Direct and indirect effects of whole body heat exposure on germ cells and spermatozoa.

A thesis submitted to the University of Adelaide in total fulfilment of the requirements for the degree of Doctor of Philosophy

by

HARSHA WECHALEKAR

School of Medical Sciences

The University of Adelaide

Adelaide, South Australia

August 2015

This thesis is dedicated to *my family*

TABLE OF CONTENTS

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holder(s) of each work.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Wechalekar, H., Setchell, B.P., Peirce, E.J., Ricci, M., Leigh, C. and Breed, W.G. (2010). Whole-body heat exposure induces membrane changes in spermatozoa from the cauda epididymidis of laboratory mice. Asian Journal of Andrology, **12(4)**, 591-598.

Wechalekar, H., Setchell, B.P., Peirce, E.J., Leigh, C. and Breed, W.G. (2011). Are male germ cells of hopping mice (*Notomys alexis*) sensitive to high environmental temperatures? Australian Journal of Zoology, **59**, 249-256.

H. Wechalekar, B. P. Setchell, K.R. Pilkington, C. Leigh, W.G. Breed and E. Peirce (2014). Effects of whole-body heat on male germ cell development and sperm motility in the laboratory mouse. Reproduction, Fertility and Development. [online] Available: http://dx.doi.org/10.1071/RD13395

ACKNOWLEDGEMENTS

I take this opportunity to express my thanks and gratitude to my supervisor, co-supervisors, colleagues, and friends, without their guidance and moral support this work would not have been possible.

To my supervisor, Dr. Eleanor Peirce, my deepest gratitude is due. She had been the guiding force all along, ever ready to lend a helping hand whenever I stumbled. I would like to thank my co-supervisors Prof. William G. Breed and Prof. Brian Setchell for their encouragement that has been motivating and comments thought provoking. Their help has been considerable and criticisms constructive. I express my sincere thanks to Dr. Mario Ricci for his needful advice and assistance. I also thank Chris Leigh for teaching histological techniques and for the help he rendered in the laboratory. Thanks to Tavik Morgenstern for production of digital images.

I would like to thank Gail Hermanis and Nadia Gagliardi for laboratory assistance. Thanks to the staff members of Adelaide microscopy for letting me use their excellent services. I would like to thank Dr. Joanne Bowen (Department of Medical Oncology, Royal Adelaide Hospital) for teaching specialized immunohistochemical technique and for using her laboratory facility.

I express my heartfelt thanks to my friends Dr. Devendra and Smita Hiwase (Department of Hematology, Royal Adelaide Hospital) for teaching and helping standardize flow cytometry techniques. They instilled in me the desire for the pursuit of perfection. I also thank Dr. Kate Pilkington (Department of Hematology, Royal Adelaide Hospital) for her assistance in preparing the flow data and analysis. I am grateful to my friend Ms. Smiti Malik for her timely help and effort in formatting this thesis.

Last but not the least; I convey my regards and gratitude to my family for their continual support.

Harsha Wechalekar

STATEMENT OF AUTHORITY AND CONTRIBUTION

Statement of Authorship

Title of Paper	Whole-body heat exposure induces membrane changes in spermatozoa from the cauda epididymidis of laboratory mice.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Publication Style
Publication Details	Wechalekar, H., Setchell, B.P., Peirce, E.J., Ricci, M., Leigh, C. and Breed, W.G. Asian Journal of Andrology, 2010,12(4), 591-598.

Principal Author

Name of Principal Author (Candidate)	Harsha Wechalekar		
Contribution to the Paper	Involved in the experimental design, conducted the animal experiments, recorded,analyzed the data, and prepared the manuscript for submission.		
Overall percentage (%)	90%.		
Signature		Date	03/08/15

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Prof. Brian Setchell		
Contribution to the Paper	Assisted in preparation of the general plan of the study in its preparation as well as editing of the manuscript.		
Signature		Date	4/8/15.

