Chemical communication for reproduction in the Tasmanian short-beaked echidna, *Tachyglossus aculeatus setosus*

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A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

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Statements by the Author

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of this thesis, nor does the thesis contain any material that infringes copyright.

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The research associated with this thesis abides by the *Australian code of practice for the care and use of animals for scientific purposes* (2004) and the rulings of the Animal Ethics Committee of the University. The research presented in this thesis was carried out under University of Tasmania Animal Ethics Approvals A100426 (2009-2012) and A12320 (2012), and Tasmanian Department of Primary Industries, Parks, Water and the Environment Permits FA10045 (2010-2011), FA11033 (2011-2012), and FA12069 (2012).

Rachel L. Harris 27th March 2014

Statement of Co-Authorship

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Chapter 2: Harris, R.L., Davies, N.W., and Nicol, S.C. (2012). Chemical composition of odorous secretions in the Tasmanian short-beaked echidna (*Tachyglossus aculeatus setosus*). *Chemical Senses* **37**, 819-836.
Contributions: Harris 50%, Davies 30%, Nicol 20%

Rachel Harris (candidate) was the primary author and contributed to the idea and its execution, conducted the field work, the majority of the laboratory analyses (with assistance from Noel Davies), statistical analyses and was primarily responsible for presentation. Noel Davies conducted some of the chemical analyses. Noel Davies and Stewart Nicol contributed to the idea, its refinement and presentation.

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Rachel Harris was the primary author and conducted the statistical analyses which led to the idea of pursuing the structure of the compound, and was responsible for its presentation. Noel Davies conducted the laboratory analyses, structural interpretation from first principles of gas chromatography and mass spectral data, and assisted with development, refinement and presentation. Stewart Nicol assisted with refinement and presentation.

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The above co-authors also contributed to the preparation of unpublished (or to be submitted) work presented as thesis chapters. Additional collaborators are acknowledged in the relevant chapters. Therefore it was appropriate to use “we” rather than “I” in the data chapters, but all work was primarily the candidate’s.

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Abstract

Communication plays a key role in coordinating all social interactions in an individual's life, but is particularly crucial for coordinating reproduction. Although chemical (olfactory) signals are ubiquitous in sexual communication in mammals, a disproportionate reliance on laboratory-based studies using a limited range of species, along with several logistical and methodological limitations, limit our broader understanding of their functions in reproduction and as sexually selected traits. I addressed these shortcomings by using an integrated, multidisciplinary approach, combining organic chemical analyses, physiology, genetics and behaviour, to investigate chemical signals, reproduction and sexual selection in a terrestrial, egg-laying mammal (monotreme), the short-beaked echidna (*Tachyglossus aculeatus*).

Although the monotreme and therian lines have been evolutionarily separate for over 150 million years, echidna scent gland secretions from the spur and cloaca showed a typical mammalian pattern in terms of high chemical diversity and complexity. I identified a large number of compounds of varying volatility, molecular weight, functional groups and aromaticity, suggesting a high reliance on olfactory communication. Similarities with other vertebrates can indicate evolutionary convergence on optimal chemicals as signals, although several obscure and even novel compounds were also identified. Consistent with other seasonal breeders, echidna chemical profiles varied between sexes and during the mating season. Profiles also differed between individuals, suggesting they could contain genetic information, although microsatellite markers were inadequate to confirm this hypothesis. Changes in male spur secretions during the mating season coincided with maximum annual plasma testosterone concentrations and could be sexually selected, functioning in mate choice or intra-sexual competition. Males were attracted to female scent, confirming male response to sex-specific scent differences is an important driver of echidna mating behaviour. Consequently, chemical sensory traits that influence the ability to locate mates seem to be favoured by natural and sexual selection.

Behavioural responses to chemical signals can be complicated by sexual conflict, although this has rarely been investigated in mammals. Male echidnas locate and mate with hibernating females; however females showed no changes in chemical profiles during hibernation. These results suggest that females do not 'actively' signal to males while hibernating, but intense male-male competition for access to females has probably driven earlier male readiness to breed, even before females might otherwise emerge from hibernation and signal to males. In females, chemical cues and reproductive physiology were not closely linked, as females showed continued mating activity during pregnancy and no detectable changes in chemical profiles at the time of fertilization or during pregnancy. Therefore, female reproductive status appears undisclosed to males, and multi-male mating may function to confuse paternity and reduce infanticide risk. These results suggest chemical signals are used differently by males and females to increase their reproductive success, often at the expense of the other sex, resulting in an evolutionary 'arms race' between signalers and receivers.

Overall, a complex interplay between chemical signals, behaviour, environmental and selective pressures is responsible for the mating behaviour observed in Tasmanian echidnas. My work highlights the benefit of using a comprehensive, multidisciplinary approach based on a free-living, 'non-model' mammalian species, representing a significant step towards understanding the influences of selective pressures, including selection and conflict, on animal communication.

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Chapter 1:

General Introduction

Chapter 1: General Introduction

Animal communication

Communication plays a critical role in the lives of animals. Individuals must send and receive information in order to find food, avoid predators and make decisions based on the behaviour, physiology and morphology of other individuals (Endler 1993; Ruxton and Schaefer 2011). Therefore, communication with conspecifics and members of other species is tightly linked with key processes including survival, reproduction, individual recognition, kin discrimination, mate choice, parental care, sociality and spatial distribution (Dawkins 1995). Consequently, animals spend a significant amount of time and energy influencing or being influenced by the behaviour of other individuals via communication (Dawkins 1995; Bradbury and Vehrencamp 2011). The study of communication is multidisciplinary, encompassing diverse research areas including physics, economics, chemistry, molecular biology, physiology, ethology, behavioural ecology, population ecology and evolutionary biology (Hebets and Papaj 2005; Bradbury and Vehrencamp 2011). In turn, studies on animal communication provide new insights into both natural and sexual selection processes and have applications in other fields such as neurobiology, psychology, linguistics, pest control, wildlife management and conservation biology (Bradbury and Vehrencamp 2011).

Communication can be difficult to define (Dawkins 1995; Wyatt 2003). Debate continues over the definitions of terms such as ‘communication’, ‘signal’ and ‘cue’, which hinders progress in this field (see discussions by Dawkins 1995; Maynard-Smith and Harper 2003; Rendall *et al.* 2009; Bradbury and Vehrencamp 2011; Ruxton and Schaefer 2011). In this thesis, ‘communication’ occurs when signals or cues from one individual stimulate the sensory system of another individual, which may result in a change in behaviour in the second individual (Dawkins 1995; Wyatt 2003). ‘Signals’ are stimuli produced by a sender and monitored by a receiver, which: (1) function to influence or provide information to another individual (Bradbury and Vehrencamp 2011); (2) may cause a change in the recipient’s behaviour (Hebets and Papaj 2005); and (3) have evolved for that purpose

(Maynard-Smith and Harper 2003). 'Cues' may also be used in communication, but unlike signals are either generated inadvertently or for purposes other than communication (Wyatt 2010; Bradbury and Vehrencamp 2011). Natural selection influences signals, receptors, recipient behaviours and signalling behaviours that minimise signal degradation and maximise the received signals relative to background 'noise' (Eisenberg and Kleiman 1972; Endler 1992; Endler 1993; Seyfarth and Cheney 2003; Hebets and Papaj 2005). However, the interests of signallers and receivers do not always coincide (Endler 1993; Seyfarth and Cheney 2003) and signals and cues may be used deceptively by signallers to the detriment of the recipient (Searcy and Nowicki 2005) or by third party recipients (e.g. predators) to the detriment of the signaller (Ryan *et al.* 1982; Zuk and Kolluru 1998). Animals use a variety of different types of signals and cues to communicate, including visual, auditory, electrical, tactile and chemical channels (Bradbury and Vehrencamp 2011).

Chemical communication

Chemical (olfactory) communication is widely regarded as the oldest evolutionarily and the most widespread form of communication among living organisms (Wyatt 2003; Johansson and Jones 2007; Bradbury and Vehrencamp 2011). Chemical communication involves sending and receiving molecules as signals or cues and has been documented in animal taxa including arthropods, amphibians, fish, reptiles, birds and mammals (Wyatt 2003; Cardé and Millar 2004; Burger 2005; Müller-Schwarze 2006; Houck 2009; Mason and Parker 2010; Breithaupt and Thiel 2011; Campagna *et al.* 2012). Chemical signals have many advantages over other communication modes, being effective at night, over long distances and able to travel around obstacles (Svensson 1996; Wyatt 2003). Importantly, chemical signals can be effective over an extended period of time and without close contact between the signaller and recipient (Eisenberg and Kleiman 1972; Alberts 1992). However, chemical signals may travel slowly due to environmental influences (Endler 1993; Svensson 1996; Wyatt 2003) and are not always receiver-specific: for example, most scent marks cannot be directed towards a particular recipient (Eisenberg and Kleiman 1972; Svensson 1996; Drea *et al.* 2013). Chemical signals have been described (likely incorrectly) as being less expensive to produce than other signal

modes (Wyatt 2003; Mason and Parker 2010), perhaps because signal molecules are often produced in minute quantities (Greenfield 1981; Cardé and Baker 1984; Phelan 1992; Johansson and Jones 2007; Kokko and Wong 2007; El-Sayed 2012). However, scent marks and scent chemicals are a finite resource (Nie *et al.* 2012) and there are significant life-history costs associated with chemical signal production (Gosling *et al.* 2000; Beynon and Hurst 2003; Byers 2005; Martín and López 2006; Harari *et al.* 2011).

Natural and sexual selection has resulted in a great diversity of chemical compounds which are used as signals, and some compounds are shared by members of different taxa (Wyatt 2003; Müller-Schwarze 2006). Chemical signals or 'semiochemicals' probably evolved from compounds having other uses or significance, such as hormones, plant products and waste or microbial by-products (Halpin 1986; Wyatt 2003; Gaskett 2007) via the process of "sensory drive" (see Endler 1992; Endler 1993; Wyatt 2003). These compounds vary in their chemical and physical properties, which influences signal life span, function and information content (Alberts 1992; Wyatt 2003). For example, short-term mate attractants or alarm signals tend to be small, volatile and airborne, while larger, nonvolatile compounds have a longer signal life and may require direct contact for perception, providing more specific information about the signaller or functioning as territorial marks (Alberts 1992; Wyatt 2003; Brennan and Zufall 2006; Gaskett 2007; Swaney and Keverne 2009). Chemicals may be emitted in the urine, faeces, reproductive organs, sweat glands, skin, cuticle, saliva or specialised scent glands, and signalling may occur passively (e.g. body odour) or deliberately (e.g. ritualised 'scent-marking' behaviour) (Eisenberg and Kleiman 1972; Wyatt 2003; Müller-Schwarze 2006). Chemical signal molecules are detected by recipient olfactory receptors via direct contact (e.g. insect antennae) or sniffing, tongue-flicking and flehmen behaviours (Wyatt 2003). In vertebrates, chemicals are detected via the main olfactory system (MOE) and the vomeronasal organ (VNO); neurobiological, morphological and anatomical details of these sensory systems are reviewed extensively elsewhere (Eisenberg and Kleiman 1972; Albone 1984; Doving and Trotier 1998; Brennan and Keverne 2004; Brennan and Kendrick 2006; Brennan and Zufall 2006; Swaney and Keverne 2009).

Chemical signals for reproduction, honesty and sexual selection

In sexually reproducing species, locating and attracting mates is critical for reproductive success (Cardé and Baker 1984). Many invertebrate and vertebrate taxa rely on olfaction to coordinate courtship and other reproductive behaviours (for reviews see Cardé and Baker 1984; Rajchard 2005; Gaskett 2007; Balthazart and Taziaux 2009; Houck 2009; Mason and Parker 2010; Petrulis 2013b; Whittaker *et al.* 2013). Chemical signals are ubiquitous in sexual communication because changes in internal chemistry (e.g. reproductive physiology) are reflected in changes in external chemistry (chemical signal) (Johansson and Jones 2007). Crucially, chemical signals are costly to produce (Gosling *et al.* 2000; Beynon and Hurst 2003; Martín and López 2006) and usually "cheat proof" (Gosling and Roberts 2001; Zala *et al.* 2004), so they are difficult to use deceptively (Voigt *et al.* 2008; Linklater *et al.* 2013). For example, scent chemicals vary with differences relating to sex (Whittaker *et al.* 2010; Rosell *et al.* 2011), diet (Ferkin *et al.* 1997; Schaefer *et al.* 2010), emotional state (Hauser *et al.* 2008; Schaefer *et al.* 2010), age (Garratt *et al.* 2011), infection (Zala *et al.* 2004), individual identity (Smith *et al.* 2001; Howard and Blomquist 2005), immune response (López and Martín 2005), symmetry (Martín and López 2006), reproductive hormones (Ferkin *et al.* 2004; Whittaker *et al.* 2011; Parker and Mason 2012), body size or condition (LeMaster and Mason 2002; Martín and López 2010) and genetic make-up (Olsson *et al.* 2003; Beauchamp and Yamazaki 2005; Brennan and Kendrick 2006; Milinski 2006; Parott *et al.* 2007; Boulet *et al.* 2009; Leclaire *et al.* 2012). Since these characteristics can vary between individuals, they are good fitness indicators (Zahavi 1975; Endler 1993) and recipient individuals may choose mates based on this information (Penn and Potts 1998; Johansson and Jones 2007; Thomas 2011). Therefore chemicals signals are subject to natural and sexual selection (Blaustein 1981; Andersson 1994; Symonds and Elgar 2008; Harari and Steinitz 2013) and can be functionally equivalent to other secondary sexual characteristics such as morphological (e.g. ornaments, bright plumage) or behavioural traits (e.g. courtship displays, calls) (Blaustein 1981; Andersson 1994; Penn and Potts 1998).

The role of odour as a sexually selected trait was first explored by Darwin (1871), who suggested that male scent "probably serves to excite or allure the female" (vol.

1, pp. 258) and the development of scent glands "is intelligible through sexual selection, if the more odoriferous males are the most successful in winning the females, and in leaving offspring to inherit their gradually-perfected glands and odours" (vol. 2, pp. 281). Sexual selection acts on several aspects of chemical communication, including the ability to detect and locate signalling individuals (often potential mates) (Wiley and Poston 1996; Shine *et al.* 2005) and the use of chemical signals as sexual ornaments for intra-sexual competition (Rich and Hurst 1998; Hayes *et al.* 2003) or attractants (Swaisgood *et al.* 2002; Martín and López 2006; Hurst 2009). The influence of sexual selection on chemical communication and reproduction has been well characterised in invertebrates, including lepidopterans (Svensson 1996), hymenopterans (Ayasse *et al.* 2001) and arachnids (Gaskett 2007). In contrast, sexually selected odours in vertebrates have not been investigated as widely or thoroughly (Blaustein 1981; Johansson and Jones 2007), as reflected in the small breadth and depth of information available compared with other sensory modes (particularly visual and auditory) and taxa (Coleman 2009), even in major sexual selection reviews (e.g. Andersson 1994, but see Johansson and Jones 2007). This discrepancy may exist because sexually selected olfactory traits are more difficult for researchers to detect and measure than other more conspicuous sensory modes (Blaustein 1981; Andersson 1994), and because of difficulties in conducting bioassays on vertebrates (see below). Furthermore, chemical signals play a crucial role in coordinating mating systems, including behaviours underlying sexual conflict (e.g. timing and frequency of mating; Gaskett 2007), but this is also poorly understood in vertebrates (but see Shine 2012; Petrulis 2013b).

Mammalian chemical signals and "the mammal problem"

Odour plays a central and often obligatory role in mammalian social and sexual behaviour (Darwin 1871; Eisenberg and Kleiman 1972; Albone 1984; Andersson 1994; Brennan and Kendrick 2006; Campbell-Palmer and Rosell 2011; Dehnhard 2011; Petrulis 2013b). The importance of chemical signals in nocturnal or solitary mammals is well known (e.g. Swaisgood *et al.* 2002; Linklater *et al.* 2013), but scent also plays a crucial role in coordinating complex behaviours in highly social species (e.g. Scordato and Drea 2007; Burgener *et al.* 2009; Leclaire *et al.* 2013).

Mammalian chemical signals function in attracting and locating potential mates (Molteno *et al.* 1998; Swaisgood *et al.* 2002), mate choice (Johansson and Jones 2007; Clutton-Brock and McAuliffe 2009) and influence parent-offspring interactions (Schaal *et al.* 2003; Burger *et al.* 2011), territorial or dominance interactions (Rosell *et al.* 1998; Rich and Hurst 1999) and social group interactions (Scordato and Drea 2007; Burgener *et al.* 2009; Leclaire *et al.* 2013). Furthermore, many species of mammal show sexually dimorphic (typically male-biased) olfactory signalling and scent gland development resulting from sexual selection (Thiessen and Rice 1976; Blaustein 1981; Penn and Potts 1998); examples include ring-tailed lemurs *Lemur catta* (Kappeler 1998; Scordato *et al.* 2007), koalas *Phascolarctos cinereus* (Salamon and Davies 1998; Tobey *et al.* 2009), greater sac-winged bats *Saccopteryx bilineata* (Voigt *et al.* 2008), African elephants *Loxodonta africana* (Rasmussen *et al.* 1996a), rabbits *Oryctolagus cuniculus* (Hayes *et al.* 2003), sugar gliders *Petaurus breviceps* (Stoddart *et al.* 1994), mandrills *Mandrillus sphinx* (Setchell *et al.* 2010) and Eurasian beavers *Castor fiber* (Rosell and Schulte 2004; Rosell and Thomsen 2006). Nonetheless, our broader understanding of the subtleties and functions of chemical signals in mammalian reproduction lags behind progress made in arthropods (Roitberg and Isman 1992; Cardé and Millar 2004; Gaskett 2007) and other vertebrates (e.g. squamates, see Mason *et al.* 1989; Martín and López 2006; Martín and López 2010; Mason and Parker 2010; Parker and Mason 2012; Shine 2012; Shine and Mason 2012), which also limits our ability to make broad, evolutionary comparisons. There are several key reasons, outlined below, which largely stem from conceptual difficulties in applying the insect-derived 'pheromone' term to mammalian systems and may be collectively referred to as "the mammal problem" (Wyatt 2010).

'Pheromones' are chemicals secreted by an individual which elicit a specific response in a conspecific recipient (Karlson and Luscher 1959), akin to the effects of hormones on internal physiology (Petrulis and Johnston 1995; Swaney and Keverne 2009). Pheromones function in mate attraction, alarm signalling and aggregation (Wyatt 2003; Symonds and Elgar 2008) and can be individual molecules or, more commonly, mixtures (Wyatt 2005; Symonds and Elgar 2008; El-Sayed 2012). Pheromone research is dominated by work in arthropods (Wyatt 2003; Cardé and Millar 2004; Symonds and Elgar 2008; El-Sayed 2012), but

several vertebrate pheromones have also been described (Mason *et al.* 1989; Sorensen *et al.* 1990; Kikuyama *et al.* 1995; Wabnitz *et al.* 1999; Houck *et al.* 2008; Shine and Mason 2012). Although different species (and taxa) may produce many of the same individual chemicals (e.g. Rasmussen *et al.* 1996b; Rasmussen *et al.* 1997), multi-compound pheromone blends and their receptors tend to be species-specific (Phelan 1997; Ache and Young 2005; Symonds and Elgar 2008; Swaney and Keverne 2009; Wyatt 2010). Chemical identification, physiological and behavioural response data are necessary to conclusively verify the function of a chemical(s) as a pheromone, leading to problems with describing pheromones in mammals (Beauchamp *et al.* 1976; Sorensen 1996; Doty 2010).

Mammalian semiochemistry can be extremely complex, making identification of pheromone compounds difficult (Wyatt 2005), although this rationale has recently been criticised (Apps 2013). Individual samples may contain many hundreds of compounds ranging from small, volatile molecules to large proteins (Beauchamp *et al.* 1976; Albone 1984; Burger 2005), comprising an overall "signature mixture" (Wyatt 2010) and not all compounds may function in communication (Dehnhard 2011; Charpentier *et al.* 2012). Despite these difficulties, several mammalian pheromones have been described and their physiological or behavioural effects demonstrated (e.g. Jemiolo *et al.* 1985; Dorries *et al.* 1995; Rasmussen *et al.* 1997; Roberts *et al.* 2010). However, several authors have argued that most putative mammalian pheromones are not true 'pheromones' (Beauchamp *et al.* 1976; Brown 1979; Wyatt 2005; Doty 2010), because the signal compound(s) does not closely match the original definition (Karlson and Luscher 1959). For example, 'pheromone' is sometimes (perhaps inappropriately) used to describe any body odour that influences interactions between conspecifics (e.g. see discussion by Johnston 2003; Johansson and Jones 2007; Thomas 2011). Even more specific terms have been coined, including allomone, kairomone, synomone and primer, releaser, signaller and modulator pheromone (Albone 1984; Wyatt 2003; Brennan and Zufall 2006), but these have seen restricted use in reference to mammals. The diversity of terms creates significant and unnecessary confusion (Albone 1984; Johnston 2003). In this thesis I have avoided using the term 'pheromone' unless it is discussed explicitly as a compound or precise blend which acts, for example, as a

sex attractant or physiological trigger (*sensu* Karlson and Luscher 1959) and have used more neutral terms such as 'chemical signal', 'odour', 'semiochemical' or 'scent' (Petrulis 2013b), with the understanding that they may include single or multiple compounds (Apps 2013).

