Commentary sheet

Thesis title: Influence of hormones and carotenoids on signalling, immunocompetence and performance in a lizard.

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Examiner 1

Page 2, paragraph 3 - Examiner writes: "*To be absolutely sure that the hormone assays are picking up the correct hormones, the kits used should have been validated for P.i. wiheimi. I would have liked to have seen parallelism undertaken with the ligand and the sample to ensure that the candidate was really picking up testosterone and corticosterone. To validate this on a close relative of the lizard species and then infer it is the same for this species is a dangerous assumption. I also would have lilted to have seen the sensitivity of the assay reported as well as the specificity of the antibody used. The candidate should consider validating the assay prior to publishing these results.*"

I agree with the concerns of the examiner, and will attempt to validate testosterone kits with *P. i.wilhelmi* prior to publishing the relevant chapters in this thesis.

Page 2 paragraph 4 – Examiner writes: "*The injection of PHA into one foot and PBS into another to assess the immune response is a classic measure of investigating immunocompetence and has been used successfully in a number of species. However, the candidate fails to tell us how many times the swelling of the pad was measured using the spessimeter. Ideally the candidate should have measured the swelling several times for both the antigen and the control and taken a mean swelling measurement. To take only one reading is erroneous.*"

I did measure the swelling of each foot twice; I have added this to the methodology on page 22, lines 8-10.

Page 2, paragraph 5 – Examiner writes: "*I would have liked to see the dosages used for the hormone implants into the animal. I do not have any idea of the dosage used until around chapter 4 or 5. Please report the dosages and explain how you derived the correct measurement of steroid added. This will be required when the chapters are crafted into papers.*"

I used dosages of (100 µg testosterone μL^{-1}), in keeping with existing protocols of manipulating testosterone in small lizards outlined by Cox et al (2005), I have now added a section outlining the making of implants including dosage on page 42, line2 12-16.

Page 2, paragraph 6 – Examiner writes: "*The statistical tests used in the chapters appear appropriate to the data analyses and the use of generalized linear models is recommended as has been used by the candidate. The graphs are informative and clear, but the numbers may need to be reduced when the chapters are converted to paper format.*"

I am aware that the number of graphs shown in each chapter is appropriate to a thesis, but would be excessive for publication. In order to publish this work I will reduce the number of graphs in each manuscript.

<u>Examiner 2</u>

Page1, paragraph 2 – Examiner writes: "*I think the reason for selecting the species of study is poorly explained at the Introduction section and could be highlighted according to behavioural and morphological traits specifically present in males*"
I have included additional motivation for the suitability of *P. i. wilhelmi* on page 9, lines 13-18.

Page 1, paragraph 2 – Examiner suggests including the paper by Fitze, Cote, San-Jose, Meylan, Isaksson, Andersson, Rossi & Clobert (2009) Carotenoid-Based Colours Reflect the Stress Response in the Common Lizard, PLoS ONE. 2009; 4(4): e5111

I have included a reference to this paper on page 111, lines 20 – 24.

<u>Examiner 3</u>

Page 1, general comments, comment 1 – Examiner writes: "*Nowhere in the analysis (figures and text) are "n" values given. I think this should be changed, especially as I was unclear exactly how samples were treated*."
Sample sizes are clearly indicated in the text (page 18, line 24; page 42 lines 21-24; page 63 line 15, page 78 line 28; page 100 lines 18-20).

Page 1, general comments, comment 2 – Examiner writes: "*The numbering of the pages is confusing. Specifically, we have pages 1 - 11 as the Introduction and then we start again at page 1 for Chapter 1. I think this should be corrected.*"
Page numbers are now sequential from the start of the introduction to the end of the text.

Page 1, general comments, comment 3 – Examiner writes: "*The figures should be labelled to reflect the Chapter, rather than having several Figure 1's and 2's.i.e. in Chapter 1 the first figure should be Figure 1.1, the second Figure 1.2 etc and in Chapter 2 the first figure should be Figure 2.1 etc.*"
I have changed this in the text.

Page 1, chapter 1, comment 1 – Examiner writes: "*There is a statement on p1, "In order for these signals to be maintained in a system they need to carry some cost" which I find difficult to understand as it implies that if there is no cost the signals won't be kept. I think this needs addressing*."
An assumption of costly signaling theory is that if there is no cost to a signal an individual can misrepresent its quality and so the system would become unreliable and therefore invalid. I have clarified this on page 12, lines 12 -13.

