



Temperature-dependent Sex Determination in the Viviparous lizard *Eulamprus tympanum*

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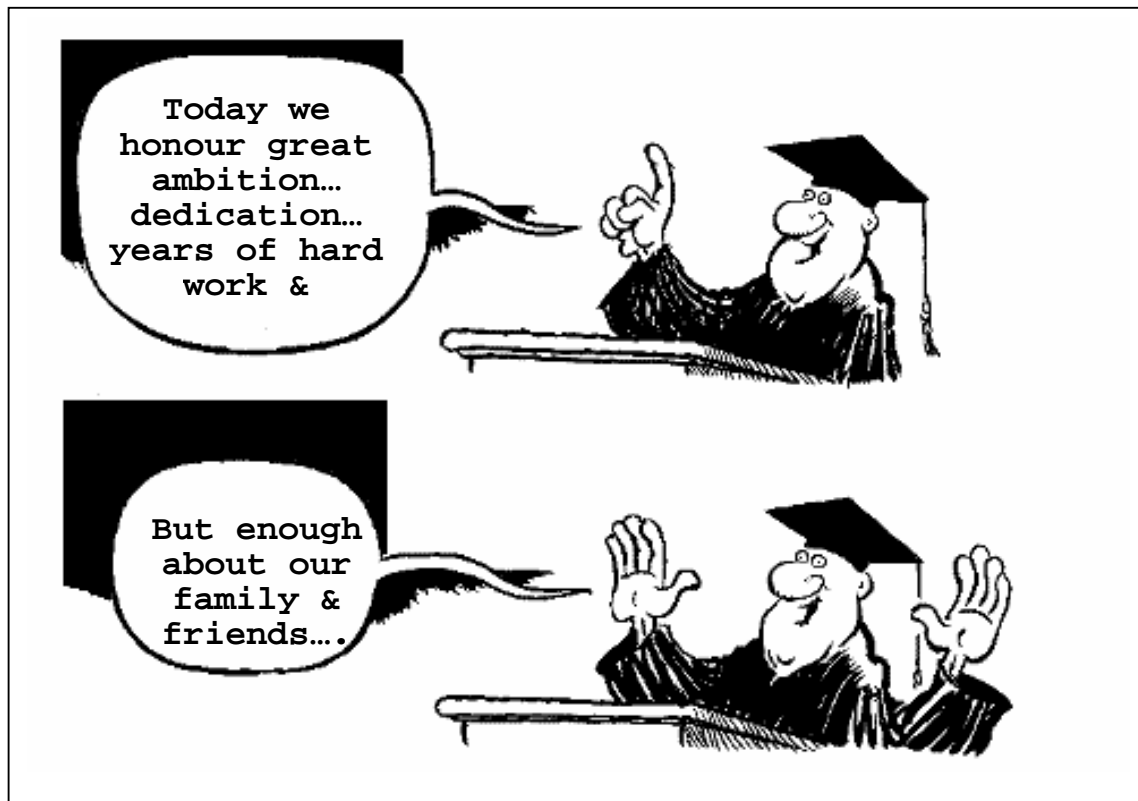
Declaration

I hereby declare that this thesis is less than 100,000 words in length and that I have not submitted a similar thesis at another University. The work contained in this thesis is my own, and contains no materials previously written by another person, except where due reference is given in the text. The research was conducted within the School of Biological Science at The University of Sydney, New South Wales, Australia.

Kylie Anne Robert

August, 2003

Dedicated to my Family and Friends



"Being a graduate student is like becoming all of the seven dwarfs. In the beginning you're Dopey & Bashful. In the middle you are Sneezzy (sick), Sleepy (coffee deprived), and Grumpy (irritable). But at the end, they call you Doc, and then you're Happy."

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Abstract

There are a remarkable variety of sex determination systems among different animal taxa. In most animals, sex is determined chromosomally. Although in an increasing number of animals sex determination has been found to be influenced primarily by the environment. Species with genotypic sex determination (GSD) have their sex determined at the time of fertilization, by genetic factors alone and those with environmental sex determination (ESD) have their sex determined by environmental factors that act after fertilization. Temperature-dependent Sex Determination (TSD), whereby the sex of the developing embryos depends on the temperature at which they develop is widespread in oviparous reptiles and occurs in all crocodylians, marine turtles and tuatara examined to date and is common in many freshwater turtles and lizards.

SECTION ONE

Temperature-dependent sex determination (TSD) was never expected to occur in viviparous reptiles, as thermoregulation by pregnant females would result in relatively stable gestation temperatures. Temperature-dependent sex determination and viviparity goes against all the basic assumptions that TSD occurs in oviparous reptiles where temperatures within a nest vary widely. However, skewed sex ratios as a result of incubation temperature indicated the possibility of TSD in the viviparous lizard *Eulamprus tympanum*. In my first experiments I show the first recorded case of a viviparous reptile with TSD. The developing embryos of the viviparous skink *E. tympanum* are subject to TSD, with gestation temperature having

a highly significant effect on sex and warmer temperatures giving rise to male offspring (Chapter 1).

Sex is fully determined at the time of birth and can be differentiated histologically into testes or ovaries (Chapter 2). The morphology and histological characteristics of the gonads of neonatal *E. tympanum* resulting from the treatment temperatures described in chapter 1 illustrate that sex in *E. tympanum* is easily distinguished at the time of birth and corresponds with the presence or absence of hemipenes. Males are histologically characterised by an elongated gonad consisting of seminiferous tubules with either no cortical epithelium or, if present at all, in a very thin band. If they are present, Müllerian ducts, showing signs of degeneration, are attached to the kidney by a shortened mesosalpinx. Females are histologically characterised by an irregularly shaped gonad consisting of a thick cortical epithelium that occasionally contains oocytes. The Müllerian ducts are obvious structures attached to the kidney by a fibrous mesosalpinx. The presence or absence of hemipenes is a reliable technique for determining sex in newborn *E. tympanum*. Sex determination is easiest to perform on neonates within the first few days of birth as hemipenes become increasingly difficult to evert as neonates age, however, with practice they are easily identified without full eversion.

SECTION TWO

The thermal biology of *E. tympanum* in the field is restricted by both the thermal properties of their habitat (Chapter 3) and behavioural modifications when faced with a predation threat (Chapter 4).

The available temperatures in the field suggest that TSD is biologically relevant in the species and not just a laboratory artefact; *E. tympanum* can attain mean selected temperatures achieved in the laboratory but the proportion of time at the temperature is restricted. Females actively thermoregulate in the field, although they are restricted in their efficiency of thermoregulation by environmental constraints, for example, microhabitat structure, weather conditions, predator avoidance and social ranking. The highly territorial nature and high densities of *E. tympanum* present in Kanangra Boyd National Park potentially force less dominant individuals into less favourable habitats that are significantly cooler. An important point is that gravid females in more favourable habitats in the period encompassing the middle third of development (the assumed sex determining period) are selecting higher temperatures, with lower variance and have greater thermoregulatory efficiency than during the rest of pregnancy, therefore, thermoregulating more precisely during this thermosensitive period (Chapter 3).

Chemosensory cues provide important information on the risk of predation. Hence, chemoreception is a common mechanism used by many species to detect the presence of, and subsequently respond to, a potential predator. The perceived risk of predation may force retreat to sub-optimal conditions, forcing a trade-off between the risk of predation and the ability to acquire resources. The basking regime maintained by gravid female *E. tympanum*, can directly alter sex ratios of offspring produced through temperature-dependent sex determination (Chapter 1). The avoidance of predator scents may restrict basking ability and in turn alter the sex of offspring produced. I measured responsiveness to chemical cues using tongue flicks as an indicator of chemical discrimination in females of different reproductive

condition. I then measured activity and basking behaviour of gravid and non-gravid females in experimental enclosures in the presence of various chemical stimuli to determine if basking opportunity is compromised by the presence of a predator scent. Females respond differently depending upon reproductive condition, with gravid females responding most significantly to a predator scent. Activity, basking frequency, and time spent in the open (basking duration) are significantly reduced in gravid females in the presence of a predator stimulus. Under laboratory conditions, gravid females modify their behaviour and forego the opportunity to bask when there is a perceived predation risk (Chapter 4).

SECTION THREE

As female viviparous reptiles can regulate the temperature of the embryo by maternal temperature selection (Chapter 1), the occurrence of TSD in *E. tympanum* opens the possibility for females to select the sex of offspring. Reproducing females may benefit by facultatively adjusting their investment into sons over daughters or vice versa, in response to population wide shifts in adult sex ratios. Female *E. tympanum*, can manipulate the sex of their offspring in response to sex imbalances in the population using temperature-dependent sex determination (Chapter 5). When adult males are scarce, females produce male-biased litters and when adult males are common, females produce female-biased litters. The cues used by a female to assess the adult population are not known, but presumably depends upon the female's experience throughout the breeding season and is the subject of further investigation (Chapter 6). The maternal manipulation of offspring sex ratio in *E. tympanum* suggests a selective advantage of temperature-dependent sex determination.

Any facultative sex ratio response needs to recognise the scarcity of one sex in order to overproduce that sex in the next generation; offspring sex ratio will vary inversely with adult sex ratio. Maternal sex allocation in *E. tympanum* is linked with population (or adult) sex ratio (Chapter 5), and one of the mechanisms by which females recognise an imbalance may be linked to visual recognition of males (Chapter 6). Females maintained throughout pregnancy without any male stimulus produce entirely male offspring (Chapter 5). In contrast females exposed to male stimulus produce both sexes (Chapter 5). Females respond differently to varying degrees of male stimulus and visual recognition of males in a population may be more important than chemoreception. In the absence of visual cues, females produce more male offspring, even when chemosensory cues are present (Chapter 6).

The study system presented here offers many advantages over oviparous species with TSD, due to *E. tympanum* being relatively short lived and fast maturing. Thus, the fitness consequences over multiple generations as a result of gestation can be investigated. Viviparity allows maternal control of embryonic temperature during gestation and a means of maternal sex allocation. Until now the maternal side of TSD and sex allocation has been where the mother deposits her eggs and the allocation of sex steroid hormones at oviposition, both of which have been difficult to study. The work presented and the study system itself should inspire great interest in TSD and viviparous reptiles.

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From a young age it was *always* evident that I was going to work with animals. Every creature great and small “*followed me home*”; something my family tolerated and accommodated admirably (and still are!.....they just had to purchase a farm). I was always fascinated with every aspect of animal biology and would sit for hours watching the chickens in the chook pen, and much to my sibling’s horror, a deceased pet often became a biology dissection. While school friends idolised movie stars, I idolised David Attenborough, wildlife documentaries today (thanks very much to cable T.V.) can still turn me into a couch potato! (Mum & Dad, personal communication).

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Preface

The aim of my thesis was to investigate the possibility, first suggested by my honours research, of temperature-dependent sex determination (TSD) in a viviparous lizard. I investigated the interaction between females and their thermal environment to gain a better understanding of the phenomenon of TSD. My specific aims were to: (1) test the possibility of TSD in the viviparous species *Eulamprus tympanum*; (2) determine and understand temperature selection and thermal biology by pregnant lizards; (3) determine the factors that affect thermal biology of *E. tympanum*, in particular – weather conditions, habitat structure and predator avoidance; and (4) determine the extent of maternal choice on offspring sex allocation.

The thesis consists of three sections each with two complementary chapters linked by a common research theme. In accordance with changing practices at The University of Sydney, each chapter is prepared as a separate stand-alone scientific manuscript and consequently there is some repetition in the various materials and methods. Additionally, each chapter contains its own introduction, discussion, and literature cited. Chapters that have been published, accepted or submitted for publication in joint authorship are identified at the beginning of the chapter. In all cases where I am not the sole author, either one or both of my supervisors are listed as co-authors for their contribution in the form of advice on experimental design, writing, and statistical guidance. In all cases I initiated and conducted the research and prepared the manuscripts for publication.

Michael B Thompson

Frank Seebacher

Signatures of co-authors certifying agreement with the contributions detailed above.

Sex determining mechanisms in plants and animals are remarkably diverse. My thesis begins with an overview and introduction to sex determination in animals and in particular environmental sex determination.

Section One

I discovered skewed sex ratios in *E. tympanum* during my Honours study, which suggested the possibility of TSD in a viviparous reptile - a first for a viviparous reptile. I conducted a series of incubation experiments to confirm the presence of skewed sex ratios in response to different thermal regimes (chapter 1) and describe the histological characteristics of the gonads and oviducts of the resulting offspring (chapter 2).

Section Two

Most reptiles maintain their body temperatures within operational ranges through behavioural thermoregulation. Some circumstances, such as weather conditions, predator avoidance, and social interactions may compromise thermoregulatory opportunities. The second section of the thesis describes the thermal biology of *E. tympanum* under controlled laboratory conditions, identifies the thermal opportunities available to lizards in their natural environment and describes

the thermal biology of females in the field (chapter 3) to establish if TSD is biologically relevant in *E. tympanum*. One of the factors that could compromise the thermal selection by lizards under field condition was investigated by a series of manipulative experiments to identify if predator avoidance may compromise basking opportunity (chapter 4). In so doing, I developed techniques to collect continuous thermal data from small lizards (Appendix 1), and to record behavioural observations using video surveillance (Appendix 2).

Section Three

Environmental sex determination has stimulated considerable research, debate and speculation from evolutionary biologists. No one can fault evolutionary biologists for a lack of creativity when it comes to devising hypotheses on adaptive significance of TSD or the adaptive significance of sex ratio variation. In Section 3, I outline maternal manipulation of offspring sex in response to population wide shifts in adult sex ratio as an adaptive advantage of TSD in a viviparous reptile, and investigate a possible maternal controlling mechanism in an attempt to advance theory into the evolution of sex determining mechanisms. Several models suggest females may benefit by facultatively skewing their investment between sons and daughters and I examine facultative sex allocation in *E. tympanum* (chapter 6). Any facultative sex ratio response needs to recognise the scarcity of one sex in order to overproduce that sex in the next generation; I examine what cues a gravid female *E. tympanum* may use (chapter 7).

Finally I provide some concluding comments on the research presented in the thesis noting the importance of the research in context with earlier work and the

future direction of studies on TSD. Most importantly, TSD and viviparity provide enormous scope for future research, and should provide an ideal model in studies on the maternal effects that may play an adaptive role in the maintenance of TSD.

INTRODUCTION

Sex Determination: A remarkable diversity

There is a remarkable variety of sex determination systems among animal taxa. In most animals, sex determination results from one of two mechanisms 1) Genotypic Sex Determination (GSD), in which sex is determined at the time of fertilization, by genetic factors independent of environmental influence or 2) Environmental Sex Determination (ESD), in which sex is determined by environmental factors that act after fertilization (Ciofi and Swingland, 1997). ESD has been studied in a variety of vertebrates and invertebrates including fishes (Conover, 1984; Conover *et al.*, 1992; Beamish, 1993; Baroiller *et al.*, 1996; Luckenbach *et al.*, 2003), crustaceans (Naylor *et al.*, 1988; Bergström, 1997; McCabe and Dunn, 1997; Rigaud *et al.*, 1997), reptiles (Bull, 1980; Ferguson and Joanen, 1983; Deeming and Ferguson, 1988; Ewert and Nelson, 1991; Janzen and Paukstis, 1991; Lang and Andrews, 1994), nematodes, rotifers, polychaetes and echiurids (Korpelainen, 1990).

Temperature-dependent Sex Determination

Sex in many of these species is determined by incubation temperature, a type of ESD known as Temperature-dependent Sex Determination (TSD), whereby the sex of the developing embryos depends on the temperature at which they develop (reviewed Bull, 1980). This mechanism of sex determination is particularly widespread in the reptiles and occurs in all crocodylians, marine turtles and tuatara

examined to date and is common in many freshwater turtles and lizards (Bull, 1980; Ewert and Nelson, 1991; Janzen and Paukstis, 1991; Cree *et al.*, 1995; Lance, 1997; reviewed Ciofi and Swingland, 1997). The sex determining mechanism is not known in many species of squamate reptiles relative to the large number of species within the group, with sex determination being described in less than 10% of extant reptilian species. No clear relationship has emerged between TSD and GSD in squamate lineages (Wibbels *et al.*, 1994; Viets *et al.*, 1994). Temperature-dependent sex determination was thought to be a natural sex determining mechanism found exclusively in species without sex chromosomes (Bull, 1980). However, TSD is now known to co-occur with GSD in several species within some lizard families including the Agamidae, Eublepharidae, Gekkonidae and possibly the Lacertidae, Varanidae and Iguanidae (Viets *et al.*, 1994; Ciofi and Swingland, 1997; Harlow and Shine, 1999; Shine, 1999).

The existence of GSD and TSD in closely related species suggests that the sex determining systems are themselves closely related (Wibbels *et al.*, 1994). Contrasting hypotheses have suggested that TSD is the primitive mode of sex determination and that GSD is derived from TSD (Ohno, 1967) or TSD is not ancestral, with temperature simply over-riding an underlying GSD mode (Raynaud and Pieau, 1985). Temperature over-rides an underlying genetic sex determining system in several species of fish (Conover and Kynard, 1981; Baroiller *et al.*, 1995; Craig *et al.*, 1996; Goto *et al.*, 1999; Devlin and Nagahama, 2002; Luckenbach *et al.*, 2003), urodeles (Dorazi *et al.*, 1995; Chardard and Dournon, 1999), and frogs (reviewed Dournon *et al.*, 1990). The two modes of sex determination co-occur within the lizard *Bassiana duperreyi* that is simultaneously affected by sex

chromosomes and incubation temperature (Shine *et al.*, 2002). It is not surprising to find multiple sex determining mechanisms given the frequency of phylogenetic transitions between GSD and ESD (Cole, 1971; reviewed Viets *et al.*, 1994). The variety of GSD mechanisms (female heterogamety, male heterogamety, homomorphic sex determination) also suggests multiple origins of sex determination (reviewed Viets *et al.*, 1994).

There are several different patterns of TSD in reptiles:

1) Low temperatures produce females, high temperatures produce males and intermediate temperatures produce both sexes (FM), in most lizards (Viets *et al.*, 1994) and some crocodiles (Ferguson and Joanen, 1983)

2) Low temperatures produce males, high temperatures produce females and intermediate temperatures produce both sexes (MF), in some turtles (Ewert *et al.*, 1994).

3) One sex is produced at both high and low incubation temperatures and intermediate temperatures produce the opposite sex (FMF), in some turtles (Ewert and Nelson, 1991), lizards (Deeming and Ferguson, 1988) and many species of crocodile (Lang and Andrews, 1994).

The mode of sex determination has been studied in only one species of viviparous squamate, the water snake *Nerodia fasciata*. Although the sex ratio was slightly skewed, it was not significantly different from an equal ratio (Osgood, 1980).

Re-examination of the data by between clutch analyses of sex ratios revealed an influence of temperature on intra-clutch sex ratios (Dunlap and Lang, 1990), leaving open the possibility of TSD in a viviparous species. TSD was never expected to occur in viviparous reptiles as thermoregulation by pregnant females would result in relatively stable gestation temperatures and goes against all the basic assumptions that TSD occurs in oviparous reptiles where temperatures within a nest varies widely. However, sex ratios skewed by incubation temperature indicated the possibility of TSD in the viviparous lizard *Eulamprus tympanum* (Robert, 1999). As female viviparous reptiles can regulate the temperature of the embryo by maternal temperature selection, the occurrence of TSD in viviparous species opens the possibility for females to select the sex of offspring.

Gonadal Differentiation and Temperature-dependent Sex Determination

Despite the differences in sex determining mechanisms, the pattern of morphological development of the gonads in all vertebrate groups is common. Embryonic differentiation of the gonads was first described by Risely (1933) in the musk turtle *Sternotherus odoratus* and since then several other studies have described the macroscopic and microscopic anatomy of the gonads (Forbes, 1956; Miller, 1963; Fox, 1977; Yntema and Mrosovsky, 1980; Yntema, 1981; Ferguson and Joanen, 1983; Raynaud and Pieau, 1985; Austin, 1988; Wibbels *et al.*, 1991). Macroscopically, the gonads lie on the dorsal abdominal wall overlying the kidneys on either side of the midline mesentery (Ferguson and Joanen, 1983). The gonads can show variation macroscopically in shape and colour, but the position and general appearance of the gonad is similar in both males and females. Female gonads are

usually longer than those of males and a regressing Müllerian duct or the complete absence of a Müllerian duct most often characterizes males. Histologically the differences are clearer. Testes are an elongated oval shape, seminiferous tubules are evident in the testis, cortical germinal epithelium is absent, and if present, Müllerian ducts show signs of degeneration (Yntema, 1981; Ferguson and Joanen, 1983). Ovaries are more irregularly shaped, cortical germinal epithelium is present and they often contain oocytes. The Müllerian ducts are obvious and supported by fibrous mesosalpinx attached to the kidney (Yntema, 1981; Ferguson and Joanen, 1983).

Sex Determining Genes and Temperature-dependent Sex Determination

There has been considerable progress in identifying the molecular basis of sex determination in species with genetic sex determination (GSD), but the molecular mechanisms responsible for TSD are unknown. A number of key genes involved in mammalian sex determination have been identified; the SRY gene on the Y chromosome is regarded as the critical testis-determining gene in eutherian mammals and, in the absence of SRY, an ovary develops (Spotila *et al.*, 1994; Lance, 1997). There is no known homologue of SRY in TSD species (Elf, 2003), although there are SRY-like genes in many reptiles and it is thought that sex differentiation in reptiles is similar to mammals i.e. the same genes and hormones act in a similar manner but, sex determination is different (Lance, 1997). SOX9 is also necessary for testis differentiation in mammalian sex determination and for the activation of anti-Müllerian hormone that is critical in the regression of the female structures derived from the Müllerian ducts (Foster *et al.*, 1994; De Santa Barbara *et*

al., 1999; Western *et al.*, 1999). SOX9 has been expressed in a marine turtle (Moreno-Mendoza *et al.*, 2001), a freshwater turtle (Spotila *et al.*, 1998), the leopard gecko (Valleley *et al.*, 2001) and the alligator (Western *et al.*, 1999), although, the regulatory mechanisms of the gene seems different in each species and expressed at a later stage than found in mammals (Western and Sinclair, 2001; Elf, 2003). Several other genes such as WT1, SF1 and DAX1 that are involved in gonadal formation and or differentiation in mammals have been characterised in reptiles with TSD (Spotila *et al.*, 1998; Fleming *et al.*, 1999; Western *et al.*, 2000) but again differ in their expression patterns (Pieau *et al.*, 2001). Aromatase receptor genes that play central roles in gonadal differentiation have been isolated from two turtles and the American alligator and identified as being responsible for differential aromatase activity with different incubation temperatures (Gabriel *et al.*, 2001; reviewed Pieau *et al.*, 2001). All the genes except SRY that are known to be involved in sex determination in mammals have their equivalents in reptiles with TSD: their expression may not only be different from mammals, but also among different reptile species (Pieau *et al.*, 2001).

Sex Steroid Hormones and Temperature-dependent Sex Determination

Ovarian differentiation in eutherian mammals can occur in the absence of estrogens, unlike reptiles that require the presence of estrogens for ovarian differentiation; in the absence of estrogen a testis develops (Lance, 1997). In reptiles with TSD, embryos will develop as females at male inducing temperatures when treated with estrogens and will develop as males at female induced temperatures when estrogen synthesis is blocked (Lance, 1997).

Yolk hormones have been measured in many birds (Winkler, 1993; Schwabl, 1993; Petrie *et al.*, 2001); and recently in oviparous reptiles (Bowden *et al.*, 2000; Lovern and Wade, 2001; Elf *et al.*, 2002a; Elf *et al.*, 2002b). It is clear that yolk steroid hormones during development influence many characteristics of the resulting offspring including sex, growth and behaviour (Elf *et al.*, 2002b; Petrie *et al.*, 2001). Incubation temperature significantly affects the hormonal environment of the developing embryo of oviparous snapping turtles, *Chelydra serpentina* (Elf *et al.*, 2002a) and the alligator, *Alligator mississippiensis* (Conley *et al.*, 1997). In the snapping turtle, those eggs developing at female producing temperatures have a hormonal environment whereby estradiol concentrations declined at a slower rate than those at mixed sex and male producing temperatures, and differences in estradiol concentrations were most pronounced during the thermosensitive period (Elf *et al.*, 2002a). Yolk steroid hormones also decrease during development in the red-eared slider turtle, *Trachemys scripta elegans* (Bowden *et al.*, 2002). Current research is examining the interactions between genes and hormones in the sex determination pathway in reptiles with TSD (Fleming and Crews, 2001; Elf, 2003).