Behavioural tests routinely used on invertebrates to ascribe functionality to compounds of interest (e.g. 'response-guided strategies' using electro-antennograms) are impossible to transfer directly to mammals and difficult to redesign (Mackintosh 1985; Burger 2005; Charpentier *et al.* 2012; Drea *et al.* 2013). Many mammalian semiochemicals are previously unknown compounds and not commercially available, making it difficult to carry out compound-specific bioassays (reviewed in Drea *et al.* 2013). Mammalian behaviour is also complex and subject to individual variability and multisensory influences (Brown 1979; Brennan and Keverne 2004; Müller-Schwarze 2006). Problems can arise when defining what constitutes a 'specific response' and whether the response was to a particular compound or chemical mixture alone (Mackintosh 1985; Brennan and Keverne 2004). Mammalian responses to odour may also be delayed or situation dependent (Johnston 2003). Furthermore, behaviour tests are frequently conducted in artificial laboratory settings with potentially little relevance to the animal's natural environment or behaviours (Halpin 1986; Harder and Jackson 2010; Nicol 2013), therefore it is crucial to also consider behavioural responses in free-ranging individuals (Wolff 2003).

Limitations and recommendations

Logistical, conceptual and methodological problems associated with studying chemical communication in mammals have important implications for research strategies and analytical approaches (Albone 1984; Apps 2013; Drea *et al.* 2013). Broad, multidisciplinary approaches are most likely to succeed in understanding signal function (Heymann 2006). Ideally, these should be grounded in a thorough, qualitative and quantitative understanding of chemical secretions, based on samples collected from as many individuals as possible and over an extended time period (Burger 2005), also incorporating physiological, behavioural and genetic information (Albone 1984; Drea *et al.* 2013). However, advances in sophisticated

chemical analytical technologies essential for the structural elucidation of pheromones and chemical signals (reviewed by Albone 1984; Burger 2005; Novotny and Soini 2008; Drea *et al.* 2013) have often outpaced the chemical knowledge of biologists interested in olfactory communication, leading to erroneous reports of synthetic or biologically impossible chemicals as mammalian signals (Charpentier *et al.* 2012). Therefore chemical signal studies should involve collaboration between biologists and chemists to prevent such inaccuracies (Burger 2005; Charpentier *et al.* 2012).

Research on mammalian reproductive chemical signals is characterised by a disproportionate reliance on information from studies on laboratory-bred rodents (Table 1.1; recent reviews include Hurst 2009; Baum and Bakker 2013; Petrulis 2013b; Petrulis 2013a) and to a lesser extent, domesticated or agricultural species (reviewed by Rekwot *et al.* 2001; Dehnhard 2011). This prevalence is largely due to logistical difficulties associated with collecting uncontaminated odorant samples and behavioural data from wild individuals and because rodents are easily studied in laboratory settings (Drea *et al.* 2013). However, it is sometimes unclear whether findings based on a limited range of species are widely applicable to other 'non-model' organisms. Detailed investigations of chemical, physiological and mechanistic aspects of chemical communication are available for a small number of other eutherian species, usually in captivity (but also includes singly- and socially-housed species in large, naturalistic, outdoor enclosures), notably ring-tailed lemurs (Hayes *et al.* 2004; Knapp *et al.* 2006; Scordato *et al.* 2007; Boulet *et al.* 2009), mandrills (Setchell *et al.* 2010; Setchell *et al.* 2011), African elephants (Rasmussen *et al.* 1996a; Rasmussen and Wittemyer 2002; Bagley *et al.* 2006; Goodwin *et al.* 2012), Asian elephants *Elephas maximus* (Rasmussen *et al.* 1997; Goodwin *et al.* 2012) and giant pandas *Ailuropoda melanoleuca* (Swaisgood *et al.* 2000; Swaisgood *et al.* 2002; Hagey and MacDonald 2003; Zhang *et al.* 2008). Few detailed studies are based on free-living, wild populations, but for exceptions see work on rabbits (Hayes *et al.* 2002), African elephants (Rasmussen *et al.* 1996a), spotted hyenas *Crocuta crocuta* (Burgener *et al.* 2009), European badgers *Meles meles* (Buesching *et al.* 2002) and Eurasian beavers (Rosell *et al.* 1998; Rosell and Thomsen 2006).

Collectively, these limitations can place restrictions on broader insights into mammalian chemical communication, because there can be subtle differences in how chemical signals are used in reproduction between species. Furthermore, the difficulties in studying mammalian chemical signalling in general mean that some orders, or even entire subclasses, of mammals are not represented at all in the chemical ecology literature (Table 1.1), which restricts our ability to make broader evolutionary comparisons between taxa, such as mammals, birds and reptiles, in terms of the different ways that chemical signals influence reproduction. A potential way forward, complimentary to laboratory-based studies, is through increased input from long-term studies on non-traditional, free-living study organisms (Müller-Schwarze 2005). Little is known about chemical signals in the egg-laying mammals (Order Monotremata), which comprise five extant species including the platypus, short-beaked echidna and critically endangered long-beaked echidnas of New Guinea. Monotremes possess a unique mosaic of reptilian and mammalian reproductive characteristics (i.e. egg-laying, milk producing) (Griffiths 1978) and diverged from the therian line approximately 161 million years ago (Phillips *et al.* 2009), hence they have been described as the 'ultimate mammalian out-group' (Nicol 2003). An investigation into olfactory communication in monotremes could prove rewarding by: (1) expanding the study of mammalian chemical signals; (2) providing new evolutionary links with other vertebrates; and (3) exploring where the echidna 'fits' in the evolution of chemical communication.

Study species – *Tachyglossus aculeatus*

I investigated the role and importance of chemical communication for reproduction in the short-beaked echidna (*Tachyglossus aculeatus*; 'echidna'). The echidna is a long-lived (45 + years), medium-sized (2-7 kg average adult mass) mammal (Nicol and Andersen 2007) and the most common extant species of monotreme (Griffiths 1978). The echidna is also Australia's most widely distributed native mammal, being found throughout mainland Australia, Tasmania, some off-shore islands and in parts of New Guinea (Augee 2008), but is divided into five subspecies according to geographic range (Griffiths 1978). The echidna's diet consists mainly of ants and termites, plus other soil invertebrates (Griffiths 1978; Sprent 2012). Both sexes

show a high degree of home range fidelity and are non-territorial (Nicol *et al.* 2011). Consistent with other myrmecophagous (ant-eating) mammals, echidnas are usually solitary (Nicol *et al.* 2011), have low body temperatures (McNab 1984) and low basal and field metabolic rates (McNab 1984; Schmid *et al.* 2003; Nicol and Andersen 2007). However, echidnas appear atypical in that brain size and complexity is greater than expected relative to their basal metabolic rate (Weisbecker and Goswami 2010; Nicol 2013) and solitary lifestyle (Hassiotis *et al.* 2003). Along with their long life span, this evidence suggests echidna behaviour may be more complex than expected (Nicol 2013), potentially requiring significant memory capacity and cognitive ability (Hassiotis *et al.* 2003).

Echidnas are seasonal breeders and mate during the Austral winter throughout their geographic range (Morrow *et al.* 2009). The mating season is preceded by a period of inactivity, ranging from brief torpor bouts in temperate areas (Rismiller and McKelvey 1996) to prolonged, deep hibernation in colder parts of its range (Beard *et al.* 1992; Beard and Grigg 2000; Nicol and Andersen 2002). Courtship behaviour varies between subspecies, but is typically characterised by intense competition among males for access to females (Morrow *et al.* 2009), and can manifest in the formation of mating aggregations (Morrow *et al.* 2009) or even 'trains', comprising one female being pursued by several (up to 10) males (Rismiller 1992; Rismiller and McKelvey 2000). Females typically lay a single egg and after hatching, the young remains inside the mother's pouch during the early part of lactation (Griffiths 1978) and is placed inside purpose-built 'nursery burrows' at different stages of lactation, depending on subspecies (Morrow *et al.* 2009). The timing of weaning also varies, occurring at approximately 150 days of age in Tasmania (Morrow *et al.* 2009; Morrow and Nicol 2012) and 200-210 days on Kangaroo Island (Rismiller and McKelvey 2009). Males provide no parental care.

Olfactory communication in *T. aculeatus*

Anatomical and behavioural features suggest chemical communication play a central role in coordinating echidna social and sexual behaviour. Echidnas can be highly nocturnal (Nicol *et al.* 2004) and use olfactory cues to locate prey items (Griffiths 1978). Their olfactory system is well-defined (Home 1802), even at

hatching, including a large olfactory epithelium (Griffiths 1978; Schneider 2011) and well-developed VNO (Broom 1895; Schneider 2011). The size and development of the echidna olfactory bulb is comparable to that of prosimians of similar size (Ashwell 2006), which are known to use olfaction to coordinate complex behaviours (Schilling 1979). Collectively, this evidence suggests a strong reliance on olfaction (Nicol 2013), while other senses appear less important for social behaviour (Griffiths 1978). Echidnas have putative scent glands located in the cloaca (Allen 1982; Russell 1985), which appear to be used in scent-marking behaviour by females during the mating season (Dobroruka 1960; Boisvert and Grisham 1988; Beard *et al.* 1992). Males also have a spur on each ankle joint (Krause 2010) and the fleshy area at the base of the spurs produces a 'waxy' secretion (Burrell 1927) which could be used for scent-marking (Morrow 2013). Chemical communication at latrines may also be important for social spacing and signalling resource use (Sprent *et al.* 2006).

Echidna mating activity appears driven by male ability to search for and locate females. Since there is intense male-male competition for access to females (Morrow *et al.* 2009), there is probably strong selective pressure on male ability to locate receptive females using scent (Johnson 1980) or even pheromones (Rismiller 1992), which would minimise search costs associated with pursuing unsuitable or unavailable females (Thomas 2011). Both sexes produce a strong odour during the mating season (Semon 1899; Rismiller and Seymour 1991; Nicol *et al.* 2004). In captivity, males show increasing interest in females leading up to mating (Ferguson and Turner 2013), suggesting males assess female reproductive status, possibly via olfactory cues from the female's cloaca (Dobroruka 1960; Boisvert and Grisham 1988). Rismiller (1992) found males in a wild population were strongly attracted to hessian bags which had previously held a female found in a mating train.

The Tasmanian subspecies – *T. a. setosus*

Tasmania represents the southern-most extreme of the echidna's geographic range, so seasonality and behaviour represent an extreme on a continuum varying over (subspecies) geographic ranges (Morrow and Nicol 2012; Morrow 2013). Tasmanian echidnas show several life history differences to mainland subspecies,

including the duration of maternal nursery burrow confinement and lactation, and undergoing deep hibernation for several months of the year (Nicol and Andersen 2008; Morrow *et al.* 2009; Nicol *et al.* 2011). Reproduction is characterised by intense sexual conflict over the timing and frequency of mating (Morrow 2013). Females are reproductively active on average only every second year and only produce a single young on average once every three to four years. In non-reproductive years, females have lower body mass, hibernate through the mating season, do not seem to attract males and do not appear to cycle (Nicol and Morrow 2012; Morrow 2013). Males mate on average three in every four years (Nicol and Morrow 2012), resulting in a skewed operational sex ratio in a given mating season (Emlen and Oring 1977; Morrow 2013). Sexual size dimorphism is low (Nicol *et al.* 2011; Nicol 2013) and reproductive females are widely dispersed (Sprent and Nicol 2012; Morrow 2013), so males are unable to monopolise females (Morrow 2013). Reproductive males undergo testes recrudescence prior to entering hibernation in January-February, emerge in May and after approximately one month of foraging and spermatogenesis begin to seek out females to mate with (Morrow 2013). It is a common male strategy to locate hibernating females and enter their hibernacula: approximately one-third of mating aggregations involve females with sub-euthermic body temperatures (T_b <28 °C) and many of these females had fresh spermatozoa recovered from their reproductive tracts (Morrow and Nicol 2009; Morrow 2013). Mating aggregations typically comprise one female with a single male, although aggregations with up to four males are common; 'trains' are rarely observed (Morrow *et al.* 2009). Females mate multiple times with several males, including during pregnancy (fertilisation to egg-laying; Morrow 2013) and may re-enter hibernation after fertilisation (Nicol and Morrow 2012).

Chemical signals appear important for successful reproduction in Tasmanian echidnas. Adult females enter hibernation approximately one month after males (Nicol and Morrow 2012) and may move to new hibernacula during periodic arousals (Nicol and Andersen 2002; Nicol *et al.* 2004), hence males cannot monitor the locations of female hibernacula prior to entering hibernation. As in other subspecies, males probably use scent to locate females, which could also result in males locating females still hibernating. Females may benefit from olfactory-based

mate assessment in the absence of extended courtship (Wiley and Poston 1996). Preliminary work suggested that scent gland secretions contained a large variety of compounds which could have several functions (Harris 2009). Although mature females do not have spurs, they produce a similar waxy secretion to that of males from the pits where their spurs were located as juveniles, and female 'spur' secretion could retain a function in communication. Although overall composition varied between sexes during the mating period, sample size and sampling design did not permit robust analysis of seasonal or individual differences (Harris 2009). Furthermore, two important issues remain unresolved: how do males use odour to locate hibernating females; and why are males attracted to pregnant females?

Study population

I studied a free-living population of *T. a. setosus* in the southern midlands of Tasmania, Australia (42°28'S, 142°14'E) approximately 50 km north of Hobart (Figure 1.1). The study site is located on a 12 km^2 section of livestock-grazing property ("Lovely Banks"; Figure 1.2) and was originally selected based on reportedly high echidna numbers and proximity to Hobart. The wild echidna population at this site has been the subject of ongoing physiological, ecological and behavioural research since 1996 and there is a resident population of approximately 120 individuals. On first capture, all individuals have passive identification transponder (PIT) tags implanted subcutaneously for identification and up to 20 individuals are fitted with radiofrequency transmitters at any time, allowing repeat observations and sample collection. On each capture, the location is recorded using a hand-held GPS unit and when possible, body mass and other relevant data are recorded, which is then added to a database. As of October 2013, a total of 7015 records had been collected from 275 individuals captured at this field site, including 103 radio-tracked animals, making this one of the most well-studied echidna populations in Australia. Continuous records on reproductive activity and home ranges are available for up to 17 years for some animals; 71 individuals have at least 20 observations, 21 individuals have over 100 observations. The maximum number of observations from a single individual was 391. However, echidnas are cryptic, semi-fossorial animals and the time between observations can be up to

10 years, making it difficult to estimate population density and life history characteristics such as survivorship and dispersal of young.

As this is currently the only study of its type in Tasmania, it is unclear how representative this population is of others in the state, although less extensive studies in two other areas, including 60 individuals in the Central Plateau (elevation 760 m asl, mean annual rainfall 645 mm) show similar timing of hibernation and reproduction (Nicol unpublished data). Mating aggregations have been found between 6 June and 6 September (including this study), so the timing of reproduction is consistent with other subspecies (Morrow *et al.* 2009). The Lovely Banks population is the only one in Australia where males have been documented entering female hibernacula (Morrow *et al.* 2009; Morrow and Nicol 2009), probably reflecting study intensity, not that this is the only population in which it occurs. Home range sizes are roughly consistent with those of other subspecies (Nicol *et al.* 2011), but vary with habitat quality (Sprent and Nicol 2012). Male home ranges are twice those of females; female home ranges are uniformly distributed with little overlap, while male home ranges are larger and overlap with those of several males and females (Nicol *et al.* 2011; Sprent and Nicol 2012).

Research aims and thesis structure

The overall aim of my research was to assess the functions and importance of chemical signals and cues for coordinating reproductive behaviour in a terrestrial monotreme, the short-beaked echidna. I used a multidisciplinary approach based on a well-characterised wild population of Tasmanian echidnas. Organic chemical analysis forms a substantial component of the research, but I have also incorporated experimental and observational behaviour, physiology, genetic analyses and multivariate statistics to analyse field data collected over a three-year period. I had four main aims, which are outlined below.

1. To characterise the chemical nature of odorous secretions from putative echidna scent glands.

A thorough investigation into the chemical compounds present in gland secretions is an important first step in understanding the functions of chemical signals in a given species (Sokolov *et al.* 1987; Zhang *et al.* 2002; Drea *et al.* 2013). Different chemical and physical properties (e.g. molecular weight, volatility, functional groups, aromaticity) make some compounds more suitable for various functions than others (Alberts 1992; Müller-Schwarze 2006), so we can make several inferences about possible functions based on the types of chemicals detected. In Chapter 2, I use several different organic mass spectrometry analytical techniques to describe potential volatile and nonvolatile semiochemicals in putative echidna scent glands (Figure 1.3). I also report the identification of several obscure and even novel vertebrate compounds (Chapter 2, Chapter 3).

2. To describe differences in chemical signals relating to the mating season and individual identity, test male responsiveness to female scent and identify which chemicals are important for differences between sexes, seasons and types of scent glands.

Sex and seasonal variations in chemical signals can be used by seasonally breeding animals to locate and assess the sex and reproductive status of potential mates, while avoiding potentially costly encounters with same-sex competitors (Wyatt 2003; Johansson and Jones 2007). Odour-based recognition of individuals is also important for social and sexual behaviours (Halpin 1986; Thom and Hurst 2004; Brennan and Kendrick 2006). However, it can be difficult to identify chemicals important for sex, seasonal or individual-specific differences in complex mammalian secretions. In Chapter 4, I first investigate changes in overall chemical mixtures or ‘profiles’ associated with sex, season, individuals and different scent glands. I have used random forests, a statistical technique novel to the field of chemical ecology, to identify chemicals important for these differences and consider how these variations influence echidna behaviour. I then use field trials to determine whether males can detect and localise female scent (Chapter 5).

3. To investigate the links between female chemical signals, hibernation and reproductive physiology and their influence on mating behaviour.

Chemical signals can have important influences on behaviours underlying sexual conflict (Gaskett 2007), but this has rarely been investigated in mammals. Reproduction and hibernation are two processes traditionally thought to be mutually exclusive; however female echidnas show significant overlap in hibernation and mating activity (Morrow and Nicol 2009). Males are attracted to hibernating females, suggesting that females produce an attractive odour while hibernating. Male echidnas do not seem to be attracted to non-reproductive females, suggesting males discriminate between females varying in reproductive status. However, females continue to attract and mate with multiple males during pregnancy. In Chapter 6, I investigate: (1) the influence of hibernation on female chemical signals (not previously described in a mammal); (2) whether female reproductive status is accurately signalled to males; and (3) effects on mating behaviour.

4. To investigate the potential for genetically-based mate choice via scent.

Chemical profiles vary between individual echidnas (Chapter 4) and could also contain genetic information. In Chapter 7, I aim to develop new echidna-specific microsatellites and compare genetic data (heterozygosity, relatedness) with chemical data (profile complexity, differences between individuals) and explore the potential role of odour in mate choice. Unfortunately, the microsatellites were too low in number and polymorphism to be suitable for the analyses. However, this chapter is useful for establishing statistical methods which may be used with stronger genetic data and tentative results are presented, hence I have included it as a chapter in the thesis.

Thesis presentation

This thesis comprises six data chapters, two appendices, a general introduction and general discussion chapter. All data chapters are written as stand-alone research papers, however when taken together they address the overall aims of the thesis. Chapters 2-4 and Appendix I have been published, or accepted for publication, in scientific journals. The remaining chapters are either in review or in preparation to submit to scientific journals. There is some variation in formatting and referencing styles between chapters, as each has been presented in the same format specified by

the journal to which it was submitted (or will be submitted). There is some unavoidable repetition between chapters, particularly in the study species description and methods sections. Due to the multidisciplinary approach of this thesis, some chapters contain significant input from supervisors and collaborators and their contributions are recognised by acknowledging them as co-authors where appropriate. However, I was responsible for all aspects of the design and completion of the study, including literature searches, fieldwork, laboratory work, statistical analyses, interpretation and manuscript preparation. Appendix I describes field observations made during this study which are discussed in Chapter 6 and the general discussion. Appendix II presents the results of behaviour trials conducted on captive animals.

Table 1.1 (continued over page). Relative contributions of mammalian species in studies of chemical communication, based on counts of papers taken from Web of Science (Thomson Reuters USA, http://thomsonreuters.com/web-of-science/) where search terms were "mammal" AND "pheromon*" OR "chemical communication" OR "olfactory communication" and the scientific or common name(s) for major groups (e.g. "rodentia" OR "mouse"), and abstracts checked for relevance. Total count (N = 474) is an underestimate since some studies would not appear using the limited search criteria, but provides an estimate of relative contribution.