Page 1, chapter 1, comment 2 – Examiner writes: "*I am unclear as to exactly how many blood samples were analysed (hence statement above about giving "n" values). On page 8 you mention that due to the small quantity of blood in samples, a subsample of plasma from approximately 15 males from each season was used. What exactly is a subsample? Is it pooled plasma from 15 animals, i.e. one sample (in which case since 45 animals were captured does n = 3). If pooled samples are used the details of exactly how that was done need to be given. For example were equal volumes of plasma taken from each individual*

animal. Also, since samples were diluted 1:60 (p9) for assay then why was it necessary to pool samples?"

I used a subsample of plasma from 15 males rather than the plasma from all 45 lizards as the volume of each plasma sample was too small to measure testosterone, corticosterone and carotenoids for each lizard. None of the samples were pooled, each was measured individually. I have clarified this in the text (page 19, lines 22-24).

Page 1, chapter 1, comment 3 – Examiner writes: "*On p9 you need to give SE (or SD) and n values for the coefficients of intra-and inter-assay variation*."

As the corticosterone assays were carried out by Dr Maria Thaker, I do not currently have access to the SE or SD values for the coefficients. It is unconventional to include SD or SE values with coefficients of variation (Thaker, M, pers. comm.) but I will consider their inclusion in manuscripts for publication.

Page 2, chapter 2, comment 1 – Examiner writes: "*Since this is the first study in the thesis that uses implants to elevate hormone (in this case testosterone) levels, I think details of the implants should be given here, rather than later (Chapter 4). 1 do have some questions about the implants which I will address later (comments on Chapter 4).*"

I have added the details of hormone implants to this chapter (page 42, lines 12 - 16).

Page 2, chapter 2, comment 2 – Examiner writes: "*It really would have enhanced the study if blood corticosterone concentrations had been measured in this study, especially since the discussion refers quite a lot to the possible confounding influence of the steroid. You mention that "my study does not investigate the effects of corticosterone directly", however, I think that more explanation of why corticosterone wasn't measured is needed.*"

As assays were required to validate the efficacy of implants I was unable to allocate any to measuring corticosterone. The results suggest a possible effect of corticosterone which was addressed in chapter 3.

Page 2, chapter 3, comment 1 – Examiner writes: "*Giving implants to females is a novel approach, compared say to castrating males and looking at the effect of reduced testosterone. However, I'm not sure that enough is made out of the role of oestrogens. Is there any comparable literature of studies using a similar approach that can be used, for example to state that levels of oestrogens are likely to be very low, due to feedback inhibition?*"

There is a dearth of studies of testosterone manipulations in females which makes it difficult to understand the far-reaching implications of testosterone manipulation on aspects of female physiology. To my knowledge there are currently no studies on the effects of elevated testosterone on oestrogens in female animals. While fascinating for the future directions of research in this group, this is beyond the scope of this thesis which focuses on constraints to signalling in male *P. i. wilhelmi* and the information gained from studying females as to how the organisational effects of testosterone may have affected males in a way which was not experienced in females. I have recommended this for future study (page 73, lines 23-25).

Page 2, chapter 4, comment 1 – Examiner writes: "*The use of implants is clearly logical (and practical) however, the efficacy of the implants has not been addressed in the thesis. It would be advisable to provide some "in vitro" evidence about exactly how the implants work, i.e. provide data about how long the implants work for (could be that they no longer work after 14 days, when blood samples were taken) or the rate of hormone delivery from the implants. If in vitro data is not available are there any similar studies in the literature that could be cited? The statement that "hormone is left in the lumen" should be corrected/expanded.*"

I used an established technique and the implants used act as slow release for several months following insertion. I have clarified the statement of the deposition of the hormone in the lumen (page 78 line 17) and included a reference concerning the long term effectiveness of these implants (page 78, lines 20-22).

Page 2, chapter 4, comment 2 – Examiner writes: "*The above "in vitro" information could also help explain the differences between the levels of testosterone and corticosterone achieved by the implants. The doses of the two hormones are the same, yet the blood concentrations are markedly different (testosterone is maintained at 2000-4000 ng/dL, whilst corticosterone is maintained at 10 - 15 ng/dL).*

Since the hormones are likely to be handled the same (i.e. have similar volumes of distribution and rates of metabolism) the discrepancies are somewhat surprising. Maybe this could be addressed in the thesis."

In Page 2, chapter 4, comment 3 the Examiner also mentions the discrepancy between hormone levels in different implant groups: "*The marked differences in testosterone levels, depending on whether corticosterone was co-administered or not, although mentioned are not explained. This should be addressed.*"

I have added a paragraph discussing potential sources of the discrepancy in hormone levels between lizards with different implant combinations (page 92, lines 4-14).