Adaptive significance of Temperature-dependent Sex Determination

Although temperature-dependent sex determination has been well studied, the adaptive significance of TSD remains controversial (Charnov and Bull, 1977; Bull, 1980; Shine, 1999a; Girondot and Pieau, 1999; Shine, 1999b; Merchant-Larios, 2001). Each sex determining mechanism seemingly enhances fitness. There have

been many adaptive hypotheses suggested on the evolutionary significance of TSD, which can be broadly placed within the following groups:

- 1) ancestral condition hypothesis - TSD is the ancestral form of sex determination and there is no adaptive significance (Shine, 1999a).
- 2) quasi-neutral hypothesis - TSD persists because it confers only minimal disadvantage in comparison with GSD (Girondot and Pieau, 1999). This hypothesis is inconsistent with the frequent evolutionary transitions between TSD and GSD in reptiles (Shine, 1999a).
- 3) sib-avoidance hypothesis - females of TSD species produce single sex clutches to avoid inbreeding among siblings (Ewert and Nelson, 1991). This hypothesis is inconsistent with the prevalence of TSD among species that store sperm and hence sequential clutches may be full sibs (Ciofi and Swingland, 1997) and in long lived species where offspring from annual cohorts breed (Shine, 1999a).
- 4) group-structured adaptation hypothesis - TSD evolved through isolated breeding units with minimal gene flow because it facilitates group fitness through sex ratio skewing (Ewert and Nelson, 1991). This hypothesis is inconsistent with isolated groups, as inbreeding should be common and heterozygosity low in comparison with GSD species, but Burke (1993) found little evidence of heterozygosity in correlation with skewed sex ratios in

chelonians suggesting there is no evidence that inbreeding is more prevalent in TSD species.

- 5) differential fitness hypothesis - the selective advantage for TSD when environmental conditions have different fitness consequences for males and females (Charnov and Bull, 1977). The sexual phenotype is matched to environmental conditions that increase the fitness of that sex over the other (Rhen and Lang, 1995; Shine, 1995). Investing differentially in offspring of each sex can enhance fitness returns. Reproducing females may benefit by facultatively adjusting their investment into sons over daughters or vice versa, in response to population wide shifts in adult sex ratios (Olsson and Shine, 2001).

Reptiles with TSD provide an important model system for the study of the evolution of sex determining mechanisms and maternal effects on differential sex allocation. Further comparative studies of the similarities and differences between mammalian and non-mammalian gene expression patterns in species with different sex determining mechanisms will help determine the developmental pathway for sex determination in species with TSD. The mechanism of sex determination provides a major constraint on the amount of adaptive sex allocation adjustment possible in most vertebrates. However, reptiles with such diverse sex determining mechanisms and some genera where TSD and GSD occur in closely related species provide excellent models for more detailed studies.

***Eulamprus tympanum* - The ideal model species for TSD and Viviparity**

The genus *Eulamprus* contains ten species of active and primarily diurnal viviparous lizards (Cogger, 1992). The montane water skink, *Eulamprus tympanum* is a medium-sized (80 mm snout-vent length) scincid lizard found in high elevation habitats of south-eastern Australia, where they are commonly found associated with fallen logs in open sclerophyll forest. Males typically emerge from wintering sites early in October with females emerging slightly later, ovulation and mating occur in late October and females give birth to between 1 and 6 young in February (Schwarzkopf, 1991).

The montane water skink provides an ideal model species for the examination of TSD in a viviparous species, primarily because:

- skewed sex ratios occur as a result of incubation temperature (Robert, 1999)
- no heteromorphic sex chromosomes have been found within the *Eulamprus* group (Donnellan, 1985)
- they inhabit montane environments with highly variable temperatures
- they are common and easily collected
- they are easily maintained and do well in captivity

My thesis begins with a study investigating temperature effects on offspring sex ratios in *E. tympanum* to establish the presence of TSD in this viviparous lizard and provide the basis of my PhD study.

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SECTION ONE

Sex determination

CHAPTER 1

Sex Determination: Temperature-dependent Sex Determination in a Viviparous lizard

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Entitled - Viviparous lizard selects sex of embryos (co-authored with M B Thompson)

1.1 Introduction

No one suspected that temperature-dependent sex determination (TSD), whereby the sex of embryos depends on the temperature at which they develop (Bull, 1980; Janzen and Paukstis, 1991; Cree *et al.*, 1995), might occur in viviparous (live-bearing) reptiles, because thermoregulation in the mother results in relatively stable gestation temperatures. But here I show that developing embryos of the actively thermoregulating viviparous skink *Eulamprus tympanum* are subject to TSD, offering the mother the chance to select the sex of her offspring and a mechanism to help balance sex ratios in wild populations.

Sex determination is the defining event that initiates which programmed cascade of events the embryo will follow in becoming male or female. In vertebrates, sex is determined either by a genotypic mechanism at the time of fertilisation, which depends only on genetic factors, or by environmental factors that act after fertilisation. Species that are subject to TSD provide an example of the latter mechanism and usually lack heteromorphic sex chromosomes. Reptiles rely on either temperature or genetic factors to influence the sex of their offspring (Ciofi and Swingland, 1997).

Eulamprus tympanum is a medium-sized scincid lizard found in high elevation habitats in south-eastern Australia, with a litter size of one to five young (Cogger, 1992). No species within *Eulamprus* has detectable heteromorphic sex chromosomes (Donnellan, 1985), therefore I investigated whether *E. tympanum* might be subject to TSD.

1.2 Materials and Methods

I maintained mothers at different laboratory temperatures and used palpation of the hemipenes (Harlow, 1996) and histology of neonatal gonads (Yntema, 1981; Yntema and Mrosovsky, 1980) to establish sex.

Pregnant viviparous *E. tympanum* (n=76) were maintained throughout pregnancy in one of four treatment temperatures; 1) females within a thermal gradient, given the opportunity to self thermoregulate for 8 h a day (n = 21), 2) females maintained at 30 °C in a constant temperature room (n = 20), 3) females maintained at 25 °C in a constant temperature room (n = 11), and 4) females that underwent gestation in the field (n = 24) and were collected just prior to parturition (refer to Chapter 2).

1.3 Results

Gestation temperature has a highly significant effect on sex ($\chi^2 : P < 0.001$), with warmer temperatures giving rise to male offspring (Fig. 1.1).

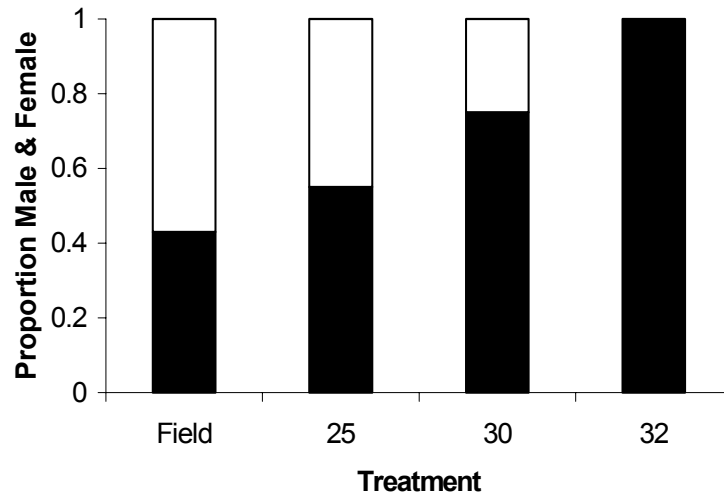


Figure 1.1 Influence of gestation temperature on the sex ratio of offspring of the viviparous lizard *Eulamprus tympanum*. Females in a thermal gradient maintaining 32 °C (n=21) gave birth to exclusively male offspring (n=55); those maintained at a constant 30 °C (n=20) gave birth to predominantly male offspring (n=58; 75% were male); those maintained at a constant 25 °C (n=11) gave birth to offspring of both sexes (n=20; 55% were male); those undergoing most of their gestation in the field (n=24) also produced a mix of sexes (n=58; 43% were male). Black portions of the bars represent male offspring; white portions represent female offspring.

1.4 Discussion

Active thermoregulation by pregnant viviparous lizards distinguishes the thermal environment of development from that in oviparous species. A combination of active thermoregulation and TSD provides the female with the opportunity to select the sex of her offspring. In the laboratory, all females provided with unlimited conditions for thermoregulation maintained body temperatures of 32 °C and produced exclusively male offspring. Equal sex ratio resulted from natural gestation in two field seasons.

We do not understand the mechanism by which females select body temperature to give equal sex ratios in the field; the implication is that thermoregulatory conditions may be restricted. Alternatively, other factors, such as unbalanced adult sex ratios, may result in mothers selectively thermoregulating to

produce offspring that shifts the population sex ratio. A viviparous skink from Tasmania adjusts the sex ratio of its offspring according to the operational sex ratio of the adult population (Olsson and Shine, 2001); presumably, TSD provides the mechanism for selection of neonatal sex by the mothers. Our population of *E. tympanum* has an adult sex ratio that is not significantly different from unity (Schwarzkopf and Shine, 1991) in the field, where they produce an equal sex ratio of neonates; however, our laboratory population was all female and produced all sons when given the opportunity to thermoregulate.

Alternative mechanisms used to explain sex bias as a result of temperature such as, differential embryonic mortality (Burger and Zappalorti, 1988), differential fertilisation (Komdeur *et al.*, 1997; Stockley, 1999) or embryonic sex reversal (Dournon *et al.*, 1990) can be discounted as an explanation for skewed sex ratios in this case. Viviparous reptiles cannot resorb abortive eggs or embryos *in utero* (Blackburn *et al.*, 1998; Blackburn *et al.*, 2003) and thus, sex-specific mortality would have been evident from non-viable eggs or embryos expelled from the oviduct (none were recorded). The number of eggs palpated at capture reflected the number of offspring produced. Similarly, differential fertilization is not relevant, because females were captured after they had ovulated in the wild and hence, any sex bias in sperm usage would be manifested in all experimental groups. Sex reversal during embryogenesis is possible, but simply one of the mechanisms capable of generating TSD. Functionally there is no difference between sex reversal during embryonic development and TSD; if sex is determined and then reversed due to temperature, ecologically the result is the same. The terms GSD and TSD thus encompass a diversity of sex influencing mechanisms. For example, GSD can arise via

heteromorphic or homomorphic sex chromosomes and TSD can arise via a delay in sexual differentiation or a reversal of some degree of prior sexual differentiation.

TSD may explain the fact that *E. tympanum*, like many other viviparous taxa, is restricted to alpine regions. The warmer temperatures further down the slopes would encourage production of exclusively male offspring and lead to the eventual extinction of those populations. A combination of alpine distribution and TSD is likely to be a problem in the event of rapid climate change or global warming, as these species may not be able to evolve rapidly enough to compensate (Janzen, 1994). For alpine species, there can be no retreat to cooler climates, so a rise in environmental temperature would result in increased production of males. Models predict a temperature rise of 4 °C by 2100 (Whetton *et al.*, 2001), which could seriously alter the sex ratio and lead to extinction of such species as *E. tympanum*.

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CHAPTER 2

Sexual differentiation of the gonads and oviducts at birth in the viviparous lizard *Eulamprus tympanum*, a species with temperature-dependent sex determination

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2.1 Introduction

Sex differentiation is the programmed cascade of events through which an undifferentiated gonad develops into a testis or ovary, the hormonal products of which then influence the development of secondary sexual traits. Sex determination is the event which sets this cascade in motion (Lance, 1997). There is a remarkable variety of sex determining systems among different animal taxa, although, most vertebrates have their sex determined by one of two mechanisms 1) genotypic sex determination (GSD), in which sex is determined at the time of fertilization by genetic factors independent of environmental influence, or 2) environmental sex determination (ESD), in which sex is determined by environmental factors that act after fertilization (Ciofi and Swingland, 1997).

ESD has been studied in a variety of animal taxa including fishes (Conover, 1984; Conover and Heins, 1987; Beamish, 1993; Baroiller *et al.*, 1995; Luckenbach *et al.*, 2003), crustaceans (McCabe and Dunn, 1997; Bergstrom, 1997; Rigaud *et al.* 1997), nematodes, rotifers, polychaetes, echiurids (Korpelainen, 1990), and oviparous (egg-laying) reptiles (Bull, 1980; Deeming and Ferguson, 1988; Ewert and Nelson, 1991; Janzen and Paukstis, 1991). Sex in many of these species is

determined by temperature, a type of ESD known as temperature-dependent sex determination (TSD), where the sex of the developing embryo is dependent on the temperature at which they develop.

Sex in many oviparous reptiles is determined by the temperature at which the egg incubates (Bull, 1980; Deeming and Ferguson, 1988; Ewert and Nelson, 1991; Janzen and Paukstis, 1991). Temperature-dependent sex determination was not considered compatible with viviparity (live bearing) in reptiles because maternal thermoregulation results in relatively stable gestation temperatures (Bull, 1980), in contrast to oviparous reptiles where eggs are deposited in a nest and developmental temperature varies with the environment. However, this study conducted in conjunction with our recently reported results (Robert and Thompson, 2001; Chapter 1) confirms that the developing embryos of the viviparous skink *Eulamprus tympanum* are indeed subject to TSD.

Despite the differences in the mechanism used in sex determination, the morphological development of the gonads in all vertebrates has been conserved throughout evolution (Western and Sinclair, 2001). Gonad development in all vertebrates begins with a sexually indifferent gonad with a medullary zone surrounded by a cortical region with associated germ cells (cortex) followed by a sex determination mechanism (e.g. genes or temperature) that initiates the cascade of differentiation into testis (characterised by well developed medullary cords and a cortical region reduced to a single layer of cells) or ovary (characterised by a proliferating cortex surrounding a vacuolated and regressed medulla) with the cellular morphology being very similar among the amniotes (Van Tienhoven, 1983; Clinton, 1998; Wibbels *et al.*, 1991). The stage when the gonads are clearly

differentiated varies with different taxa. In mammals and birds the gonads are clearly differentiated in embryos well before birth and hatching respectively. In many fish, including elvers, the gonads are not differentiated at hatching but differentiate at species specific body size (Colombo and Grandi, 1996; Luckenbach *et al.*, 2003). Gonadal differentiation follows similar patterns in both frogs and salamanders, with differentiation in most occurring during larval stages (Hayes, 1998). There are some exceptions in some ranid frog species (Witschi, 1930), where some remain undifferentiated until metamorphosis (Pucket, 1940) while others metamorphose with ovaries then transform into males through an intersex stage after 6 to 12 months (Witschi, 1921 and 1930).

The chronology of sex differentiation has been well studied in oviparous reptiles with TSD. Sexual differentiation generally takes place in the middle third of embryonic development (Yntema, 1979; Pieau and Dorizzi, 1981; Yntema and Mrosovsky, 1980; Hewavisenthi and Parmenter, 2002). The developmental stage when sex is determined differs between species due to the different staging schemes and reproductive modes, for example 26 stages of development are described in freshwater turtles (Pieau and Dorizzi, 1981), up to 40 stages in lizards (Dufaure and Hubert, 1961; Hubert, 1985), and 43 stages in snakes (Hubert and Dufaure, 1964; Hubert, 1985), and most oviparous lizards deposit their eggs at an advanced stage of development that corresponds with stage 30 in a viviparous species (Dufaure and Hubert, 1961; Hubert, 1985). Comparative staging among different species is difficult due to the numbering of equivalent stages of development differing (Hubert, 1985). Irrespective of the stage, differentiation is complete and irreversible when initial sex specific changes are evident in the gonad

(Wibbels *et al.*, 1991), that is, the temperature sensitive period ends at a time when the gonads begin to sexually differentiate (“an all or none” effect). The gonads of most turtles, alligators and lizards are histologically differentiated into testis or ovaries by the time of hatching (Pieau, 1971; Yntema and Mrosovsky, 1980; Ferguson and Joanen, 1983; Miller, 1985). However, a few species have been classed as intersex at the time of hatching due to ambiguous gonads and both sets of sex ducts. Some hatchlings of the European Pond turtle (*Emys orbicularis*) have been classed as intersex due to the gonads not being clearly differentiated at hatching, although by one month of age they are clearly differentiated (Pieau and Dorizzi, 1981). The chronology of gonadal differentiation may be less advanced in *Emys orbicularis* than other turtles, resulting in hatchlings that are slightly more immature. In the Red-eared slider turtle and the leopard geckos offspring have been classed as intersexes since they are not clearly differentiated as one sex or the other at hatching when treated with exogenous androgen during development causing gonadal females to develop male-typical intromittent structures (Crews *et al.*, 1991; Tousignant and Crews, 1994). One study has examined sexual differentiation in a viviparous reptile (Miller, 1963), although gonadal differentiation is assumed to follow the general pattern exhibited by oviparous reptiles (Wibbels *et al.*, 1991) and amniotes in general (Van Tiehoven, 1983), with sex being clearly differentiated at birth.

Reptiles do not possess external genitalia and most lack secondary sexual dimorphism, so sex identification is not always obvious (DeNardo, 1996). The methods used to identify sex in squamate reptiles vary with age and species, and the various methods differ in their simplicity and/or their accuracy. Sex identification in some species can be made with varying degrees of accuracy based entirely on

secondary sexual characteristics that distinguish males from females, for example, colour differences, size differences, femoral or preanal pores in many iguanid and gekkonid species, spurs in boid snakes, or ornamentation such as rostral horns in some chameleons (DeNardo, 1996). Secondary characteristics are usually not developed in neonates and juvenile individuals, which need to be sexed using alternative methods. The most common method for identifying sex in squamate reptiles is to manually evert the hemipenes (DeNardo, 1996; Harlow, 1996). The presence of hemipenes identifies the animal as male and the lack of hemipenes female. The assignment of female from a negative result can often lead to misdiagnosis of males and some females possess glands or hemiclitori that can be mistaken for hemipenes (Harlow, 1996; Hare, 2001). Probing the cloaca to identify an inverted hemipenis is most commonly used to sex adult snakes and some large lizards (DeNardo, 1996). The ability of the probe to be inserted and the depth of insertion distinguish males from females. Misdiagnosis can also be made using this method in some species when females have well developed blind diverticula (DeNardo, 1996). The most accurate technique for sex identification is by visual examination of the gonads and histological examination of gonad and associated structures microscopically (Pieau, 1971; Yntema, 1981; Yntema and Mrosovsky, 1980). This technique is most invasive and requires removal of gonads and associated structures for histological examination in small species or by laparoscopic examination through a surgical incision in larger species (Cree *et al.*, 1991). However, the invasive techniques may be necessary to confirm sex determined using other non-invasive techniques.

The purpose of this chapter is to describe the characteristics of the gonads and oviducts at birth in *Eulamprus tympanum*, the first viviparous species discovered

to have TSD (Robert and Thompson, 2001; Chapter 1). I used histological examination of the gonads to assess how sexually differentiated the gonads are at birth and to confirm palpation as a robust means of sexual identification in newborn *Eulamprus tympanum*.

2.2 Materials and Methods

2.2.1 Animals and their maintenance

Pregnant viviparous *E. tympanum* (n=76) were maintained throughout pregnancy in one of four treatment temperatures; 1) females within a thermal gradient, given the opportunity to self thermoregulate for 8 h a day, 2) females maintained at 30 °C in a constant temperature room, 3) females maintained at 25 °C in a constant temperature room, and 4) females that underwent gestation in the field and were collected just prior to parturition.

Lizards in the first three treatment groups were collected immediately following ovulation and mating in late October (ovulation was evident by palpation for eggs and recently mated by fresh mating scars). Lizards in the last treatment were collected just prior to giving birth in late December and early January. All lizards were collected from Kanangra Boyd National Park (33°55'S, 150°3' E; elevation 1,300m), NSW, Australia.

Lizards were housed at The University of Sydney individually in enclosures (400 mm x 230 mm) with 20-30 mm depth of soil, flat rocks and leaf litter for shelter. Lizards in the first treatment (thermal gradient) were supplied with a heat source (40 W incandescent light bulb) suspended over one end of their enclosure, providing a

thermal gradient of 20 to 40 °C for 8 h a day, falling to 20 °C overnight. All lizards were supplied with water *ad libitum* and fed a maintenance diet of four mealworms, *Tenebrio molitor*, dusted in calcium gluconate and commercial reptile multivitamins (Herptivite™, Rep-Cal Research Los Gatos, CA) every three days (Robert, 1999).

2.2.2 Sex determination

Immediately following birth, newborn offspring were weighed, measured and sexed externally by the presence or absence of hemipenes by palpation (Harlow, 1996; Hare, 2001). Palpation involved holding the neonate under a dissecting microscope, ventral surface up with the forefinger placed anterior to the cloaca. A blunt probe was then rolled from approximately 5 mm caudal to the cloaca, up the tail towards the cloaca. The pressure applied from the forefinger and the rolling probe everts one or both hemipenes in males and occasionally hemiclitori in females.

Neonates were then killed by an intrathoracic injection of Nembutal®. The gross morphology of the gonads and associated ducts was observed under a dissecting microscope (Ferguson and Joanen, 1983) then both the right and left gonad kidney complexes were removed, fixed in Bouin's solution and then stored in 70% ethanol (Humason, 1972). One gonad, kidney and associated ducts from each neonate was embedded in paraffin wax, sectioned in transverse cuts at 7 µm, mounted and stained with haematoxylin and eosin (H & E stain) (Humason, 1972). Sex was assessed by examination under light microscope according to criteria established by Miller and Limpus (1981), Yntema (1981), Ferguson and Joanen (1983), and Wibbels *et al.* (1991).

2.3 Results

Females maintained in thermal gradients produced 55 offspring, all of which were male, females maintained at a constant 30 °C produced 58 offspring of which 75% were male, females maintained at a constant 25 °C produced 20 offspring of which 55% were male, and females maintained in the field produced 58 offspring of which 43% were male (Robert and Thompson, 2001; Chapter 1).

Males

Palpation evicted either large white projections that were classed as definite males or smaller pink projections that were classed as putative males prior to histological examination (Fig. 2.1[2,3, and 5]), male at 5 months of age). Dissection revealed that neonatal gonads lie on the dorsal abdominal wall overlying the kidneys on either side of the midline mesentery. They are cream in colour, elongated finger-like structures lying in close to the adrenal gland. Müllerian ducts are usually absent, although occasionally small traces of the degenerating duct are visible due to the neonate's immaturity (Fig. 2.2).

Histologically, the gonads are clearly differentiated at birth (Fig. 2.3 [1-3]). Histology confirmed those assigned as putative males by palpation to be males. The testes are an elongated oval shape with either no cortical epithelium or cortical epithelium present as a thin band. Seminiferous tubules are clearly evident in the testis and, if present at all, the Müllerian ducts show signs of degeneration and are attached to the kidney by a shortened mesosalpinx.

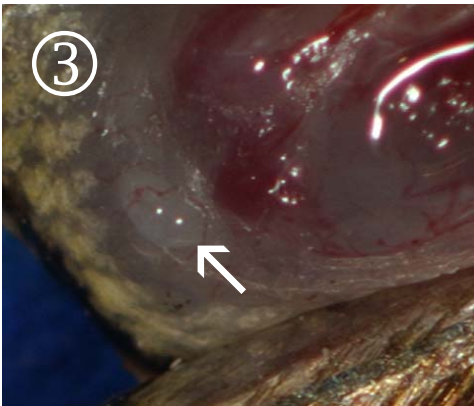


Figure 2.1 Sexing neonatal *Eulamprus tympanum* at 5 months of age, hemipenes become increasingly difficult to evert as neonates age however, hemipenes are easily identified without full eversion (1) female, (2) male showing right hemipene partially everted, (3) close up view of right hemipene, (4) female, (5) male, hemipene is the opaque structure below surface prior to eversion.



Females

Palpation resulted in either no projections and were classed as definite females or very short transparent projections and classed as putative females prior to histological examination (Fig. 2.1[1, and 4]), female at 5 months of age). In gross morphology the gonads of female neonates are very similar in position and appearance to those of a male. The Müllerian ducts are the most obvious difference; they are long transparent structures that run along the dorsolateral margin of the gonad and ultimately become the oviducts (Fig. 2.2).

Histological examination confirmed those assigned as putative females by palpation to be female. Histologically, the ovary has a more irregular shape than the testis and has a thick cortical epithelium occasionally containing oocytes (Fig. 2.4 [4-5]). The Müllerian ducts are unmistakable and are supported by a fibrous mesosalpinx attached to the kidney.

