.
- Robson, M. A. & Miles, D. B. 2001. Locomotor Performance and Dominance in Male Tree Lizards, *Urosaurus ornatus*. *Functional Ecology*, **14**, 338-344.
- Romero, L. M., Cyr, N. E. & Romero, R. C. 2006. Corticosterone responses change seasonally in free-living house sparrows (*Passer domesticus*). *General and Comparative Endocrinology*, **149**, 58-65.
- Romero, L. M. & Remage-Healey, L. 2000. Daily and Seasonal Variation in Response to Stress in Captive Starlings (*Sturnus vulgaris*): Corticosterone. *General and Comparative Endocrinology*, **119**, 52-59.
- Ros, A. F. H., Bouton, N., Santos, R. S. & Oliveira, R. F. 2006. Alternative Male Reproductive Tactics and the Immunocompetence Handicap in the Azorean Rock-Pool Blenny, *Parablennius parvicornis*. *Proceedings: Biological Sciences*, **273**, 901-909.
- Rubenstein, D. R. & Hauber, M. E. 2008. Dynamic feedback between phenotype and physiology in sexually selected traits. *Trends in Ecology & Evolution*, **23**, 655-658.
- Safran, R. J., Adelman, J. S., McGraw, K. J. & Hau, M. 2008. Sexual signal exaggeration affects physiological state in male barn swallows. *Current Biology*, **18**, R461-R462.
- Saino, N., Galeotti, P., Sacchi, R. & Møller, A. P. 1997. Song and immunological condition in male barn swallows (*Hirundo rustica*). *Behavioral Ecology*, **8**, 364-371.
- Saino, N., Incagli, M., Martinelli, R. & Møller, A. P. 2002. Immune response of male barn swallows in relation to parental effort, corticosterone plasma levels, and sexual ornamentation. *Behavioral Ecology*, **13**, 169-174.
- Saino, N. & Møller, A. P. 1994. Secondary sexual characters, parasites and testosterone in the barn swallow, *Hirundo rustica*. *Animal Behaviour*, **48**, 1325-1333.
- Saino, N. & Møller, A. P. 1995. Testosterone correlates of mate guarding, singing and aggressive behaviour in male barn swallows, *Hirundo rustica*. *Animal Behaviour*, **49**, 465-472.
- Saino, N., Møller, A. P. & Bolzerna, A. M. 1995. Testosterone effects on the immune system and parasite infestations in the barn swallow (*Hirundo rustica*): an experimental test of the immunocompetence hypothesis. *Behavioral Ecology*, **6**, 397-404.
- Saino, N., Ninni, P., Calza, S., Martinelli, R., Bernardi, F. D. & Møller, A. P. 2000. Better Red Than Dead: Carotenoid-Based Mouth Coloration Reveals Infection in Barn Swallow Nestlings. *Proceedings: Biological Sciences*, **267**, 57-61.
- Saino, N., Stradi, R., Ninni, P., Pini, E. & Møller, A. P. 1999. Carotenoid Plasma Concentration, Immune Profile, and Plumage Ornamentation of Male Barn Swallows (*Hirundo rustica*). *The American Naturalist*, **154**, 441-448.

- Saino, N., Suffritti, C., Martinelli, R., Rubolini, D. & Møller, A. P. 2003. Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (*Hirundo rustica*). *Behavioral Ecology*, **14**, 318-325.
- Saks, L., Ots, I. & Hõrak, P. 2003. Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. *Oecologia*, **134**, 301 - 307.
- Salvador, A. & Veiga, J. P. 2000. Does testosterone or coloration affect growth rates of adult males of the lizard *Psammodromus algirus*? *Canadian Journal of Zoology*, **78**, 1463-1467.
- Saraiva, J. L., Gonçalves, D. M. & Oliveira, R. F. 2010. Environmental modulation of androgen levels and secondary sex characters in two populations of the peacock blenny *Salarias pavo*. *Hormones and Behavior*, **57**, 192-197.
- Setchell, J., Charpentier, M., Abbott, K., Wickings, E. & Knapp, L. 2009. Is Brightest Best? Testing the Hamilton-Zuk Hypothesis in Mandrills. *International Journal of Primatology*, **30**, 825-844.
- Setchell, J. M., Smith, T., Wickings, E. J. & Knapp, L. A. 2010. Stress, social behaviour, and secondary sexual traits in a male primate. *Hormones and Behavior*, **58**, 720-728.
- Seutin, G. 1994. Plumage Redness in Redpoll Finches Does Not Reflect Hemoparasitic Infection. *Oikos*, **70**, 280-286.
- Shorey, H. H. 1976. *Animal communication by pheromones*. New York: Academic Press inc.