Page 3, chapter 5, comment 1 – Examiner writes: "*The lack of evidence for an increase in blood levels of β-carotene with it's supplementation is somewhat disconcerting. I realise that there is little to be done, but other indices of elevated body carotene (e.g. total liver concentrations) should have been explored. Is there evidence from the literature that the method used by you actually does increase carotene delivery?*"

The method that I used to deliver carotenoids is a novel one, and unfortunately due to the logistical constraints of doing fieldwork in Mpumalanga and lab analysis in Pretoria meant that I was unable to assay carotenoids in the field. The method of using a carrier-oil to deliver substances to lizards through their skin is well established as an alternative to implants. In future studies I do recommend sacrificing a subsample of test subjects in order to measure carotenoid concentrations in different tissues (page 110, lines 20-22), but due to the obvious effects of the treatment compared to the control group of lizards shows that this is a valid method of carotenoid supplementation.

Page 3, chapter 5, comment 2 – Examiner writes: "*The levels of β-carotene measured in Chapter 1 seem to be significantly lower than the pre-supplementation values in this study. Is there any explanation?*"

I addressed this issue on page 110, lines 8 – 9, given the effect of supplementation on response variables it is likely that β-carotene was mobilised quickly following uptake.

Page 3, chapter 5, comment 3 – Examiner writes: "*The statement (p99) that "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" seems to imply that the assays were unreliable. Is this what was meant to be conveyed?*"

While I am confident that the technique employed in the assay for β-carotene, plasma levels are not necessarily an all-encompassing measure of carotenoids, as I have addressed on page 110. I recommend the measure of carotenoid levels in other tissues in future studies. It must be acknowledged that working with small lizards does limit the options available to measure these without sacrificing the animal which makes repeated-measures designs impossible.

Page 3, chapter 5, comment 4 – Examiner writes: "*How long was β -carotene supplementation given for? The text implies it was for two weeks, but it needs to be explicitly stated. In which case what was the basis of choosing two weeks (or however long it was given for)?*"

B-carotene was supplemented for 14 days. I have changed 'two weeks' to '14 days; on page 100, line 16.

Page 3, minor typographical/grammatical errors (new page numbers in brackets)

1) *Page iii (Abstract), last line "...vastly improved influenced ..."*

I have removed 'influence' Done (page iii, line 31).

2) *Page 3, 5th line from top "Testosterone is recognised ..."*

Done (page 3, line 6).

3) *Page 5, 10th line from top" ... according a various ..."*

Changed 'a' to 'to' (page 5 line 10).

4) *Page 8, 3rd line from top "...that are limited ..."*

Done (page 8, line 3).

5) *Page 6 (Chapter I), 7th line from top "...season (in spring before rains) - remove brackets?*

I believe that the brackets are necessary to provide clarity in this case. (page 17, line 8).

6) *Page 6, 7th line from bottom "..that there would be an associated ..."*

Done (page 17, line 21).

7) *Page9, 11th line from bottom "..had stopped moving.." - is a strange phrase to use.*

I have replaced 'had stopped moving' with 'had settled' (page 20 line 20)

8) *Page 28, 14th line from top "will" should be "with".*

Done (page 39, line 14).

9) *Page 50, 7th line from bottom "...may have testosterone levels that co-vary with testosterone in males.." One of the testosterones is incorrect?*

Both are correct as I am referring to the fact that testosterone levels in females may co-vary with testosterone levels in males. I have clarified this in the text (page 61, line 12).

10) *Page 51, last line ".. for further details concerning.."*

Done (page 62, line 30).

11) *Page 62, top line "..are under activational.."Take out the "a".*

Done (page 72 line 1).

12) *Page 65, 12th line from top "...which allows the animal to cope ..."*

Done (page 75, line 3).

13) *"generate" not "general".Page 66,14th line from bottom "."constrains not "constraints".*

Done (page 76, line 17).

14) *Page 68, 11th line from bottom "..for further details see. .."*

Done (page 79, line 23)

15) *Page 86, 6th line from top "..hypothesis.."*

This refers to three different hypotheses (ICHH, SL-ICHH and corticosterone only) and so should be plural (page 91, line 1).

16) *Page 88, 10th line from bottom "..and juveniles are drab brown.."*

Done (page 99, line 20)

17) *Page 90,2nd line from top "testosterone" should be "carotenoids"*

Done (page 101, line 2).

18) *Page 111, 8th line from bottom "corticosterone" not "corticsoterone"*

Done (page 121, line 27).

19) *Page 114, 9th line from top "generate" not "general".*

Done (page 124 line 10).

Helen Place

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