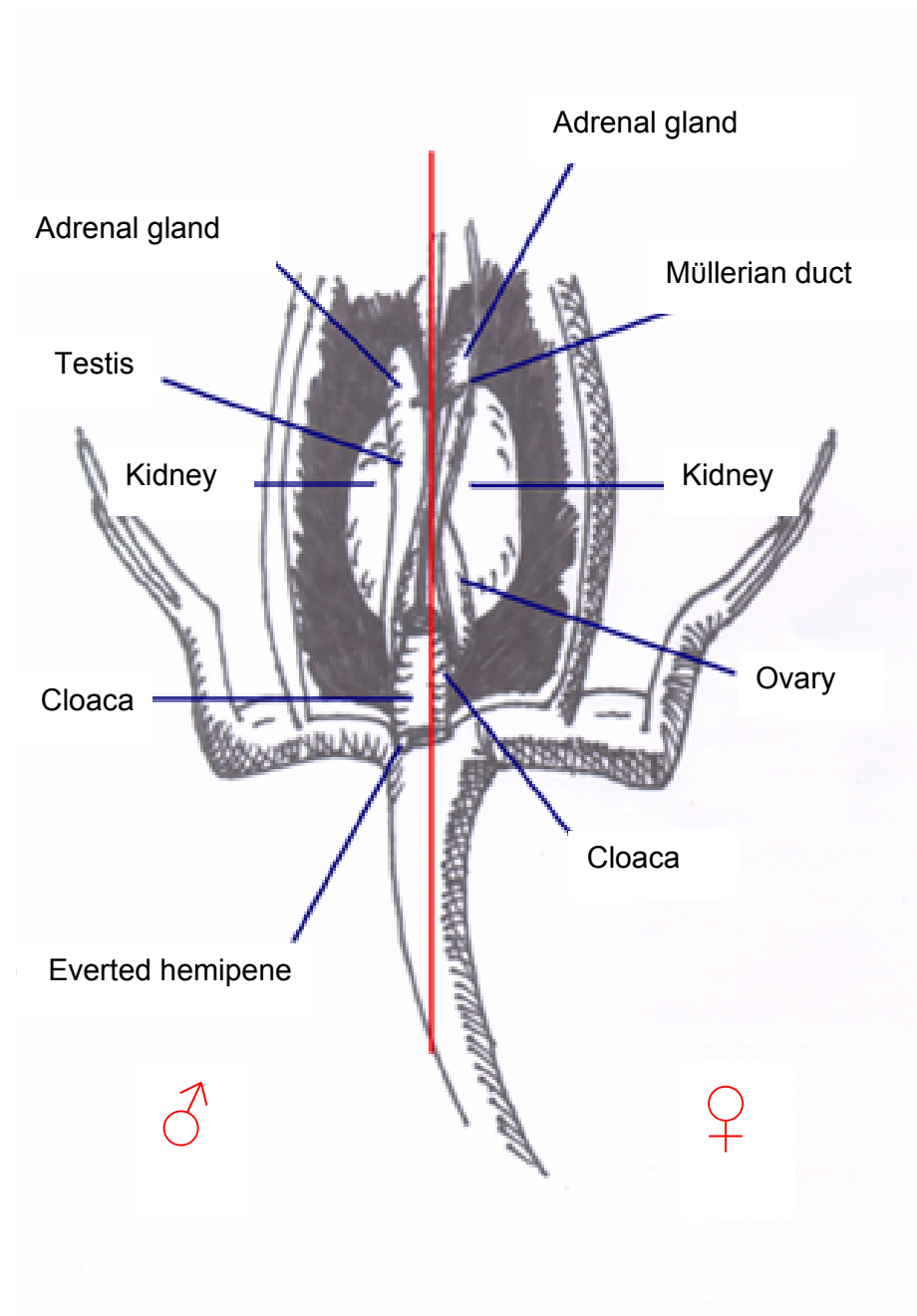


Figure 2.2 Schematic diagram of ventral dissection of neonatal *Eulamprus tympanum* to illustrate major reproductive structures: (♂) male gonads (♀) female gonads

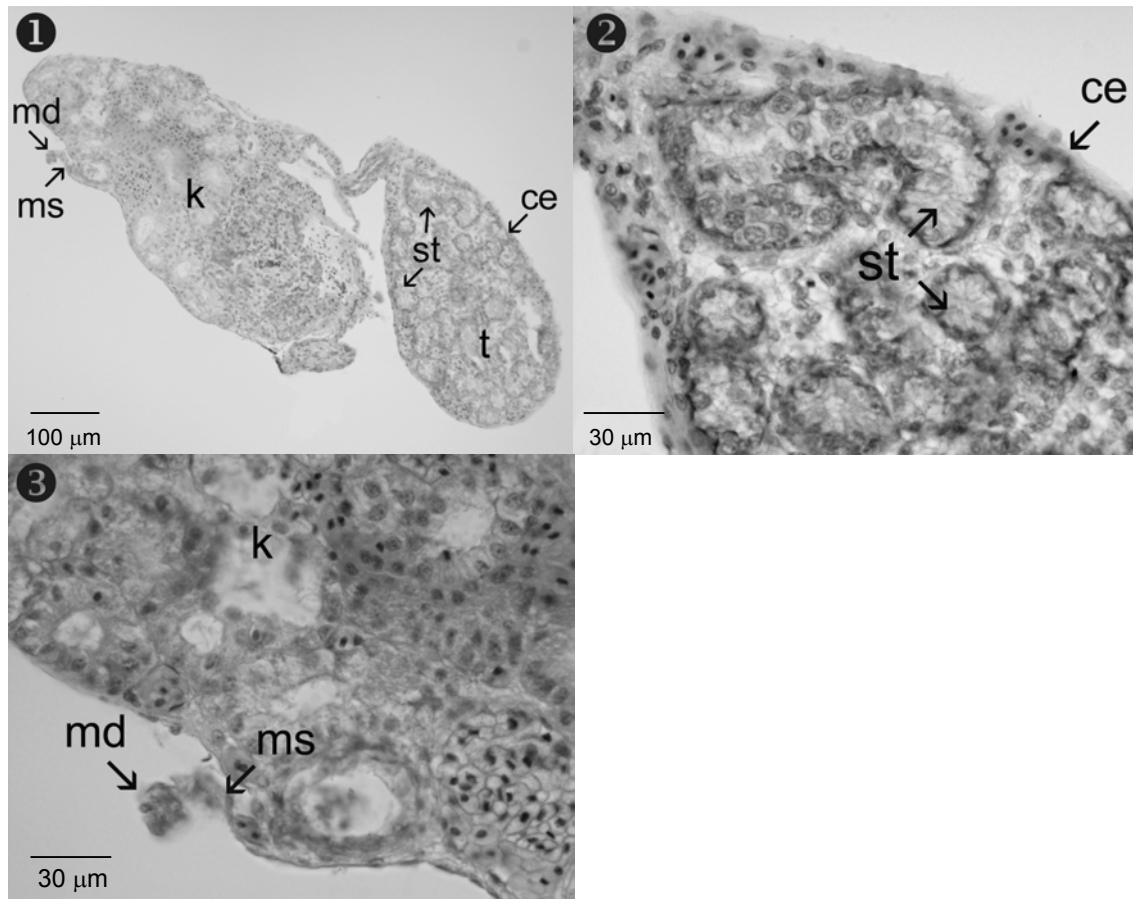


Figure 2.3 Transverse sections of male gonads at birth in *E. tymanum* (1), gonad-kidney complex, (2), detailed view of testis, (3), and degenerated Müllerian duct (ce = cortical epithelium, k = kidney, md = Müllerian duct, ms = mesosalpinx, st = seminiferous tubule, t = testis)

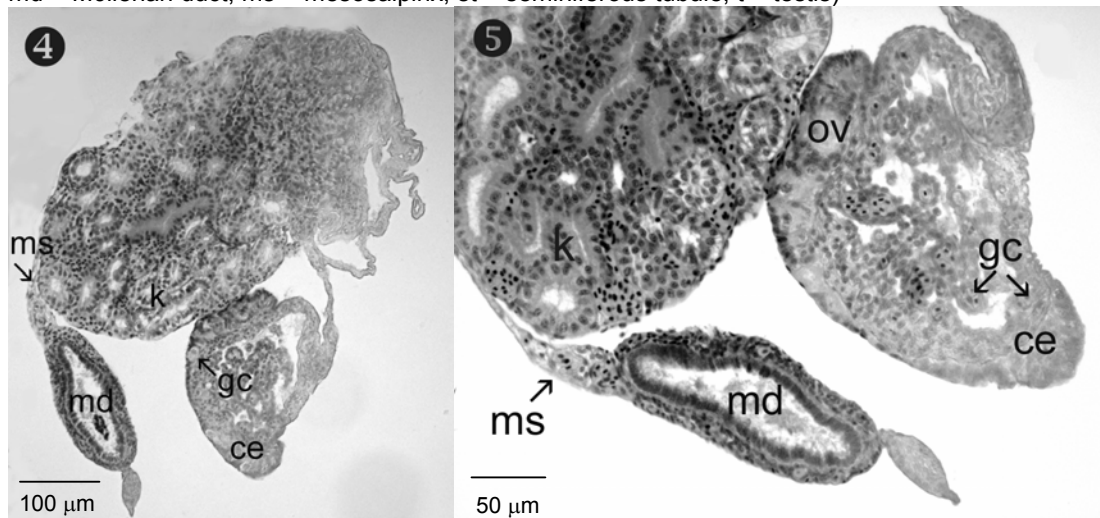


Figure 2.4 Transverse sections of female gonads at birth in *E. tymanum* (4), gonad-kidney complex, (5), detailed view of ovary and mullerian duct (ce = cortical epithelium, gc = germ cell, k = kidney, md = Müllerian duct, ms = mesosalpinx, ov = oocyte)

2.4 Discussion

Sexual differentiation occurs in a similar fashion in all vertebrates but sex determination or the process that entrains differentiation is clearly different (Lance, 1997). In mammals and birds, chromosomes inherited at fertilisation determine whether an individual develops as a male or female (genotypic sex determination) (Wibbels *et al.*, 1994), while in many reptiles temperature throughout embryonic development determines the differentiation of male or female organs (Bull, 1980). Once sex determination mechanisms are activated, sex differentiation begins. The stage when the gonads are clearly differentiated into testis or ovaries varies, but the basic pathway of sexual differentiation is common (Wibbels *et al.*, 1991). Sex is fully determined at the time of birth and histologically can be differentiated into testes or ovaries in *E. tympanum*. Some males from the warmer treatments had Müllerian ducts that had not completely degenerated and could be classed as immature rather than intersex, this was also suggested in the freshwater turtle *Trachemys scripta* (Wibbels *et al.*, 1991) where 14 day old hatchlings showed more maturity. This more than likely reflects embryogenesis being sped up and duct regeneration incomplete at birth rather than sex not being clearly differentiated (Wibbels *et al.*, 1991). The main concern with histological examination is not to rely on the presence or absence of Müllerian ducts alone due to some immature individuals retaining ducts.

Factors affecting sex determination in many species of reptile remains unknown and the mechanism and adaptive advantages of TSD remain elusive (Ciofi and Swingland, 1997). Heteromorphic sex chromosomes have not been identified in any species within the *Eulamprus* group (Donnellan, 1985) and the sex ratio of

offspring differs from equality over a range of temperatures in *Eulamprus tympanum*, indicating TSD (Robert and Thompson, 2001; Chapter 1).

Identification of the sex of newborn *Eulamprus tympanum* based on the presence or absence of hemipenes was checked by subsequent gross morphological and histological examination and in each case the initial identification was confirmed. By palpation, males had large white projections or smaller pink projections, females had no projections or very short transparent gland-like projections (hemiclitori). On dissection, the gross morphology of the reproductive organs was not as distinguishable; the gonads of both male and female offspring are similar in position and appearance, with the most obvious characteristic distinguishing sexes being the presence of a Müllerian duct. The subsequent histological examination confirmed males by the presence of testis containing seminiferous tubules and by the lack of or by a degenerating Müllerian duct. Those classed as females by palpation and gross morphology were confirmed histologically as female by the presence of a distinct Müllerian duct and ovaries with a thick cortical epithelium that occasionally contained oocytes.

The histology of the gonads is the definitive method of determining sex (DeNardo, 1996). Here I used histology to confirm the use of the less invasive technique of everting hemipenes as a reliable technique in determining sex in newborn *E. tympanum*. Distinguishing hemipenes from hemiclitori becomes more apparent with practice, with hemiclitori evident by a shorter more conical transparent structure compared to the hemipenis which is a white projection that is longer and more spherical in shape. Sex determination is easiest when performed on neonates within the first few days of birth, when hemipenes are easier to evert. Hemipene

eversion becomes increasingly difficult as neonates age, although with experience full eversion is not required to identify hemipenes and females are less likely to exhibit hemiclitori. The increasing difficulty to evert hemipenes as neonates grow and musculature increases has also been reported in agamids (Harlow, 1996), varanids (Hairston and Burchfield, 1992) and pythons (Barker and Barker, 1994).

There have been a number of alternative mechanisms suggested to explain the sex bias as a result of temperature in *E. tympanum* (Valenzuela *et al.* 2003), specifically differential embryonic mortality (Burger and Zappalorti, 1988), differential fertilisation (Komdeur *et al.*, 1997; Stockley, 1999) or embryonic sex reversal (Dournon *et al.*, 1990). Viviparous reptiles cannot resorb abortive eggs or embryos *in utero* (Blackburn *et al.*, 1998; Blackburn *et al.*, 2003) and thus, sex-specific mortality would have been evident from non-viable eggs or embryos expelled from the oviduct (none were recorded). The number of eggs palpated at capture reflected the number of offspring produced. Thus, differential mortality can be discounted as an explanation for skewed sex ratios in this case. Similarly, differential fertilization is not relevant, because females were captured after they had ovulated in the wild and hence, any sex bias in sperm usage would be manifested in all experimental groups. Sex reversal during embryogenesis is possible, but simply one of the mechanisms capable of generating TSD. Functionally there is no difference between sex reversal during embryonic development and TSD; if sex is determined and then reversed due to temperature, ecologically the result is the same. The terms GSD and TSD thus encompass a diversity of sex influencing mechanisms. For example, GSD can arise via heteromorphic or homomorphic sex chromosomes and TSD can arise via a delay in sexual differentiation or a reversal of some degree of prior sexual differentiation.

The occurrence of TSD in viviparous reptiles has wide ranging implications for evolutionary, behavioural and conservation studies. A clear understanding of how temperature determines sex in reptiles with TSD remains elusive and currently the molecular regulation of sex determination is known in detail from only a few species with genetic sex determination (Spotila *et al.*, 1994).

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**SECTION
TWO**

Thermal biology

CHAPTER 3

Thermal Biology of *Eulamprus tympanum*: can females maintain mean selected temperatures under field conditions?

Submitted for publication in Journal of Thermal Biology (co-authored with M B Thompson and F Seebacher)

3.1 Introduction

Endotherms usually maintain their body temperature within a narrow limit using high rates of aerobic metabolism leading to heat production, while ectotherms use a range of behavioural strategies such as modifying activity patterns and microhabitat selection to maintain a thermal preference. Most aspects of behaviour and physiology in ectotherms are sensitive to body temperature and body temperature is the single most important ecophysiological variable affecting performance in ectotherms. Body temperature, for example, influences locomotion (Bennett, 1980), foraging ability (Greenwald, 1974; Ayers and Shine, 1997), metabolic rates (Patterson and Davies, 1989; Beyer and Spotila, 1994), reproduction (Navas and Bevier, 2001), embryonic development (Bull, 1980; Shine and Harlow, 1993), immune function (Mondal and Rai, 2001) and rates of feeding and growth (Van Damme *et al.*, 1991). Thermoregulation enables ectotherms to maximise energy acquisition in a thermally heterogeneous environment.

The range of temperatures available to animals in their natural environment varies widely with geography, habitat structure, weather conditions, season, and altitude (Withers, 1992). Microhabitats can differ significantly in their thermal properties and may be exploited by animals, for example, by remaining within

vegetation, crevices or within burrows when exposed habitats are thermally unfavourable.

Eulamprus tympanum is a diurnal viviparous skink that is often found associated with fallen logs in open sclerophyll forest (Cogger, 1992). *Eulamprus tympanum* occurs in high densities with as many as 244 individuals per hectare recorded in Kanangra Boyd National Park (Schwarzkopf, 1991). *Eulamprus tympanum* is territorial with larger lizards exerting greater dominance (personal observation) and competition for basking positions may force less dominant individuals into unfavourable microhabitats. Individuals forced into more shaded habitats may be thermally restricted in their ability to thermoregulate efficiently and may be unable to achieve selected thermal preferendum.

The thermoregulatory history of a gravid female during gestation and or nest site selection can strongly affect offspring characteristics such as phenotype (Shine and Harlow, 1993; Shine *et al.*, 1997; Shine and Downes, 1999; Wapstra, 2000), size and quality (Shine *et al.*, 1995), locomotor performance (Elphick and Shine, 1998; Elphick and Shine, 1999), survival (Burger and Zappalorti, 1988; Rock and Cree, 2003), behaviour (Burger, 1991; Flores *et al.*, 1994; Downes and Shine, 1999), and particularly offspring sex ratio (Burger and Zappalorti, 1988; Harlow, 2000; Robert and Thompson, 2001; Shine *et al.*, 2002). Increased body temperatures accelerate the rate of embryogenesis and decrease the length of gestation (Shine, 1980; Schwarzkopf and Shine, 1991; Shine & Harlow, 1993; Robert, 1999). Many squamates modify their behavioural thermoregulation during pregnancy by either selecting temperatures greater (Stewart, 1984; Beuchat, 1986; Charland and

Gregory, 1990; Rock *et al.*, 2000; Rock *et al.*, 2003) or less (Smith and Ballinger, 1994; Mathies and Andrews, 1997) than non-gravid females and males, changing their heating and cooling rates (Labra and Bosinovic, 2002) or by maintaining higher temperatures longer than non-reproducing individuals (Schwarzkopf and Shine, 1991; Borges, 1999).

Gravid female *E. tympanum* bask more often than males and non-gravid females, although basking body temperatures are not significantly different from non-reproducing individuals (Schwarzkopf, 1991). Body temperatures of basking individuals measured soon after capture indicates that *E. tympanum* in the field can reach temperatures achieved under laboratory conditions (Spellberg, 1972; Schwarzkopf, 1991). Gravid females often bask partially concealed by a shelter (K Robert, personal observation) and are very difficult to capture, so body temperatures measured in only those captured may not be representative of the population of gravid females.

Although temperature-dependent sex determination (TSD) has been illustrated in *E. tympanum* under laboratory conditions (Robert and Thompson, 2001; Chapter 1), establishing whether TSD is biologically relevant to the species or if it is in fact a laboratory artefact, requires knowledge of available temperatures and selected temperatures under natural conditions.

I measured the mean selected body temperature of gravid *E. tympanum* under conditions where basking opportunity is not restricted (laboratory thermal gradient) to characterise the selected thermal preference to compare to both

available temperatures and selected temperatures under field conditions.

Considering *E. tympanum* are present in high densities and they are highly territorial, some individuals may be forced into unfavourable habitats, so selected temperatures were measured in pregnant lizards in favourable (sunny with < 50% canopy cover) and unfavourable (shady with > 50% canopy cover) habitats, using continuous measurement of body temperature (Robert and Thompson, 2003; Appendix 1).

3.2 Materials and Methods

3.2.1 Temperature selection under laboratory conditions

3.2.1.1 Animals and their maintenance

Female lizards ($n = 6$) were collected immediately following ovulation and mating in late October (ovulation was evident by palpation for eggs and recent mating by fresh mating scars) from Kanangra Boyd National Park (33°55' S, 150°3' E; elevation 1,300m), NSW, Australia. Lizards were housed individually at The University of Sydney in enclosures (400 mm x 230 mm) with 20-30 mm depth of soil, flat rocks and leaf litter for shelter. Heat was provided using a 40 W incandescent light bulb suspended over one end of their enclosure, which provided a thermal gradient from 23 - 40 °C for 12 h a day (0600 h to 1800 h), falling to 20 °C overnight. All lizards were supplied with water *ad libitum* and fed a maintenance diet of four mealworms - *Tenebrio molitor* dusted in calcium gluconate and commercial reptile multivitamins (Herptivite™, Rep-Cal Research Los Gatos, CA) every three days (Robert, 1999) when not within an experimental treatment.

3.2.1.2 Temperature measurement

Ambient temperatures within enclosures were measured directly below the incandescent light bulb (hot spot) and furthest from the globe (cold spot) using Tiny-Tag Data Loggers with thermocouples positioned on the substrate surface.

Lizards ($n = 6$) were fitted with modified miniature temperature data loggers of no more than 10% of the lizards body mass (Robert and Thompson, 2003; Appendix 1) attached with cyanoacrylic glue (superglue) to their dorsal surface just behind the head between the shoulder blades (Fig. 3.1) and body surface temperatures (T_b) were measured every 5 minutes for 2 separate 5 day experimental periods between 1st December 2001 and the 11th January 2002. External data loggers were chosen as an alternative to internal measurements from cloacal thermocouples to ensure consistency with field measurements (section 3.2.3). The small size of the lizards meant that use of internal transmitters for field measurement would have been difficult and miniature temperature data loggers were modified and attached externally (Fig. 3.1; Robert and Thompson, 2003; Appendix 1).

External data loggers generally provide accurate estimates of internal body temperatures in small ectotherms that have limited physiological capacity to control rates of heat exchange (Bakken, 1992). To calibrate external measurements using the modified loggers (Thermochron iButtons, Dallas Semiconductor), three lizards were fitted with cloacal K-type thermocouples coated in laboratory Parafilm "M"® and Vaseline® lubricant, inserted into the cloaca of the lizard and fastened to the body with a strip of Likeskin® tape. Each thermocouple was attached to a digital

thermometer to measure internal body temperatures simultaneously every minute as a comparison to externally attached loggers. Lizards were placed in incubators at 15 °C and left for 10 minutes to equilibrate. Incubator was then re-set to 20 °C, and the lizard (internal and external) and incubator temperature were measured simultaneously every minute for 20 minutes. This procedure was repeated by re-setting the incubator to 25 °C, 30 °C and then 35 °C.

3.2.1.3 Temperature analysis

Thermal behaviour in the laboratory was characterised by the following parameters: mean selected body temperature ($T_{I_{sel}}$), mean upper ($U_{I_{sel}}$) and lower limits ($L_{I_{sel}}$) of $T_{I_{sel}}$ calculated from temperatures bounding the interquartile



Figure 3.1. Modified data loggers were attached to the dorsal surface of female *Eulamprus tympanum*, between the shoulder blades. The exposed wires (IO & GND) allowed data retrieval while the logger remained attached to the lizard (see Appendix 1 for details).

range (middle 50% of temperatures) (Hertz *et al.*, 1993), mean selected minimum (T_{lmin}) and maximum (T_{lmax}) and the variance of temperatures selected (V_{lsei}). All variables were calculated as means maintained during the middle third (1000 h to 1400 h) of the entire light period (0600 h to 1800 h), to allow for differences in emergence time, heating, and cooling rates. Thus, ensuring the temperature recorded reflects the lizard's selected thermal preference, rather than passive heating or cooling processes as a result of the heat source switching on or off. Data were gathered over two separate experimental periods for three consecutive days following two days of customisation to the logger (two 5 day experimental periods). Therefore, variables were calculated from a total of 6 days of data for each lizard ($n = 6$ lizards).

3.2.2 Temperature availability in the field

3.2.2.1 Canopy structure

The amount of canopy cover can have a direct influence on the availability and distribution of understorey light and hence thermal conditions. Hemispherical canopy photographs were taken skyward using a standard SLR camera fitted with short focal length (8 mm) fish-eye lens with a full 180 ° field of view, from the forest floor. Photographs were analysed with Gap Light Analyzer (GLA) software (available from: http://www.rem.sfu.ca/forestry/downloads/gap_light_analyzer.htm) to determine canopy structure and gap light transmission indices. The software converts images into bitmaps and transforms pixel positions into angular coordinates, the division of pixel intensities into sky and non-sky classes, and the

computation of sky-brightness distributions. These data are combined to produce estimates of light transmission, solar radiation, canopy openness, leaf area index and sunfleck frequency distribution (Chazdon and Field, 1987; Frazer *et al.*, 1999). The data provided a correlation of solar radiation with available temperatures measured with data loggers (Thermochron iButton, Dallas Semiconductor).

Hemispherical (180° fish-eye lens) photographs were taken at 16 positions (8 in shaded positions and 8 in sunny positions) in Kanangra Boyd National Park where *Eulamprus tympanum* occurred. A visual assessment was made of sunny and shaded habitat and a position was randomly chosen by throwing an identifiable item into either habitat type and then the position of the first lizard observed in the vicinity of the item was used as a sample point for measuring % canopy cover and % solar radiation. Miniature temperature data loggers (Thermochron iButtons, Dallas Semiconductor) were fastened to the log surface (where the lizard was observed) and temperatures were recorded every 15 min to calculate a daytime average for the period of 0900 h to 1700 h.

3.2.2.2 Microhabitat temperatures

The thermal conditions within the microhabitats available to lizards were measured by placing miniature data loggers in 3 positions; buried at a depth of 70 mm, within log shelter sites, and on the surface of an exposed log (16 loggers for each of the positions; a total of 48 loggers). Half of the loggers for each position were placed in shaded positions and half in sunny positions.

The microhabitat temperatures measured were used as the distribution of operative temperatures T_e based on the assumption that data loggers provide sets of operative temperatures similar to models (Vitt and Sartorius, 1999), and models generally provide accurate estimates of operative temperatures for small ectotherms (Bakken, 1992; Shine and Kearney, 2001).

3.2.2.3 Weather conditions

Weather records collected by the Bureau of Meteorology Oberon (Jenolan Caves Road) weather station (33°44'24"S, 149°52'56", elevation 1190 m) provided monthly long-term weather records from a 50 year period (1953 - 2003) to calculate minimum and maximum averages for the area.

3.2.3 Temperature selection under field conditions

3.2.3.1 Animals and their maintenance

Field enclosures (to assist logger retrieval) were constructed within Kanangra Boyd National Park (33°55' S, 150°3' E; elevation 1,300 m) from flat galvanised iron sheets riveted together to form circular enclosures measuring 3.5 m in diameter and 450 mm in height (Fig. 3.2). The bases of the enclosures were buried to a depth of at least 50 mm to prevent escape by skinks. The enclosures (8 in total) were situated so 4 were positioned in sun (< 50% canopy cover) and 4 in shaded (> 50% canopy cover) areas and, where possible, constructed around existing fallen logs. Female lizards

(n = 8) were collected immediately following ovulation and mating in late October and randomly allocated to the nearest enclosure (one lizard per enclosure). All enclosures were visually monitored every two weeks to ensure a sufficient supply of natural food and water was available.