Subclass, Order	**Approx. # references**	**% of total**	**Representative examples**		
			Species	**Type of study***	**References**
Eutheria	**457**	**96.4**			
Rodentia (mice, rats; majority in laboratory setting)	133	28.1	*Mus musculus*	B, C, G	Singer *et al.* (1997)
				C, P	Novotny *et al.* (1999)
				G, M	Spehr *et al.* (2006)
				B, G	Thonhauser *et al.* (2013)
Carnivora (felids, canids, ursids)	86	18.1	*Ailuropoda melanoleuca*	C	Zhang *et al.* (2008)
				B	Swaisgood *et al.* (2002)
			Crocuta crocuta	B, C	Burgener *et al.* (2009)
			Lutra lutra	C	Kean *et al.* (2011)
			Ursus arctos	C	Rosell *et al.* (2011)
Rodentia (excluding mice, rats, e.g. voles, hamsters, beavers)	76	16.0	*Castor fiber*	B, C	Tinnesand *et al.* (2013)
				A	Rosell and Schulte (2004)
			Microtus pennsylvanicus	B, P	Ferkin *et al.* (2004)

Table 1.1. (continued)

Subclass, Order	Approx. # references	% of total	Representative examples		
			Species	**Type of study***	**References**
Primates (excluding humans, e.g. marmosets, lemurs, mandrills)	70	14.8	*Callithrix jacchus*	C	Smith *et al.* (2001)
			Mandrillus sphinx	C	Setchell *et al.* (2010)
			Lemur catta	C	Scordato *et al.* (2007)
				C, G	Boulet *et al.* (2009)
				B, C, G	Charpentier *et al.* (2010)
			Propithecus edwardsi	C, G	Morelli *et al.* (2013)
Artiodactyla (pigs, deer, camels, giraffes, antelope)	40	8.4	Alces alces	C	Whittle *et al.* (2000)
Proboscidea (elephants)	21	4.4	*Loxodona africana*	C	Goodwin *et al.* (2012)
				B	Bagley *et al.* (2006)
				C, P	Rasmussen *et al.* (1996a)
			Elephas maximus	B, C, P	Rasmussen *et al.* (1997)
Lagomorpha (rabbits, hares, pika)	19	4.0	*Oryctolagus cuniculus*	B, P	Arteaga *et al.* (2008)
				B,C	Hayes *et al.* (2003)
Chiroptera (bats)	7	1.5	*Leptonycteris curasoae*	A, B	Munoz-Romo and Kunz (2009)
			Myotis bechsteinii	C, G	Safi and Kerth (2003)

Table 1.1. (continued)

Subclass, Order	Approx. # references	% of total	Representative examples		
			Species	**Type of study***	**References**
Perissodactyla (horses, rhinoceros)	4	0.8	*Diceros bicornis*	B	Linklater *et al.* (2013)
Sirenia (manatees, dugongs)	1	0.2	*Trichechus manatus latirostris*	A	Bills *et al.* (2013)
Marsupialia	**17**	**3.6**			
Diprotodontia (koalas, possums, macropods)	8	1.7	*Macropus eugenii*	B, P	Schneider *et al.* (2010)
			Phascolarctos cinereus	C	Tobey *et al.* (2009)
			Trichosurus vulpecula	C	McLean *et al.* (2012)
Didelphimorphia (opossums)	5	1.1	*Monodelphis domestica*	B, P	Harder *et al.* (2008)
Dasyuromorphia (antechinus, quolls)	4	0.8	*Antechinus agilis*	B, G	Parott *et al.* (2007)
			Antechinus stuartii	C	Toftegaard *et al.* (1999)
Monotremata	**0**	**0.0**			

* A: anatomical; B: behavioural; C: chemical; G: genetic; M: molecular/biochemical; P: physiological.

Mammalian orders returning no search hits: *Eutheria*: Afrosoricida, Cetacea, Cingulata, Dermoptera, Erinaceomorpha, Hyracoidea, Macroscelidea, Pholidota, Pilosa, Scandentia, Soricomorpha, Tubulidentata; *Marsupialia*: Microbiotheria, Notoryctemorphia, Paucituberculata, Peramelemorphia; *Monotremata*: Monotremata.

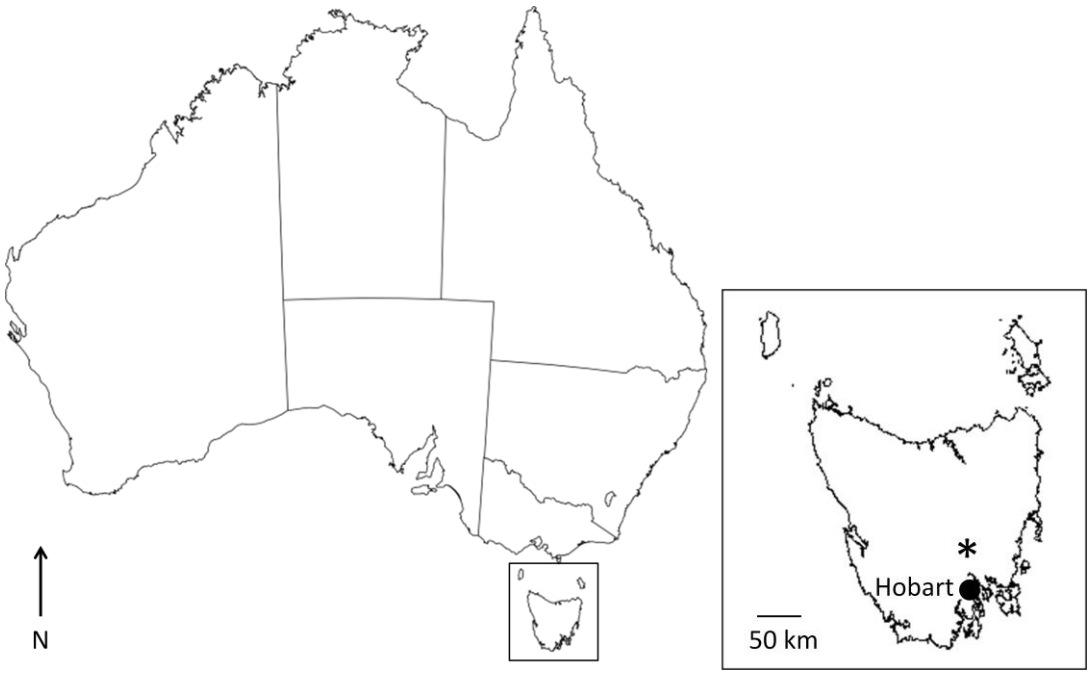

Figure 1.1. Location of field site (inset, indicated by *) in the southern midlands of Tasmania, Australia (42°28'S, 142°14'E). Images modified from www.online-field-guide.com and www.lib.unimelb.edu.au.

Figure 1.2. Photographs of typical vegetation at the 'Lovely Banks' study site in southern midlands of Tasmania, Australia (42°28'S, 142°14'E). Photos: R. Harris.

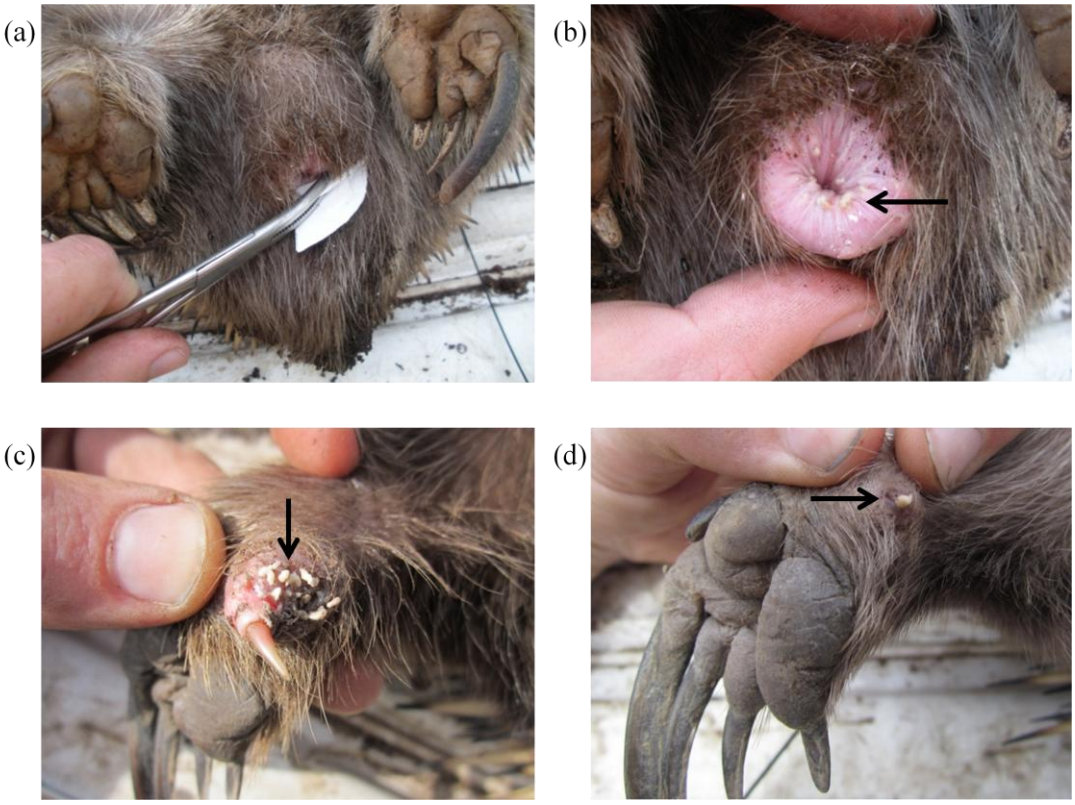

Figure 1.3. Photographs of odorant samples collected from male and female short-beaked echidnas (*Tachyglossus aculeatus setosus*): (a) cloacal swab; (b) cloacal wax secretion (arrow); (c) spur wax secretion (arrow; male); 'spur' wax secretion (arrow; female). Photos: R. Harris.

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SECTION I:

CHEMICAL COMPOSITION

Chapter 2:

Chemical composition of odorous secretions in the Tasmanian short-beaked echidna (*Tachyglossus aculeatus setous*)

Chapter 2: Chemical composition of odorous secretions in the Tasmanian short-beaked echidna (*Tachyglossus aculeatus setosus*)

Abstract

The short-beaked echidna is believed to use olfactory cues from a cloacal scent gland to attract and locate mates during the breeding season. We investigated the chemical composition of echidna secretions, including cloacal swabs and solid, "waxy" exudates from the cloaca and spurs. Scent samples from 37 individuals were collected over a one year period and analyzed using a range of different analytical techniques. A total of 186 compounds were identified, including volatile carboxylic acids, aldehydes, ketones, fatty acids, methyl esters, ethyl esters, terpenes, nitrogen and sulphur-containing compounds, alcohols and aromatics. Long chain and very long chain monounsaturated fatty acids, sterols and sterol esters were identified as the major constituents of solid exudates, some of which have not previously been described from any animal skin gland. There was a high degree of composition overlap between male and female cloaca swabs; however there is significant variation, which could mediate echidna mating behavior. Many of the volatile and nonvolatile chemicals detected are used for communication in other species, suggesting that chemical signals have important and diverse functions in echidna social interactions.

Introduction

Chemical signals are vitally important for mediating social and sexual interactions in a wide range of animal species (Wyatt 2003; Brennan and Kendrick 2006). Chemical signals originating from dedicated scent glands, urine, feces, saliva, reproductive organs, and other body secretions convey information between conspecifics, often in the absence of the signalling animal (Wyatt 2003; Müller-Schwarze 2006). Chemical signals can transmit complex information on individual identity (Smith et al. 2001), genetic quality (Charpentier et al. 2010), sex and reproductive status (Scordato et al. 2007; Rosell et al. 2011), social status (Burgener et al. 2009; Setchell et al. 2010) and body condition (Buesching et al. 2002). These signals play a vital role in mediating behaviours essential for reproductive success, such as mate attraction (LeMaster and Mason 2002), mate choice (Johansson and Jones 2007) and kin recognition (Charpentier et al. 2010).

Mammalian chemical signals are typically extremely complex, and this has contributed to the relatively poor understanding of chemical, functional and ecological aspects of mammalian chemical ecology in comparison with those of other taxa (Burger 2005). However, a large amount of semiochemical information is now available for laboratory mammals, such as rats and mice, and the literature base of compounds described in "nonmodel" species' secretions is also expanding. Mammalian scent-gland secretions typically contain a very large number and range of compounds, including aldehydes, alcohols, ketones, esters, sterols, acids, proteins and aromatics (reviewed by Burger 2005). Variations in chemical and physical properties between these different groups of compounds (Alberts 1992; Müller-Schwarze 2006) mean that no single method of chemical analysis is best able to detect all of them. Further, the biologically active component(s) of a chemical signal could be one or many of these compound types (Andersen and Vulpius 1999). Many studies have implemented gas chromatography-mass spectrometry methods to describe a large number of compounds present in mammalian secretions; however, very few use multiple analytical techniques to give a more comprehensive description of the chemicals produced by a given species. It is possible that not doing so could result in an underestimate of the number and diversity of chemicals used for communication in some species.

Currently, the majority of mammalian chemical ecology research is concentrated on eutherian mammals (Burger et al. 2001; Scordato et al. 2007; Burgener et al. 2009), and to a lesser extent, marsupials (Salamon and Davies 1998; Toftegaard et al. 1999; Tobey et al. 2009). Very little is known of chemical signals in the monotremes, the ultimate mammalian "out-group" (Nicol 2003). Monotremes diverged from other mammals 161-217 million years ago (Phillips et al. 2009), and an investigation of chemical signals in this unique group of animals is likely to reveal new insights into the evolution of olfactory communication.

The short-beaked echidna (Figure 2.1; *Tachyglossus aculeatus*) is the most common extant monotreme (Griffiths 1978), and Australia's most widely distributed native mammal (Augee 2008). Echidnas are usually solitary, and chemical signals are believed to be used for attracting and locating mates during the breeding season (Griffiths 1978; Rismiller 1992; Nicol et al. 2004). The breeding season occurs from June to September, which in colder parts of the echidna's range immediately follows several months of hibernation (Beard et al. 1992; Nicol and Andersen 2002). In the Tasmanian subspecies (*T. a. setosus*), males emerge from hibernation approximately one month before females, and the mating season is characterized by intense male intra-sexual competition for access to mates as females emerge (Morrow et al. 2009; Morrow and Nicol 2009). Some females have been found in mating aggregations while still hibernating (Morrow and Nicol 2009). Both sexes give off a pungent, musky odor when found in mating aggregations (Nicol et al. 2004). The presence of secretory glands located in the cloaca wall (Allen 1982; Russell 1985), cloaca "wiping" behavior (Boisvert and Grisham 1988; Beard et al. 1992), and field experiments (Rismiller 1992) suggest that a "pheromone" (Rismiller 1992) or chemical attractant present in cloacal secretions might be used for advertising female reproductive status, and that males are strongly attracted to this scent.

In addition to cloaca glands, male echidnas also possess a gland-spur apparatus in each of the hind limbs, composed of a keratinous spur located on the ankle joint, with its central canal connected via a duct to a "crural" gland located in the popliteal fossa (Krause 2010). Although echidna venom contains some of the peptides found in platypus (*Ornithorhynchus anatinus*) venom (Koh et al 2010),

unlike the platypus spur, the echidna spur is not firmly attached and cannot be used aggressively. A creamy secretion has been observed at the base of the spurs, and the crural gland's size and secretory activity is seasonal, being most active during the mating season. This suggests that the crural gland and associated glands at the base of the spur may function in scent production related to mating (Krause 2010).

No thorough investigation into the chemical composition of echidna scent gland secretions has been carried out. Only 5 compounds were identified in a swab from the chest area of an echidna in a previous semiochemical study, where a single individual was used as a comparative out-group (Zabaras et al. 2005). The aim of the present study was to describe the chemical composition of echidna secretions in detail using a range of analytical techniques, including gas chromatography-mass spectrometry (GC-MS). We also asked whether there are any differences in chemical profiles between sexes during the mating season. We predicted that sex-specific differences in cloaca chemical profiles should be evident during the mating season, as this would help to explain much of the reproductive behaviour outlined above.

Materials and methods

Study site and sample collection

A total of 514 odorant samples were collected from 173 captures of 21 adult male and 16 adult female echidnas between January and December 2010 from a wild population in the Tasmanian southern midlands, approximately 55 km north of Hobart (42°28'S, 142°14'E). The field site consists of undulating grazing pastures, dry schlerophyll forest patches and wooded gullies dominated by *Eucalyptus amygdalina* (Harris and Kitchener 2005). The site has variable topography with elevation ranging from 200–400 m above sea level. Rainfall averages 430 mm annually, mean minimum and maximum temperatures are 10 °C to 24 °C in summer and 2 °C to 11 °C in winter (Australian Bureau of Meteorology).

All animals were captured by hand after first either being spotted walking around the site, radio-tracked, or found with radio-tracked individuals (such as in a mating group). On initial capture, all echidnas at this field site were fitted with passive

implantable transponder (PIT) tags (LifeChip, Destron-Fearing, USA) for identification. Selected individuals were fitted with radio-transmitters (Biotelemetry, Australia; Titley Scientific, Australia) attached to the spines of the lower back with 2-part epoxy glue, allowing serial sampling. As many as 19 individuals (10 males, 9 females) had radio-transmitters attached during the study period.

Odorant samples were collected approximately once per month from radio-tracked animals, and more frequently during the mating period (June – September). Although we aimed to collect odorant samples from approximately equal numbers of radio-tracked male and female individuals, echidnas are cryptic, semi-fossorial animals, and frequently select shelter sites where they are inaccessible. Additional individuals which were not radio-tracked were sampled opportunistically as described earlier. Therefore, the number of samples collected varies quite considerably between individuals. For example, 15 animals were only sampled once, 11 animals were sampled 2 to 5 times, and 11 animals were sampled more than 6 times over at least a 5-month period. The greatest number of times any individual was sampled from over the entire study period was 16. Males and females are represented by at least one sample in each month.

Two types of samples, swabs and solid secretions, were collected from echidnas while the animals were under light inhalation anaesthesia (4% isoflurane in oxygen). Swab samples (N = 173) were collected by wiping the cloaca with a half-circle of glass fibre filter paper (Advantec, Japan, ø 47 mm), held with surgical forceps (Salamon and Davies 1998; Hayes et al. 2004). We also collected "control" samples (N = 173) by wiping a representative body area lacking dedicated scent glands (the right foreleg) with filter paper using the same technique as for cloacal swabs. Control swabs were collected for the purpose of identifying compounds which were contaminants present on the animal, and compounds which were not exclusive to cloaca secretions. Fresh urine and fecal material were removed before sampling by wiping the area with facial tissue, until any obvious contamination had been removed. Subsequent GC-MS analyses of swabs collected after this procedure was conducted showed that we were able to remove, or at least minimise, urine and fecal contamination without removing potentially important volatile compounds

from the area to be sampled. Throughout the year, both males and females produced a solid, waxy exudate from glands located in the cloaca and base of the spur. Females lose their spurs before maturity, but still produce a small amount of secretion from the pits where the spurs were previously located. Wax secretion samples (N = 170) were collected by gently squeezing the cloaca or base of spur, where present, and collecting a small (approximately 1–3 mg) sample of the resulting odorous exudate with forceps. Forceps were cleaned with ethanol and dried between collecting each sample. All filter paper and solid exudate samples were immediately stored in individual glass vials (20 mL scintillation vials with foil-lined urea screw caps, PerkinElmer, USA, and 2 mL screw-capped vials with PTFE/silicone septa, Waters, UK, respectively). All samples were stored on ice for a maximum of 8 h while in the field, and then at -20 °C in the laboratory until they could be analyzed, which for most samples was approximately 3 to 6 months after collection.

GC-MS analysis of filter paper swabs

Each filter paper sample was analyzed using combined GC-MS following thermal desorption using a thermal desorption unit (TDU; Unity, Markes, UK). A section of filter paper was placed inside a stainless steel desorption tube (Mi 098519, Markes, UK), and manually inserted into the TDU sampling port. The sampling port was heated from 40 °C to 150 °C, and then held at 150 °C for 10 min; the trap was then rapidly heated from -10 °C to 290 °C and remained at this temperature for 3 min. Filter paper samples were injected in split mode with a desorption tube pressure of 18 ± 0.2 psi. GC-MS analyses were performed using a Varian CP-3800 gas chromatograph with a Varian 1200L triple-quadrupole mass spectrometer (Varian, USA). The transfer line temperature was held at 290 °C and the source temperature was 220 °C. A 30 m Varian VF-5ms phenyl-dimethylpolysiloxane column (250 μm ID, 0.25 μm film thickness) was used with helium as the carrier gas operating at 40 °C for 2 min, then increased at 8 °C/min to 290 °C (total run time 35.25 min). The scan range was *m/z* 20–300 for the first 3 min, then *m/z* 35–500 for the remainder. Blank filter paper swabs, facial tissue samples and control swabs were analyzed to identify artifacts and contaminants.

GC-MS analyses of solid exudates

Solid exudates were analyzed using several different analytical techniques in order to identify their major volatile and nonvolatile constituents.

Volatile composition was determined using solid phase microextraction (SPME) headspace GC-MS analysis with the instrument described above and a 75 μm Carboxen™ polydimethylsiloxane partially crosslinked fibre (Supelco, USA). SPME analyses were carried out on samples in their original glass storage vials. The fibre was advanced through the vial septum and exposed to the sample headspace for 10 min at 25 °C, then withdrawn and manually injected into the GC port for 5 min (column oven temperature 2 °C for 5 min, then 10 °C/min to 250 °C over 24.8 min; total run time 29.8 min). The injector temperature was 280 °C, the source temperature was 220 °C, the transfer line temperature was 290 °C, and the scan range was *m/z* 35–400. Blank samples, using an empty sample vial, were run to identify artifacts and contaminants. The same fibre was used for all SPME headspace analyses, and all analyses were carried out in split mode.

Solid exudates were derivatized by extracting a small portion (~ 0.5 mg) of each sample in approximately 200 μL of chloroform. A 50 μL aliquot was then combined with 50 μL of *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1 % trimethylchlorosilane (TMCS) (Sigma-Aldrich, Australia) and allowed to react for 10 min at 60 °C, to form trimethylsilyl (TMS) fatty acid derivatives. Each sample was then injected into the GC-MS port using a Varian CP 8400 autosampler (1 μL; initial column oven temperature 100 °C, temperature increase 8 °C/min to 310 °C, final temperature 310 °C for 9 min, total run time 35.25 min). GC-MS analyses were carried out in split mode, the source temperature was 230 °C and the scan range was *m/z* 35 to 550. The first 8 min were excluded from further analyses as peaks in this time period were derived from the solvent. Blank samples, using 50 μL of chloroform reacted with BSTFA + 1 % TMCS, were run to identify artifacts and contaminants.

On-column injections were done using a fused silica needle (200 μm outside diameter) (0.5 μL of TMS-derivatized male spur exudate; initial temperature 50 °C; temperature increase 8 °C/min; final temperature 310 °C for 15 min; total run time

49.50 min). The source temperature was 230°C and the scan range was *m/z* 35–550. Direct probe chemical ionisation (CI) was done using a Kratos Concept ISQ high resolution mass spectrometer, on a 2 μL aliquot of a male spur exudate extracted in chloroform. Methods and conditions used by Lusby et al. (1984) were replicated in order to attempt to yield molecular ions for sterol esters. CI spectra were obtained at a source block temperature of 120 °C, the scanning range was *m/z* 35–1000 and ammonia was used as the reagent gas.