Name of Co-Author	Prof. William G. Breed		
Contribution to the Paper	Assisted in preparation of the general plan of the study with preparation as well as editing of the manuscript.		
Signature		Date	August 4TH, 2015

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Dr. Eleanor Peirce
Contribution to the Paper	Assisted in preparation of the general plan of the study in its preparation as well as editing of the manuscript.
Signature	Date 4/8/2015

Name of Co-Author	Dr. Mario ricci
Contribution to the Paper	Assisted in editing the manuscript and in preparation of the general plan of the experiment
Signature	Date 6/8/2015

Name of Co-Author	Mr. Chris Leigh
Contribution to the Paper	Assisted with tissue sampling, processing and various other histological techniques.
Signature	Date 6/8/15

Statement of Authorship

Title of Paper	Are male germ cells of hopping mice (Notomys alexis) sensitive to high environmental temperatures?
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Publication Style
Publication Details	Wechalekar, H., Setchell, B.P., Peirce, E.J., Leigh, C. and Breed, W.G. Australian Journal of Zoology, 2011, 59, 249-256.

Principal Author

Name of Principal Author (Candidate)	Harsha Wechalekar		
Contribution to the Paper	Conducted the animal experiments, recorded and analyzed the data, and prepared the manuscript for submission.		
Overall percentage (%)	85%		
Signature		Date	03/08/15

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Prof. Brian Setchell		
Contribution to the Paper	Assisted with the general plan of the study, data interpretation and preparation of the manuscript.		
Signature		Date	4/8/15

Name of Co-Author	Prof. William G. Breed		
Contribution to the Paper	Assisted with the general plan of the study and helped in conducting the experiments, data interpretation and preparation of the manuscript.		
Signature		Date	August 4th, 2015

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Dr. Eleanor Peirce
Contribution to the Paper	Assisted in preparation and editing of the manuscript.
Signature	Date 4/8/2015

Name of Co-Author	Mr. Chris Leigh
Contribution to the Paper	Assisted with the animal experiments, tissue sampling and histological techniques.
Signature	Date 6/8/18

Statement of Authorship

Title of Paper	Effects of whole-body heat on male germ cell development and sperm motility in laboratory mouse.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Publication Style
Publication Details	Reproduction, Fertility and Development, 2014 Sep 11. doi: 10.1071/RD13395.

Principal Author

Name of Principal Author (Candidate)	Harsha Wechalekar		
Contribution to the Paper	Conducted the animal experiments, recorded and analysed the data, and prepared the manuscript for submission.		
Overall percentage (%)	90%.		
Signature		Date	03/08/15

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Prof. Brian Setchell		
Contribution to the Paper	Assisted in preparation of the general plan of the study, in conducting experiments, and in data interpretation and preparation of the manuscript.		
Signature		Date	4/8/15

Name of Co-Author	Prof. William G. Breed		
Contribution to the Paper	Assisted in the general plan of the study, data interpretation and editing of the manuscript.		
Signature		Date	August 4th, 2015

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Ms. K.R. Pilkington
Contribution to the Paper	Assisted in interpretation and preparation of flow cytometry data in the manuscript including the flow graphs.
Signature	Date 1/9/2015

Name of Co-Author	Dr. Eleanor Peirce
Contribution to the Paper	Assisted in preparation and editing of the manuscript.
Signature	Date 4/8/2015

Name of Co-Author	Mr. Chris Leigh
Contribution to the Paper	Assisted with the animal experiments, tissue sampling and histological techniques.
Signature	Date 6/8/15

ABSTRACT

Exposure to extreme temperature conditions such as occurs in certain occupations is known to induce male infertility. In humans and most of the eutherian mammals, it has been shown that whole body heat stress decreases fertility and produces defective embryos. However, the mechanisms producing fertility defects post exposure to whole body heat are not yet fully determined. Hence, the present study aimed at gaining some insight into the mechanisms producing fertility defects after whole body heat stress.