- Sinervo, B., Miles, D. B., Frankino, W. A., Klukowski, M. & DeNardo, D. F. 2000. Testosterone, Endurance, and Darwinian Fitness: Natural and Sexual Selection on the Physiological Bases of Alternative Male Behaviors in Side-Blotched Lizards. *Hormones and Behavior*, **38**, 222-233.
- Slater, C. H. & Schreck, C. B. 1997. Physiological Levels of Testosterone Kill Salmonid Leukocytes in Vitro. *General and Comparative Endocrinology*, **106**, 113-119.
- Smits, J. E., Bortolotti, G. R. & Tella, J. L. 1999. Simplifying the Phytohaemagglutinin Skin-Testing Technique in Studies of Avian Immunocompetence. *Functional Ecology*, **13**, 567 - 572.
- Stahl, J., Tolsma, P. H., Loonen, M. J. J. E. & Drent, R. H. 2001. Subordinates explore but dominants profit: resource competition in high Arctic barnacle goose flocks. *Animal Behaviour*, **61**, 257-264.
- Staub, N. L. & De Beer, M. 1997. The Role of Androgens in Female Vertebrates. *General and Comparative Endocrinology*, **108**, 1-24.
- Steffen, J. E., Hill, G. E. & Guyer, C. 2010. Carotenoid Access, Nutritional Stress, and the Dewlap Color of Male Brown Anoles. *Copeia*, **2010**, 239-246.
- Stoddard, P. K. & Salazar, V. L. 2011. Energetic cost of communication. *Journal of Experimental Biology*, **214**, 200-205.
- Stuart-Fox, D. M., Moussalli, A., Marshall, N. J. & Owens, I. P. F. 2003. Conspicuous males suffer higher predation risk: visual modelling and experimental evidence from lizards. *Animal Behaviour*, **66**, 541-550.
- Summers, C. H., Watt, M. J., Ling, T. L., Forster, G. L., Carpenter, R. E., Korzan, W. J., Lukkes, J. L. & Overli, O. 2005. Glucocorticoid interaction with aggression in non-mammalian vertebrates: Reciprocal action. *European Journal of Pharmacology*, **526**, 21-35.
- Surai, P. F., Speake, B. K., Wood, N. A. R., Blount, J. D., Bortolotti, G. R. & Sparks, N. H. C. 2001. Carotenoid discrimination by the avian embryo: a lesson from wild birds. *comparative Biochemistry and Physiology*, **128**, 743 - 750.
- Svensson, E., Raberg, L., Koch, C. & Hasselquist, D. 1998. Energetic Stress, Immunosuppression and the Costs of an Antibody Response. *Functional Ecology*, **12**, 912-919.
- Svensson, P. A. & Wong, B. B. M. 2011. Carotenoid-based signals in behavioural ecology: a review. *Behaviour*, **148**, 131 - 189.
- Tella, J. L., Figuerola, J., Negro, J. J., Blanco, G., Rodríguez-Estrella, R., Forero, M. G., Blásquez, M. C., Green, A. J. & Hiraldo, F. 2004. Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds. *Journal of Evolutionary Biology*, 156 - 164.

- Terio, K. A., Marker, L. & Munson, L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. *Journal of Wildlife Diseases*, **40**, 259-266.
- Thompson, C. W. & Moore, M. C. 1991. Throat colour reliably signals status in male tree lizards, *Urosaurus ornatus*. *Animal Behaviour*, **42**, 745-753.
- Thompson, C. W. & Moore, M. C. 1992. Behavioral and hormonal correlates of alternative reproductive strategies in a polygynous lizard: Tests of the relative plasticity and challenge hypotheses. *Hormones and Behavior*, **26**, 568-585.
- Tokarz, R. R. 1987. Effects of corticosterone treatment on male aggressive behavior in a lizard (*Anolis sagrei*). *Hormones and Behavior*, **21**, 358-370.
- Tokarz, R. R., McMann, S., Seitz, L. & John-Alder, H. 1998. Plasma Corticosterone and Testosterone Levels during the Annual Reproductive Cycle of Male Brown Anoles (*Anolis sagrei*). *Physiological Zoology*, **71**, 139-146.
- Tokarz, R. R., McMann, S., Smith, L. C. & John-Alder, H. 2002. Effects of Testosterone Treatment and Season on the Frequency of Dewlap Extensions during Male-Male Interactions in the Lizard *Anolis sagrei*. *Hormones and Behavior*, **41**, 70-79.
- van Oers, K., Buchanan, K. L., Thomas, T. E. & Drent, P. J. 2011. Correlated response to selection of testosterone levels and immunocompetence in lines selected for avian personality. *Animal Behaviour*, **81**, 1055-1061.
- Veiga, J. P., Salvador, A., Merino, S. & Puerta, M. 1998. Reproductive effort affects immune response and parasite infection in a lizard: a phenotypic manipulation using testosterone. *Oikos*, 313 - 318.