3.2.3.2 Temperature measurement

Lizards were fitted with miniature temperature data loggers (Robert and Thompson, 2003; Appendix 1) (Fig. 3.1) attached to their dorsal surface just behind the head between the shoulder blades (as used in the laboratory thermal gradients, section 3.2.1) and body temperatures (T_b) were measured every 15 minutes from 1st November 2001 until 11th January 2002. Data loggers were downloaded and re-started every 20 days while remaining attached to the lizard.



Figure 3.2. Field enclosure constructed in Kanangra Boyd National Park measuring 3.5 m in diameter and 450 mm in height.

During the fortnightly monitoring, all lizards were checked to ensure loggers remained attached. Loggers that had fallen off were re-attached with date and time noted so that data could be excluded from analyses.

3.2.3.3 Temperature analysis

Thermal behaviour in the field was characterised by mean selected daytime temperatures ($T_{f_{sel}}$), mean selected variance ($V_{f_{sel}}$), thermal maxima (T_{max}), thermal minima (T_{min}) during the daytime period, for each habitat (sunny and shaded) over the three months (November, December, and January). An index of thermoregulation was calculated for each habitat type and month (Hertz *et al.*, 1993)

$$E = 1 - (d_b / d_e)$$

where E is the effectiveness of temperature regulation, and d_b is the deviation of field body temperatures ($T_{f_{sel}}$) from upper ($U_{l_{sel}}$) and lower ($L_{l_{sel}}$) laboratory selected temperatures, and d_e is the deviation of environmental operative temperatures (T_e) from upper ($U_{l_{sel}}$) and lower ($L_{l_{sel}}$) limits of laboratory selected temperatures (middle 50% of selected temperatures). When there is no thermoregulation d_b and d_e will be similar and E will be close to zero. When more precise thermoregulation is occurring d_b will be smaller than d_e and E will be close to one (Hertz *et al.*, 1993).

All thermal variables were calculated as the means during the middle third (1000 h to 1500 h) of the entire light period (0500 h to 1900 h), thus allowing for differences in emergence time, heating, and cooling rates. The light period was

calculated from Geoscience Australia National Mapping Division's Sunrisenset program V2.2 (<http://www.ga.gov.au/nmd/geodesy/astro/sunrise.jsp>), which calculates daily sunrise and sunset times for specified areas based on latitude and longitudinal position.

3.2.4 Statistical analysis

The data were checked for normality (Kolmogorov-Smirnov Test) and equal variances (Levene Median Test) prior to analysis. Data that deviated from these assumptions were log transformed to improve homogeneity of variances. Data that did not meet the assumptions and did not improve with transformation were analysed by non-parametric two-way analysis of variance applied on ranked data using the Scheier-Ray-Hare extension of the Kruskal-Wallis Test (Sokal and Rolf, 1997). All hypotheses were tested for statistical significance at the $P < 0.05$ level, and results are reported as means \pm SE.

3.3 Results

3.3.1 Temperature selection under laboratory conditions

The mean maximum temperature available during the light period (0600 h to 1800h) was 40.2 ± 0.17 °C directly below the heat source (hot spot), while the mean minimum available during the same period was 23.4 ± 0.05 °C furthest from the heat source (cold spot).

External measurements using iButton's provided an accurate measurement of body temperature (T_b) in *E. tympanum*; external and internal measurements were highly correlated (correlation coefficient: 0.99, $P < 0.01$). The difference between internal and external temperatures was an average increase of 0.65 ± 0.04 °C in the external measurements and the difference is not statistically significant (Mann-Whitney test: 6074.5, $P = 0.213$). Internal body temperatures lag slightly behind external measures (mean 3.3 minutes). Thus, external measurement provides a good predictor of internal body temperatures in *E. tympanum*.

Gravid females within thermal gradients achieved a $T_{I_{sel}}$ of 31.2 ± 0.16 °C and $V_{I_{sel}}$ of 2.70 ± 0.16 °C during the middle third of the day (1000 h to 1400 h). Mean upper ($U_{I_{sel}}$) and lower limits ($L_{I_{sel}}$) of $T_{I_{sel}}$ were calculated as 30.1 ± 0.10 °C and 32.2 ± 0.34 °C respectively (Fig. 3.3).

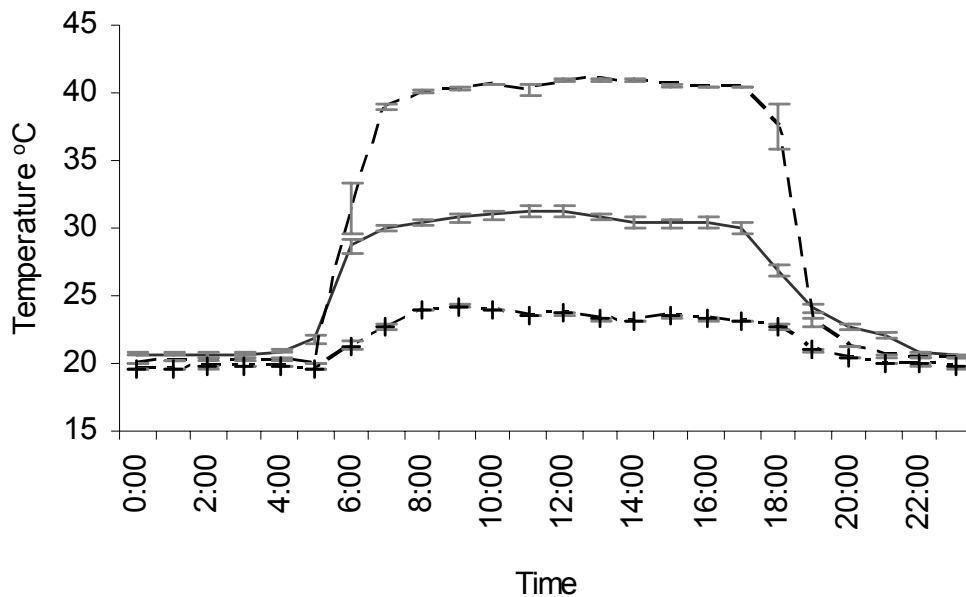


Figure 3.3. Mean (\pm SE) $T_{I_{sel}}$ every hour for gravid female *Eulamprus tympanum* ($n = 6$) within thermal gradients (*solid line*). The available temperatures directly under the hot spot (*dashed line*) and furthest from the hot spot (*dashed & crossed line*) are also plotted.

3.3.2 Temperature availability in the field

3.3.2.1 Canopy structure

Sunny habitats had a mean canopy cover of $49.7 \pm 1.4\%$ and shaded habitats had a mean canopy cover of $69.7 \pm 1.6\%$. Total (direct and diffuse) solar radiation calculated from the Gap Light Analyzer in sunny habitats averaged $68.5 \pm 2.7\%$, and $41.4 \pm 3.2\%$ in shaded habitats.

Solar radiation is positively correlated with substrate surface temperatures (correlation coefficient: 0.80, $P < 0.01$). Substrate surface temperature increases with increased solar radiation and a reduction in canopy cover (Fig. 3.4).

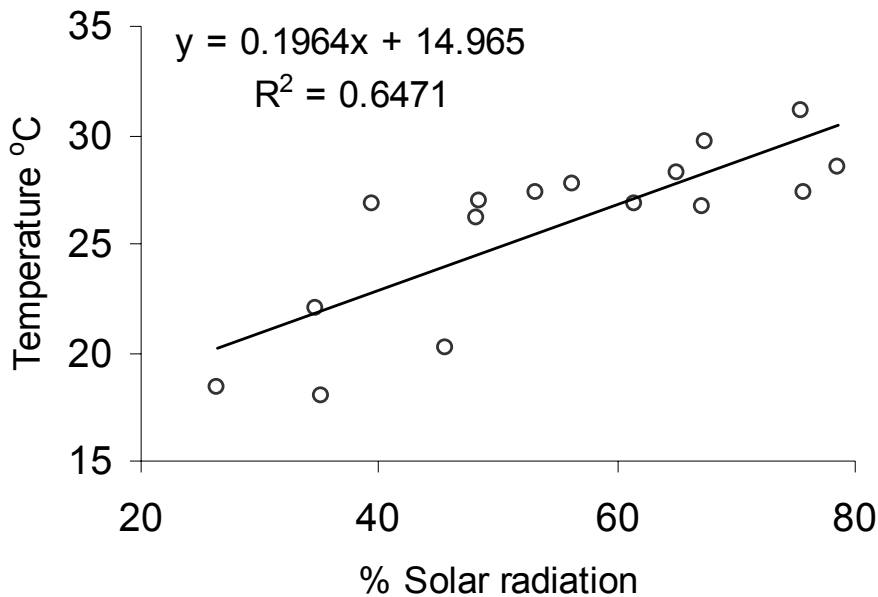


Figure 3.4 Percent total solar radiation (direct and diffuse) (x-axis) plotted against substrate surface temperature (y-axis).

Daytime mean hourly surface temperatures (0900 to 1700 h) significantly differ with canopy structure (8 loggers and 9 hourly averages, $n=72$ for each position; T-test: $t_{142} = 4.59$, $P < 0.0001$); shaded positions are significantly cooler during the day than sunny positions (Fig. 3.5). The mean daytime surface temperature in a shaded position was $23.2\text{ }^{\circ}\text{C}$ while a sunny position was $28.3\text{ }^{\circ}\text{C}$.

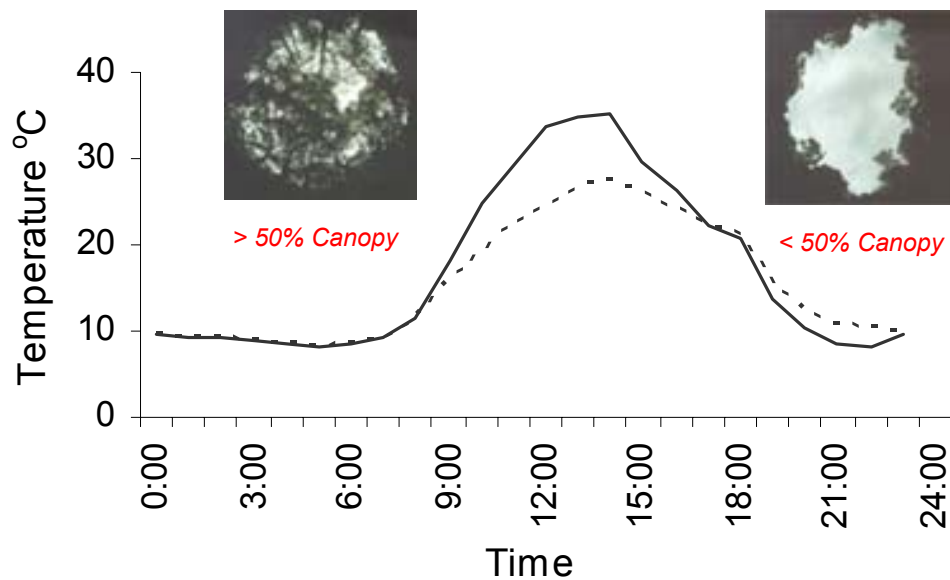


Figure 3.5. Hourly average substrate temperatures in areas with $< 50\%$ canopy cover (sunny habitat, *solid line*) and areas with $> 50\%$ canopy cover (shaded habitats, *dashed line*). Hemispherical photographs representing shaded ($>50\%$ Canopy) and sunny ($<50\%$ Canopy) habitats.

3.3.2.2 Microhabitat temperatures

Available temperatures in shaded environments measured 24 h a day during the gestation period (November to January) using loggers buried at a depth of 70 mm, within log shelter sites and on exposed log surfaces in sunny and shaded environments are significantly cooler than sunny environments (Fig. 3.6). In all months, T_e for sunny habitats ($18.2 - 23.4\text{ }^{\circ}\text{C}$) was consistently higher than that of shaded habitats ($14.9 - 19.9\text{ }^{\circ}\text{C}$) (Table 3.1).

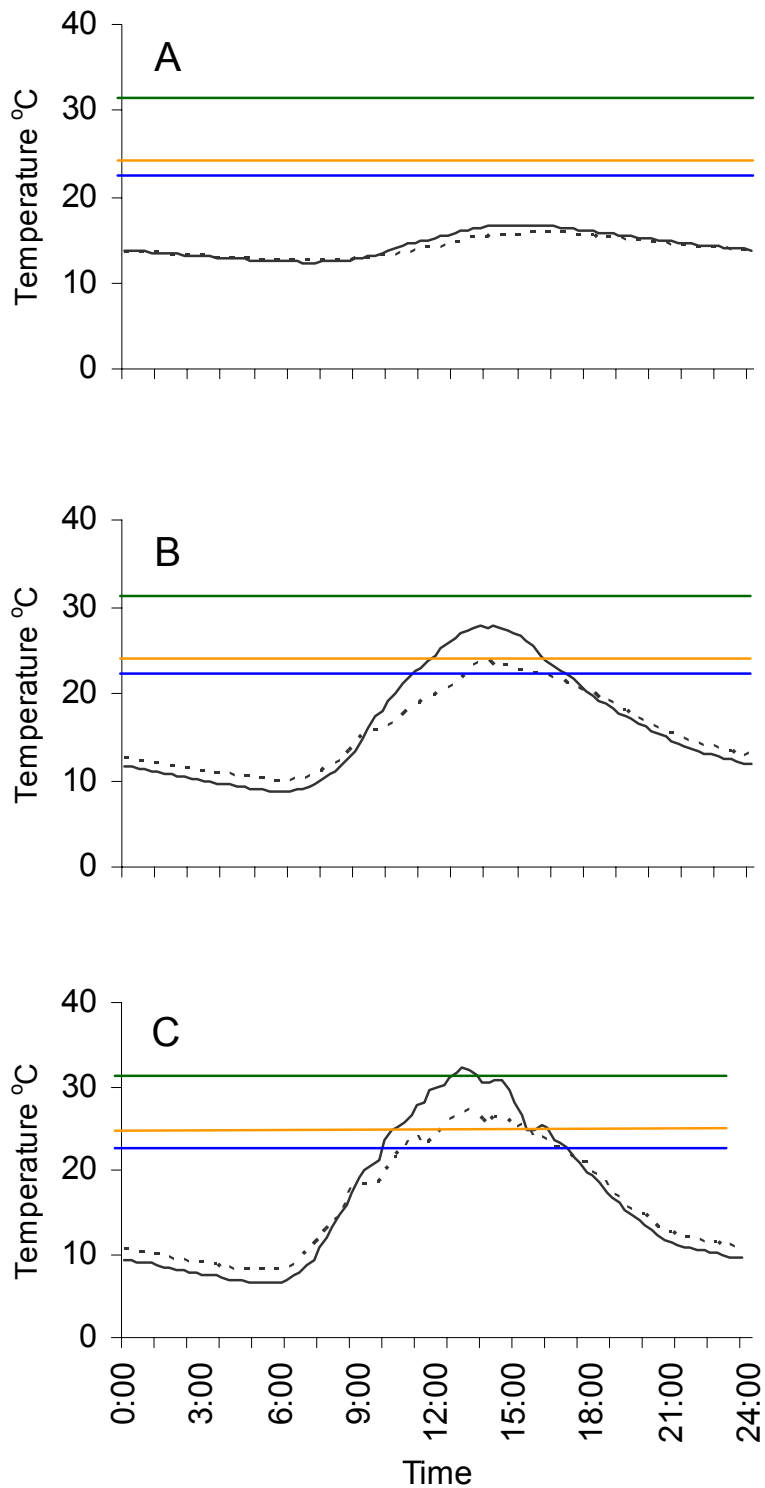


Figure 3.6 Mean available (T_e) environmental temperatures (A) buried, (B) within log, (C) log surface. Sunny sites (*solid line*) and shaded sites (*dashed line*). Mean selected body temperatures (during the middle third of the day) are represented by horizontal coloured lines; in the lab (*green*), sunny habitats in the field (*orange*), and shady habitats in the field (*blue*).

3.3.2.3 Weather conditions

Weather conditions in Kanangra Boyd National Park typify an Australian montane environment with below freezing temperatures and snow common throughout winter months. Mean maximum temperatures do not exceed 25 °C in the hottest month (January) and 10 °C in the coolest month (July) (Fig. 3.7). Daily fluctuations in temperatures of over 15 °C are not uncommon and below freezing temperatures and snow can occur as late as December (personal observation).

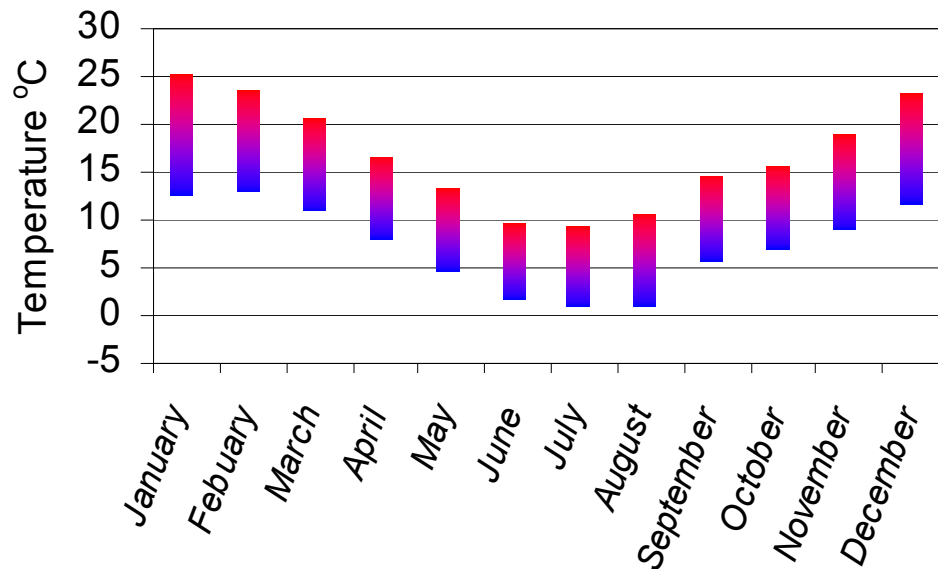


Figure 3.7 Air temperatures (plotted as the range between average minimum and average maximum) each month calculated from the Bureau of Meteorology, Oberon (Jenolan Caves Rd) weather station over a 50 year period, 1953-2003 (33°44'24"S, 149°52'56", elevation 1190 m).

3.3.3 Temperature selection under field conditions

Average daily temperatures (T_{av}) significantly differed across months (2-way ANOVA Sq-transformed: $F_{2,137} = 19.9$, $P < 0.0001$), although habitat (sun or shade) did not affect the selected daily average temperatures of lizards (2-way ANOVA sq-transformed: $F_{1,137} = 0.926$, $P = 0.338$) (Fig. 3.8).

Table 3.1. Means \pm SE of thermal variables measured during the middle third of the light period (1000 - 1500 h Field; 1000 - 1400 h Lab). Sample size (number of *Eulamprus tympanum*) are given in parentheses with number of sampling days [d], Sample size for field operative temperatures (number of loggers) in parentheses with number of sampling days [d].

	Month						ANOVA on ranks (Scheirer-Ray-Hare extension of Kruskal-Wallis test)		
	November		December		January		Statistic for Habitat	Statistic for Month	Statistic for Interaction
	Sun	Shade	Sun	Shade	Sun	Shade			
Field operative	<i>(n = 12[11])</i>	<i>(n = 12[11])</i>	<i>(n = 24[11])</i>	<i>(n = 24[11])</i>	<i>(n = 24[11])</i>	<i>(n = 24[11])</i>			
T_e	18.2 \pm 0.63	14.9 \pm 0.39	23.0 \pm 0.76	19.8 \pm 0.58	23.4 \pm 0.82	19.9 \pm 0.61	$H_1 = 26.86,$ $P < 0.0001^*$	$H_2 = 59.10,$ $P < 0.0001^*$	$H_2 = 0.063,$ $P < 0.975$
$T_{\max(e)}$	23.1 \pm 1.01	18.9 \pm 0.64	26.2 \pm 0.97	22.7 \pm 0.75	27.4 \pm 1.05	23.5 \pm 0.80	$H_1 = 26.03,$ $P < 0.0001^*$	$H_2 = 27.82,$ $P < 0.0001^*$	$H_2 = 0.171,$ $P < 0.90$
$T_{\min(e)}$	12.1 \pm 0.16	10.3 \pm 0.14	18.3 \pm 0.46	15.2 \pm 0.33	18.8 \pm 0.56	16.3 \pm 0.47	$H_1 = 28.31,$ $P < 0.0001^*$	$H_2 = 199.95,$ $P < 0.0001^*$	$H_2 = 0.509,$ $P < 0.75$
$V_{(e)}$	21.1 \pm 2.55	10.6 \pm 1.23	7.3 \pm 0.84	6.6 \pm 0.61	9.9 \pm 1.00	6.5 \pm 0.60	$H_1 = 4.25,$ $P < 0.025^*$	$H_2 = 4.55,$ $P < 0.10$	$H_2 = 2.38,$ $P < 0.25$
Field selected	<i>(n = 4[30])</i>	<i>(n = 4[30])</i>	<i>(n = 3[31])</i>	<i>(n = 3[31])</i>	<i>(n = 3[10])</i>	<i>(n = 2[10])</i>			
T_{fsel}	22.7 \pm 0.63	21.3 \pm 0.62	26.3 \pm 0.47	22.1 \pm 0.47	25.4 \pm 0.60	23.5 \pm 1.12	$H_1 = 19.61,$ $P < 0.0001^*$	$H_2 = 10.81,$ $P < 0.005^*$	$H_2 = 11.46,$ $P < 0.005^*$
T_{\max}	29.1 \pm 0.71	27.6 \pm 0.76	31.2 \pm 0.48	28.2 \pm 0.62	30.0 \pm 0.69	30.0 \pm 1.38	$H_1 = 3.43,$ $P < 0.10$	$H_2 = 2.03,$ $P < 0.50$	$H_2 = 12.61,$ $P < 0.005^*$
T_{\min}	15.4 \pm 0.52	15.5 \pm 0.50	18.4 \pm 0.59	16.2 \pm 0.40	16.3 \pm 0.68	16.9 \pm 0.86	$H_1 = 0.306,$ $P < 0.75$	$H_2 = 10.58,$ $P < 0.001^*$	$H_2 = 3.53,$ $P < 0.25$
V_{fsel}	21.6 \pm 1.67	17.3 \pm 1.57	16.1 \pm 1.53	16.1 \pm 1.45	15.9 \pm 1.79	21.9 \pm 4.23	$H_1 = 0.121,$ $P < 0.75$	$H_2 = 2.71,$ $P < 0.50$	$H_2 = 3.54,$ $P < 0.25$
Lab selected			<i>(n = 6 [6])</i>						
$T_{\text{I sel}}$	31.2 \pm 0.21		$T_{\text{I max}}$	33.0 \pm 0.55					
$L_{\text{I sel}}$	30.1 \pm 0.10		$T_{\text{I min}}$	29.9 \pm 0.16					
$U_{\text{I sel}}$	32.2 \pm 0.34		$V_{\text{I sel}}$	2.4 \pm 1.00					

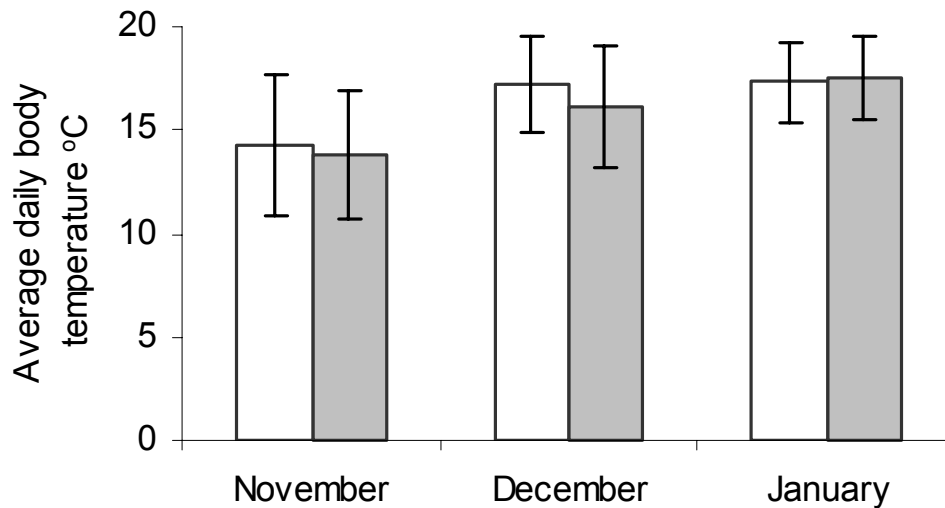


Figure 3.8. Daily average (\pm SE) body temperature in gravid female *Eulamprus tympanum* over the gestation period (November to January) in either favourable (sunny, *open bars*) or unfavourable (shady, *shaded bars*) habitats in the field.