Electrospray ionisation (ESI)-MS was done using a Finnigan LCQ ion trap mass spectrometer fitted with an ESI source. Methods and conditions used by Kalo et al. (2006) were replicated in order to yield ammonium adducts of free fatty acids, free sterols and sterol esters. Positive ion ESI spectra were obtained by direct infusion of a 50:50 dilution of a male spur exudate extracted in chloroform with methanol at ~20 μL/min. The ESI needle voltage was 4 kV, the sheath gas (nitrogen) pressure was 70 psi and capillary temperature was 200 °C. The scanning range was *m/z* 250–1000. 100 mM ammonium acetate was used for infusion ESI. MS/MS data were obtained for selected ions observed in the main spectrum with collision energy of 17% and isolation width of 2 mass units. Finnigan Navigator software was used. A sterol ester standard (cholesteryl oleate, Sigma-Aldrich, Castle Hill, Australia) in a 50:50 dilution of chloroform and methanol was used to validate the method before applying it to echidna secretion samples. On-column injections, direct probe CI and ESI-MS analyses were done in 2009 using a sample collected in that year as part of a preliminary study (Harris 2009).

Double bond positions

Double bond positions in long chain fatty acids were assigned using methods described in Carlson et al. (1989), except we used chloroform as the solvent. Briefly, a 200 μL aliquot of a male cloaca exudate extracted in chloroform was combined with 200 μL dimethyl disulfide (DMDS; Sigma-Aldrich, Australia) and 100 μL iodine solution, and left to react overnight at 40 °C. The mixture was then diluted with chloroform and 5% sodium thiosulfate added to remove the iodine. The organic phase was transferred to a new vial, and the remaining aqueous layer re-extracted in chloroform. The organic phases were then combined and dried over anhydrous sodium sulphate, then blown almost to dryness with nitrogen. The

sample was then reacted with BSTFA + 1 % TMCS, and analyzed by GC-MS using the same methods and temperature regimes as for TMS-derivatized samples.

Peak identification

Chromatogram peaks were initially identified by comparing mass spectral and gas chromatogram data with existing databases (National Institute for Standards and Technology 2008). Retention time, molecular weight, peak purity and peak shape were also considered when identifying peaks. Structures of unidentified compounds were added to an "in-house" database and included in subsequent searches. These unidentified compounds could then be recognized in other samples by comparing mass spectra and retention times. When identifying known compounds in new samples, we required that retention times be within 0.2 min, and if we were unable to identify the diagnostic mass spectral ions of a compound corresponding to a given peak, it was deemed absent from the sample. Kovats' retention indices were calculated for the majority of all compounds identified and compared with published Kovats' data in the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/). Some methyl and ethyl esters were positively identified by co-elution of known standards. The difference in Kovats' indices between branched and straight chain isomers was used to directly assign iso and anteiso branching in some cases (Khorasheh et al. 1989).

Statistical analyses

We present preliminary analyses investigating compositional differences between sexes during the mating season. We analyzed peak data from a subset of cloaca filter paper swabs ($N = 56$) collected from males ($n = 17$) and females ($n = 12$) found in mating aggregations. This data set contains multiple samples for some individuals. When selecting compounds to be included in our analyses, we excluded artifacts identified in blank and control swabs. We then excluded compounds which occurred in less than 5% of all cloaca swabs analyzed, since these are either artifacts or unlikely to be important as semiochemicals. The 58 compounds retained for statistical analyses are indicated in Table 2.1. Due to the nature of our sample collection method, we could not control for the amount of secretion collected and analyzed. Therefore we calculated relative peak abundances as a percentage of the sum of all selected peaks, rather than attempting to quantify absolute concentration.

The area of each targeted peak was calculated using automated search methods, using the Varian instrument software (MS Data Review V6.41). The automated peak matches and area calculation reports were manually checked for accuracy and corrected when necessary. To circumvent the problems of pseudo-replication and increased risk of Type I error associated with using multiple samples from the same individuals, we calculated average peak abundances for repeat samples. We generated a Bray-Curtis similarity index after applying a square-root transformation to the dataset. We used a multidimensional scaling (MDS) plot and a single factor permutational multivariate analysis of variance (PERMANOVA) to examine whether there is a difference in chemical profiles between males and females during the mating season. A *P* value was obtained using 9999 permutations and Type I (sequential) sums of squares. The significance level was set at $\alpha = 0.05$. All statistical analyses were carried out using Primer V6.1.12 with the PERMANOVA+ V1.02 add-on package.

Results

Compounds identified in echidna secretions

Echidna secretions were extremely chemically complex, and very large amounts of chromatographic and mass spectral data were generated as a result of our analyses. A total of 469 different compounds were detected across all sampling methods and sample types. Of these, 186 compounds were determined to be from the animal and tentatively identified (Table 2.1). A very high proportion of the remainder were artifactual.

GC-MS analysis of filter paper swabs

Filter paper swabs were analyzed by thermal desorption and GC-MS. We identified a total of 31 compounds which were present in blank swabs and facial tissue samples. These included phthalates, silicone-containing compounds (likely originating from the silicone trap in the instrument), alcohols, alkanes, plasticizers, aromatic esters and other unidentified compounds. Phthalates and silicon-containing compounds occurred in all animal swabs analyzed. All compounds identified in blank and facial tissue samples were excluded from all further analyses. A further 67 compounds were identified in control samples. Many of these were synthetic

aromatics and siloxanes, which are likely contaminants from the time of sampling and/or analysis in the laboratory. These compounds were excluded from any further analyses. Naturally occurring compounds detected in control and cloacal swabs included diterpenes, cholestadienes and aldehydes. The composition of cloacal swabs were extremely variable, and typically comprised a complex mixture of compounds including aldehydes, alcohols, aromatics, hydrocarbons, terpenoids, short- and long-chain carboxylic acids, sterol derivatives and ethyl esters (Figure 2.2).

GC-MS analyses of solid exudates

GC-MS analyses of derivatized solid exudates revealed the presence of a series of sterols and methyl esters, as well as fatty acids ranging in length from 14 to 34 carbon atoms, several being monounsaturated (Figure 2.3). Double bond positions were determined for fatty acids (Table 2.1, Figure 2.3). SPME headspace analysis detected 7 short-chain ethyl esters (ethyl propanoate, ethyl butanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate), as well as 6 other volatile carboxylic acids already identified using thermal desorption of filter paper swabs.

Detection of sterol esters in solid exudates

Thermal desorption and on-column injections indicated that cholesta-2,4-diene and cholesta-3,5-diene were the products of sterols undergoing elimination reactions during the hot injection (Okerholm et al. 1968; Gerst et al. 1997). On-column injections also indicated that the source(s) of the cholestadienes were likely to be sterol esters, which eluted much later than the sterols and were too large for GC analysis. Direct probe CI and ESI mass spectra were used to attempt to determine molecular mass. Direct probe CI failed to yield a clear molecular ion, despite replicating conditions used by Lusby et al. (1984). Fragment ion mass spectra indicated the presence of sterol components, but the intact esters decomposed at relatively low temperatures (120 °C). ESI-MS analysis proved successful at yielding molecular adduct ions for several sterol esters present in the sample, along with fragment ions representing long chain fatty acid components. The high molecular weight peaks present in the ESI-MS spectra indicated there were several sterol esters present in the solid sample, plus a series of long chain fatty acids

(Figures 2.4, 2.5). Fatty acid chain lengths were calculated (Table 2.2), and matched those detected by earlier GC-MS analyses of derivatized exudates and paper swabs.

Sex differences

Most of the 58 compounds we considered for statistical analyses occurred in samples from both sexes. We found 3 compounds which were only found in female swabs collected during the mating season (dimethyl disulfide, isobutanoic acid, pentanoic acid), and 5 compounds only found in male swabs (dimethyl sulfone, 2-methylpropanol, ethyl n-henicosanoate, ethyl tetracos-15-enoate, and ethyl n-tetracosanoate). However, when we compared all cloacal swabs collected in the entire study period, only 1 compound was sex-specific (ethyl n-tetracosanoate, although this only appeared in 3 male samples). There is a considerable degree of overlap in cloacal swab composition between males and females (Figure 2.6), however some separation is evident (PERMANOVA: pseudo-$F_{1,28} = 4.25$, $P < 0.001$).

Discussion

Our analyses of echidna swabs and solid exudates revealed a complex mixture of compounds present in the majority of samples, some of which have previously been identified in the secretions of other mammalian species, while others have never previously been reported as animal products.

A total of 4 compounds reported here have been identified in echidna secretions in a previous study: acetic acid, 1,8-cineole, a C8 aldehyde (octanal) and a C10 aldehyde (Zabaras et al. 2005). Octanal, nonanal and other generic aldehydes are commonly found in a wide variety of mammalian secretions (Burger 2005). 1,1-bis-(*p*-tolyl)-ethane was detected in the majority of samples collected from other species analyzed by Zabaras *et al.* (2005), but was not detected in this study. Zabaras *et al.* (2005) reported that 1,1-bis-(*p*-tolyl)-ethane has structural similarities with DDT and has not previously been found as a natural compound, and we consider it to likely be an artifact. It must also be pointed out that the compounds found by Zabaras *et al.* (2005) were identified in swabs reportedly from sternal glands, however we can find no evidence to suggest that echidnas have such glands.

Swabs from the sternal area were not found to be significantly different from "control" samples from other areas of the body, such as the feet and legs (unpublished data).

Our odorant collection methods likely contributed to the detection of the large number of compounds reported in this study. By using swabs from the cloaca area, we collected compounds derived from the animal's skin, fur, feces, urine, reproductive organs and scent glands. In doing so, we collected compounds representative of the full suite of cloaca-related chemicals available to be detected by other animals. Several naturally occurring compounds were identified in both cloacal and control swabs collected from animals in this study, including terpenoids, aldehydes and cholestadienes, although peak sizes were much smaller in control samples. Aldehydes and cholestadienes (likely derived from sterols) are common constituents of mammalian skin surfaces (Stewart and Downing 1991; Burger 2005); however we included these compounds in our statistical analyses of cloacal swabs as they may still be important for communication. Samples which were contaminated with urine during the sampling procedure were easily identified by the presence of urea, *N*,*N*-dimethyl urea, tetramethylthiourea, trimethyl urea and cyanuric acid, which we excluded from further analyses. The presence of contaminant compounds from feces is more difficult to determine. Several compounds are likely to be derived from the echidna's diet (see *Terpenoids*), while others could be the result of bacterial action in the gut. The volatile components of scent marks in many mammalian species have been shown to be of bacterial origin (Alberts 1992). Further work is being conducted to identify compounds derived from echidna feces.

Volatiles

The volatile fraction of echidna cloacal secretions was dominated by volatile carboxylic acids, plus sulphur-containing compounds, phenol derivatives and short-chain aldehydes. These compounds likely contribute to the pungent, musky odor produced by both sexes when found in mating aggregations (Nicol et al. 2004). The sulphur-containing compounds are especially strong-smelling, and have previously been identified in the scent gland secretions of mustelids (Zhang et al. 2002; Wood et al. 2005) and marmosets *Callithrix jacchus* (Smith et al. 2001). Volatile

carboxylic acids are common constituents of mammalian secretions (Burger 2005). Their volatility makes them ideal short-term attractant signals (Alberts 1992), potentially functioning in sexual recognition (Burger et al. 2001) or mate attraction (Tobey et al. 2009). Our preliminary analyses indicate that the volatile compounds are most abundant during the mating season (unpublished data), and may allow male echidnas to locate females at this time of year.

Nitrogen-containing compounds such as 1-Methyl-2-pyrollidinone and a methyl-2,4-imidazolidinedione isomer were infrequently detected, but their peak sizes were relatively large in some samples. 1-Methyl-2-pyrollidinone has been identified in the scent gland secretions of wolverines *Gulo gulo* (Wood et al. 2005), tigers *Panthera tigris* (Burger et al. 2008) and marmosets *C. jacchus* (Smith et al. 2001). Trimethylamine is abundant in dog (*Canis familiaris*) and coyote (*C. latrans*) anal sacs (Preti et al. 1976). Aromatic, nitrogen-containing compounds are easily formed by bacterial degradation of proteins (Smith et al. 2001). Further work will be needed to determine whether proteins and their derivatives play as important a role in olfactory communication in echidnas as in other species (Belcher et al. 1990; Hurst et al. 1998).

Nonvolatiles

One of the most significant outcomes of this study was the identification of relatively large (*m/z* 800–900) sterol esters, comprising a sterol and a saturated or unsaturated long chain fatty acid (up to 34 carbons in length). To the best of our knowledge, due to the length of their fatty acid components, these compounds are considerably larger than other sterol esters reported as animal products. Sterol esters with fatty acid components of up to 24 carbon atoms in length are found in the preputial gland secretion of musk deer *Moschus moschiferus* (Sokolov et al. 1987), and sterol esters of up to 18 carbon atoms in sternum lipids are found in the brushtail possum *Trichosurus vulpecula* (Woolhouse et al. 1994). Large, nonvolatile compounds such as waxes (typically long chain alcohol esters of long chain fatty acids) and lipids function as fixing or waterproofing agents, ensuring that scent marks persist in the environment and thus enhances their detectability (Sokolov et al. 1987; Alberts 1992; Burger et al. 2008). Examples include the lipid fraction of male Bengal tiger (*P. tigris*) marking fluid, which is dominated by squalene and

fatty acids (Poddar-Sarkar 1996; Burger et al. 2008), major urinary proteins in the mouse *Mus musculus* (Hurst et al. 1998; Beynon and Hurst 2003), 2-phenoxyethanol in rabbit (*Oryctolagus cuniculus*) chin gland secretions (Hayes et al. 2003) and squalene in lemur (*Lemur catta*) brachial gland secretions (Scordato et al. 2007). We believe that the large sterol esters detected in this study may have a similar role in the echidna. Sterol esters could function as storage or fixing agents, releasing the more volatile secretion components that function as sexual attractants. If the life of olfactory signals in scent marks is extended by using a nonvolatile medium, then this should increase the potential number of animals that detect and are then attracted to the scent source (for example, the female echidna during the mating season). Echidnas also use latrines during the nonbreeding period of their active season (Sprent et al. 2006), and latrines have important communication functions in other species (Sneddon 1991; Begg et al. 2003). Squalene and large sterol esters may prolong the life of scent marks left at echidna latrines, potentially signalling individual presence and resource use. Waxes may also function as a source of free fatty acids (for example, through bacterial transformation) as indicated by the similar composition of free and bound (in esters) fatty acids (Sokolov et al. 1987).

A series of saturated, monounsaturated, and polyunsaturated long chain fatty acids were identified in echidna secretions in this study, with chain lengths ranging from 14 to 34 carbon atoms. These occurred as free fatty acids as well as in sterol and ethyl esters. Shorter chain fatty acids with chain lengths ranging from 6 to 13 carbon atoms were also detected, although less frequently. Very long chain fatty acids (> 22 carbon chain length) are present in small amounts in most animal tissues (Poulos 1995), but not often reported in mammalian scent gland secretions (Sokolov et al. 1987). In contrast, long chain fatty acids (12–22 carbon chain length) often make up a significant proportion of scent gland secretions (Poddar-Sarkar 1996; Wood et al. 2005). These fatty acids are likely to be important as nonvolatile chemical signals, as they have high degrees of molecular diversity, thus potentially increasing the information content of a chemical signal (López and Martín 2005). For example, fatty acids up to 26 carbon chain length have been identified in femoral gland secretions in some squamate lizards, where they may function in intra- and inter-specific discrimination (Alberts et al. 1992; Escobar et al. 2001).

Long chain fatty acids make up a significant proportion of giant panda (*Ailuropoda melanoleuca*) anogenital gland secretions, and erucic acid (among others) has been hypothesized to be a female panda pheromone (Zhang et al. 2008). In badgers (*Meles meles*), long chain fatty acids present in subcaudal gland secretions contribute to significant variations in secretion composition relating to group membership, sex and reproductive status (Buesching et al. 2002). Nonvolatile cues may be important for signalling between male and female echidnas during the mating season, due to the variety of different compounds detected. While odorous volatiles may initially attract wide-ranging males to a female, higher molecular weight compounds such as fatty acids may provide more specific information on reproductive status, body condition or individual identity.

A series of ethyl esters were identified in the echidna secretions with fatty acid components ranging from 3 to 26 carbon atoms in length, and our results indicate that these are the same fatty acid chains as identified in TMS-derivatized samples. Ethyl esters of fatty acids with chain lengths ranging from 14 to 20 carbon atoms have been found in male panda urine, which may code for genetic relatedness (Liu et al. 2008). Fatty acid methyl esters are present in sternal gland secretions and sternal gland hair in mandrills (*Mandrillus sphinx*), which contain information on age, sex and dominance status (Setchell et al. 2010), and may provide a cue of individual genetic identity (Setchell et al. 2011). Unidentified long chain esters contribute to species-specificity in gland secretions in lemurs (Hayes et al. 2004), and shorter chain ester series' appear to be sex-specific in peccaries (Waterhouse et al. 1996; Waterhouse et al. 2001). It is possible that long chain fatty acids and their methyl and ethyl esters may play an important role in signalling during the breeding and nonbreeding season in echidnas. Variations in composition of fatty acids, methyl ester series', and ethyl ester series' may code for individual identity, sex or reproductive status. Further work will be needed to investigate this.

Sterols may also contribute to signals containing information on individual identity or reproductive status in echidnas. Several sterols and steroid derivatives in both cloacal and spur secretions were detected, including cholesterol, desmosterol, cholestan-3-ol, coprostenol and cholest-4-en-3-one. Cholesterol is an extremely common component of mammalian scent gland secretions (Stewart and Downing

1991), and is found in a wide range of species including brown bears *Ursus arctos* (Rosell et al. 2011), hamsters *Phodopus sungorus sungorus* (Burger et al. 2001), giant pandas *A. melanoleuca* (Liu et al. 2008) and wolverines *G. gulo* (Wood et al. 2005). Like the sterol esters found in echidnas, cholesterol is thought to be used as an unreactive matrix that delivers important semiochemicals (López and Martín 2005). Steroids present in chemical signals may also be related to sex steroids (Escobar et al. 2001), and thus could potentially provide information on sex or reproductive status. The range of sterols found in echidna secretions suggests they may act as a nonvolatile medium for more volatile signal components, and may provide information on reproductive status. We did not find any evidence of triglycerides or phospholipids in echidna secretions.

Terpenoids

A large number of mono-, sesqui- and diterpenes were detected in echidna swab samples, including kaurene, aromadendrene and globulol. Terpenes are often detected in mammal secretions, although usually as secondary metabolites from their diet (koalas *Phascolarctos cinereus* (Salamon and Davies 1998), beavers *Castor canadensis* (Tang et al. 1995)). Globulol, limonene and kaurene, are likely to be present on the echidna's body through direct contact with their environment, as they were also detected in control samples. Some of the compounds we detected, including iridomyrmicin, are found in *Iridomyrmex* sp. (Cavill et al. 1982) and other ants which form part of the diet of echidnas at our study site (Sprent 2012), and could be present in residual fecal material collected on cloacal swabs. Terpenes may also occur in mammalian secretions with no obvious dietary or environmental source. For example, 1,8-cineole, a eucalyptus derivative, is present in koala sternal gland secretions (Salamon and Davies 1998; Tobey et al. 2009), but also occurs in giant panda scent marks (Hagey and MacDonald 2003).

Juvabione (I, Figure 2.7) is of particular interest, as it is found in North American *Abies* fir trees and considered to be an insect hormone mimic (Manville and Kriz 1977). It was dubbed "the paper factor" after its first isolation from paper towels made from this fir genus (Sláma and Williams 1965). However, there are no *Abies* trees at our study site. Juvabione was frequently identified in echidna swabs, did not appear in blanks or facial tissues, and was often the largest peak present. We have

been unable to source synthetic juvabione or *Abies* heartwood samples to confirm our identification, but both the mass spectrum and Kovats' retention index were excellent matches to published values (Pichette et al. 1998). We initially considered this to be a possible artifact, and we have exhaustively explored all other possible sources to explain the presence of juvabione in the echidna swabs. Terpenoid insect pheromones have previously been found in mammalian secretions: the aphid alarm pheromones (*E,E*)-α-farnesene and (*E*)-β-farnesene are found in female African elephant urine (Goodwin et al. 2006), the bumblebee sesquiterpene (*E*)-2,3-dihydrofarnesol is found in the temporal gland secretion in male African elephants (Goodwin et al. 1999). If juvabione is produced by the echidna, its function remains unclear.

Sex differences

Our results indicate that there is sex-specific variation in the odor profiles of male and female echidnas during the mating season. This provides new evidence supporting literature speculation that echidnas use olfactory cues for mate attraction (Griffiths 1978; Rismiller 1992). Chemical cues are known to advertise sex in a wide range of mammals (Wyatt 2003; Johansson and Jones 2007), and are particularly important in solitary animals that have little or no social contact with conspecifics (Hagey and MacDonald 2003; Rosell et al. 2011). Field experiments have shown that male echidnas are strongly attracted to the scent of females in mating groups, and this effect is not observed outside the breeding season (Rismiller 1992). Volatile compounds conveying information on sex may be particularly important for enabling males to locate mates, as volatile cues can convey information over large distances (Alberts 1992). Further investigations also need to be carried out, ideally incorporating data from controlled behavior experiments, reproductive physiology, and genetic analyses to further elucidate the function and seasonal variability of these signals.