- Viau, V. 2002. Functional Cross-Talk Between the Hypothalamic-Pituitary-Gonadal and -Adrenal Axes. *Journal of Neuroendocrinology*, **14**, 506-513.
- Vleck, C. M., Vortalino, N., Vleck, D. & Butcher, T. L. 2000. Stress, corticosterone and heterophil to lymphocyte ratios in free-living Adelie penguins. *The Condor*, **102**, 392 - 400.
- von Schantz, T., Bensch, S., Grahm, M., Hasselquist, D. & Wittzell, H. 1999. Good Genes, Oxidative Stress and Condition-Dependent Sexual Signals. *Proceedings: Biological Sciences*, **266**, 1-12.
- Wada, H., Hahn, T. P., Breuner, C. W. & Wada, H. 2007. Development of stress reactivity in white-crowned sparrow nestlings: Total corticosterone response increases with age, while free corticosterone response remains low. *General and Comparative Endocrinology*, **150**, 405-413.
- Wade, J. 1997. Androgen Metabolism in the Brain of the Green Anole Lizard (*Anolis carolinensis*). *General and Comparative Endocrinology*, **106**, 127-137.
- Weedon, B. C. L. 1965. Chemistry of the carotenoids. In: *Chemistry and biochemistry of plant pigments* (Ed. by Goodwin, T. W.), pp. 75 - 122. London: Academic Press.
- Weiss, S. L. & Moore, M. C. 2004. Activation of aggressive behavior by progesterone and testosterone in male tree lizards, *Urosaurus ornatus*. *General and Comparative Endocrinology*, **136**, 282-288.
- Westneat, D. F., Hasselquist, D. & Wingfield, J. C. 2003. Tests of association between the humoral immune response of red-winged blackbirds (*Agelaius phoeniceus*) and male plumage, testosterone, or reproductive success *behavioral Ecology and Sociobiology*, **53**, 315 - 323.
- Whiting, M. J. & Alexander, G. J. 2001. Oil Spills and Glue: a Comment on a Sticky Sampling Problem for Lizards. *Herpetological Review*, **32**, 78 - 79.
- Whiting, M. J. & Greef, J. M. 1997. Facultative Frugivory in the Cape Flat Lizard, *Platysaurus capensis* (Sauria: Cordylidae). *Copeia*, **4**, 812-818.
- Whiting, M. J., Nagy, K. A. & Bateman, P. W. 2003. Evolution and maintenance of social status-signaling badges. In: *Lizard Social Behaviour* (Ed. by Fox, S. F., McCoy, J. K. & Baird, T. A.), pp. 47-82. Baltimore: John Hopkins University Press.
- Whiting, M. J., Stuart-Fox, D. M., O'Connor, D., Firth, D., Bennett, N. C. & Blomberg, S. P. 2006. Ultraviolet signals ultra-aggression in a lizard. *Animal Behaviour*, **72**, 353-363.
- Whittingham, L. A. & Schwabl, H. 2002. Maternal testosterone in tree swallow eggs varies with female aggression. *Animal Behaviour*, **63**, 63-67.

- Wingfield, J. C. 2003. Control of behavioural strategies for capricious environments. *Animal Behaviour*, **66**, 807-816.
- Wingfield, J. C., Hegner, R. E., Dufty, A. M., Jr. & Ball, G. F. 1990. The "Challenge Hypothesis": Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. *The American Naturalist*, **136**, 829-846.
- Winne, C. T., Willson, J. D., Andrews, K. M. & Reed, R. N. 2006. Efficacy of Marking Snakes with Disposable Medical Cautery Units. *Herpetological Review*, **37**, 52 - 54.
- Woodley, S. K. & Moore, M. C. 1998. Female territorial aggression and steroid hormones in mountain spiny lizards. *Animal Behaviour*, **57**, 1083 - 1089.
- Zahavi, A. 1975. Mate selection--A selection for a handicap. *Journal of Theoretical Biology*, **53**, 205-214.
- Zuk, M. 1996. Disease, Endocrine-Immune Interactions, and Sexual Selection. *Ecology*, **77**, 1037-1042.
- Zuk, M. & Kolluru, G. R. 1998. Exploitation of Sexual Signals by Predators and Parasitoids. *The Quarterly Review of Biology*, **73**, 415-438.
- Zysling, D. & Demas, G. 2007. Metabolic stress suppresses humoral immune function in long-day, but not short-day, Siberian hamsters (*Phodopus sungorus*). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, **177**, 339-347.
- Zysling, D. A., Greives, T. J., Breuner, C. W., Casto, J. M., Demas, G. E. & Ketterson, E. D. 2006. Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (*Junco hyemalis carolinensis*). *Hormones and Behavior*, **50**, 200-207.