Selected thermal maxima (T_{fmax}) are not significantly different with either month or habitat, but there is an interactive effect of month and habitat on selected maximum body temperatures (Table 3.1). Selected thermal minima (T_{fmin}) differed between months but not habitat, and there is no interactive effect of month and habitat on the selected minimum body temperatures in *E. tympanum* (Table 3.1).

The field selected body temperatures of gravid female *E. tympanum* ($T_{f sel}$) are significantly different between months and habitat (Fig. 3.9), and there is an interactive effect of habitat and month on the mean selected daytime body temperatures in *E. tympanum* (Table 3.1).

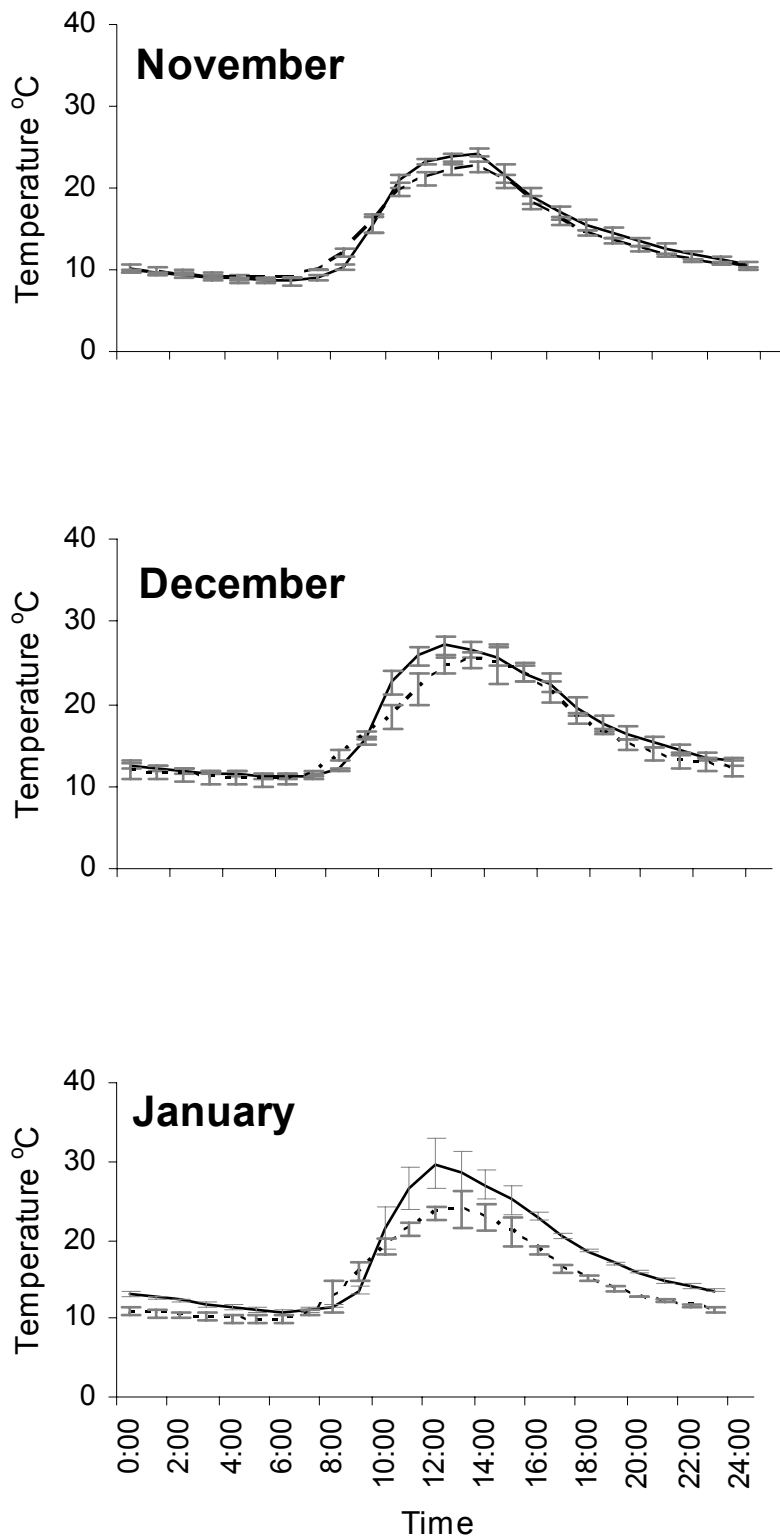


Figure 3.9 Mean (T_{fse1}) selected hourly temperatures (\pm SE) of gravid female *Eulamprus tympanum* each month in sunny habitats (solid line) and shaded habitats (dashed line).

Hourly field selected ($T_{f_{sel}}$) and lab selected ($T_{l_{sel}}$) temperatures are significantly different (ANOVA: $F_{2,17} = 49.5$, $P < 0.0001$), with lab selected temperatures significantly greater at $31.2^{\circ} \text{C} \pm 0.21$ compared to females restricted to sunny habitat at $24.4 \pm 0.37^{\circ} \text{C}$ and females restricted to shaded habitat at $21.8 \pm 0.37^{\circ} \text{C}$ (Table 3.1, Fig. 3.6 and 3.9). Variances are much greater under field conditions ($V_{f_{sel}}$) than under lab conditions ($V_{l_{sel}}$) (Table 3.1) In the field, d_b is smaller than d_e , in all months and habitat, so lizards are thermoregulating, although they are somewhat restricted by the environment. Thermoregulatory efficiency (E) is significantly different between habitats in December but not in November or January (Table 3.2)

Females in sunny habitats had higher selected temperatures ($T_{f_{sel}}$), higher variances ($V_{f_{sel}}$) but reduced thermoregulatory efficiency (E) in comparison with shady habitats during the coolest month (November). In the warmest month (January), females in sunny habitats had higher selected temperatures ($T_{f_{sel}}$) lower variances ($V_{f_{sel}}$) but reduced thermoregulatory efficiency compared to shady habitats. In December, lizards in sunny habitats had higher selected temperatures ($T_{f_{sel}}$) compared to shady habitats, variances ($V_{f_{sel}}$) were equal but thermoregulatory efficiency (E) was much greater in sunny habitats in comparison with shaded habitats.

Table 3.2 Mean \pm SE for body temperatures ($T_{f_{sel}}$) and operative temperatures (T_e), deviations of body temperature (d_b) and operative temperature (d_e) from upper (U_{sel}) and lower (L_{sel}) selected temperatures used to evaluate the effectiveness of temperature regulation in *Eulamprus tympanum* (E) during the middle third of daylight hours (1000 - 1500 h Field; 1000 - 1400 h Lab). Where effectiveness of thermoregulation: $E = 1 - (d_b / d_e)$, with no thermoregulation $E = 0$ ($d_b = d_e$) and careful thermoregulation $E = 1$ ($d_b < d_e$) (criteria set by Hertz *et al.* 1993).

Habitat & season	$T_{f_{sel}}$	L_{sel}	U_{sel}	T_e	d_b	d_e	E	Statistic <i>t-test</i>
November - Sunny	22.7 \pm 0.63	30.1 \pm 0.10	32.2 \pm 0.34	18.2 \pm 0.63	7.6 \pm 0.64	11.9 \pm 0.63	0.36 \pm 0.05	$P = 0.5612$
November - Shady	21.3 \pm 0.62	30.1 \pm 0.10	32.2 \pm 0.34	14.9 \pm 0.39	8.7 \pm 0.60	15.2 \pm 0.39	0.43 \pm 0.04	
December - Sunny	26.3 \pm 0.47	30.1 \pm 0.10	32.2 \pm 0.34	23.0 \pm 0.76	4.2 \pm 0.44	7.1 \pm 0.76	0.41 \pm 0.06	$P = 0.0068^*$
December - Shady	22.1 \pm 0.47	30.1 \pm 0.10	32.2 \pm 0.34	19.8 \pm 0.58	8.2 \pm 0.57	10.3 \pm 0.58	0.20 \pm 0.06	
January - Sunny	25.4 \pm 0.60	30.1 \pm 0.10	32.2 \pm 0.34	23.4 \pm 0.82	4.8 \pm 0.66	6.7 \pm 0.82	0.29 \pm 0.10	$P = 0.1042$
January - Shady	23.5 \pm 1.12	30.1 \pm 0.10	32.2 \pm 0.34	19.9 \pm 0.61	6.7 \pm 1.10	10.2 \pm 0.61	0.34 \pm 0.11	

3.4 Discussion

In the laboratory, gravid female *E. tympanum* maintains a mean selected temperature (T_{isel}) of 31.2 °C while body temperatures in the field are restricted by the thermal properties of their habitat. Temperatures within habitats with less than 50 % canopy cover (sunny habitats) are significantly warmer than those habitats with greater than 50% canopy cover (shady habitats). The effectiveness of thermoregulation (E) illustrates that the majority of daily body temperatures fall below T_{isel} . Although, females are actively thermoregulating (d_b consistently less than d_e), they are restricted to moderate effectiveness of thermoregulation ($E = 0.20 - 0.43$) due to the vast majority of T_e readings falling below lower (L_{isel}) limits of T_{isel} . Thus, environmental constraints limit thermoregulatory precision in *E. tympanum*, despite thermoregulatory effort.

In the laboratory where basking availability is reduced, a closely related species *Eulamprus quoyii*, compensates by modifying basking behaviour to maintain higher body temperature (Borges, 1999). *Eulamprus tympanum* in shady habitats also modifies behaviour by thermoregulating slightly more efficiently than lizards in sunny habitats in both November and January.

Selected body temperatures during the activity period (T_{fisel}) varied with month, as is often observed with seasonal changes (Van Damme *et al.*, 1986; Van Damme *et al.*, 1987; Patterson and Davies, 1989; Patterson, 1992). *Eulamprus tympanum* are territorial and aggressive towards con-specifics (personal observation), and are present in dense populations (Schwarzkopf, 1991), which creates significant

competition for logs in open habitats with suitable refuge. Larger individuals are more dominant over smaller individuals, even when the smaller individual is the resident (K Robert, unpublished data). Gravid females stay closer to shelter sites and bask more than males and non-gravid females, particularly in the later stages of pregnancy (Schwarzkopf and Shine, 1991; Chapter 4). Considering the influence of maternal basking on embryonic development, offspring phenotypes, size, and quality, occupation and maintenance of an optimal position would place strong selective pressures on the basking behaviour of females.

If the assumption is made that sex determination in *E. tympanum* occurs in the middle third of development, as is commonly observed in many species with temperature-dependant sex determination (Yntema, 1979; Pieau and Dorizzi, 1981; Yntema and Mrosovsky, 1982; Wibbels *et al.*, 1991; Wibbels *et al.*, 1994 and references therein) the time corresponding with the period of sex determination in the field for *E. tympanum* would be December (ovulation and mating occur throughout October and birth occurs throughout February). December and January have the warmest available temperatures, with lizards in December having the highest selected body temperatures, lowest selected thermal variance and the greatest thermoregulatory efficiency in the field when in sunny habitats (< 50% canopy cover). During this thermosensitive period of sex determination, and when not restricted to shaded environments, gravid females are thermoregulating more precisely than in other months.

There are many costs eg., greater predation risk due to increased exposure, and benefits eg., reduced gestation period associated with thermoregulation, that

determine the extent to which individuals attempt to thermoregulate. In highly variable montane environments where available temperatures regularly fall below mean selected temperatures, it is important for gravid females to maintain body temperatures best suited to embryogenesis, hence basking opportunity would be of highest priority. Gravid female *E. tympanum* bask more than non-reproductive individuals, even utilising overcast days (Schwarzkopf and Shine, 1991). Increased basking by gravid females also occurs in several other viviparous reptiles (Shine, 1979; Shine, 1980; Beuchat, 1986; Beuchat, 1988; Daut and Andrews, 1993). An assumption of increased basking is greater vulnerability to predation, but this has not been recorded in *E. tympanum*, where females bask more but predation is not significantly greater (Schwarzkopf and Shine, 1991). Behavioural modification in response to a predation threat may compromise basking opportunity (Chapter 4), with females foregoing the opportunity to bask when perceiving a predation risk.

Lizards may use less of the actual available basking time due to behavioural decisions being shaped by a compromise between costs and benefits of basking and by thermal constraints caused by weather conditions. Thermal availability in the montane habitat of Kanangra Boyd National Park varies temporally, spatially and seasonally and precise thermoregulation by *E. tympanum* may be difficult or impossible at certain times or under certain conditions when they are restricted in their capacity to attain preferred temperatures.

Eulamprus tympanum can attain the mean selected temperature of an unrestricted thermal environment (laboratory thermal gradient) under field conditions as indicated by the mode of selected field temperatures for both habitats over all

months was 30.5 °C, and T_{\max} in sunny environments ranged from 29.1 - 31.2 °C depending upon month. Temperature-dependent sex determination is biologically relevant in *E. tympanum*, as the range of temperatures over which sex is influenced is available in their natural environment. Mean daily temperatures (T_{fsei}) in this highly heterogenous environment may not be a good indication of the thermal conditions experienced by developing embryos. Mean daily temperatures in nests of the freshwater turtle, *Chrysemys picta*, with temperature-dependent sex determination (TSD) is a poor predictor of hatchling sex ratio due to nest temperature fluctuations (Schwarzkopf and Brooks, 1985). Mathematical models designed to incorporate daily fluctuations in temperature demonstrate that sex determination in species with TSD depends on the daily proportion of development that occurs above a threshold temperature rather than the proportion of time spent above the temperature (Georges, 1989; Georges *et al.*, 1994). For example, 1h at 1 °C above the threshold will not be equivalent to 1 h at 4 °C above the threshold (Georges *et al.*, 1994). Re-examination of T_{fsei} from lizards in sunny habitats during a 1 h period (1200 - 1300 h) illustrates this point, average T_{fsei} for this 1 h period (from 4 lizards over 71 days of measurement) is 25.9 ± 0.2 °C. However, 37% of T_b are over 30 °C and 69% of T_b are over 25 °C. Temperatures in the laboratory over 25 °C and over 30 °C produce male biased litters (Robert and Thompson, 2001; Chapter 1). In environments where temperatures fluctuate widely, the threshold temperature between male production and female production will depend on both the mean temperature and the magnitude of temperature fluctuation (Georges *et al.*, 1994). Further research is needed in both the laboratory and the field to establish threshold temperatures under constant and fluctuating conditions to fully test the biological relevance of TSD in *E. tympanum* in

the field, especially when predicting responses to changing environmental conditions.

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CHAPTER 4

Is basking opportunity in *Eulamprus tympanum* compromised by the presence of a predator scent?

Submitted for publication in Behavioural Ecology and Sociobiology
Entitled - Is basking opportunity in the montane water skink, Eulamprus tympanum, a species with temperature-dependant sex determination, compromised by the presence of a predator scent? (co-authored with M B Thompson)

4.1 Introduction

The avoidance of predator chemical cues is widespread throughout a variety of animal taxa including, mammals, birds, fish, molluscs, amphibians and reptiles (reviewed Kats and Dill, 1998). Chemoreception is one of the mechanisms a lizard uses to detect and respond to the presence of a predator (Thoen *et al.*, 1986; Dial *et al.*, 1989; Cooper, 1990; Downes, 2002). The ability to detect predator scents is a distinct advantage in indicating the presence of a predator without the need to confront the predator (Van Damme and Castilla, 1996). The response of lizards to a predator scent can be innate or learned (Burghardt, 1977; Phillips and Alberts, 1992; Van Damme *et al.*, 1995), resulting in responses that may differ with age (Head *et al.*, 2002) or previous experience (Shine and Downes, 1999; Downes, 2002) and reproductive state (Cooper *et al.*, 1990; Downes and Bauwens, 2002). Lizards may alter activity, basking rates, and habitat use when perceiving a predation risk (Greene, 1988; Downes and Shine, 1998; Downes, 2001), and a trade-off between the perceived risk of predation and acquisition of resources can occur.

The montane water skink, *Eulamprus tympanum*, is a medium sized live-bearing (viviparous) lizard that occurs at high elevations in south-eastern Australia

(Cogger, 1992). Basking regime in *E. tympanum* can directly affect offspring sex ratios because of temperature-dependent sex determination (Robert and Thompson, 2001; Chapter 1). Predator avoidance may be one of the factors restricting basking opportunity in pregnant females and hence the thermal conditions experienced by developing embryos and thereby affect sex of offspring.

Eulamprus tympanum is usually associated with fallen logs in open sclerophyll forest (Schwarzkopf, 1993) where they are at risk of predation from several species of birds and elapid snakes, particularly kookaburras (*Dacelo novaeguineae*) (Barker and Vestjens, 1989; Blomberg and Shine, 2000), black snakes (*Pseudechis porphyriacus*) and copperhead snakes (*Austrelaps ramsayi*) (Shine, 1991; Blomberg and Shine, 2000).

In this study, I determine chemical discrimination of a predator scent by *E. tympanum* with respect to reproductive condition of females, and I determine whether activity and basking behaviour of gravid and non-gravid females is affected by different chemical stimuli.

4.2 Materials and Methods

4.2.1 Animals and their maintenance

The study consisted of two experimental trials; (1) chemical discrimination trial and (2) activity and basking behaviour trial, to determine whether predator avoidance is a factor affecting basking opportunity and, hence, offspring sex ratio in pregnant *E. tympanum*. The study was conducted in December and January, 2001-3. Non-gravid

females (n = 35), and gravid females (n = 35) were collected from Kanangra Boyd National Park (33°55' S, 150°3' E; elevation 1,300m) immediately following ovulation and mating in late October in 2001 and 2002 (ovulation was evident by palpation for eggs and recent mating by fresh mating scars). Additional gravid females (n = 20) were collected just prior to giving birth in late December and early January of each year. Following birth, these females became available as post-partum females in chemical discrimination trials.

Lizards were individually housed at The University of Sydney in enclosures (400 mm x 350 mm) with 20-30 mm depth of soil, flat rocks and leaf litter for shelter. Heat was provided using a 40W incandescent light bulb suspended over one end of their enclosure, which provided a thermal gradient from 20 to 40 °C for 8 hours a day, falling to 20 °C overnight. A light regime of 12 h:12 h was provided. All lizards were supplied with water *ad libitum* and fed a maintenance diet of four mealworms (*Tenebrio molitor*) dusted with calcium gluconate and commercial reptile multivitamins (Herptivite™, Rep-Cal Research Los Gatos, CA) every three days (Robert, 1999).

4.2.2 Chemical stimuli

The chemical stimuli were prepared the week before the first trial and frozen for storage in separate 5 ml glass vials (50 vials of each scent) to ensure the same scent consistency was used in each trial. The control was distilled water, the novel (or pungency) control was a 1:1 solution of commercial cologne "Givenchy III" and distilled water, the prey solution was prepared using 2.5 g of mealworms blended with 250 ml of distilled water and strained to remove large particles, and the predator

scent was prepared using 7 g of fresh black snake (*Pseudechis porphyriacus*) faeces mixed in 250 ml distilled water and strained through a 1 mm sieve to remove large particles. One vial of each scent type was defrosted on the evening prior to the trial.

Black snake was chosen as the predator stimulus as it is a known predator of *E. tympanum* (Schwarzkopf, 1991; Shine, 1991) and olfaction would play a more important role in detecting a snake as opposed to an avian predator that ambushes its prey from above (Barker and Vestjens, 1989). Snake faeces was chosen as the predator scent stimulus as opposed to other chemical stimuli from the snake, such as shed skin or an integument wash, as faeces were easily obtained from a captive specimen and fresh faeces also represents an actively feeding individual.

4.2.3 Discrimination of chemical cues by lizards

Lizards were fasted for three days prior to each trial to ensure post-absorptivity (Robert and Thompson, 2000). Cotton tip swabs (Softex) were attached to a 150 mm long 5 mm diameter doweling rod, dipped in scent solution and excess fluid blotted dry and then presented to the experimental lizard by slowly approaching the experimental lizard's home cage and placing the cotton swab 10-20 mm anterior to its snout. I recorded the number of tongue flicks over 60 seconds from the time the lizard was presented with the scent. I assumed that fleeing from the swab during the trial was a stronger response than any number of tongue flicks (Cooper and Burghart, 1990; Downes and Shine, 1999). Therefore, in each trial in which the lizard did not flee, the number of tongue flicks in 60 seconds was recorded. If the lizard fled from the swab, the trial was scored as the greatest number of tongue flicks emitted from any lizard plus 60 minus the latency to flee in seconds (criteria set by Cooper

and Burghardt, 1990). Each lizard was tested only once and randomly assigned to a scent type to ensure independent data and eliminate bias from habituation to the test conditions (personal observation). Comparisons made between the same individuals (Downes and Bauwens, 2002) in a randomised order was not possible due to females being viviparous and reproducing a single litter less than annually (Schwarzkopf, 1991), so the order of first presentation could not be exploited by multiple reproductive conditions in one season as a result of producing consecutive litters (criteria set by Downes and Bauwens, 2002).

4.2.4 Activity and basking behaviour

Circular arenas constructed of sheet metal (1m in diameter and 150 mm high Fig. 4.1) were used for experimental trials. At one end was a clay tile with a ceramic heating globe (basking spot) suspended above it, at the opposite end was a series of three PVC pipe shelters (retreat sites) and the base consisted of washed river sand (sand, shelters and tiles were changed and arenas cleaned between each trial to avoid contamination). The arenas (3 in total) were housed in separate constant temperature rooms at 20 °C with light cycles as previously described in housing conditions. One scent was assigned to each room to ensure no cross contamination. Each room was fitted with a miniature surveillance camera (Mini-4040b monochrome camera) equipped with motion detect capabilities and was connected to a PC computer via a 4 channel PC digital video surveillance recording system (Govideo-DVR4) running Grandtec Guard Anywhere software version 1.3 (Appendix 2).

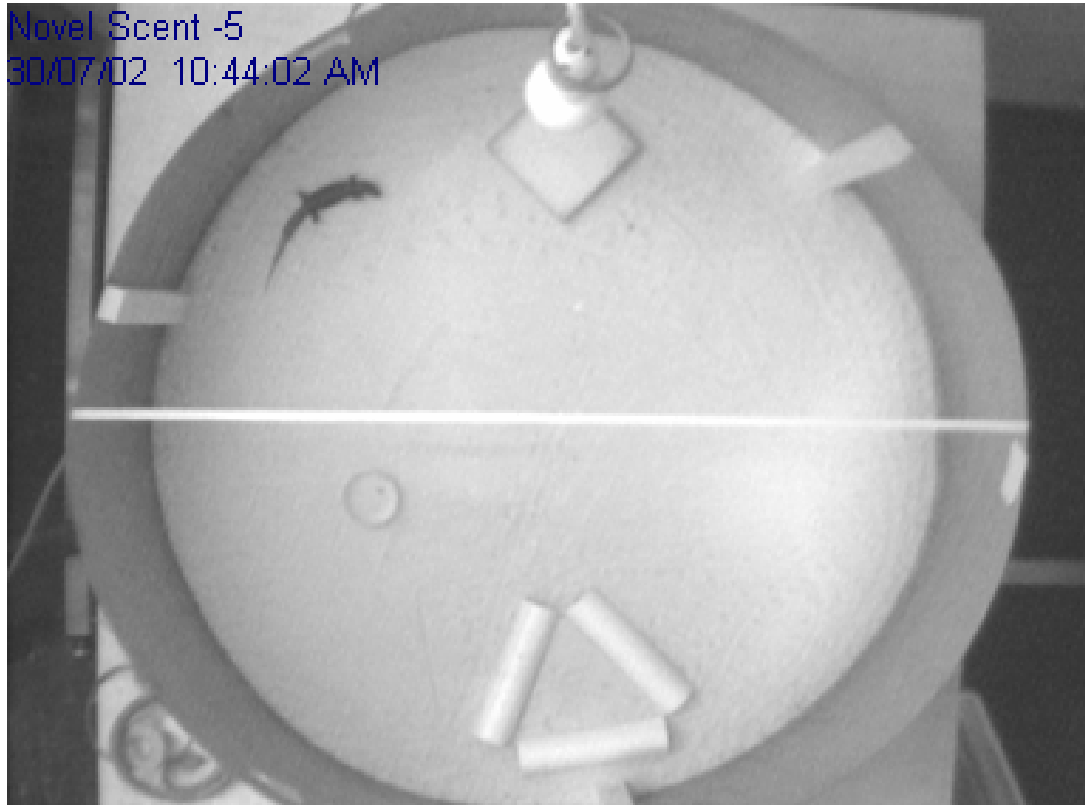


Figure 4.1 Experimental enclosure used to measure activity rates and basking behaviour. A clay tile placed below a suspended ceramic globe provided the basking spot and a series of three PVC pipe shelters provided a shelter site. The tape dividing the enclosure was used to measure activity rates by scoring the number of times the lizard crossed the centre-line.