Mating behavior observations at our field site (Morrow et al. 2009; Morrow and Nicol 2009) suggest that female echidnas start to produce a chemical signal which attracts males *prior* to her final emergence from hibernation. Further analyses will be required to investigate what triggers the production of such a signal, and when this change occurs. We could find little evidence for a female-specific compound or

potential "pheromone." Instead, our preliminary data suggest that sex-specific differences in cloacal secretions are quite subtle. Our sampling procedure may have contributed to this result - bacterial breakdown products or "generic" compounds collected in cloacal swabs could potentially "mask" the effect of compounds important for distinguishing between males and females. It appears more likely that, as in many species, echidna female-specific signals are made up of mixtures of several compounds, which encode information on sex (Hagey and MacDonald 2003), reproductive status (Scordato et al. 2007), and potentially individual identity (Smith et al. 2001). More obvious sex-related differences may be present in the cloaca and spur secretions, and these will be examined once sample collection and chemical analyses are completed. We also observed that males typically produced more secretion from the base of their spurs than females from the same body area, which may reflect differences in function (for example, males may use spur secretions to signal to other males during competitive mating encounters). Any functional variations in these secretions may be revealed following further analyses of sex and seasonal differences in these secretions.

The results presented in this study indicate that the composition of Tasmanian echidna secretions is chemically complex and contains compounds not previously detected in the secretions of other animals. There are indications of differences between sexes during the mating season, which will need to be examined in more detail. This study was based on samples collected from a single location, and is by no means representative of the entire species. Secretion composition can vary between populations (Hayes et al. 2002), and differences in the relative amounts of different compounds depending on climatic and geographical differences between subspecies may be expected.

Figure 2.1. Tasmanian short-beaked echidna (*Tachyglossus aculeatus setosus*) with radio-transmitter attached to lower back. (Photo: R Harris).

Table 2.1 (continued over page). Compounds fully or partially identified in cloacal swabs and solid exudates from male and female short-beaked echidnas *Tachyglossus aculeatus setosus*.

Number in Figs 2.1, 2.2[f]	Compound	Identification[g]	Kovats' retention index
*	Trimethylamine	a	
*	2-Methylpropanal	a,b	614
*	3-Methylbutanal	a,b	652
*	Acetic acid	a,b	659
	2-Methylbutanal	a,b	665
*	3-Methylbutanol	a,b	680
	1-2-Dimethylcyclopentane	a,b	705
*	Propanoic acid	a,b	716
*	Dimethyldisulfide	a,b	725
	Ethyl propanoate	a,b	728
*	Isobutanoic acid	a,b	750
	2-Butenoic acid	a,b	770
	n-Hexanal	a,b	777
	1,3-Butanediol	a,b	779
*	*n*-Butanoic acid	a,b	779
	Ethyl butanoate	a,b	780
	4-Hydroxy-4-methyl-2-pentanone	a,b	825
*	3-Methylbutanoic acid	a,b	832
	Ethyl 2-methylbutanoate	a,b	837
*	2-Methylbutanoic acid	a,b	840
	Ethyl 3-methylbutanoate	a,b	842
	Cyclohexanone	a,b	872
	n-Heptanal	a,b	874
*	2-Butoxyethanol	a,b	877
*	*n*-Pentanoic acid	a,b	886
*	Dimethylsulfone	a,b	897
	Benzoquinone	a,b	927
	5-Methyl-2(3)-dihydrofuranone	a,b	932
*	Dimethyltrisulfide	a,b	950
*	Phenol	a,b	960
	4-Methyl-3-pentenoic acid	a,b	967
	2-Pentylfuran	a,b	977
	Ethyl *n*-hexanoate	a,b	984
*	*n*-Octanal	a,b	985
*	*n*-Hexanoic acid	a,b	994
	α-Phellandrene	a,b	1014
	Limonene	a,b	1032
	1,8-Cineole	a,b	1036
*	1-Methyl-2-pyrrolidinone	a,b	1039
	n-Heptanoic acid	a,b	1072

	Dihydromyrcenol	a,b	1072
*	Cresol	a,b	1077
*	*n*-Nonanal	a,b	1103
	2-Ethylhexanoic acid	a,b	1132
	2-Nonenal	a,b	1156
*	Octyl acetate	a,b	1158
	n-Octanoic acid	a,b	1169
	Menthol	a,b	1173
*	Benzoic acid	a,b	1184
	Ethyl *n*-octanoate	a,b	1198
*	Benzothiazole	a,b	1200
*	*n*-Decanal	a,b	1202
	Nonanoic acid	a,b	1266
*	Benzeneacetic acid	a,b	1274
	2,4-Decadienal	a,b	1290
	Methyl 2-hydroxymethylbenzoate isomer	a	1300
*	*n*-Undecanal	a,b	1301
	2-Ethylhexyl butanoate	a,b	1310
	Benzenepropanoic acid	a,b	1351
	n-Decanoic acid	a,b	1363
	Ethyl n-decanoate	a,b	1395
*	*n*-Dodecanal	a,b	1403
	Dihydronepetalactone	a,b	1405
	Aromadendrene	a,b	1440
	n-Undecanoic acid	a,b	1461
	Iridomyrmecin	a,b	1484
*	*n*-Tridecanal	a,b	1506
*	Diethyltoluamide	a,b	1555
	n-Dodecanoic acid	a,b	1559
1	Ethyl *n*-dodecanoate	a,b	1597
	Globulol	a,b	1602
*	Diphenylamine	a,b	1610
	n-Tetradecanal	a,b	1611
	n-Tridecanoic acid	a,b	1658
2	Methyl *n*-tetradecenoate	a,b	1710
	A dihydroxy dimethyl methyl benzoate	a	1704
	n-Pentadecanal	a,b	1710
	Methyl *n*-tetradecanoate	a,b,c	1723
	Heptadecene	a	1725
	n-Tetradecanoic acid	a,b	1764
	Ethyl 7-tetradecenoate	a,b	1779
3*	Ethyl n-tetradecanoate	a,b,c	1791
	n-Hexadecanal	a,b	1825
	Methyl *n*-pentadecanoate	a,b	1824
	7-Tetradecenoic acid (TMS)	a,b,d	1833
4*	Ethyl pentadecanoate, branched	a	1836
5	*n*-Tetradecanoic acid (TMS)	a,b	1845

6*	Ethyl 13-methyltetradecanoate	a,b	1853
7*	Ethyl 12-methyltetradecanoate	a,b	1861
	n-Pentadecanoic acid	a,b	1867
8*	Ethyl *n*-pentadecanoate	a,b,c	1890
	n-Pentadecenoic acid (TMS)	a,b	1907
9	Methyl *n*-hexadecenoate	a,b	1908
10*	Ethyl hexadecanoate, branched	a	1930
11	Methyl *n*-hexadecanoate	a,b,c	1930
	n-Pentadecanoic acid (TMS)	a,b	1943
	(9*Z*)-9-Hexadecenoic acid	a,b	1944
12*	Ethyl 14-methylpentadecanoate	a,b	1954
13*	Ethyl (9*Z*)-9-hexadecenoate	a,b,c	1970
	n-Hexadecanoic acid	a,b	1977
	Kaurene	a,b	1978
	Ethyl 11-hexadecenoate	a,b	1980
14*	Ethyl *n*-hexadecanoate	a,b,c	1992
15*	Juvabione	a,b	2021
16	(9*Z*)-9-Hexadecenoic acid (TMS)	a,b,d	2022
	11-Hexadecenoic acid (TMS)	a,b,d	2032
17*	Ethyl heptadecanoate, branched	a	2036
18	*n*-Hexadecanoic acid (TMS)	a,b	2042
19*	Ethyl 15-methylhexadecanoate	a,b	2055
20*	Ethyl 14-methylhexadecanoate	a,b	2064
	Dehydrojuvabione	a,b	2093
21*	Ethyl *n*-heptadecanoate	a,b	2094
22	Methyl (9*Z*,12*Z*)-9,12-octadecadienoate	a,b	2096
23*	Methyl (9*Z*)-9-octadecenoate	a,b	2102
	Methyl 11-octadecenoate 1	a,b	2108
	Methyl 11-octadecenoate 2	a,b	2114
	Methyl *n*-octadecanoate	a,b,c	2128
24*	Ethyl octadecanoate, branched	a	2132
	n-Octadecenoic acid	a,b	2139
	Ethyl 16-methylheptadecanoate	a,b	2156
25*	Ethyl (9*Z*,12*Z*)-9,12-octadecadienoate	a,b,c	2162
26*	Ethyl (9*Z*)-9-octadecenoate	a,b,c	2168
	Ethylhexylcinnamate	a,b	2170
	n-Octadecanoic acid	a,b	2173
27*	Ethyl *n*-octadecanoate	a,b,c	2195
28	(9*Z*,12*Z*)-9,12-Octadecadienoic acid (TMS)	a,b	2207
29	(9*Z*)-9-Octadecenoic acid (TMS)	a,b.d	2213
	11-Octadecenoic acid 1 (TMS)	a,b,d	2219
	11-Octadecenoic acid 2 (TMS)	a,b,d	2223
	13-Octadecenoic acid (TMS)	a,b,d	2231
30	*n*-Octadecanoic acid (TMS)	a,b	2240
31*	Ethyl *n*-nonadecanoate	a,b	2295
	Ethyl *n*-icosadienoate	a,b	2349
	Methyl dehydroabietate	a,b	2353

32	Ethyl 13-icosenoate	a,b	2361
	Ethyl 15-icosenoate	a,b	2379
33*	Ethyl *n*-icosanoate	a,b	2397
34	*n*-Icosadienoic acid (TMS)	a,b	2407
	11-Icosenoic acid (TMS)	a,b,d	2411
35	Unknown (*m/z* 215, 331) (TMS)	a,b	2416
	13-Icosenoic acid (TMS)	a,b,d	2419
	15-Icosenoic acid (TMS)	a,b,d	2430
36	*n*-Icosanoic acid (TMS)	a,b	2437
37*	Ethyl *n*-henicosanoate	a,b	2498
38	Ethyl docosanoate, branched	a	2531
39*	Ethyl *n*-docosanoate	a,b	2598
	13-Docosenoic acid (TMS)	a,b,d	2610
	15-Docosenoic acid (TMS)	a,b,d	2619
	17-Docosenoic acid (TMS)	a,b,d	2629
	n-Docosanoic acid (TMS)	a,b	2634
	Cholestatriene 1	a	2750
40*	Ethyl 15-tetracosenoate	a,b	2774
*	Ethyl *n*-tetracosanoate	a,b	2797
41	15-Tetracosenoic acid (TMS)	a,b,d	2810
	Squalene	a,b	2815
	17-Tetracosenoic acid (TMS)	a,b,d	2818
	19-Tetracosenoic acid (TMS)	a,b,d	2828
	n-Tetracosanoic acid (TMS)	a,b	2832
42*	Cholesta-2,4-diene	a,b	2848
	Cholest-2-ene	a,b	2862
	A hexadecenoic acid ester	a	2878
	Cholesta-4,6-dienol	a	2888
43*	Cholesta-3,5-diene	a,b	2911
44	Ethyl 17-hexacosenoate	a,b	2975
45	17-Hexacosenoic acid (TMS)	a,b,d	3011
	19-Hexacosenoic acid (TMS)	a,b,d	3018
46	*n*-Hexacosanoic acid (TMS)	a,b	3032
	Cholestatriene 2	a	3042
47	Coprostenol (TMS)	a	3137
48	Cholesterol (TMS)	a,b	3151
49	5α-Cholestan-3β-ol (TMS)	a,b	3162
	Desmosterol (TMS)	a,b	3190
	Unknown sterol (*m/z* 255, 345) (TMS)	a	3202
50	19-Octacosenoic acid (TMS)	a,d	3210
	21-Octacosenoic acid (TMS)	a,d	3218
	n-Octacosanoic acid (TMS)	a	3229
51	Cholest-4-en-3-one (TMS)	a	3274
	Sitosterol (TMS)	a	3339
52	21-Triacontenoic acid (TMS)	a,d	3410
	23-Triacontenoic acid (TMS)	a,d	3417
	n-Triacontanoic acid (TMS)	a	3426

53	22-Hentriacontenoic acid (TMS)	a,d	3508
54	23-Dotriacontenoic acid (TMS)	a,e	3612
55	24-Tritriacontenoic acid (TMS)	a,e	3714
56	25-Tetratriacontenoic acid (TMS)	a,e	3811

[f]Compounds are listed in order of Kovats retention indices. Selected compounds (numbered) are labelled in Figures 2.2 and 2.3. *Asterisk*: compounds included in statistical analyses.

[g]Reliability of proposed identification is indicated by the following: a, mass spectrum agrees with mass spectral database; b, calculated Kovats retention index agrees with literature data (where available); c, mass spectrum and Kovats retention index match with known standards; d, double bond position determined by DMDS derivatization; e, double bond position estimated by extrapolation from observed patterns in preceding unsaturated fatty acids.

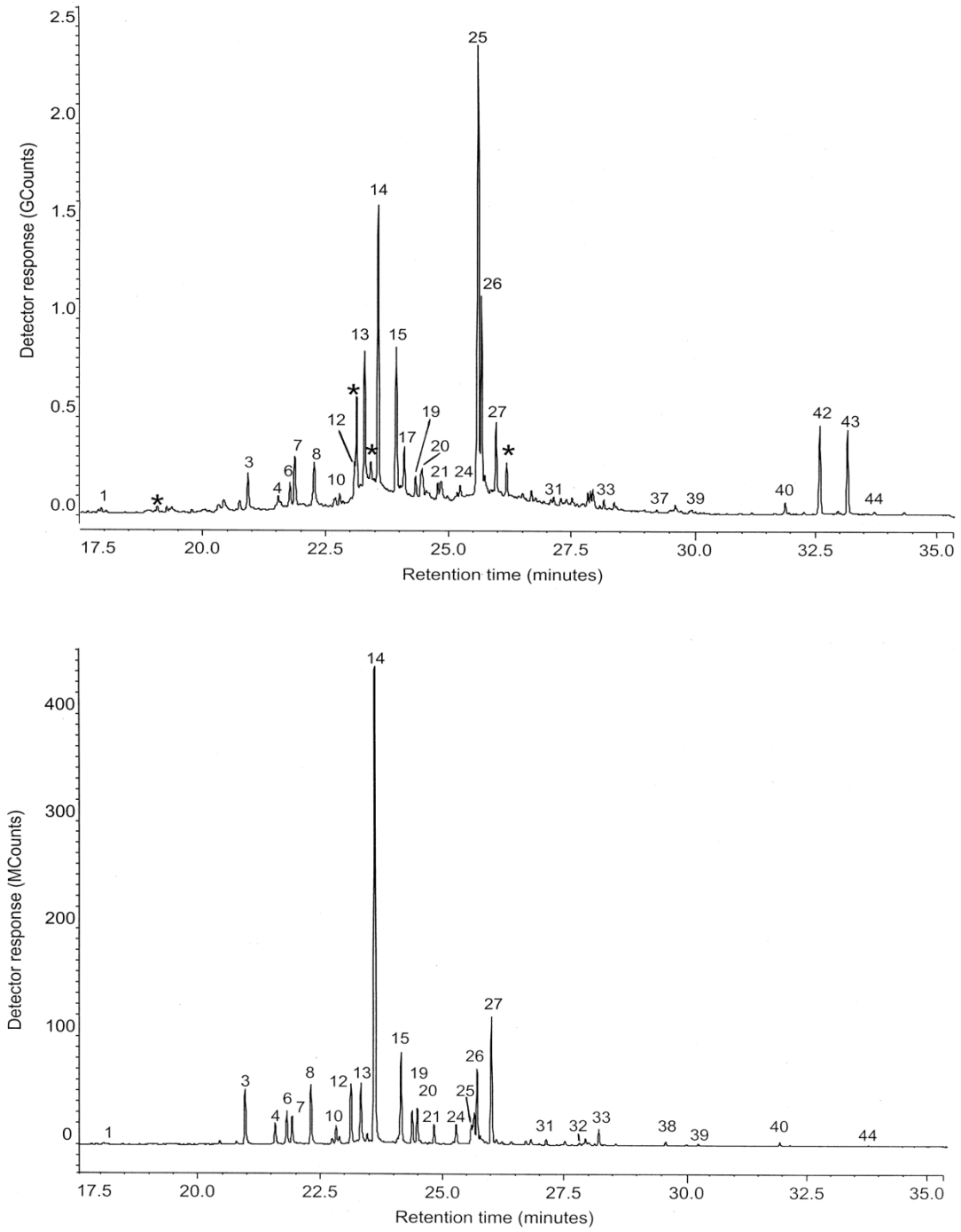


Figure 2.2. Representative thermal desorption total ion current chromatogram of a male echidna cloacal microfiber filter paper swab (upper) and chromatogram showing position of ethyl esters (lower; *m/z* 88, 101). *Asterisk*: unidentified or artifact. Peak numbers correspond to those given in Table 2.1.

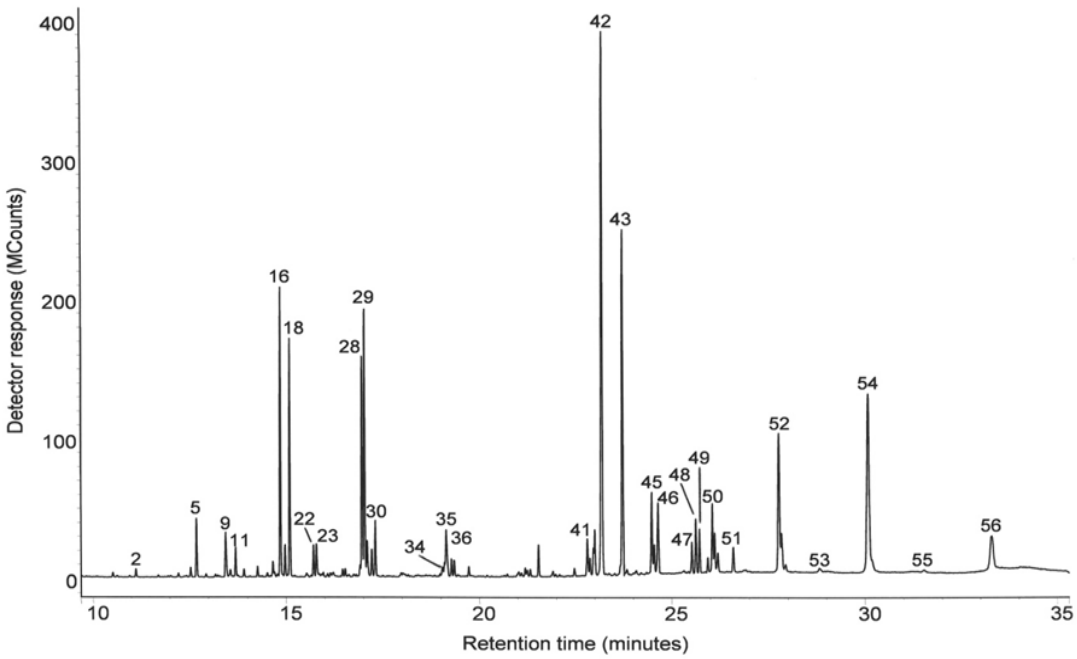


Figure 2.3. Total ion current chromatogram obtained by GC-MS analysis of TMS-derivatized components of a male echidna spur exudate. Peak numbers correspond to those given in Table 2.1.

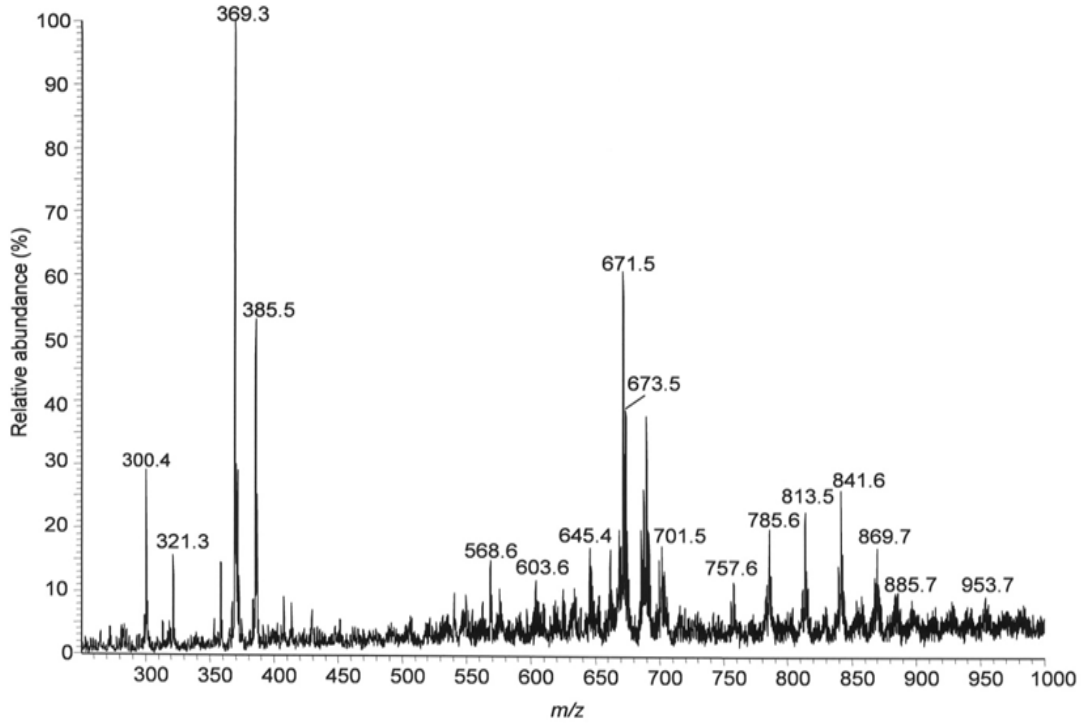


Figure 2.4. Full ESI-MS mass spectrum of a male echidna spur exudate. Note higher molecular weight ions (*m/z* 600–1000) indicating sodiated intact sterol esters containing long chain monounsaturated fatty acids.