Laboratory mice were exposed to constant temperatures higher than their core body temperature of 37-38^{o}C for 8h on three consecutive days. A decrease in testis weight occurred as early as 7 days post exposure to heat with weights comparable to controls by 21 days post heat exposure. Histology of the testicular tissue showed germ cell apoptosis affecting the pachytene spermatocytes and the spermatids. Germ cell apoptosis was investigated by TUNEL 16h, 7, 14 and 21 days following the heat treatment. Apoptosis was found to be stage-specific affecting early and late stages of the seminiferous epithelium cycle on day 7 and 14 post exposure to heat stress with involvement of all stages of seminiferous epithelium 16h following heat stress. Reduction in germ cell apoptosis was evident 21 days post exposure. To determine the mechanism of germ cell apoptosis, caspase-3 proteins were detected in apoptotic germ cells following heat stress which showed strong positivity. Hence, we concluded that whole body heat stress results in caspase-3 mediated germ cell apoptosis. Furthermore, changes in cauda epididymal spermatozoa were investigated to determine sperm apoptosis following heat stress. Sperm apoptosis was detected by the exteriorization of phosphotidylserine (PS) on the outer layer of the plasma membrane. The results showed an early and late apoptosis in caudal epididymal spermatozoa 16hr following heat treatment. A second experiment was conducted to determine the time of appearance of changes in spermatozoa following whole body heat stress for which the duration of exposure to heat of

37-38ºC was reduced to one-day period. This showed more spermatozoa in the early phase of apoptosis with fewer dead spermatozoa, which is suggestive of a time and temperature dependant pattern of sperm apoptosis.

Arid-adapted *Notomys alexis* (hopping mice) were also investigated to determine whether differences in response to whole body heat have evolved in extreme climate conditions. Therefore hopping mice were exposed to similar temperatures as laboratory mice to investigate heat influences on germ cells and spermatozoa. Like the laboratory mice, germ cells and spermatozoa of hopping mice also showed apoptosis. However, unlike in laboratory mice, stage specificity in apoptosis could not be determined in hopping mice because of the presence of more than one cell association within the cross sections of the seminiferous tubules. Similar to laboratory mice, germ cell apoptosis in hopping mice was caspase-3 mediated. The vasculature of hopping mice was also determined to look for any variations in the cooling mechanisms, which could have resulted in germ cell apoptosis. We found the absence of a coiled testicular artery suggestive of a lack of such a cooling mechanism in hopping mice that could have resulted in germ cell and sperm apoptosis. We also investigated changes in germ cells following experimental cryptorchidism, which showed tubular degeneration with low numbers of germ cells lining the seminiferous epithelium. Thus the findings indicated that whole body heat had a detrimental effect on developing germ cells and spermatozoa.

It is also known that heat stress changes other bodily functions to maintain body homeostasis. Heat stress is associated with an activation of the hypothalamic-pituitary axis, which is followed by an increase in blood cortisol levels in the circulation as a result of increased synthesis of corticosteroids from the adrenocortical cells. Hence the current study also determined changes, if any, in the adrenal glands, especially the adrenocortical cells of laboratory and hopping mice following whole body heat exposure.

Heat stress resulted in the formation of vacuoles, dilated capillaries and interstitial fibrosis in cortical areas of the adrenal glands in both the species. In addition, large syncytial bodies were evident in hopping mice adrenal cortical cells. Thus it is evident from the study that germ cell and sperm apoptosis are a result of either a direct effect of heat on germ cells and/ or due to changes in the body hormonal milieu following whole body heat stress.

Thus this study showed an activation of caspases in producing germ cell apoptosis and externalization of PS in inducing sperm apoptosis following whole body heat stress with changes in the adrenocortical cells following heat stress. This study demonstrated that an arid-adapted species *Notomys alexis*, although evolved in extreme environmental conditions, also is affected by high temperatures. Hence, this study gives some insight in the reasons for reduced fertility following whole body heat stress.