The experimental lizard was placed within the arena retreat site, and the retreat site was isolated from the arena by a plastic box forming a cover and left overnight. On the morning of the trial (0800 h), the clay tile (basking spot) was treated by spotting with 2 ml of the prepared chemical stimulus (control, novel control or predator scent) and the retreat site cover removed. The surveillance system was set to record from 0900 h till 1700 h on motion detect. Each lizard was tested only once and randomly assigned to a room, hence one scent type. Pilot trials were initially set in a randomised order to test lizards repeatedly with different scents,

however, while trialling camera equipment there was an obvious effect of repeated measurement on the same individual, as lizards became more familiar with the test arena with each repeated trial.

Video was then scored between 0930 h and 1600h as personal observations of lizards in the field showed activity levels of lizards spanned this time frame. Activity rate was scored by the number of times the lizard crossed the marked centre-line (Fig. 4.1). Basking behaviour was characterised by two parameters; 1) the number of times the lizard was recorded on the basking spot (basking frequency) and 2) the time spent in the open (basking duration) recorded by scoring the lizard's position every 5 minutes to calculate the percentage of time spent in the open. The duration of time on the basking spot was not used as a measure of basking duration as temperature measurement within enclosures indicated that lizards would still be capable of efficiently thermoregulating at a distance of 200 to 250 mm from the basking spot. Gravid females have recorded mean selected body temperatures in the laboratory of 31.2 °C (Chapter 3) and 32 °C (Schwarzkopf and Shine, 1991) and available temperatures measured within experimental arenas averaged 40.1 ± 1.34 °C directly below the heat source, 20.2 ± 0.2 °C furthest from the heat source, and 30.6 ± 0.63 °C at a distance of 200 to 250 mm from the heat source.

4.2.5 Data analysis

The data were checked for normality (Kolmogorov-Smirnov Test) and equal variances (Levene Median Test) prior to analysis. Some data deviated from these assumptions and were log-transformed to normalise variances. Data in the chemical discrimination trials were highly non-normal in some instances with a large number of zeros (when no tongue flicks were recorded) and therefore analysed using non-parametric two-way analysis of variance applied on ranked data, using the Scheirer-Ray-Hare (S-R-H) extension of the Kruskal-Wallis test (Sokal and Rolf, 1997). The data that passed the relevant assumptions were analysed using parametric two-way analysis of variance (ANOVA). All hypotheses were tested for statistical significance at the $P < 0.05$ level.

4.3 Results

4.3.1 Chemical discrimination

Chemical discrimination by females depends upon their reproductive condition, with a significant interaction occurring between scent type and reproductive condition (Table 4.1). Gravid females have a stronger response to the predator stimulus than all non-reproductive females (Fig. 4.2).

Table 4.1 Two-factor ANOVA on measured behavioural variables in gravid female *Eulamprus tympanum*; ^a non-parametric two-way ANOVA applied on ranked data using the Scheirer-Ray-Hare extension of the Kruskal-Wallis test; ^b log-transformed prior to analysis to normalise variances.

Variable	Interaction		Factors			
	Statistic	P-value	Scent type		Reproductive condition	
			Statistic	P-value	Statistic	P-value
Tongue-Flicks	$H_6 = 20.99$ ^a	< 0.005 *	$H_3 = 6.56$ ^a	<0.100	$H_2 = 1.012$ ^a	<0.750
Delayed emergence	$F_{2,24} = 2.15$ ^b	0.1382	$F_{2,24} = 2.62$ ^b	0.0901	$F_{1,24} = 5.31$ ^b	0.0301 *
Activity	$F_{2,24} = 4.89$	0.0165 *	$F_{2,24} = 3.15$	0.0610	$F_{1,24} = 11.93$	0.0021 *
Basking frequency	$F_{2,24} = 1.62$	0.2189	$F_{2,24} = 6.26$	0.0065 *	$F_{1,24} = 15.62$	0.0006 *
Basking duration	$F_{2,24} = 4.52$	0.0215 *	$F_{2,24} = 1.52$	0.2383	$F_{1,24} = 8.17$	0.0215 *

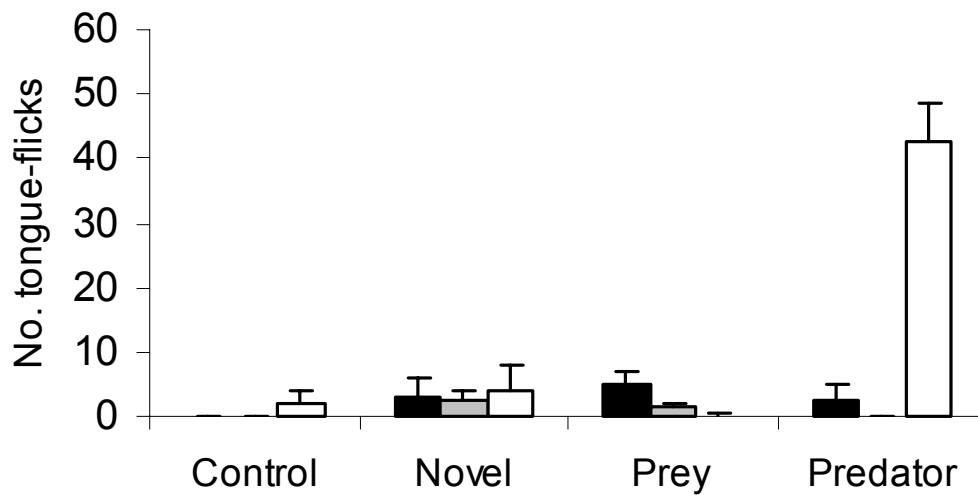


Figure 4.2 Mean (\pm SE) number of tongue-flicks over 60 s in response to control, novel, prey and predator chemical cues by female *Eulamprus tympanum* of different reproductive condition: gravid (n = 20) (open bars), non-gravid (n =20) (shaded bars) and postpartum (n =20) (black bars).

4.3.2 Activity and basking behaviour

Time till first emergence from shelter sites was significantly greater in gravid females than in non-gravid females (Fig. 4.3a). Gravid females significantly delayed their time of first emergence from evening shelters when chemical scents were present (Table 4.1) Activity was significantly greater in non-gravid females than in gravid females (Fig. 4.3b). Gravid females significantly reduce their activity in the presence of a predator scent, and there is a significant interaction between scent and reproductive condition (Table 4.1).

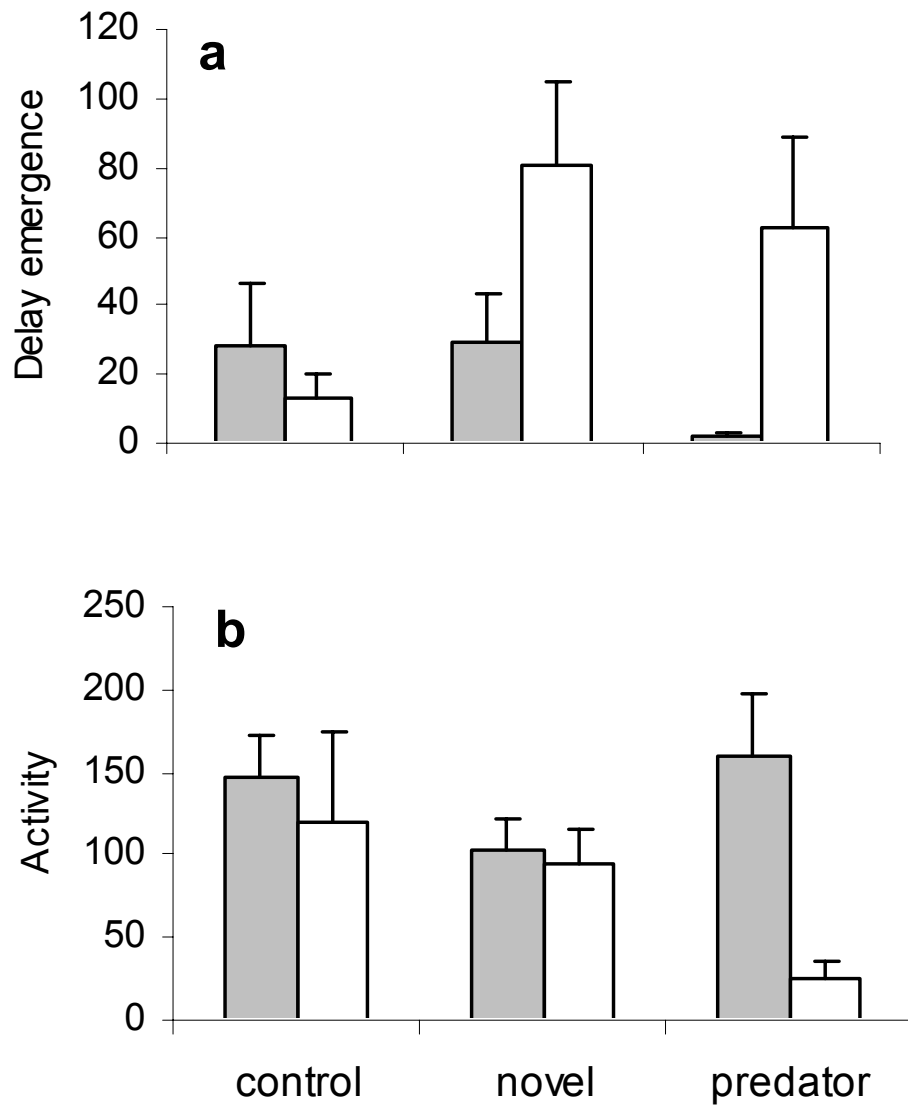


Figure 4.3a, b Activity and basking behaviour in *Eulamprus tympanum*; **a.** Time delayed till first emergence (minutes) from evening shelter site (mean \pm SE). **b.** Activity recorded as the number of times the lizard crosses the enclosure centre-line during the experimental period (0930 h – 1600 h) (mean \pm SE) for gravid females (*open bars*) ($n = 15$) and non-gravid females (*shaded bars*) ($n = 15$) in the presence of a control, novel or a predator scent.

Lizards of both reproductive conditions significantly reduce their basking frequency in the presence of a predator scent (Fig. 4.4a). Gravid females significantly reduce their basking in the presence of a predator scent, with a significant interaction between scent and reproductive condition (Table 4.1). The duration of basking or the percentage of time spent in the open (Fig. 4.4b) is significantly less in gravid females than in non-gravid females with a significant interaction between scent and reproductive condition (Table 4.1).

4.4 Discussion

Eulamprus tympanum responds differently to chemical stimuli depending on reproductive condition. Additionally, the change in time to emerge, activity and basking behaviour in gravid females could potentially compromise their basking opportunities and hence the thermal conditions experienced by their developing embryos.

Non-reproductive lizards showed little discrimination to scents and most notably to that of a known predator, a behaviour also found in a closely related species *Eulamprus heatwolei*, and may be a reflection on the predator's ecology (Head *et al.*, 2002). Chemical cues may remain long after this predator has left the area due to it being a wide ranging active forager (Shine, 1991; Head *et al.*, 2002). Alternatively the lack of response in the chemical discrimination trials may result from a modification of behaviour, as observed in the activity and basking trials, where activity and basking behaviour is modified in the presence of a predator scent. Non-gravid females reduced their basking frequency although maintain their duration of

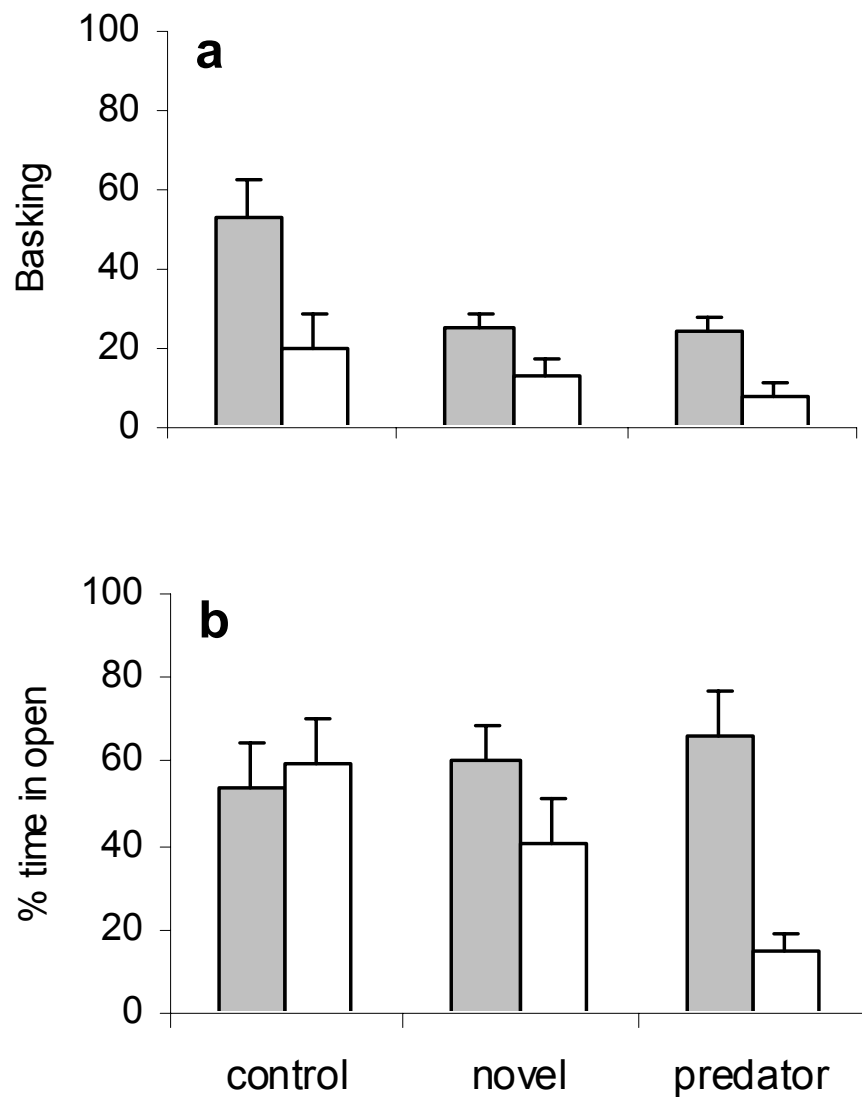


Figure 4.4a, b Activity and basking behaviour in *Eulamprus tympanum*; **a.** Basking frequency recorded as the number of times the lizard was recorded on the basking spot (mean \pm SE). **b.** Basking duration recorded as % of time the lizard was in the open during the experimental period (mean \pm SE) for gravid females (*open bars*) ($n = 15$) and non-gravid females (*shaded bars*) ($n = 15$) in the presence of a control, novel or a predator scent.

basking in the presence of a predator scent by avoidance of the scented basking spot and shifting to alternate basking positions.

Gravid females show a significant response to predator chemical cues, possibly because of their greater vulnerability to predation due to a greater inability to escape through a reduction in running speeds (Shine, 1980; Cooper *et al.*, 1990; Sinervo *et al.*, 1991; Olsson *et al.*, 2000; Miles *et al.*, 2000) and their increased exposure through gravid females basking more than non-gravid females and males (Shine, 1980; Schwarzkopf and Shine, 1991; Schwarzkopf and Shine, 1992; Downes and Bauwens, 2002). Despite their reduced ability to escape and their increased visibility with increased basking, gravid females do not suffer increased predation relative to non-gravid females or males when exposed to predators in experimental field enclosures (Schwarzkopf, 1991), which may reflect their stronger discrimination of predator chemical cues indicated by this study and a shift in behavioural response. Gravid *E. tympanum* delay their emergence from evening shelters, reduce their activity, and their basking behaviour in the presence of predator scent in the laboratory. In the field gravid females frequently bask close to a known escape route or are only partially emerged from a retreat site, especially in the later stages of pregnancy. Gravid females in this later stage of pregnancy also become more wary of a predation threat (at least by a human predator) and become far more difficult to capture; a missed capture almost always results in the female remaining within refuge (personal observation). In the absence of a perceived predation threat, gravid females may bask more often although they shift from an exposed basking position to basking in closer proximity to an escape route (personal observation) and delay their time to flee when faced with an approaching threat, to flee over shorter

distances (Schwarzkopf, 1991). Gravid *Lacerta vivipara* also reduce their activity and shift towards more cryptic behaviour in an attempt to avoid detection or reduce encounters with predators (Bauwens and Thoen, 1981). Inactivity when predation risk is high may reduce risk of predation, especially with an active, visually orientated predator (Skelly, 1994), but will have implications for thermoregulation.

Interestingly, gravid females show little discrimination towards prey chemical cues until after parturition and supports the suggestion that females feed infrequently during pregnancy (Schwarzkopf, 1991) and rely upon stored energy such as lipids in the proximal portion of the tail (Doughty, 1996; Doughty and Shine, 1998). Gravid *E. tympanum* have significantly smaller gut content volumes than non-gravid individuals and a strong relationship exists between embryonic mass and the volume of stomach contents (Schwarzkopf, 1991), suggesting that females feed less as gestation increases because of the increased reduction in body cavity space. This ability to rely upon stored energy would reduce the need to forage, decreasing the need to be active in the later stages of pregnancy and hence reducing their vulnerability to predation.

The presence of predator scents at preferred basking position of a gravid female could compromise her basking opportunity with the perceived risk of predation forcing retreat to sub-optimal conditions. The influence of maternal basking on embryonic development and offspring phenotypes suggest strong selective pressures on females basking behaviour (Shine and Harlow, 1993; Wapstra, 2000). The basking ability of *E. tympanum* is important in determining offspring sex ratios because this species has temperature-dependent sex determination (Robert and

Thompson, 2001; Chapter 1). Females within less favourable environments with reduced basking opportunities remain significantly cooler (up to 3 °C cooler) than females in more favourable environments (Chapter 3). The thermal regime maintained by the mother throughout pregnancy directly affects the sex of offspring produced and predator avoidance may be one of the factors that could influence the sex of offspring produced by preventing females from thermoregulating at male producing temperatures.

Considering that foraging is of little importance in gravid females, especially late in gestation, the most important resource would be the maintenance of preferred body temperatures. Under field conditions, gravid females remain closely associated with a retreat site and often remain partially emerged rather than fully emerged if the retreat is in optimal conditions (personal observation). Under laboratory conditions, gravid females give up the opportunity to bask when there is a perceived predation risk. Further experimentation conducted under field conditions to determine whether gravid females will abandon a preferred basking position in the presence of a predator scent is necessary to test fully the hypothesis that basking opportunity is compromised by the presence of a chemical cue from a predator.

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TSD & Viviparity: a selective advantage?

SECTION THREE

CHAPTER 5

Facultative sex allocation in the viviparous lizard *Eulamprus tympanum*: A selective advantage

Published in Australian Journal of Zoology 51: 367-370

Entitled- Facultative sex allocation in the viviparous lizard Eulamprus tympanum, a species with temperature-dependent sex determination (co-authored with M B Thompson & F Seebacher)

5.1 Introduction

Most animals produce equal numbers of sons and daughters, although the ability to choose offspring sex may be an advantage in changing environments with different selective pressures on males and females (Hamilton, 1967; Trivers and Willard, 1973; Charnov, 1982). Sex choice could enhance fitness by skewing offspring sex ratio towards the sex with the best reproductive success (Trivers and Willard, 1973; Werren and Charnov, 1978). Temperature-dependent sex determination (TSD), where the temperature of development determines the sex of the offspring produced, is widespread among oviparous (egg-laying) reptiles and may provide a mechanism for mothers to choose the sex of their offspring through nest site selection (Bull, 1980). However, maternal manipulation of offspring sex by nest site selection is controversial and may be implausible because many oviparous reptiles with TSD are long-lived with late maturation, so that any nest site decision would need to predict the population sex ratio many years into the future when those offspring mature (Rosenburg and Niewiarowski, 1998; Morjan, 2003). Many studies linking maternal manipulation by nest site selection use repeatability of microenvironment or non-random selection of nest sites as evidence (Vogt and Bull, 1984; Schwarzkopf and Brooks, 1985; Janzen and Morjan, 2001) without monitoring

other potential limiting factors such as predators, so repeatability of nesting microenvironments may reflect a response to other consistent driving pressures of nest site selection rather than heritability (Spencer and Thompson, 2003). These concerns would not apply to fast maturing, short lived species. The exciting recent discovery of a viviparous (live-bearing) lizard that is relatively short lived (8 yrs), fast maturing (2-3 yrs) (Blomberg, 1994; Doughty, 1996) and can manipulate the sex of its offspring directly by TSD (Robert and Thompson, 2001; Chapter 1) means that this species, and others with similar life histories, could gain selective advantages from manipulating the sex of their offspring. Temperature-dependent sex determination provides the mechanism for *E. tympanum* to directly alter population sex ratios through active thermoregulation, rather than by nest site selection as in oviparous species. The mother's basking behaviour throughout pregnancy directly controls the thermal conditions experienced by the developing embryo and hence offspring sex (Robert and Thompson, 2001; Chapter 1). Gestation temperature has a highly significant effect on sex, with warmer temperatures giving rise to male offspring (Robert and Thompson, 2001; Chapter 1).

In this study, I altered the sex ratios experienced by pregnant females to test the hypothesis that females use TSD to adjust offspring sex ratio in response to sexual imbalances within the population.

5.2 Materials and Methods

5.2.1 Animals and their maintenance

Pregnant females (n = 12) collected from the Kanangra Boyd National Park (33°55' S, 150°3' E; elevation 1,300m) immediately following ovulation and mating

(ovulation was evident by palpation for eggs and recent mating by fresh mating scars) were assigned to laboratory enclosures (450 mm x 350 mm) with 20-30 mm depth of soil, flat rocks and leaf litter for shelter, with either a high proportion of males ($n = 6$) (consisting of 3 males to 1 female in each enclosure) or all females ($n = 6$) (consisting of 4 females in total for each enclosure). Heat was provided using a 60W incandescent light bulb suspended over one end of their enclosure, which provided a thermal gradient from 20 to 40 °C for 8 hours a day, falling to 20 °C overnight. All lizards were supplied with water *ad libitum* and fed a maintenance diet of four mealworms - *Tenebrio molitor* dusted in calcium gluconate and commercial reptile multivitamins (Herptivite™, Rep-Cal Research Los Gatos, CA) every three days (Robert, 1999). In addition, a low male treatment was conducted where females ($n = 6$) were maintained singly in enclosures (1.5 m in diameter) in the field at the site of collection and returned to the lab just prior to parturition. The thermal regime experienced under field conditions varied with weather conditions. As part of another study, females had miniature temperature data loggers (Robert, 2003; Appendix 1) attached to measure thermal behaviour with the resulting average temperatures ranging from 9 °C overnight to 31 °C throughout the day (Chapter 3).

5.2.2 Sex determination

Immediately following birth, newborn offspring were sexed externally by the presence or absence of hemipenes by palpation (Harlow, 1996; Chapter 2). Palpation involved holding the neonate under a dissecting microscope, ventral surface up with the forefinger placed anterior to the cloaca. A blunt probe was then rolled from approximately 5mm caudal to the cloaca, up the tail towards the cloaca. The pressure applied from the forefinger and the rolling probe everts one or both

hemipenes in males and occasionally hemiclitori in females. Palpation for hemipenes is a reliable technique in sexing newborn *E. tympanum* (Chapter 2).

5.2.3 Data analysis

Sex ratios are expressed as the proportion of sons in a litter and analysed by ANOVA analysis on arcsin square root of proportions. All hypotheses were tested for statistical significance at the $P < 0.05$ level.

5.3 Results

The proportion of male offspring in the experimental treatments are significantly different (ANOVA: $F_{1,16} = 31.11$, $P < 0.001$). Females maintained in female only groups produced more male offspring than female (Fig. 5.1) and eleven of the twelve litters were male biased (Table 5.1). In contrast, females maintained with males produced offspring of mixed sexes (Fig. 5.1) and four of the six litters were female biased (Table 5.1).

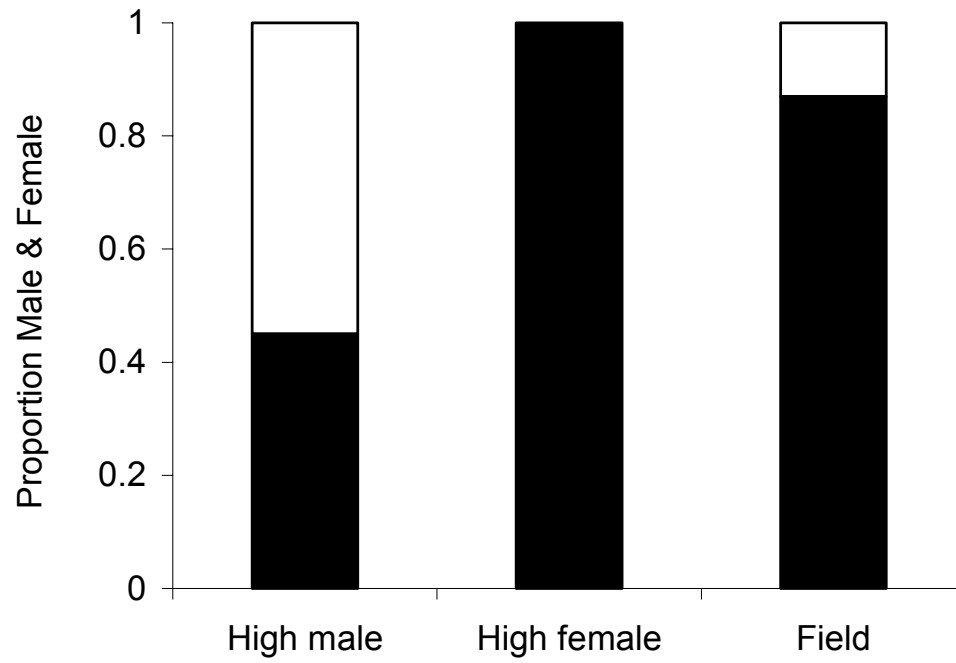


Figure 5.1 Influence of adult sex ratio on offspring sex ratio in the viviparous skink *Eulamprus tympanum*. Black portions of the bars represent male offspring; white portions represent female offspring.