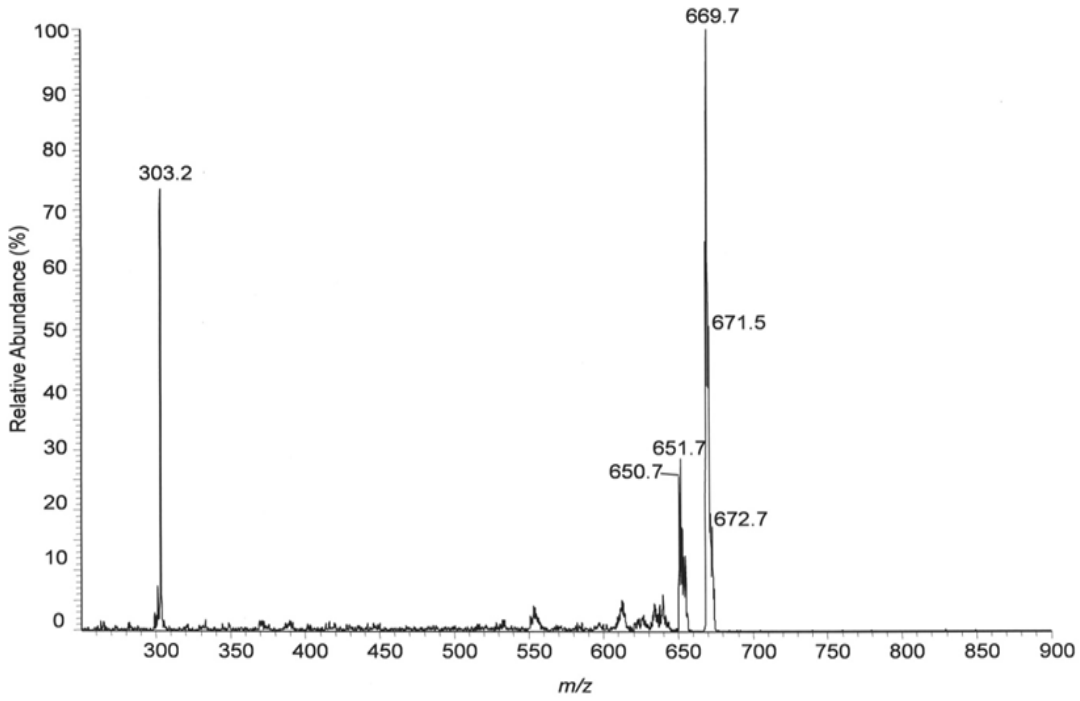


Figure 2.5. ESI MS/MS spectrum showing product ions of *m/z* 671.50 (Na adduct of intact sterol ester); *m/z* 303 = Na adduct ion for fatty acid fragment (C18:2, M_r = 280).

Table 2.2. Calculation of fatty acid chain length based on ESI MS/MS chromatogram and sodium (Na) adduct formation with a standard (cholesteryl oleate). Predicted sodiated molecules (in bold type) are observed in the ESI mass spectrum of the crude exudate. The range of fatty acids detected by ESI MS/MS match the free fatty acids identified as TMS derivatives by GC-MS.

Compound	**Sterol ester (mol. weight)**	**Sterol ester (Na adduct; *m/z*)**	**Fatty acid (Na adduct; *m/z*)**	**Fatty acid (mol. weight)**	**Likely fatty acid**
unknown	648	**671**	**303**	280	C18:2
cholesteryl oleate	650	**673**	**305**	282	C18:1
unknown	678	**701**	**333**	310	C20:1
unknown	706	**729**	**361**	338	C22:1
unknown	734	**757**	**389**	366	C24:1
unknown	762	**785**	**417**	394	C26:1
unknown	790	**813**	**445**	422	C28:1
unknown	818	**841**	**473**	450	C30:1
unknown	846	**869**	**501**	478	C32:1
unknown	874	**897**	**529**	506	C34:1
unknown	902	**925**	**557**	534	C36:1
unknown	930	**953**	**585**	562	C38:1

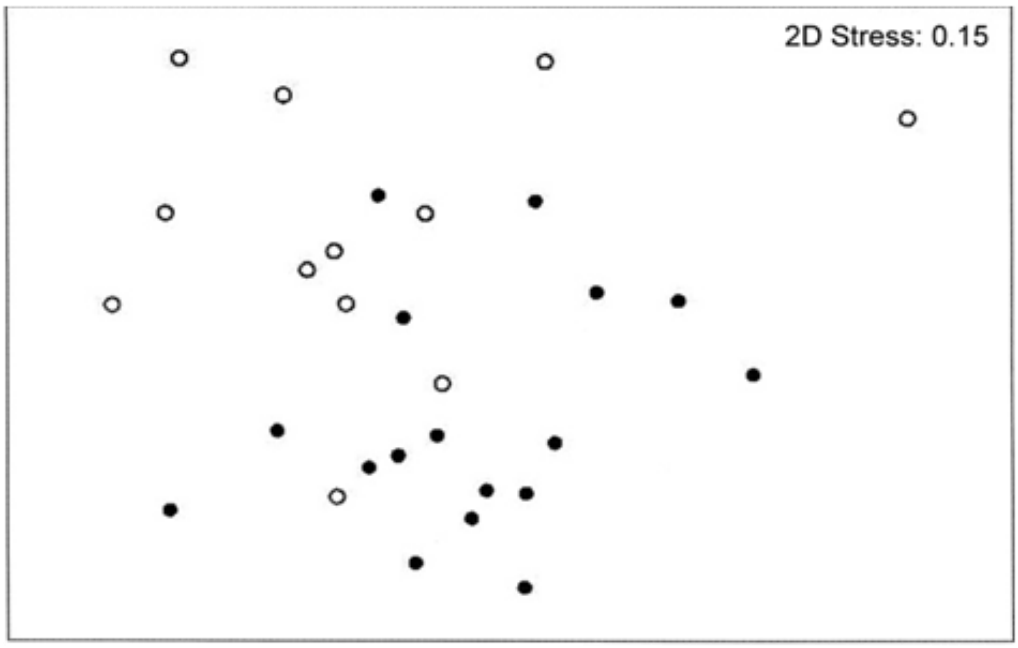
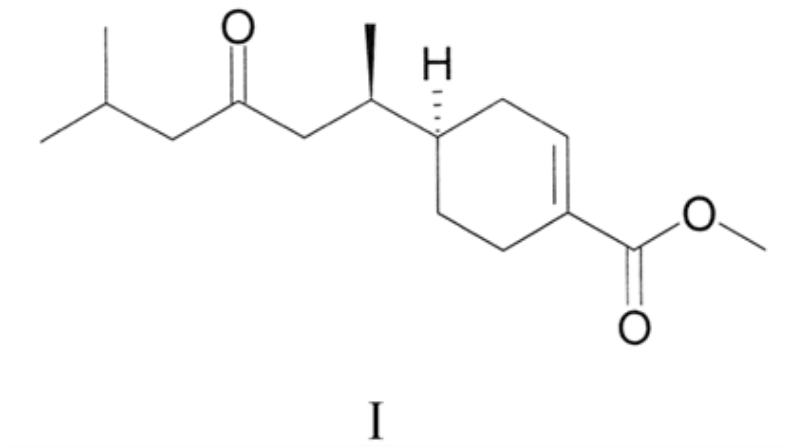


Figure 2.6. MDS ordination plot of chemical profiles showing some differentiation between male (closed circles) and female (open circles) echidna cloacal filter paper swabs during the mating season. The differentiation between samples is significant (PERMANOVA: pseudo-$F_{1,28} = 4.25$, $P < 0.001$). Axes are dimensionless.



I

Figure 2.7. Structure of juvabione (I).

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Chapter 3:

Identification of desmostanol as a novel vertebrate sterol in short-beaked echidna secretions

Chapter 3: Identification of desmostanol as a novel vertebrate sterol in short-beaked echidna secretions

Abstract

Mass spectra and chromatographic data are presented to support the identification of cholest-24-en-3β-ol (desmostanol) in odorous secretions in Tasmanian short-beaked echidnas. This sterol has previously been described only in marine invertebrates and phytoplankton, and may have a role in chemical communication in the echidna.

Introduction

A large number of mammalian species are known to use chemical or olfactory signals to communicate, particularly during the mating season. Mammalian chemical signals are typically complex, and may contain both volatile and nonvolatile compounds including phenols, terpenoids, fatty acids, aldehydes, esters and sterols (reviewed in Burger 2005). Such compounds may have important functions in coordinating mating behaviour, particularly in advertising species, sex, reproductive status, receptivity and individual identity to potential mates (Wyatt 2003; Johansson and Jones 2007). Nonvolatile compounds of high molecular weight, including sterols, sterol derivatives and their esters, are thought to function as inert matrices that release more volatile chemicals (Sokolov *et al.* 1987; Alberts 1992). Sterols and steroid derivatives may also act as 'pheromones', triggering mating behaviour in vertebrates (Dorries *et al.* 1997) and invertebrates (Hamilton *et al.* 1989).

Short-beaked echidnas (*Tachyglossus aculeatus*; hereafter 'echidna') are the most common species of extant monotreme (Griffiths 1978), and Australia's most widely distributed native mammal (Augee *et al.* 2008). Olfactory cues originating from the female are thought to be important for allowing males to locate widely dispersed mates during the breeding season (Griffiths 1978; Rismiller 1992). Secretion glands located in the cloacal wall (Allen 1982; Russell 1985), and in the fleshy area at the base of the spurs in males (Rismiller 1993; Krause 2010) are potential sources for chemical signals important in echidna mating behaviour. Females have been observed 'scent-marking' by dragging their cloaca along objects in the wild (Beard *et al.* 1992) and in captivity (Dobroruka 1960; Boisvert and Grisham 1988), and males appear to be attracted to female scent (Boisvert and Grisham 1988; Rismiller 1992). Echidna secretions from the cloaca and base of the spurs (including females, from the pits where spurs were previously located until lost at maturity) are particularly rich in relatively nonvolatile compounds including methyl and ethyl esters, long-chain fatty acids, sterols and sterol esters (Harris *et al.* 2012; Chapter 2). Several unusual compounds are also present, including juvabione, an insect hormone mimic found in North American fir trees, and high-molecular-weight

sterol esters, larger than any previously described in a vertebrate species (Harris *et al.* 2012; Chapter 2).

We have previously described an unidentified sterol present in cloacal and spur secretions of the Tasmanian echidna, 'unknown sterol (*m/z* 255, 345)' (Harris *et al.* 2012; Chapter 2). In more recent statistical analyses, this compound has been found to be important for differentiating between spur and cloacal secretions, and between males and females (Harris, Cameron, Davies, Holland, and Nicol, unpubl. data; Chapter 4). Here, mass spectral and chromatographic data to support the putative identification of the unknown sterol as cholest-24-en-3β-ol, 'desmostanol', are presented.

Materials and methods

Study site and animals

Fieldwork was conducted at our study site in the Tasmanian southern midlands (42°28'S, 142°14'E), approximately 55 km north of Hobart. The wild population of echidnas at this site has been the subject of ongoing physiological, ecological and behavioural studies since 1996, and over 270 individuals have been identified during this time. Details of the field site and field procedures, including radio tracking and capture of animals, are described elsewhere (Nicol *et al.* 2011; Harris *et al.* 2012; Chapter 2). Animals were captured by hand after either being radio tracked, spotted walking around the property, or when found close to radio-tracked individuals (i.e. in a mating group).

Odorant sample collection

Wax secretion samples were collected from adult male and female echidnas while the animals were positioned on their backs and under light inhalation anesthesia (4 % isoflurane in oxygen). Samples were collected by gently squeezing the base of the spur (where present) and the cloaca until a waxy exudate could be collected using surgical forceps. The waxy 'spur secretion' described in this study originates from the fleshy area at the base of the spurs, where present. The contents of echidna 'venom' which can be collected from the tips of the spurs is described elsewhere (Koh *et al.* 2010). Forceps were cleaned with ethanol and dried between samples.

Secretion samples (1-3 mg) were stored in individual 2-mL screw-capped vials with PTFE/silicone septa (Waters, UK) on ice while in the field. Samples were then kept frozen at -20 °C until they could be analysed. All animals were returned to their place of capture after sampling and recovery from the anesthetic.

Sample treatment and analyses

The unknown sterol has previously been found in both spur and cloacal wax secretions, (Harris *et al.* 2012; Chapter 2), and recent statistical analyses indicate it is most abundant in female 'spur' secretions (Harris *et al.* unpublished data). A representative sample of spur secretion collected from an adult female echidna was analysed using combined gas chromatography–mass spectrometry (GC–MS) (for details see Harris *et al.* 2012; Chapter 2). Briefly, the sample was extracted in approximately 200 μL of chloroform overnight. A 50-μL aliquot was then reacted with 50 μL of *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1 % trimethylchlorosilane (TMCS) (Sigma-Aldrich, Australia) and allowed to react for 10 min at 60 °C, to form trimethylsilyl (TMS) fatty acid and sterol derivatives. The sample was then analysed by GC–MS within 2 hrs of being derivatised. GC–MS analysis was conducted using a Varian CP-3800 benchtop gas chromatograph (Varian, Palo Alto, CA, USA) and a Bruker 300-MS TQ mass spectrometer (Bruker, Preston, Vic., Australia). Acetylation was carried out using acetyl chloride, the sample was then analysed isothermally at 280 °C to calculate relative retention times (RRT; relative to cholesteryl acetate) using the same equipment as above. Blank samples, using 50 μL of chloroform reacted with BSTFA + 1 % TMCS, were run to identify artefacts and contaminants.

Chromatogram peaks were identified by comparing mass spectral and gas chromatogram data with existing databases (National Institute for Standards and Technology 2008) and published values, and by matching calculated Kovats' retention indices with published values in the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry).

Results and discussion

Mass spectra

Mass spectra for desmosterol (cholesta-5,24-dien-3β-ol) and the unknown sterol are shown in Fig. 3.1. The two mass spectra show largely analogous fragmentation, with the mass spectrum for desmosterol containing a distinctive *m/z* 343 ion for the loss of 113 (C_8H_{17}), which would normally be indicative of a saturated sterol side chain. However, this loss from the unsaturated side chain has been shown to be due to a complex 2H rearrangement, when there is a delta-24 double bond (Brooks 1979). Also, a loss of 84 (C_6H_{12}) to give the ion at *m/z* 372 is characteristic of delta-24 double bond-containing sterols (Brooks 1979). The *m/z* 129 ion and (M^+-129) ion at *m/z* 327 are characteristic of an unsaturated ring structure with a double bond at the delta-5 position (Brooks 1979).

The unknown sterol has a difference of +2 daltons for all major peaks compared with the desmosterol spectrum, including the molecular ion (*m/z* 458). The *m/z* 129 ion and (M^+-129) are absent in the unknown sterol spectrum. These results indicate that the unknown sterol ring structure contains one fewer double bonds than desmosterol (Fig. 3.2), but retains the delta-24 double bond (as indicated by the characteristic losses of 84 and 113). The sterol can therefore be assigned as cholest-24-en-3β-ol ('desmostanol').

Mass spectra data for the acetylated unknown sterol are in general agreement with published data for 5α-cholest-24-enyl acetate (Teshima *et al.* 1982); mass: 428 (8 %, M^+), 413 (12 %), 353 (6 %), 345 (4 %), 344 (17 %), 343 (7 %), 316 (18 %), 315 (66 %), 283 (3 %), 255 (32 %), 229 (4 %) and 215 (13 %). Our calculated RRT (1.13) is identical to that reported on OV-1 for 5α-cholest-24-enyl acetate by Teshima *et al.* (1982).

Kovats' retention indices

Further evidence was obtained from gas chromatographic retention indices. Our calculated Kovats' retention index for the unknown sterol matches closely with published data for 5α-cholest-24-en-3β-ol (Edmonds *et al.* 1977). We also calculated offsets in our calculated Kovats' retention indices (Harris *et al.* 2012;

Chapter 2) for desmosterol and the unknown sterol (Table 3.1). We compared the offset with that for cholesterol and cholestanol, as these two known compounds are also related by the removal of the delta-5 double bond: this was the same as the difference between desmosterol and the putative desmostanol, consistent with the effect of removing the delta-5 double bond in each case.

Very few other references to this compound exist in the literature (Barrett *et al.* 1995; Li *et al.* 1996), and none provide any physical data. We have been unable to source a standard to confirm our identification, but the relative retention time, mass spectral and Kovats' retention index data for the trimethylsilylated and acetylated extracts nevertheless provide solid evidence for this identification.

Desmostanol has previously been described in only a few species of marine organisms, including a sponge (*Hymeniacidon perleve*) from Scotland (Edmonds *et al.* 1977), a diatom (*Rhizosolenia setigera*) from Victoria, Australia (Barrett *et al.* 1995), and a chiton (*Liolophura japonica*) from Japan (Teshima *et al.* 1982). The function of desmostanol in echidnas is unclear; however its occurrence in echidna secretions raises the question as to why this compound has never previously been described in any other vertebrate species.

Waxy secretions from the cloaca and base of the spurs in echidnas are rich in several other sterols, some of which are ubiquitous in animal secretions (i.e. cholesterol), while others are relatively uncommon (i.e. coprostenol) (Harris *et al.* 2012). Sterols and sterol derivatives make up a significant component of the scent gland secretions of several mammalian species, including bactrian camels (*Camelus bactrianus*) (Ayorinde *et al.* 1982), and musk deer (*Moschus moschiferus*) (Sokolov *et al.* 1987), as well as the fat tissue in pigs (*Sus scrofa*) (Bonneau 1982; Dorries *et al.* 1995). Due to their relatively large molecular weight and low volatility, sterols and their derivatives are unlikely to be used as wide-ranging attractants, but may be used for close-contact signals (Alberts 1992; Brennan and Kendrick 2006). The relative abundances of many of the sterols found in echidna secretions, including desmostanol, vary significantly between secretions from different glands, between males and females, and some also show seasonal variations associated with the mating period (Harris, Cameron, Davies, Holland, and Nicol, unpubl. data; Chapter

4). Therefore, sterols appear to be an important component of nonvolatile signals that may help to coordinate mating behaviour in echidnas, such as by advertising sex, reproductive status or individual identity.

This study has provided supporting evidence for our identification of cholest-24-en-3β-ol, (desmostanol) in waxy secretions produced by short-beaked echidnas. This is the first report of this rare sterol in a vertebrate, and provides further evidence for the chemical and taxonomic diversity of compounds used in olfactory communication by different animal species.

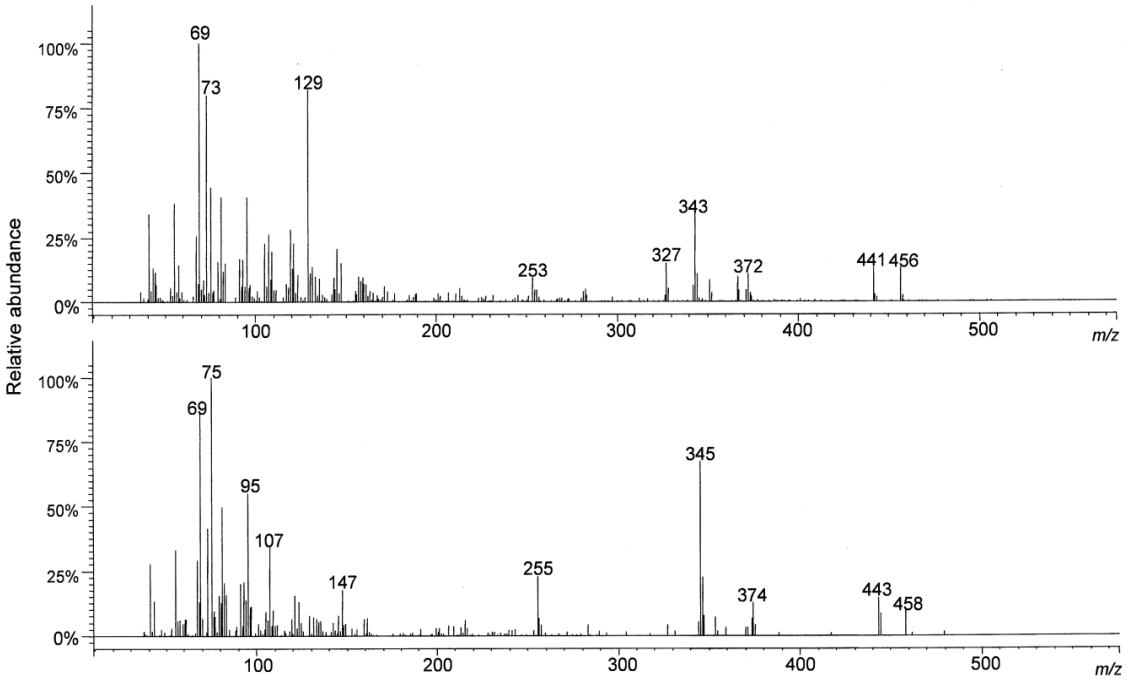

Figure 3.1. Mass spectra for trimethylsilyl ethers of desmosterol (upper) and desmostanol (lower).

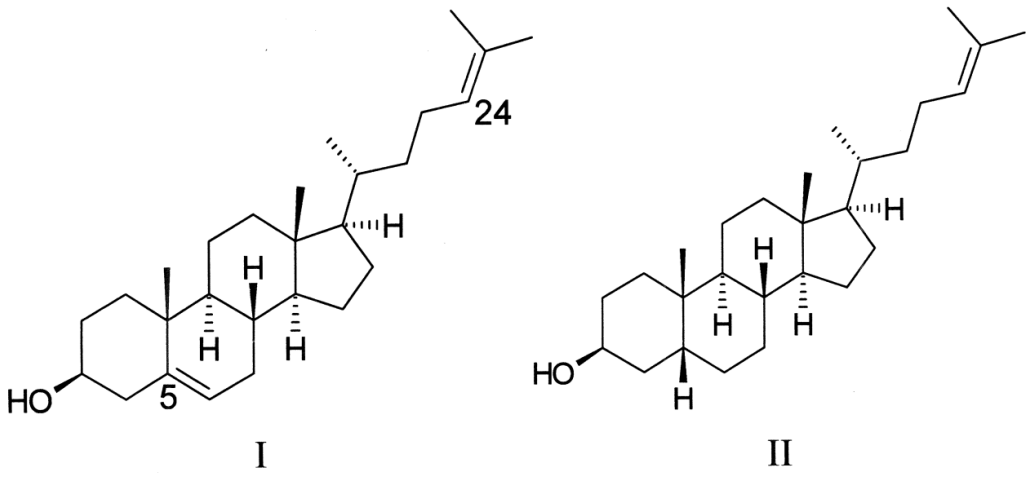

Figure 3.2. Structures of desmosterol (I) and desmostanol (II). Carbon atoms mentioned in the text are numbered on the desmosterol structure (numbering also applicable to desmostanol structure).

Table 3.1. Kovats' retention indices, and offsets in Kovats' retention indices between cholesterol and cholestanol, and desmosterol and desmostanol.
Calculated Kovats' retention indices values are from Harris *et al.* (2012), see Chapter 2.

Compound name	**Kovats' retention index**	**Kovats' offset**
Cholesterol (TMS)	3151	
Cholestanol (TMS)	3162	+ 11
Desmosterol (TMS)	3190	
Desmostanol (TMS)	3202	+ 12

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SECTION II:

CHEMISTRY, SEASONALITY AND BEHAVIOUR

Chapter 4:

Chemical signals in the echidna: differences between seasons, sexes, individuals and gland types

Chapter 4: Chemical signals in the echidna: differences between seasons, sexes, individuals and gland types

Abstract

Seasonally reproducing animals show many behavioural and physiological changes during the mating period, including increased signalling for mate attraction. Mammals often rely on chemical signals for communication and coordination of mating and other social behaviours, but our understanding of the subtleties and functions of mammalian signalling could be increased with more input from non-model systems. We used gas chromatography–mass spectrometry and nonparametric statistics to investigate the volatile and non-volatile composition of odorous secretions in an egg-laying mammal (monotreme), the short-beaked echidna *Tachyglossus aculeatus*. We collected a total of 778 odorant samples from 69 wild, sexually mature individuals over 3 years at our field site in southern Tasmania. Animals were sampled during the breeding and non-breeding seasons, as well as during hibernation. Odorants included swabs from the cloaca and 'waxy' secretions from putative scent glands in the cloacal wall and at the base of the spurs. Chemical profiles varied between different gland secretions and by sex and season. Female spur and cloacal wax secretion profiles had higher relative abundances of sterols, whereas male wax secretion profiles had more long chain fatty acids. Male spur secretions changed significantly during the mating season and could function in intra-sexual competition or female mate choice. Echidna scent gland secretions also varied between individuals, suggesting olfactory cues could be used for individual recognition. Our results indicate that echidna secretions contain information that could be used by individuals to attract and locate mates during the breeding season. We also provide evidence for the potential importance of compounds traditionally classified as 'non-volatile', including sterols and fatty acids, as cues for individual recognition or mate assessment.

Introduction

In sexually reproducing animals, individual fitness can be increased by the ability to detect and respond to signals from conspecifics and to effectively advertise characteristics such as species, sex, reproductive status and identity (Johansson and Jones 2007; Shine and Mason 2012). Species may communicate using visual, auditory, tactile or chemical signals (Andersson 1994), sometimes in combination (Candolin 2003). Chemical communication is the dominant mode of signalling in many mammals (Albone 1984). Odorous mixtures typically present in faeces, urine or other body secretions have advantages over other signal modes, being long-lasting, wide-ranging, highly specific, effective in darkness and effective in the absence of the signalling animal (Wyatt 2003). Combinations of 'volatile' and 'nonvolatile' scent components are found in mammalian secretions (Burger 2005) and help to coordinate mating behaviours (Wyatt 2003). Volatiles, usually small, odorous compounds (e.g. short-chain fatty acids) can function as wide-ranging mate attractants, while larger nonvolatiles (e.g. sterols) lack a strong odour and may require direct contact for detection, providing more detailed information such as on individual identity (Alberts 1992).

Many seasonally breeding mammals show sex differences or temporal changes in chemical signals (Scordato, Dubay & Drea 2007; Tobey, Nute & Bercovitch 2009; Rosell *et al.* 2011), which are associated with reproductive physiology (Ferkin *et al.* 1994; Rasmussen, Hall-Martin & Hess 1996) and attract potential mates (Swaisgood, Lindburg & Zhang 2002; Thomas 2011). Chemical signals may also be individual-specific (Scordato *et al.* 2007; Burgener *et al.* 2009), sexually-selected (Blaustein 1981; Penn & Potts 1998) and function in mate assessment and choice (Johansson & Jones 2007). Multidisciplinary approaches which describe quantitative and qualitative aspects of secretions are particularly useful for understanding the functions of mammalian chemical signalling (Burger 2005; Drea *et al.* 2013) and our understanding of the complexities and subtle differences in chemical communciation among species could be improved by studying a broader range of taxa with different ecological and physiological characteristics. As part of a larger study incorporating chemical, behavioural, physiological and genetic data, we investigated chemical signals in relation to mating behaviour in a highly

seasonal, terrestrial monotreme, the short-beaked echidna (*Tachyglossus aculeatus*; 'echidna').

Anatomical and behavioural evidence suggests echidnas rely on chemical communication for social and sexual behaviour, and we asked whether variations in chemical signals also support this hypothesis. The first detailed anatomical study of echidnas noted the large size of the olfactory nerves (Home 1802) and the relative size and complexity of the echidna brain suggests significant olfactory memory or cognitive ability (Nicol 2013). The size and folding of the olfactory bulb is comparable to that of prosimians (Ashwell 2006), which use chemical communication to mediate complex social behaviours (e.g. Scordato & Drea 2007), and the echidna is the only mammal known to have a gyrified olfactory bulb, probably an adaptation for analysing a large odorant repertoire (Ashwell 2013). This evidence suggests olfaction is highly developed in echidnas and could regulate a variety of different behaviours, but contrasts with literature suggesting monotreme behaviour is simplistic (reviewed by Nicol 2013).

Olfactory cues appear important for coordinating echidna mating behaviour (Griffiths 1978; Rismiller 1992) and possibly individual recognition (Augee, Bergin & Morris 1978). Echidnas are usually solitary (Nicol *et al.* 2011), but mating aggregations comprising a single female with multiple males have been recorded throughout Australia (Morrow, Andersen & Nicol 2009), including 'trains' on Kangaroo Island (Rismiller & Seymour 1991; Rismiller 1992). Mating occurs in winter (June-September) throughout their geographic range (Morrow *et al.* 2009) and depending on subspecies this is preceded by a period of inactivity, ranging from brief torpor bouts to prolonged hibernation (Rismiller & McKelvey 1996; Nicol & Morrow 2012). In the Tasmanian subspecies, *T. a. setosus*, males emerge from hibernation earlier than do females, then locate and mate with females that are still hibernating (Morrow & Nicol 2009), possibly using olfactory cues (Harris, Davies & Nicol 2012). Since males enter hibernation before females, hibernating females are usually completely concealed and often shift during periodic arousals (Nicol & Andersen 2002; Nicol, Vedel-Smith & Andersen 2004), it seems unlikely that males use visual cues or map female hibernacula locations to find mates. However, males are attracted to female scent (Chapter 5; Rismiller 1992) and both sexes give off a

pungent 'musky' odour during the mating season (Rismiller & Seymour 1991; Nicol *et al.* 2004) so odour appears important for locating mates. Chemical signals could originate from cloacal glands in both sexes and volatile composition differs between males and females during the mating period (Harris *et al.* 2012). Differences between seasons and individuals have not been examined. Additionally, males produce a 'waxy' secretion from putative scent glands at the base of spurs on their hind limbs (Harris *et al.* 2012). This secretion is different from the aqueous 'venom' from the crural glands which can be expressed from the tips of the spurs (Krause 2010; Wong *et al.* 2013). Females lose their spurs at maturity but produce a small amount of similar secretion from the pits where spurs were located (also referred to here as 'spur secretion'; Harris *et al.* 2012), which could function in chemical communication.

We investigated the volatile and nonvolatile components of Tasmanian echidna odorants using gas chromatography-mass spectrometry (GC-MS) and compared differences in composition with different characteristics of donor animals. We predicted that odour profiles would vary between seasons, sexes, individuals and secretions from different scent glands.

Materials and methods

Field site and animals

Fieldwork was conducted in the Tasmanian southern midlands north of Hobart (42°28'S, 142°14'E) between January 2010 and September 2012. The wild echidna population here has been the subject of ongoing ecological and physiological studies since 1996 and over 270 individuals have been captured and tagged during this time (details see Nicol *et al.* 2011; Harris *et al.* 2012). Up to 23 echidnas were fitted with radiofrequency transmitters at any time for serial odorant sampling (Nicol *et al.* 2011). Radio-tracked animals (14 males, 15 females) were sampled approximately monthly and up to three times per week during the mating season. Other individuals (n=40) were either spotted or found near a radio-tracked animal (e.g. in a mating aggregation) and sampled opportunistically. Samples were collected from sexually mature adults in years that they showed mating activity (Table 4.1).

Sample collection and chemical analyses

We collected three types of odorants as described previously (Harris *et al.* 2012): (1) cloacal swabs (*N*=331), (2) cloacal wax secretions (*N*=168) and (3) spur wax secretions (*N*=279). Samples were stored at -20 °C for a maximum of two years before they were analysed. We re-analysed some samples to verify volatile compound presence over storage time. We used control swabs from the right foreleg (i.e. without scent gland secretions) and blank swabs to identify artefacts and contaminants (Harris *et al.* 2012), which were excluded from further analyses.

We used two types of GC-MS analyses to describe both volatile (b.p.<300 °C) and nonvolatile scent components. Details of analyses and compound identification protocols are described elsewhere (Harris *et al.* 2012). Cloacal swabs were analysed by GC-MS after thermal desorption, minimising interference with early-eluting, volatile components (McLean, Davies & Wiggins 2012). Cloacal swab GC-MS analyses were performed using a Varian CP-3800 benchtop gas chromatograph (Varian, Palo Alto, CA, USA) and 1200L triple-quadrupole mass spectrometer (Varian, Palo Alto, CA, USA) or Bruker 300-MS TQ mass spectrometer (Bruker, Preston, Victoria, Australia). The Varian instrument was replaced by the Bruker during the study, but all temperature programs and scanning conditions were held constant. Spur and cloacal wax secretion samples were analysed by GC-MS as trimethylsilyl (TMS) derivatives, which improved peak shape and separation of less volatile, polar compounds including sterols and fatty acids. All wax secretion GC-MS analyses were performed using the Varian gas chromatograph and Bruker mass spectrometer.

We excluded from further analyses compounds which appeared in less than 5% of samples (*n*=76; see also Harris *et al.* 2012), as they were unlikely to be important for discriminating between groups. We retained 68 compounds found in cloacal swabs for our analyses (Supporting Information Table 4.S1), including C_2-C_6 and C_{14}-C_{18} fatty acids, phenolics, ethyl esters of C_{14}-C_{26} fatty acids, nitrogen- and sulphur-containing compounds, aldehydes and cholestadienes. Thirty-three compounds were found in >50% of cloacal swabs, 13 of these occurred in >75% of samples. We retained 56 compounds found in spur and cloacal wax secretions, including C_{14}-C_{34} saturated and unsaturated fatty acids, sterols and methyl esters;

nine of these also occurred in cloacal swabs (Supporting Information Table 4.S1). Forty-one compounds were present in >75% of wax secretions analysed and 36 present in >95% of samples.

We calculated relative peak areas of compounds in each sample using automated search methods (Varian MS Data Review, version 6.41). To overcome problems with peak co-elution, for each compound we estimated the total ion current (TIC) peak area using the peak areas of 2-3 quantitative ions (QIs) from the best quality spectrum available (McLean *et al.* 2012). We used retention time (±0.2 min) and TIC reference spectrum as search criteria and the automated reports were manually checked for accuracy. Relative abundances of compounds are expressed as peak areas relative to the total area of all quantified peaks in a given sample.

Statistical analyses

Chromatographic data are characterised by a large number of variables (chemicals) derived from a relatively small number of samples and often do not meet the assumptions or sample size requirements of 'traditional' multivariate statistical methods (Burgener *et al.* 2009; Martin & Drijfhout 2009). To test for differences between *a priori* sample groups (year, gland type, sex, season, individual, mass spectrometer) we used a combination of three nonparametric methods: non-metric multidimensional scaling (MDS) plots; permutational multivariate analyses of variance (PERMANOVA; Anderson 2001) and random forests (Breiman 2001). These methods make fewer assumptions about the nature and quality of data compared with parametric methods, but may be interpreted in a similar manner (Anderson 2001; McCune, Grace & Urban 2002; Lunetta *et al.* 2004).

To test for temporal differences in chemical profiles, we divided samples into *a priori* 'seasons' based on echidna ecology and the animal's behaviour, physiology and time of year when sampled: *hibernating* (cool skin temperature, slow response to touch, body temperature (T_b) <28 °C (but commonly <10 °C; Nicol and Andersen 2002), usually March-June); *breeding* (mid-June to mid-September, observed in mating aggregations during this period); or *non-breeding* (active, euthermic ($T_b \approx 32$ °C), usually October-February). These groups loosely correspond to time of year but timing varies slightly between individuals. We included breeding

season sample data for females which were euthermic, torpid, pregnant and non-pregnant, as all attract males (Morrow & Nicol 2009; Morrow 2013). Cloacal swabs were analysed separately from both types of wax secretions, because they were chemically analysed using different methods (thermal desorption vs. TMS-derivatives) and could not be directly compared. We removed five outliers from the cloacal swab dataset (one female, four males; each sample with < eight compounds). These were probably poor quality samples, due to small compound peak sizes and relatively large numbers and abundances of contaminants and artefacts.

Relative abundance data for each odorant type were square-root transformed and converted to a Bray-Curtis distance matrix prior to the MDS and PERMANOVA analyses. We used single-factor and mixed model nested PERMANOVAs to test for differences between sexes, seasons, individuals and gland types. These main effects may vary with mass spectrometer (cloacal swab dataset only), year or storage time, so we first included 'Mass Spectrometer' and 'Year' as fixed factors to adjust for any confounding effects. Although a potentially important variable (Drea *et al.* 2013), storage time was confounded by year so could not be tested reliably (but results were the same when 'Year' was replaced with 'Storage time' in the models). There were also some significant interactions involving 'Year' (Supporting Information Table 4.S2), although these were not related to a particular biological hypothesis and are difficult to interpret, but could be related to either environmental variations or storage time. For simplicity we present results of PERMANOVA models without these interactions, which largely do not change interpretation of main effects. We then included 'Sex' and 'Season' as fixed factors and individual identity nested within sex, 'Animal ID(Sex)', to account for multiple samples from most individuals. We used pair-wise comparisons to investigate significant Sex*Season interactions. *P*-values were obtained using 9999 permutations of residuals under a reduced model with Type I (sequential) sums of squares. MDS plots and PERMANOVAs were generated using PRIMER (version 6) with the PERMANOVA+ add-on package (Anderson, Gorley & Clarke 2008). Significance was set at $\alpha \leq 0.05$ for all statistical tests.

We used random forests to identify compounds potentially important for differences between gland types, sexes, seasons and individuals. Random forests, an extension of classification tree analysis, make no implicit assumptions about relationships between variables or data distribution and can cope with large numbers of variables (Lunetta *et al.* 2004). Random forests report an overall model error rate and an importance score for each variable (chemical), which measures its weighting for accurate classifications (Lunetta *et al.* 2004; Cutler *et al.* 2007). We used random forests comprising 1000 classification trees to partition the raw chemical relative abundance data into groups (gland type, sex, season, individual). Random forests for discriminating between individuals were based on animals sampled at least five times; the mean interval between sampling from the same individual was 38 days (minimum two days, maximum 18 months). We used separate forests for males and females to identify chemicals important for predicting seasons and individuals, since different chemicals may vary seasonally or between individuals in each sex. As a conservative approach we report the rank top four chemicals based on the unscaled variable importance measure (Strobl, Malley & Tutz 2009). We compared model accuracy with expected accuracy using the "default" classifier (assigning everything to the most common sample class). Forests were generated using the *randomForest* package (Liaw & Wiener 2002) in R (R Core Team 2012).

Results

Mass spectrometers

Cloacal swab profiles differed between the two mass spectrometers (Table 4.2). More compounds were detected in samples analysed using the Bruker instrument (mean ± standard deviation: 39.7±9.6, N=145) than the Varian (29.4±9.15, N=181; unequal variances two-tailed t-test, t_{304}=9.79, P<0.0001). We then split the dataset according to mass spectrometer to test for sex, seasonal and individual effects.

Glandular differences

All 56 compounds identified in wax secretions were found in both spur and cloaca samples, but there were differences in overall composition (Fig. 4.1, Table 4.2). Random forests predicted the response variable (gland type) based on chemical profiles and classification accuracy was high (95.1%); C_{26}-C_{28} fatty acids,

desmosterol and desmostanol appeared most important for these differences (Table 4.3). Spur and cloacal secretions were then analysed separately for differences between sexes, seasons and individuals.

Sex and seasonal differences

Cloacal swab profiles varied significantly between sexes, seasons and years in both mass spectrometer datasets (Fig. 4.2; Table 4.2). Random forest classification accuracy for predicting sex was 68.5% (Varian) and 66.9% (Bruker), expected accuracy from the default classifiers was 61.3% (Varian) and 53.8% (Bruker). Two compounds were consistently important for sex differences and were more abundant in male than female samples: (*9Z,12Z*)-9,12-octadecadienoate (ethyl linoleate) and a branched ethyl ester of heptadecanoic acid. Classification accuracy for predicting season was ~60% (Varian) and ~80% (Bruker) for both males and females, although in the Bruker dataset this was not appreciably better than selecting the most common class by default. No chemicals were consistently identified as important for seasonal differences. The significance of some interaction terms differed between mass spectrometers (Table 4.2, Supporting Information Table 4.S2).

All 56 compounds were present in both male and female cloacal and spur wax secretions, but varied in their relative abundances. Cloacal secretions from males and females were different year round and varied seasonally and between years (Table 4.2, Fig. 4.3): female samples contained relatively higher abundances of sterols than did male samples (classification accuracy for predicting sex: 85.7%; Table 4.3; Fig. 4.4). Male cloacal secretions increased in relative abundances of C_{14}-C_{24} fatty acids during the breeding season (classification accuracy for predicting season: 84.4%; Table 4.3). Female cloacal secretions seemed to increase in relative abundances of fatty acids and desmosterol during the breeding season, but classification accuracy was not appreciably better than the default classifier (Table 4.3). There was no difference in the number of compounds present between sexes (males: 45.1±4.02; females: 44.8±4.04; unequal variances two-tailed *t*-test, t_{162}=0.49, *P*=0.62).

There was a significant sex*season interaction for spur wax secretions (Table 4.2): males and females were different year round (classification accuracy for predicting sex: 96.4%; Fig. 4.3; Fig. 4.4; Supporting Information Table 4.S3), but male secretions showed stronger seasonal changes than did female secretions, reflected in the higher classification accuracy for predicting season for males than females (Table 4.3) and clear separation of male breeding season samples (Fig. 4.3). Male spur secretion contained more compounds (males: 48.61±4.15; females: 44.5±3.02; unequal variances two-tailed *t*-test, t_{276}=9.60, P<0.0001) and higher abundances of C_{16}-C_{18} fatty acids than in females and C_{14}-C_{16} fatty acids increased during the breeding season (Table 4.3). Female spur secretion contained relatively more sterols and steroid derivatives which appeared to differ seasonally, although model accuracy for predicting season was not appreciably better than the default classifier (Table 4.3).

Individual differences

Cloacal swab profiles varied between individuals (Table 4.2), but random forest classification accuracy for predicting individual was higher than for the default classifier (sexes pooled due to low sample size; Varian dataset: 21.2%: N=151 samples, n=12 individuals; Bruker dataset: 28.4%; N=74, n=9), but no chemicals were consistently identified as important. Spur and cloacal wax secretion profiles also varied between individuals (Table 4.2; Fig. 4.5); classification accuracy was high for both spur (70.1%; N=207, n=19) and cloacal wax secretions (51.3%; N=113, n=12). Desmosterol, cholest-4-en-3-one and unsaturated C_{18} fatty acids were important for individual differences in spur wax secretions; desmostanol, cholesterol and C_{15}-C_{16} fatty acids were important for individual differences in cloacal wax secretions.

Discussion

Volatile and nonvolatile scent components differed between seasons, sexes, individuals and gland types in echidnas. Such variations suggest chemical signals could be used for individual identification or for identifying sex and reproductive status in conspecifics, so are probably important for coordinating reproductive behaviour. Scent chemicals from the spur and cloaca showed different seasonal

patterns in males and females, suggesting functional differences. However, there also appears to be some redundant information, since for example, sex differences were detected in all three sample types.

Echidnas appear to follow a typical mammalian pattern in which sex and reproductive status are advertised by changes in overall chemical 'profiles' (Johnston 2003; Wyatt 2003; Thomas 2011). Alternatively, a sex- or season-specific chemical could have been present, but below instrumental detection limits or quantification thresholds. Volatile cloacal profiles varied seasonally and between sexes, so could function as wide-ranging mate attractants or to signal reproductive condition (Alberts 1992; Johansson & Jones 2007). However, it was difficult to identify which volatiles were important for sex or seasonal differences because of detection differences between mass spectrometers (e.g. the superior sensitivity of the Bruker instrument improved detection of diagnostic ions). As in other species, sex-differences in volatiles could help male echidnas to quickly and efficiently locate widely dispersed females (Swaisgood *et al.* 2002; Johansson & Jones 2007; Thomas 2011). Nonvolatile composition of both spur and cloacal wax secretions also varied significantly with sex and season, but was most obvious for spur secretions. Although many of the less volatile compounds identified in echidnas are probably too large to be airborne cues or long-range sex attractants (Alberts 1992), they could advertise sex, identity or physiological status and require direct contact for detection (Swaisgood *et al.* 2002; Wyatt 2003). For example, nonvolatile female cues could function in mate recognition and stimulate male courtship behaviour. Subtle seasonal changes (perhaps not detected by all of our statistical analyses) could also provide information on female reproductive status. The stimuli for any seasonal changes in female chemical signals are unclear, particularly in Tasmania where female hibernation and mating activity can overlap (Morrow & Nicol 2009).