Table 5.1 Comparison of sex ratios within litters for female *Eulamprus tympanum* maintained in either high female populations (n = 12) or high male populations (n = 6).

Litter size	Treatment			
	High male populations		High female populations	
	<i>Male offspring</i>	<i>Female offspring</i>	<i>Male offspring</i>	<i>Female offspring</i>
2	-	-	2	0
3	-	-	3	0
	-	-	3	0
	-	-	3	0
4	-	-	3	1
	1	3	4	0
	3	1	4	0
	3	1	4	0
	-	-	4	0
	-	-	2	2
5	1	4	5	0
	2	3	-	-
	2	3	-	-
6	-	-	6	0
Totals	12	15	43	3

5.4 Discussion

Ecological theory predicts a population to be in equilibrium when reproductive resources are divided evenly between the production of sons and daughters (Fisher, 1929). However, there are ecological and social circumstances when producing one sex over the other has an advantage, for example, in response to local mate competition (Hamilton, 1967; Werren, 1980), local resource competition (Clark, 1978; Clutton-Brock and Lason, 1986; Komdeur *et al.*, 1997), local resource enhancement (Schwartz, 1988; Komdeur *et al.*, 1997), and sex specific fitness (Trivers and Willard, 1973). In natural systems where it is common for populations to suffer unpredicted periods of exceptional mortality or survival, one sex can be affected more due to behavioural or physiological differences (Werren and Charnov, 1978). Any facultative sex ratio response needs to recognise the scarcity of one sex in order to overproduce that sex in the next generation; offspring sex ratio will vary inversely with adult sex ratio (Werren and Charnov, 1978). Maternal sex allocation in *E. tympanum* is linked with population (or adult) sex ratio (Fig. 5.1), but the mechanism by which females recognise a shortage of one sex over the other is not known and will be the basis of further investigation (Chapter 6). Female viviparous snow skinks, *Niveoscincus microlepidotus*, facultatively adjust offspring sex ratios depending upon their experience during the mating season (Olsson and Shine, 2001). Females courted and mated by many males would perceive the adult male population as plentiful and would in turn overproduce female offspring in an attempt to balance population sex ratios (Olsson and Shine, 2001). The maternal manipulation of offspring sex ratio in *E. tympanum* illustrates a selective advantage of TSD in a viviparous species and further studies will help identify the mechanisms used by

females to recognise a shortage of one sex over the other. Temperature-dependent sex determination extends across such diverse taxa that no one explanation for the selective advantage of TSD may be possible, although maternal effects potentially play an adaptive role in many of these species.

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CHAPTER 6

Cues used by gravid female *Eulamprus tympanum* to make a sex allocation decision

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6.1 Introduction

For any facultative sex ratio response, an individual needs to recognise the scarcity of one sex in order to shift the sex ratio of offspring in the next generation. Hence, offspring sex ratio will vary inversely with adult sex ratio (Werren and Charnov, 1978). Maternal sex allocation in *Eulamprus tympanum* is linked with population (or adult) sex ratio (Robert *et al.*, 2003; Chapter 5), but the mechanism by which females recognise a shortage of one sex over the other is not known. In viviparous snow skinks, *Niveoscincus microlepidotus*, females courted and mated by many males produce a female biased sex ratio in their offspring, which results ultimately in a more balanced population sex ratio (Olsson and Shine, 2001). Females are under selection to perceive biases in the adult sex ratio and shift the sex ratio of offspring in the reversed direction.

Temperature-dependent sex determination (TSD) provides a mechanism for *E. tympanum* to directly alter population sex ratios through active thermoregulation, with the mothers basking behaviour during pregnancy directly controlling the thermal conditions experienced by the developing embryo and hence offspring sex (Robert and Thompson, 2001; Chapter 1). Gestation temperature and population sex ratios experienced during gestation have significant effects on juvenile sex ratios in *E.*

tympanum, with warmer temperatures and a higher ratio of adult females within a population giving rise to more male offspring (Robert and Thompson, 2001; Chapter 1; Robert *et al.*, 2003; Chapter 5). Gravid females must assess adult population sex ratios and then use active thermoregulation to produce offspring sex ratios that vary inversely to adult sex ratios. Here I examine some cues (vision and scent) a female may use to establish the presence of adult males within a population.

6.2 Materials and Methods

6.2.1 Animals and their maintenance

The study was conducted in December and January, 2002-3 as a consequence of an investigation on maternal sex allocation resulting from an alteration of population sex ratios (Robert *et al.*, 2003; Chapter 5). Gravid females ($n = 18$) were collected from Kanangra Boyd National Park (33°55' S, 150°3' E; elevation 1,300m) immediately following ovulation and mating in late October in 2002 (ovulation was evident by palpation for eggs and recent mating by fresh mating scars, Chapter 2). Lizards were assigned to one of three treatments differing in exposure to male cues: 1) females ($n=6$) with visual and olfactory contact with males, 2) females ($n=6$) with visual but no olfactory contact, 3) females ($n=6$) that had olfactory contact but no visual contact with males. Laboratory enclosures (550 mm x 400 mm) with 10-20 mm of fine bark chip, flat rocks and leaf litter for shelter, had centre dividers positioned to provide visual or olfactory barriers. Visual barriers consisted of either clear perspex (visual access) or opaque perspex (visual denial) and olfactory barriers were either holes drilled in perspex (olfactory access) (0.3 mm diameter) or un-drilled perspex (olfactory denial). In addition, those with olfactory

access were supplemented each fortnight with substrate material from the male's enclosure and those with olfactory denial were supplemented with clean bark chip. Lizards in the olfactory denial enclosures also had fresh air pumped from outside the room via aquarium pumps (flow rate calculated as 13.02 ml/min using soap bubble technique set by Withers, 1977) to ensure that male chemical cues within the room did not enter the enclosure. Heat was provided using a 20 W incandescent light bulb suspended over one end of the enclosure, to provide a thermal gradient from 20 to 30 °C for 8 hours a day, falling to 20 °C overnight. A light regime of 12 h: 12 h was provided. All lizards were supplied with water *ad libitum* and fed four mealworms (*Tenebrio molitor*) dusted with calcium gluconate and commercial reptile multivitamins (Herptivite™, Rep-Cal Research Los Gatos, CA) every three days (Robert, 1999).

6.2.2 Sex determination

Immediately following birth, newborn offspring were sexed externally by the presence or absence of hemipenes by palpation (Harlow 1996; Chapter 2).

6.2.3 Data analysis

Sex ratios are expressed as the proportion of sons in a litter and analysed by ANOVA analysis on arcsin square root of proportions and further analysed using the recommended method (Wilson and Hardy, 2002) of logistic regression analysis. All hypotheses were tested for statistical significance at the $P < 0.05$ level.

6.3 Results

The proportion of males in litters over the three experimental treatments was not significantly different (ANOVA: $F_{2,11} = 0.543$, $P = 0.596$; LOGIT: $\chi^2_6 = 6.358$, $P = 0.384$)(Fig. 6.1). However, females maintained without visual access to males produce more male offspring with 3 of the 5 litters being male biased (Table 6.1 and 6.2), females maintained with visual access but no olfactory access to males produced offspring of mixed sexes with 2 of the 3 litters being male biased (Table 6.1 and 6.2), and females maintained with visual and olfactory access to males produced offspring of mixed sexes, and 3 of the 6 litters were male biased (Table 6.1 and 6.2).

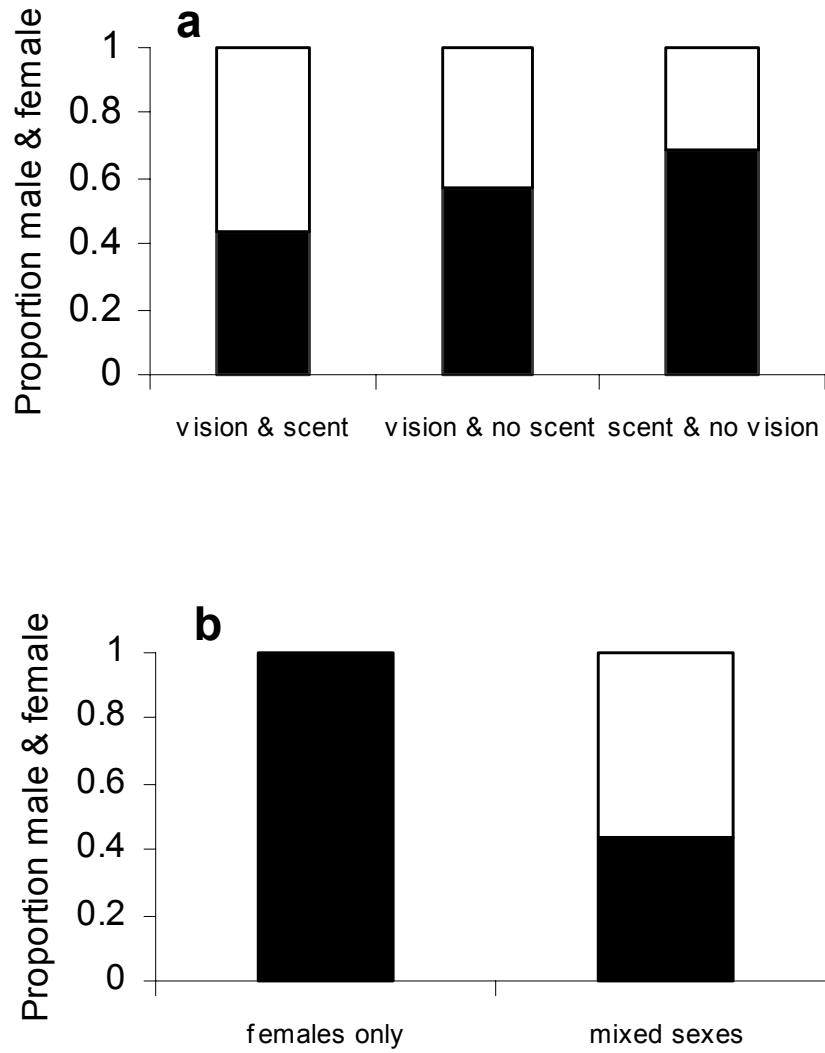


Figure 6.1 Influence of male cues on offspring sex ratio in the viviparous skink *Eulamprus tympanum*. **a)** effect of visual and chemosensory cues (this study), **b)** effect of presence or absence of males (Robert *et al.*, 2003; Chapter 5). Black portions of the bars represent male offspring; white portions represent female offspring.

Table 6.1 Comparison of sex ratios within litters for females maintained in 1) enclosures visual but no olfactory (scent) access to males (n = 3 females), 2) enclosures with visual and olfactory access to males (n = 6 females) or 3) enclosures with olfactory but no visual access to males (n = 5 females).

Litter size	Treatment					
	Visual & no scent		Visual & scent		Scent & no vision	
	Male offspring	Female offspring	Male offspring	Female offspring	Male offspring	Female offspring
1	1	-	1	-	-	-
2	-	-	1	1	1	1
	-	-	-	2	2	-
3	2	1	2	1	2	1
	1	2	2	1	-	-
4	-	-	-	-	1	3
5	-	-	1	4	5	-
Totals	4	3	7	9	11	5

Table 6.2 Sex ratio expressed as a proportion of sons per litter for females maintained in 1) enclosures visual but no olfactory (scent) access to males (n = 3 females), 2) enclosures with visual and olfactory access to males (n = 6 females) or 3) enclosures with olfactory but no visual access to males (n = 5 females).

Litter number	Treatment		
	Visual & no scent	Visual & scent	Scent & no vision
1	1.0	1.0	0.5
2	0.67	0.5	1.0
3	0.34	0	0.67
4	-	0.67	0.25
5	-	0.67	1.0
6	-	0.2	-

6.4 Discussion

Although chemoreception plays an important role in the detection of predators (Chapter 4), *E. tympanum* appears to be visually orientated when it comes to recognising the opposite sex. Despite not being statistically significant, in the absence of visual cues, females produce more male offspring, even when chemosensory cues from males are present. Females maintained throughout pregnancy without any male stimulus (females only) (Robert *et al.*, 2003; Chapter 5), produce entirely male offspring (Fig. 6.1b). In contrast, females exposed to all male stimuli (visual, chemoreceptive and interactive) (Fig 6.1b) produce equal sex ratios (Robert *et al.*, 2003; Chapter 5). Females respond differently to varying degrees of male stimulus and the visual recognition of males within a population may be more important than chemoreception. However, a more comprehensive data set is needed to fully test the hypothesis that vision plays a more important role in the detection of males within a population. Analysis with a relatively small sample size of mothers is guarded, and the use of logistic regression analysis in future studies is recommended (Wilson and Hardy, 2002).

Female courtship in several fish is elicited exclusively by male visual cues and not by chemosensory cues. The peacock blenny (*Salaria pavo*) responds to visual cues and not chemosensory cues (Goncalves *et al.*, 2002) and choice in gravid female cichlids (*Lake malawi mbuna*) is based primarily on visual information (Jordan *et al.*, 2003). Vision provides the primary cue for mate choice in the strawberry poison frog (*Dendrobates pumilio*) (Summers *et al.*, 1999) and the damselfly (*Coenagrion puella*) can distinguish males from females by morphological structures

and colour patterns (Gorb, 1998). In contrast, chemosensory cues play more important roles than vision in mate choice and recognition in some species, for example, mate recognition in two species of newt (*Triturus montandori* and *Triturus vulgaris*) (Cogalniceanu, 1994) and in elephants (Rasmussen *et al.*, 2003) depends entirely on olfactory signals and not by visual cues. In male garter snakes olfaction and vision play important roles in mate choice and recognition (Lemaster and Mason, 2002; Shine *et al.*, 2003). It is likely that combinations of cues are important in both mate choice and recognition in many species.

Population sex ratios are predicted to be in equilibrium when each sex has equal reproductive value and parents divide their reproductive investment equally between the sexes (Fisher, 1929). Sex allocation theory identifies a number of situations when investing differentially in offspring of each sex can enhance parental fitness when different selective pressures act on males and females (Hamilton, 1967; Trivers and Willard, 1973; Clark, 1978; Werren and Charnov, 1978; Charnov, 1982).

The mechanism of sex determination in most vertebrates has been suggested as the major constraint determining the extent of sex ratio adjustment, with genetic sex determination resulting from random meiotic division (West and Sheldon, 2002). The mechanism of sex ratio manipulation in vertebrates is poorly understood and many of the suggested mechanisms infer high costs with a loss of invested resources or time, for example selective culling of offspring at some point during development or biased allocation or mortality between the sexes after birth/hatching (Williams, 1979; Franks, 1990; Krackow, 1995). A recent study of the viviparous lizard *Lacerta vivipara* suggests a masculinization and feminization effect on

offspring due to intrauterine position during gestation (Uller and Olsson, 2003), not unlike that found in mammals (reviewed in Clark and Galef, 1998). The steroids produced by one embryo crosses fetal membranes to be absorbed by an adjacent embryo (Even *et al.*, 1992). A fetus positioned between two of the opposite sex will be influenced by the steroid hormones produced by that sex, some of these effects may have fitness related consequences such as lowered reproductive output (Vom Saal, 1989) and reduced attraction to the opposite sex (Clark *et al.*, 1992). These negative effects may be an incurred cost of producing biased sex ratios and a constraint on sex ratio manipulation in viviparous species with a litter size of three or more (Uller, 2003). The intrauterine position of embryos during gestation in *Eulamprus tympanum* may also be influenced by steroid leakage however the effect on neonates may not be as pronounced as occurs in mammals with large litters. *Eulamprus tympanum* has a restricted yet variable litter size of 1 - 5 young and has a maximum of 3 embryos in an oviduct at a time (personal observation). I would suggest the cost to females producing biased litters would be minimal in *E. tympanum* although it would be interesting to measure.

Support for sex allocation theory is striking in some taxa and less convincing in others. Less convincing is particularly true for most vertebrates (Emlen, 1997; West and Sheldon, 2002), in which many of the assumptions of sex allocation theory are violated (Frank, 1990; Komdeur and Pen, 2002). Much of the ideas and the most striking examples of sex ratio adjustment occur in organisms with haplodiploid sex determination, little or no parental care, no cost associated with the controlling mechanism, no overlapping generations and a fixed amount of resources for reproduction (Hamilton, 1967; Frank, 1990). In contrast, many of the taxa used to

explain sex allocation theory in vertebrates exhibit genotypic sex determination, are characterised by extended parental care, incur high costs associated with controlling sex ratios with a loss of invested resources or time, and parental investment may have an overlapping effect on the parent's ability to invest in next generation (Frank, 1990; Komdeur and Pen, 2002). The best examples of sex ratio manipulation will be seen in species in which the fitness benefits of facultative sex ratio adjustment are high and the costs are low (Werren, 1980; West and Sheldon, 2002).

Skewed sex ratios in reptiles are commonly observed in species with TSD and *E. tympanum* may provide a useful model species for further studies on the direct fitness consequences of sex ratio manipulation on both parents and offspring. The life history of *E. tympanum* has many advantages over many other vertebrates in that they are relatively short lived and fast maturing, meaning that measuring fitness in successive generations is possible. The delayed maturation and long reproductive lifespan of many vertebrate groups preclude experimental studies on the ways sex allocation might influence fitness and selective advantage; *E. tympanum* and species with similar life histories can overcome this obstacle. Unlike many other vertebrates, *E. tympanum* also fits many of the general assumptions of sex allocation theory and the association between a female's ability to recognise population sex ratios and TSD facilitates maternal manipulation of offspring sex ratios.

This study suggests that a female *E. tympanum* may base her sex allocation decision on a visual assessment of the proportion of adult males within the population. The maternal manipulation of offspring sex ratio in *E. tympanum* could

serve as a mechanism to maintain balanced sex ratios in populations and may be a selective advantage of TSD in viviparous species.

There have been few experimental studies on vertebrates that manipulate an aspect that affects sex ratios. Most studies have involved birds, a group that provides an inconsistent picture of sex ratio variation (Komdeur and Pen, 2002; Westneat *et al.*, 2002). Manipulative experimental studies in birds have included translocation experiments to examine offspring produced by females in relation to habitat quality (Komdeur *et al.*, 1997; Hörnfeldt *et al.*, 2000), pairing females with mates of varying attractiveness (Sheldon *et al.*, 1999), and manipulating maternal diet and condition (Bradbury and Blakey, 1998; Nager *et al.*, 1999; Velado, 2002). In all of these studies a major question remains. By what mechanism do female birds use to manipulate offspring sex?

Another advantage of sex allocation studies using *E. tympanum* is the knowledge of how sex is manipulated, in comparison to other vertebrates studied, where virtually nothing is known about how a female controls the sex of her offspring (Sheldon, 2000). The most extreme and precise sex ratio variation will be seen in species in which the fitness benefits of facultative sex allocation are high and the parental costs are low (West and Sheldon, 2002). Parental costs in *E. tympanum* are low in comparison to other vertebrates, particularly those with extended parental care or lactation and there is abundant evidence to support fitness benefits associated with developmental temperatures that differentially affect offspring sexes in many reptiles (Burger and Zappaloriti, 1988; Shine *et al.*, 1995; Shine and Harlow, 1993; Elphick and Shine, 1998; Borges, 1999; Downes and Shine, 1999; Robert,

1999; Shine and Downes, 1999; Olsson and Shine, 2001). Parental costs for manipulating the sex ratio of offspring in *E. tympanum* are also low in comparison to other vertebrates.

Sex ratios and the factors affecting sex ratios vary considerably within and among species. Experimental studies on sex ratio variation and the theory explaining sex ratio variation are at present confusing and further studies using more empirical work using appropriate species like *E. tympanum* may help explain maternal sex ratio manipulation and test the theory of sex ratio variation.

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SUMMARY & DIRECTIONS FOR FUTURE RESEARCH

Temperature-dependent sex determination and viviparity: a unique model system

This thesis presents an important study extending temperature-dependent sex determination (TSD) to another reproductive mode, a viviparous (live bearing) lizard (Robert and Thompson, 2001; Chapter 1) and the maternal control of offspring sex through choice of thermal environment (Robert *et al.*, 2003; Chapter 5). Viviparity allows maternal control of embryonic temperature throughout development and thus a means of sex allocation. This work and the study system itself should inspire a new wave of interest in TSD in reptiles. Until now studies of the maternal aspects of TSD have been 1) where the mother lays the eggs and 2) the allocation of sex steroid hormones to eggs, which has been exceedingly difficult to study. Research on TSD in oviparous (egg laying) reptiles has been limited in examining the maternal control of sex ratio, due to difficulties in obtaining repeated nests from the same individual over years and because variables other than temperature can affect nest site choice.

My study system offers many advantages over the traditionally studied oviparous species with TSD (the crocodiles and turtles) and could possibly answer many important questions. Future research will help address the many unanswered questions regarding the evolution of TSD, the adaptive significance of TSD and maternal manipulation of sex ratios. The life history of *E. tympanum* has many advantages over the previously studied oviparous species with TSD, in that they are relatively short lived and fast maturing, meaning that measuring fitness in successive

generations is possible. The delayed maturation and long reproductive lifespan of many of the oviparous species preclude experimental studies on the ways sex allocation might influence fitness and selective advantage. The low cost associated with sex ratio manipulation in *E. tympanum* provides an ideal model species to test the theories of sex allocation. Future research will help address the many unanswered questions regarding the evolution of TSD, the adaptive significance of TSD and maternal manipulation of sex ratios.

There is strong evidence for temperature regulation of the production of sex steroid hormones as an integral part of the sex determining pathway in oviparous reptiles with TSD (Elf *et al.*, 2002b). In addition, the amount of steroid hormone deposited in eggs (both avian and reptilian) show considerable variability and possible maternal control (Schwabl, 1993; Petrie *et al.*, 2001; Elf *et al.*, 2002a; Elf *et al.*, 2002b). The embryonic intrauterine position in some mammals is affected by sex-related steroid transfer between siblings (reviewed by Clark and Galef, 1998) and a recent study of the viviparous lizard *Lacerta vivipara* illustrates a feminization and masculinization effect on offspring from sex biased litters suggesting intrauterine position and steroid transfer also occurs in viviparous lizards (Uller and Olsson, 2003). The hormonal environment during embryonic development in viviparous reptiles with TSD may provide a good model in establishing maternal contribution of yolk hormones and hormonal transfer between both siblings and mother.

Temperature-dependent sex determination is widespread in reptiles (Bull, 1980), but its adaptive significance remains controversial (Shine, 1999; Girondot and Pieau, 1999). Research on the adaptive significance of TSD has primarily focussed

on long lived, slow maturing species (turtles and crocodilians) that constrain experimental studies on differential fitness as a result of incubation temperature due to the difficulties in making multigenerational analysis. In comparison, *Eulamprus tympanum* is a relatively short lived and fast maturing species that provides a model that could overcome this obstacle.

Until now the fitness consequences of developmental temperature effects on sex were not related to population sex ratio (Roosenburg and Niewiarowski, 1998) and hence the selective advantage of TSD remained elusive. An important direction of research now would be to identify the traits that *E. tympanum* use to maximise offspring fitness: is there a fitness difference between males and females from different developmental temperatures? A female that can adjust her offspring's sex to the sex best suited to environmental conditions could enhance her maternal fitness.

Females may be able to manipulate the sex of their offspring through the differential allocation of sex steroid hormones during the entire period of sexual differentiation, unlike oviparous species that need to allocate hormone or hormone precursors to follicles or eggs prior to oviposition. Future research is needed to measure both circulating maternal hormones and embryonic yolk hormones throughout development to determine if reptiles can allocate hormones or hormone precursors differentially. *Eulamprus tympanum* is an ideal model for these studies.

Not only does the occurrence of TSD in *E. tympanum* open up abundant possibilities for future research on the many questions associated with TSD, but also

opens up the examination of TSD in other viviparous species that were originally thought to be incompatible with TSD.

There are many questions that are important to examine, either to provide answers to questions that I have raised or to expand on areas that I was unable to explore.

The following important questions would make profitable studies:

- 1) How widespread is TSD in other viviparous species? A logical step would be to examine the closely related *Eulamprus heatwolei* that occurs in adjoining and often overlapping habitat with *E. tympanum*.
- 2) How widespread is TSD in other lineages? How often has it evolved?
- 3) At what stage does the temperature sensitive period of sex differentiation occur in *E. tympanum*? For the purpose of my study I made the assumption that sex determination in *E. tympanum* would be the same as in oviparous species.
- 4) What role do sex steroid hormones play in sex determination?
- 5) What is the interplay between sex steroid hormones and genes that have been identified as playing a role in sexual differentiation (for example, SOX9, SRY-like genes, DAX1, SF1 etc.)
- 6) Is there any maternal control or adjustment of sex steroid hormones at any time during pregnancy?
- 7) If the perceived risk of predation is extended either in length of time or by greater intensity, do females take greater risks or continue to forgo the benefits of basking?

- 8) What is the thermal profile of females and the resulting sex ratios of offspring produced under circumstances where basking opportunity is restricted? For example by a predation threat or a social hierarchy that restricts access to basking positions (larger females show a greater dominance over smaller females in captivity, personal observation)
- 9) What effect does social position place on an individual female in their ability to manipulate offspring sex?
- 10) What are the fitness consequences of TSD in *E. tympanum*?

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APPENDICES



Appendix 1

Reconstructing Thermochron iButtons to reduce size and weight as a new technique in the study of small animal thermal biology

*Published in Herpetological Review 34(2): 130-132.
(co-authored with M B Thompson)*

A1.1 Introduction

Recent developments in small electronic temperature recorders designed for use in the food industry have the potential to revolutionize the collection of thermal data in small animals. Collecting temperature data in small reptiles is most often restricted to laboratory studies in thermal gradients (Licht *et al.*, 1966; Greenberg, 1976; Sievert and Hutchison, 1991), by the use of physical models placed in the field (Porter *et al.*, 1973) or by inserting a cloacal thermocouple/thermometer soon after capture in field studies (Avery, 1982; Schwarzkopf and Shine, 1991). Thermal gradients measure selected thermal preferences in an artificial environment, models estimate operative temperatures in the field, and cloacal thermocouple/thermometer measurements in field studies can only measure active individuals or those that take refuge in accessible areas. Each of the above techniques alone does not provide a true representation of the animal's "natural" or "field" thermal biology, particularly in periods of inactivity, hibernation or within inaccessible refugia.

With modification, the DS1921 Thermochron iButtons manufactured by Dallas Semiconductor, (Texas, U.S.A.) (www.ibutton.com/ibuttons/thermochron.html), a small lightweight real time and temperature recording device, can be attached externally or implanted internally into small animals. The unmodified DS1921

Thermochron iButton weighs approximately 3.0 g, is 17 mm in diameter and 6 mm in thickness. By disassembling units and then reconstructing without the external stainless steel housing, the weight can be halved to approximately 1.5 g or less and the dimensions reduce to the size of the circuit board which is 14 mm in diameter and the thickness depends upon the new battery size.

The DS1921 Thermochron iButton integrates a thermometer, real time clock and memory for storing temperature readings. It accurately measures temperatures ± 1 °C from as low as -20 °C to $+85$ °C in 0.5 °C increments. Temperature readings are available in two formats which run concurrently 1) time vs. temperature mode that records temperature and date-stamps when it occurred, 2) histogram mode that measures the frequency of temperature in 2 °C increments rather than specific times of occurrence. The DS1921 Thermochron iButton can log up to 2048 consecutive temperature readings in read-only memory, after which readings can be either stopped or enabled to roll over. The histogram mode has 63 bins of memory set at 2 °C intervals, and each bin can store 65,000 readings (Greaves, 1999). The DS1921 Thermochron iButton's can be launched and downloaded with the use of a reader (Blue Dot Receptor[®]) connected to an existing PC computer with free software downloaded from Dallas Semiconductor (available at www.iButton.com). Recordings are made at user defined time and rates, with a maximum start delay of 45 days and recording rate ranging from 1 every minute to one every 255 minutes. The life expectancy of the DS1921 Thermochron iButton is more than 10 years or greater than 1 million samples, whichever occurs first (Greaves, 1999). To date our reconstruction of the DS1921 Thermochron iButton has not altered the performance or the life expectancy and not a single unit has failed.

A1.2 Construction of the DS1921 Thermochron iButton

The DS1921 Thermochron iButton is constructed (Fig. A1.1) of two stainless steel cups that make up the housing separated by a plastic grommet. The plastic grommet isolates the IO (data) and GND (ground) sections of the circuit board externally and the VBAT (power) and GND connections internally. Springs are welded to the positive and negative terminals of the battery. It is important that the positive and negative terminals do not touch or the battery will short out causing rapid battery drain. The battery “snaps” into the plastic grommet. The circuit board is positioned in the bottom of the inner housing with the crystal facing up. The semicircle trace on the front side of the circuit board is VBAT. The other large trace is GND, corresponding to the springs on the battery. The back of the circuit board is one large IO trace, which makes contact with the bottom of the inner housing. The grommet and battery slides on top of the inner housing with the VBAT and GND terminals aligned. This combination then slides into the outer housing and the GND battery spring makes contact to the outer housing. The outer housing is crimped over the grommet and inner housing, holding the construction together.

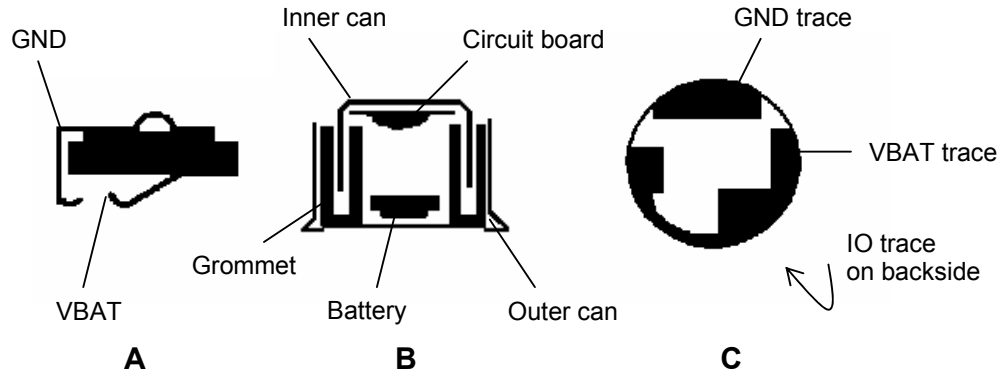


Figure A1.1 Construction of the DS1921 Thermochron iButton, (A) Battery (B) Complete unit (C) Circuit board. See text for explanation of components.

A1.3 De-housing of the DS1921 Thermochron iButton

To reduce the size and weight of the DS1921 Thermochron iButton for use on small animals the iButton can be opened to remove the internal components and reconstructed without the stainless steel housing that accounts for a large proportion of the weight. A vise should be used to hold the DS1921 Thermochron iButton and a small tooth hacksaw (32 teeth/inch) can be used to make vertical cuts through the outer housing in 3 or 4 evenly spaced cuts around the housing. By using a pair of needle nosed pliers, the outer housing should be pulled away from the inner, not unlike peeling a banana.

A1.4 Reconstruction of the DS1921 iButton without the housing

Outside of the housing the circuit board should be soldered to the battery (Fig. A1.2). To avoid damage to the battery it would be best to use new batteries with solder tabs attached which are available upon request from most major battery

suppliers. The DS1921 uses a 3 Volt lithium BR1225 battery; a smaller battery such as BR1216 or CR1025, can be substituted, but they have a shorter lifetime. The bottom tab is VBAT and the tab that comes over the top of the battery is GND. If the battery is not insulated, a piece of plastic tape should be used to prevent the GND tab to the side of the battery which would cause the battery to short. It is important to line the tabs up with the traces on the circuit board and solder them.

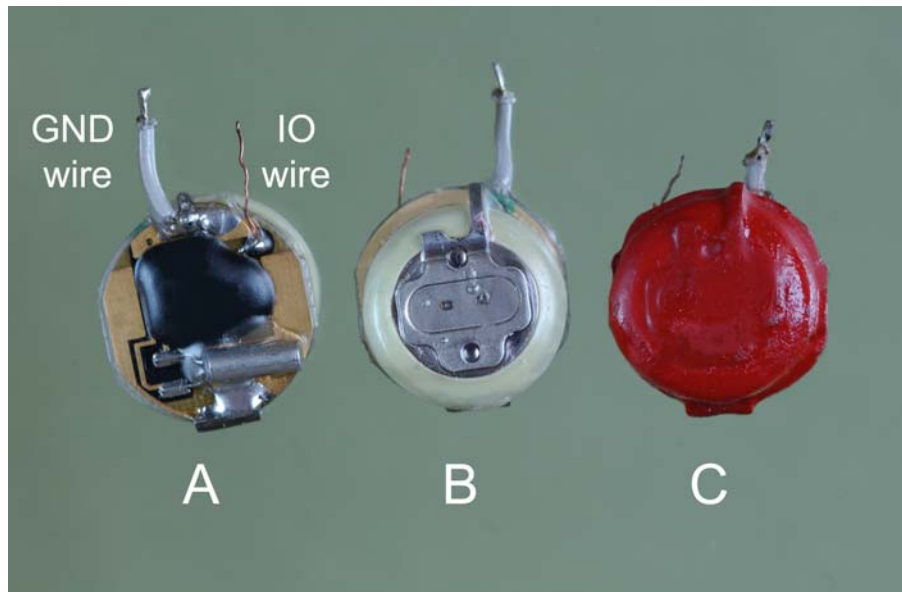


Figure A1.2 Reconstructing the DS1921 Thermochron iButton without housing (A) Circuit board soldered to battery with rigid exposed wires attached (B) Insulated battery soldered to circuit board viewed from above (C) Modified unit complete and coated in red Plastidip[®].

To attach the unit to the reader (Blue Dot Receptor[®]) a rigid wire needs to be soldered to the IO trace and another to the GND trace on the reconstructed unit (Fig. A1.2). One wire should be thicker in diameter or colour coded for easy identification when attaching to the reader. These two wires provide exposed leads for attachment to the reader (Fig. A1.3). The wire attached to the IO trace connects to the inner contact on the reader and the wire attached to the GND trace connects to the outer

contact on the reader. To connect the unit to the reader, one end of a wire needs to be coiled around the inner contact of the reader and the other end with a small alligator clip can then be attached to the exposed IO wire on the unit. Another wire should be coiled around the outer contact of the reader with the opposite end attached to the exposed GND wire on the unit also with a small alligator clip (Fig. A1.3). After testing the soldered unit, it should be sealed to ensure it is watertight with Plastidip[®] (Minnesota, U.S.A.) (www.PLASTIDIP.com) with the exposed ends of the IO and GND wires emerging from the Plastidip[®] (Fig. A1.2).



Figure A1.3 Reader (Blue Dot Receptor[®]) with wires that attach to exposed leads on the modified units via alligator clips.

A1.5 Testing the modified unit

We tested the modified unit on six gravid female *Eulamprus tympanum* over several weeks in laboratory enclosures. Lizards were housed individually in 620 x 400 mm enclosures with 20-30 mm depth of soil, flat rocks and leaf litter for shelter. Lizards were provided with heat from 100 W incandescent bulbs at one end of their

enclosure to allow them to thermoregulate. Enclosures provided a thermal gradient of 20 to 40 ± 1 °C during daylight hours and 20 ± 1 °C during the night. The modified units were attached to the backs of lizards with Super Glue[®] (cyanoacrylic glue) behind the head, between the shoulder blades (Fig. A1.4) and gave continuous measurements of temperature at 5-minute intervals (Fig. A1.5). Measurements can be downloaded and units can be re-launched while the unit remains attached to the animal. The unit will detach from reptiles upon shedding or acetone can be applied to the site of attachment to remove the unit prior to shedding.



Figure A1.4 A female *Eulamprus tympanum* fitted with a modified Thermochron iButton.

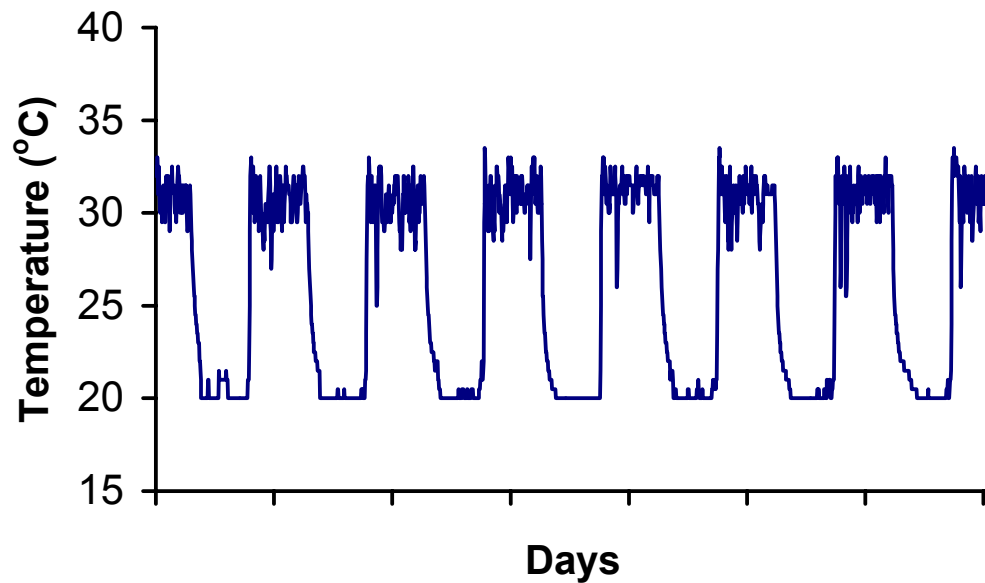


Figure A1.5 Thermal profile of a captive female *Eulamprus tympanum* over one-week at a 5-minute sample rate.

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Appendix 2

An inexpensive video surveillance technique for wildlife studies

Submitted for publication in Wildlife research.

A2.1 Introduction

Most wildlife behavioural studies require time consuming direct observations of animals (Altmann, 1974) or the use of expensive closed-circuit television (CCTV) cameras and time-lapse video equipment (Wratten, 1994). Direct observation of animals is limited by how practical observations are of the species and how easily the species is habituated to an observer (Stewart *et al.*, 1997). The use of video applications in wildlife research have been well documented as a useful technique (Pulliainen, 1971; Wratten, 1994; Sykes *et al.*, 1995; Stewart *et al.*, 1997) and video surveillance equipment has been used increasingly in studies with great success (Asa *et al.*, 1998; Brown *et al.*, 1998; Hughes, 1998; Mc Quillen and Brewer, 2000; Roberts and Anderson, 2002). However, the technique has not been exploited to its full potential by biologists, due primarily to the high initial cost and length of time to extract data from cassettes (Sykes *et al.*, 1995; Stewart *et al.*, 1997; Brown *et al.*, 1998). Time-lapse video is widely used by developmental biologists (Kulesa and Fraser, 1998; Peippo *et al.*, 2001; Rezaie *et al.*, 2002) but has enormous scope in applications for wildlife research and until recently has been cost prohibitive. The main advantages of a video surveillance system are gaining a permanent record of events that can be replayed as many times as necessary to retrieve data, reduction in observer bias and missed observations, easy habituation by the study animal and the ability to document difficult to observe events that are not easily detected using

direct observations. Video surveillance is a technique that is useful for behavioural studies, recording activity at a focal site (such as entrance or exit to a shelter site or burrow, the removal of baits by target and non-target species), identification of individuals, documenting predation events, and detection of nocturnal, crepuscular or elusive species (Stewart *et al.*, 1997; Brown *et al.*, 1998; Deufel and Cundall, 1999).

A2.2 Materials and Methods

In this study, I used inexpensive miniature monochrome (MINI-M20A) video surveillance cameras attached to an existing PC computer via a 4 channel PC digital surveillance recording system (Fig. A2.1) (Go Video DVR4, PCI PC capture card and software) available from Allthings Sales and Service (www.allthings.com.au) (Kelmscott, Western Australia). The entire set-up can be purchased as a complete package and attached to an existing IBM compatible computer from as little as \$450 AUD consisting of a 4 channel PCI PC capture card, software and 4 monochrome cameras and a Plug-in DIY AV 20 metre cable/adaptor set for 4 cameras. Several optional colour camera upgrades also available. Alternatively each component can be purchased separately and a system built to suit individual or experimental needs. There are numerous closed circuit television and surveillance system specialists that can supply and install the identical system described, however quotations received ranged from \$2,306 to \$5,360 AUD in comparison.



Figure A2.1 System components showing video surveillance set-up using a 4 channel PC digital video surveillance recording system.

A2.3 Equipment details

Go Video-DVR4 is a PC compatible 4 channel digital video recording surveillance system for use with colour or monochrome cameras. It consists of a PCI PC capture card and Grand Guard anywhere software (Grandtec, Taiwan) that allows connection of up to four video cameras (capture cards are also available for up to 16 cameras) to display/record simultaneously on a IBM compatible PC computer (Fig. A2.1). The surveillance system uses motion detect with adjustable detection sensitivity for each channel (if continuous recording is required motion detect sensitivity is set at 100%). Areas not to be observed or disregarded for motion detect can be defined by masking an area and audible warnings can be set for each channel to notify of movement. Each video input can be adjusted for brightness, contrast, saturation, hue, image quality and configured to record camera identification, date and time. Higher resolution recording of 384 (H) x 288 (V) per camera (110 592 pixels per camera) in comparison to conventional 4 channel quad/VCR recording system of quad and VHS VCR 160 (H) x 288 (V) per camera

(46 080 pixels) or SUPER-VHS VCR 265 (H) x 288 (V) per camera (76 320 pixels). Video capture rate is 3 to 5 frames per second and is dependent upon computer hardware, number of channels in use, image size. The frame rate can be increased when being re-played to speed up data gathering. Video is captured as AVI files that can be compressed and saved on the computer hard drive or burnt onto compact disc for storage and future analysis. A snap shot option captures frozen images that can be saved as BMP or JPEG images (Fig. A2.2). The system requires a Pentium 200 or above (Pentium 500 or above is recommended), PCI 2.1 compliant mother board, 64 MB RAM or above, Microsoft windows 95 or 98 operating system (capture cards are also available for other Windows operating systems, ME/2000/XP), one PCI slot, and at least 1GB hard disk space per camera.

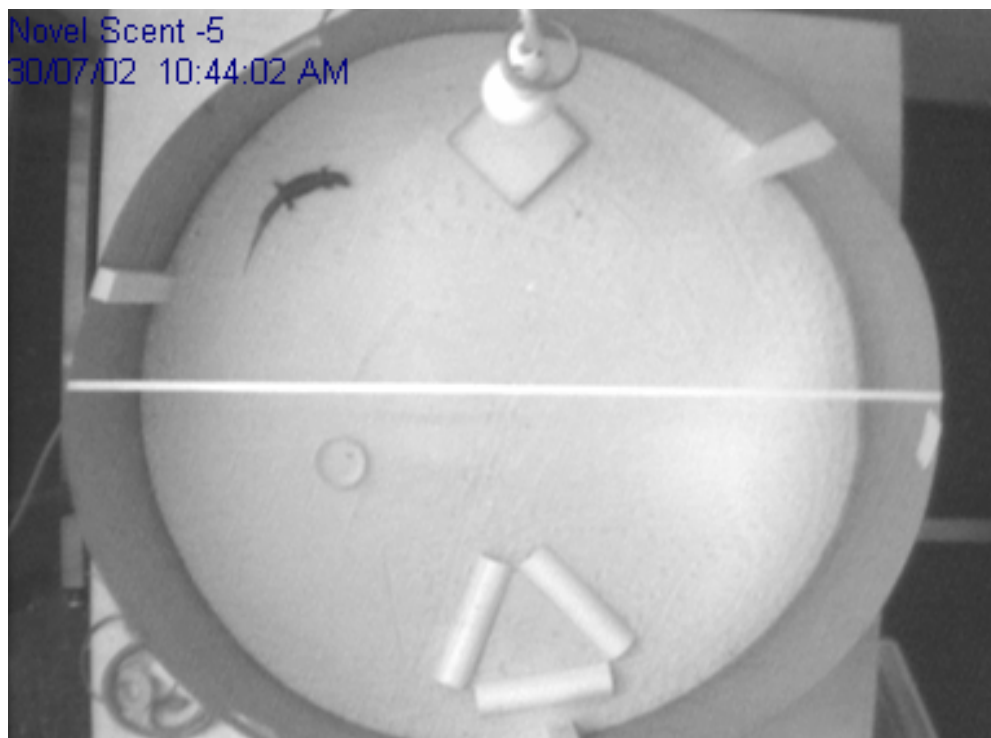


Figure A2.2 Snapshot image of experimental enclosure showing camera identification, date and time display.

MINI-M20A cameras are ¼" low smear image sensor monochrome infra red sensitive surveillance cameras with wall or ceiling mount. They come complete with a 3.6mm lens but you can choose the lens that best suits your application.

Plug-in DIY AV 20 metre cable/adaptor set includes 20 metres of audiovisual cable with all the appropriate moulded plugs and sockets required for self-installation.

A2.4 Testing the set-up

I tested the video set-up using three separate temperature controlled rooms with cameras mounted to the ceiling approximately 1.3 m above experimental enclosures. I placed medium sized lizards, *Eulamprus tympanum* (130-150 mm total length) within 1 m diameter enclosures and the lizards' behaviour was recorded from 0900 to 1700 h on 95% motion detect (Fig. A2.3). To test the suitability of the set-up for my purposes, I chose to record position of lizard at 5 minute intervals and include an estimate of activity rate. The experimental enclosure was divided in half with a piece of string and the number of times the lizard crossed this line became my measure of activity rate. The clarity of the picture was more than sufficient for the purpose of this study. Each AVI file was compressed (zipped) and burnt onto compact disc for storage and later analysis. The software included with the surveillance package Presto! Video Works (Newsoft®) was used for video replay and data collection (Fig. A2.4). Video frame rate was adjusted to 10 frames per second to speed up the scoring process.

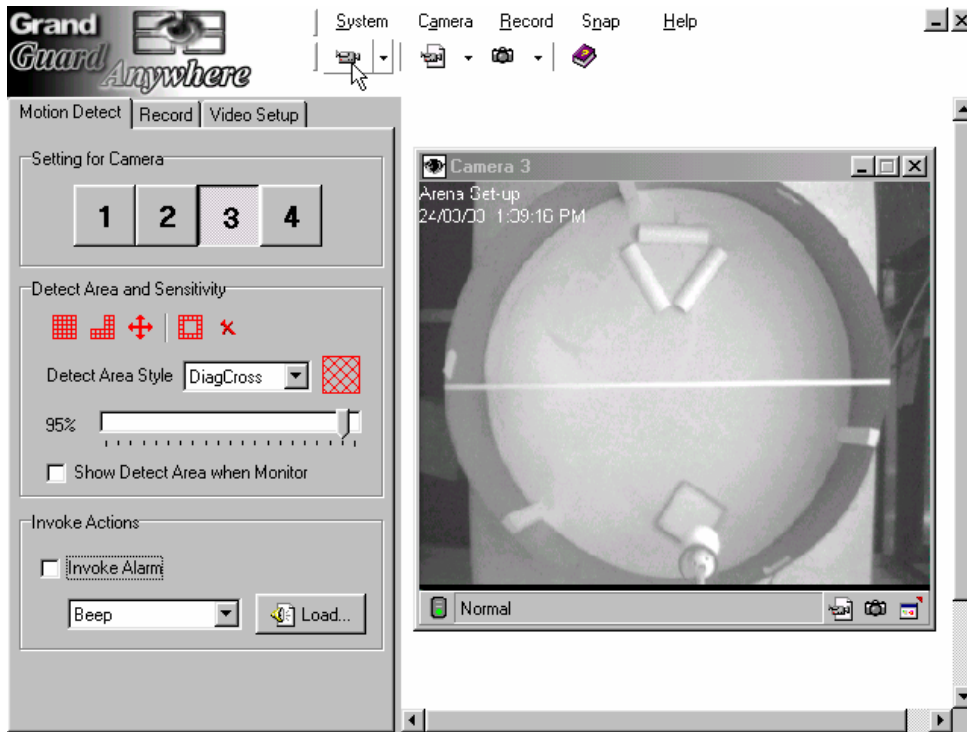


Figure A2.3 On screen view of one camera (1 to 4 cameras can be viewed simultaneously) with set-up menu for motion detect.



Figure A2.4 On screen view of video replay using Presto! Video Works software supplied with system.

A2.5 Discussion

The use of miniature cameras and video surveillance software has wide applications for wildlife and behavioural research. Previously, the major disadvantage with this technology has been the initial set-up cost and the time required to playback video tapes for data collection. New technologies have developed inexpensive cameras and computer software equipped with motion detect sensing to eliminate periods of no activity that in turn speed up data retrieval upon playback. A compact disc burner is recommended to burn all compacted (zipped) AVI files for storage and future data analysis to reduce storage requirements on the hard drive and to ensure a safe back-up should a different analysis or further analysis be required.

The entire set-up could be modified or components upgraded to collect field data by running on a computer in weather proof housing or a laptop computer with a USB video capture box using a portable power source (generator or solar power). Cameras can either be placed in weather proof housings or upgraded to outdoor surveillance cameras. There is also the option of wireless cameras and receivers.

A video surveillance technique for wildlife has not been exploited to its full potential in behavioural and ecological studies despite a history of documented use in such studies (Wratten, 1994; Sykes *et al.*, 1995). The more recent interest in both home and commercial security needs and the rapid improvements in low cost equipment have made the technology readily accessible to biologists and may now see a more widespread application of the technique.

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