Nonvolatile chemical signals in spur wax secretions appear to be important for communication in male echidnas and to be under endocrine control. Changes in male spur secretion during the mating period coincide with peak testosterone concentration (Nicol, Andersen & Jones 2005; Morrow 2013) and crural gland size and activity (Krause 2010; Morrow 2013). The echidna spur-crural gland apparatus was previously thought to be vestigial (Augee 2008), but the crural gland

transcriptome appears associated with steroid and fatty acid production (Wong *et al.* 2013), which are abundant in spur secretions. Testosterone appears to influence signal composition, scent gland activity and marking behaviour (e.g. Ferkin *et al.* 1994; Rasmussen *et al.* 1996), potentially advertising social dominance, aggression or age (Arteaga *et al.* 2008; Tobey *et al.* 2009; Setchell *et al.* 2010). Testosterone could also influence chemical signals and reproductive success in male echidnas, although experimental studies and genetic information (e.g. paternity) are needed to test these hypotheses.

Many species showing little obvious sexual dimorphism are dimorphic in their odour signals (Blaustein 1981; Andersson 1994). Sexual selection seems to result in sexually dimorphic chemical signals, scent gland development or marking behaviour in several mammals (Penn & Potts 1998; Scordato & Drea 2007; Voigt *et al.* 2008). Sexual size dimorphism is low in echidnas (Nicol *et al.* 2011) and spur secretions appear to be sexually selected: only males have spurs as adults; males produce more secretion than do females; male spur secretion contains more compounds than does that of females and changes significantly during the mating period, whereas seasonal differences in female spur secretion were more subtle. Male spur secretions were particularly rich in C_{14}-C_{18} fatty acids, which increased during the mating season. Such compounds or their derivatives may be costly to produce and could advertise competitive ability or quality to potential mates and competitors (Johansson & Jones 2007; Boulet *et al.* 2010).

Volatile and nonvolatile chemical profiles varied between individual echidnas, but individuals could only be predicted accurately using nonvolatile profiles. In other species, individual-specific chemical signals may advertise individual presence (Johnston 2003), mediate social dynamics (Scordato & Drea 2007; Burgener *et al.* 2009) or help individuals to avoid encounters with dominant individuals (Johnston 2003; Setchell *et al.* 2010). Echidnas have high home range fidelity (Nicol *et al.* 2011) and individual recognition via nonvolatile chemical cues at latrines could advertise home range and resource use (Sprent, Andersen & Nicol 2006). We suggest chemical signals are important functions in echidna reproductive and social behaviours, and help explain the echidna's large and complex cerebral cortex and olfactory bulb.

Table 4.1. Details of odorant samples collected from 69 wild, reproductively active male and female echidnas and subsequently analysed by GC-MS. Sample sizes reported as counts of odorant samples collected (number of individuals sampled).

Sample type[a]	**Sex**	**Time of year sampled**			
		Hibernation	**Breeding**	**Non-breeding**	**Total**
Cloacal swab	Male	20 (10)	116 (40)	47 (11)	183 (40)
	Female	12 (7)	104 (26)	32 (11)	148 (29)
Cloaca wax	Male	5 (4)	50 (22)	22 (9)	77 (22)
	Female	4 (4)	73 (21)	14 (6)	91 (21)
Spur wax	Male	16 (10)	105 (40)	44 (11)	165 (40)
	Female	10 (7)	72 (25)	32 (12)	114 (28)

[a] Sample size is not equal between the three sample types: cloacal swabs were collected each time an animal was captured, whereas wax secretion samples from the cloaca and spur were dependent on the presence of sufficient secretion to be collected at a given time. Animals produced little, if any, cloacal wax secretion during hibernation. The number of samples collected also varies between animals, as echidnas are cryptic, semi-fossorial, and frequently select shelter sites where they are inaccessible.

Table 4.2. Results of PERMANOVAs comparing chemical profiles of odour samples collected from adult male and female echidnas. Interactions involving the nested term 'Animal ID (Sex)' could not be tested as not all individuals were sampled in each season or year, resulting in an unbalanced PERMANOVA design (Anderson *et al.* 2008). All wax secretion samples were analysed using the Bruker mass spectrometer. Significant results in bold type.

Sample	Term	df	SS	MS	Pseudo-*F*	*P* (perm)
Cloacal swab	Mass Spec.	1	34459	34459	26.16	<**0.001**
	Residuals	324	427000	1317	-	-
Bruker mass spec.	Year	1	8823	8823	6.90	<**0.001**
	Sex	1	5352	5352	2.20	**0.04**
	Season	2	8214	4107	3.81	<**0.001**
	Animal ID (Sex)	47	66383	1412	1.56	<**0.001**
	Sex*Season	2	1497	749	0.83	0.60
	Residuals	91	82153	903	-	-
Varian mass spec.	Year	1	12246	12246	9.50	<**0.001**
	Sex	1	11053	11053	3.59	<**0.01**
	Season	2	8671	4336	3.52	<**0.001**
	Animal ID (Sex)	32	46494	1453	1.22	<**0.01**
	Sex*Season	2	6335	3167	2.65	<**0.01**
	Residuals	142	169550	1194	-	-
Wax secretion	Gland type	1	13839	13839	63.07	<**0.001**
	Residuals	445	97642	219	-	-
Spur	Year	2	6189	3094	15.63	<**0.001**
	Sex	1	21309	21309	35.92	<**0.001**
	Season	2	4302	2151	18.33	<**0.001**
	Animal ID (Sex)	67	14854	222	2.52	<**0.001**
	Sex*Season	2	1303	711	8.09	<**0.001**
	Residuals	204	17940	88	-	-
Cloaca	Year	2	5214	2607	12.34	<**0.001**
	Sex	1	1676	1676	3.96	<**0.001**
	Season	2	1389	695	5.06	<**0.001**
	Animal ID (Sex)	42	9200	219.0	1.88	<**0.001**
	Sex*Season	2	394	197	1.69	0.06
	Residuals	118	13752	117	-	-

Table 4.3 (continued over page). Summary of chemical variables identified by random forests (RF) as potentially important for sex, seasonal and individual differences between spur and cloacal wax secretion samples collected from male and female short-beaked echidnas. Rank top four chemicals reported. 'Direction' indicates which group (gland type, sex or season; e.g. 'breeding season') had the higher (+) or lower (-) relative abundance of each compound. Numbers in parentheses: expected model accuracy using the default classifier.

Sample	Test	RF % correct	Chemicals	Direction
Wax secretion	Gland type	95.1 (62.4)	Desmostanol (TMS)	+ spur
			n-Octacosanoic acid (TMS)	+ spur
			17-Hexacosenoic acid (TMS)	+ cloaca
			Desmosterol (TMS)	+ spur
Spur	Sex	96.4 (59.1)	Desmostanol (TMS)	+ female
			(9*Z*)-9-Hexadecenoic acid (TMS)	+ male
			Desmosterol (TMS)	+ female
			Cholesterol (TMS)	+ female
	Season (M)	87.9 (61.8)	11-Hexadecenoic acid (TMS)	+ breeding
			n-Tetradecanoic acid (TMS)	+ breeding
			(9*Z*)-9-octadecenoic acid (TMS)	+ breeding
			n-Hexadecanoic acid (TMS)	+ breeding
	Season (F)	67.5 (63.2)	Desmosterol (TMS)	+ breeding
			Cholesta-2,4-diene	- breeding
			Cholesta-3,5-diene	- breeding
			Cholesterol (TMS)	+ breeding
	Individuals (M)	69.1 (19.0)	Desmosterol (TMS)	-
			Cholest-4-en-3-one (TMS)	-
			Cholestan-3-ol (TMS)	-
			n-Hexacosanoic acid (TMS)	-
	Individuals (F)	72.4 (21.6)	Cholest-4-en-3-one (TMS)	-
			23-Dotriacontenoic acid (TMS)	-
			(9*Z*, 12*Z*)-9-Octadecadienoic acid (TMS)	-
			n-Docosanoic acid (TMS)	-
Cloaca	Sex	85.7 (54.2)	Desmosterol (TMS)	+ female
			Cholesterol (TMS)	+ female
			Icosanoic acid (TMS)	+ female
			(9*Z*)-9-octadecenoic acid (TMS)	+ male
	Season (M)	84.4 (61.0)	Tetradecanoic acid (TMS)	+ breeding
			11-Hexadecenoic acid (TMS)	+ breeding
			19-Tetracosenoic acid (TMS)	+ breeding
			(9*Z*)-9-octadecenoic acid (TMS)	+ breeding
	Season (F)	82.6 (80.2)	Icosanoic acid (TMS)	+ breeding
			Desmosterol (TMS)	+ breeding
			(9*Z*)-9-octadecenoic acid (TMS)	+ breeding
			23-Dotriacontenoic acid (TMS)	+ breeding

	Individuals (M)	50.0 (27.4)	*n*-Hexadecanoic acid (TMS) -
			Coprostenol (TMS) -
			Cholestan-3-ol (TMS) -
			11-Hexadecenoic acid (TMS) -
	Individuals (F)	34.9 (31.2)	*n*-Pentadecanoic acid (TMS) -
			Desmostanol (TMS) -
			11-Hexadecenoic acid (TMS) -
			15-Icosenoic acid (TMS) -

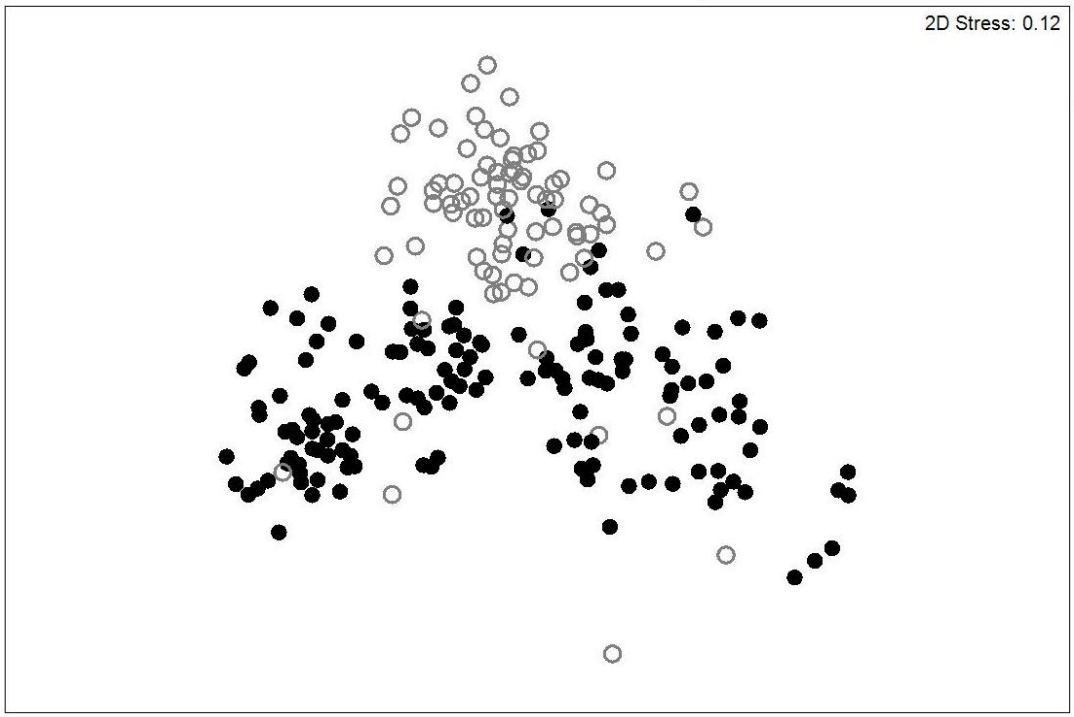

Figure 4.1 MDS plot showing differentiation in chemical profiles of spur (●) and cloacal (○) wax secretions from adult male and female echidnas; data from 2011-2012 shown. Axes are dimensionless. Stress = 0.12. Stress is a measure of 'goodness of fit', which evaluates how well the 2D configuration represents the observed distance matrix in relation to the original data. Values between 0.10 and 0.2 indicate the plot corresponds to a usable representation of the data (McCune *et al.* 2002; Burgener *et al.* 2009).

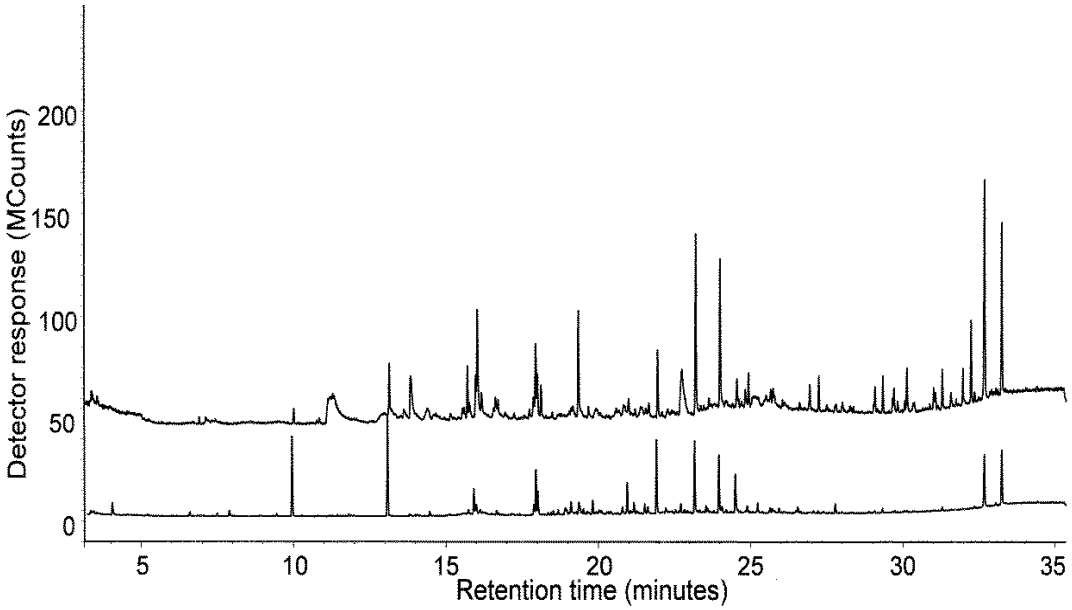

Figure 4.2. Example TICs of cloacal swabs collected from female echidna 2957 during the breeding (upper) and non-breeding (lower) season in 2010. Both samples analysed by thermal desorption and then GC-MS using the Varian gas chromatograph and mass spectrometer.

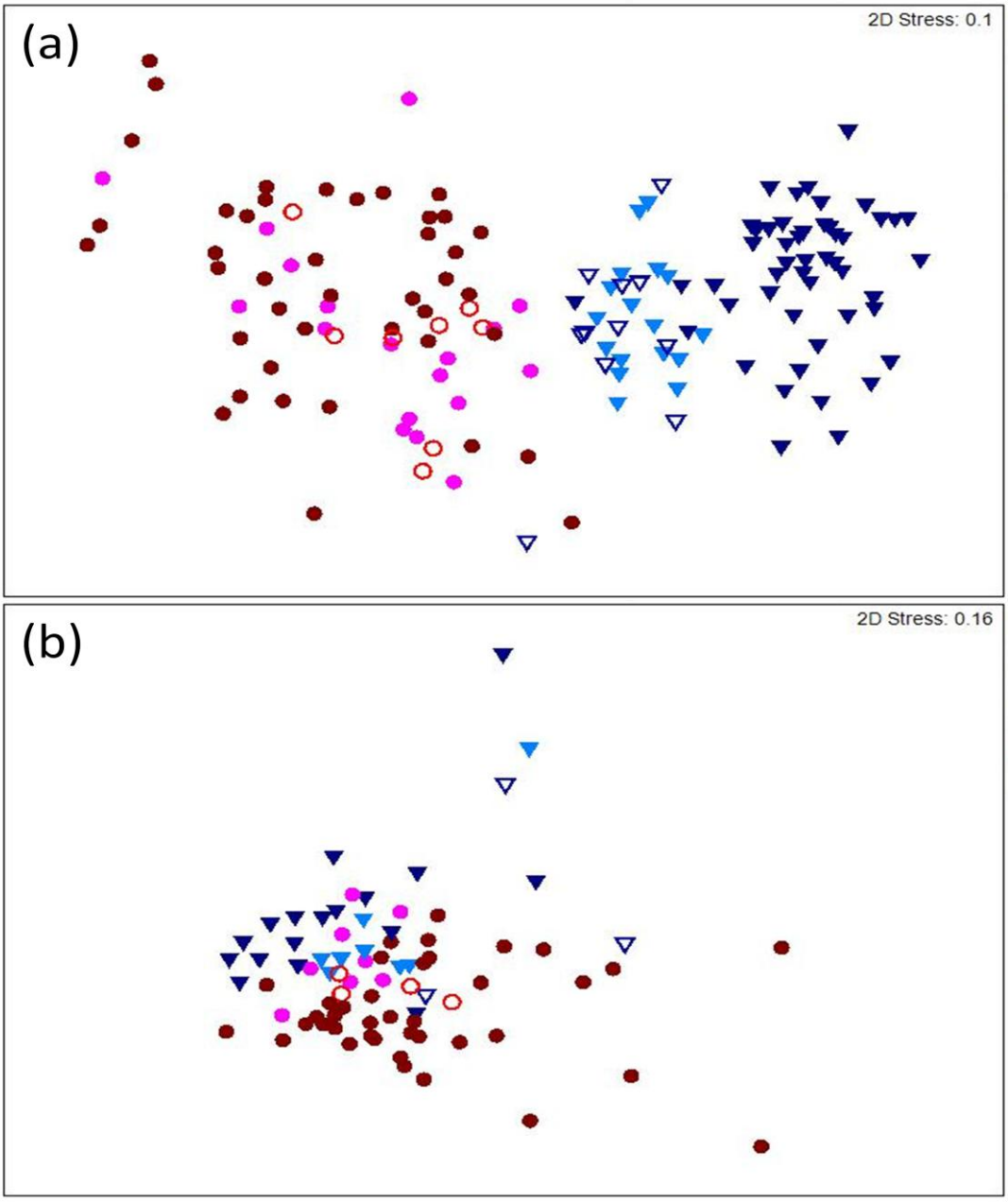

Figure 4.3. MDS plot showing sex and seasonal differentiation between male (breeding ▼, nonbreeding ▼, hibernating ▽) and female (breeding ●, nonbreeding ●, hibernating ○) echidna glandular secretions; (a): spur; (b): cloaca; data from 2011-2012 shown. Stress = 0.1 (spur), 0.16 (cloaca).

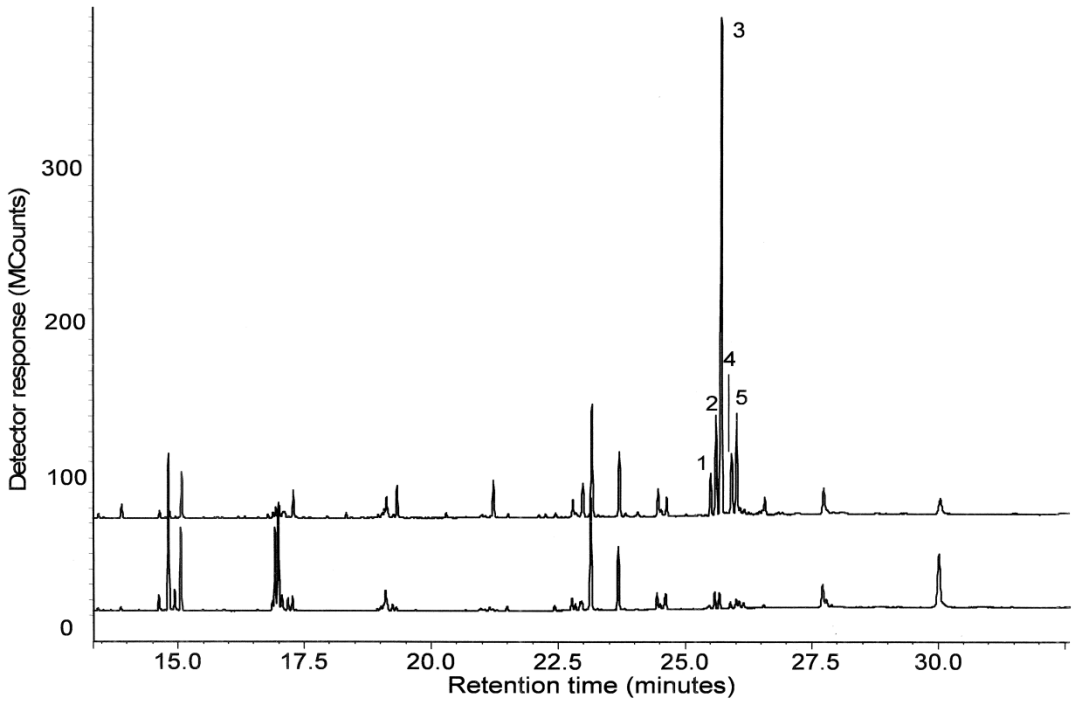

Figure 4.4. Example TIC of TMS-derivatised spur wax secretion from (a) female (animal 0F24) and (b) male (animal 2E00) echidnas during the 2011 mating season. Labelled peaks correspond to sterols, some identified as important for differences between sexes and seasons: (1) coprostenol, (2) cholesterol, (3) cholestanol, (4) desmosterol, (5) desmostanol.

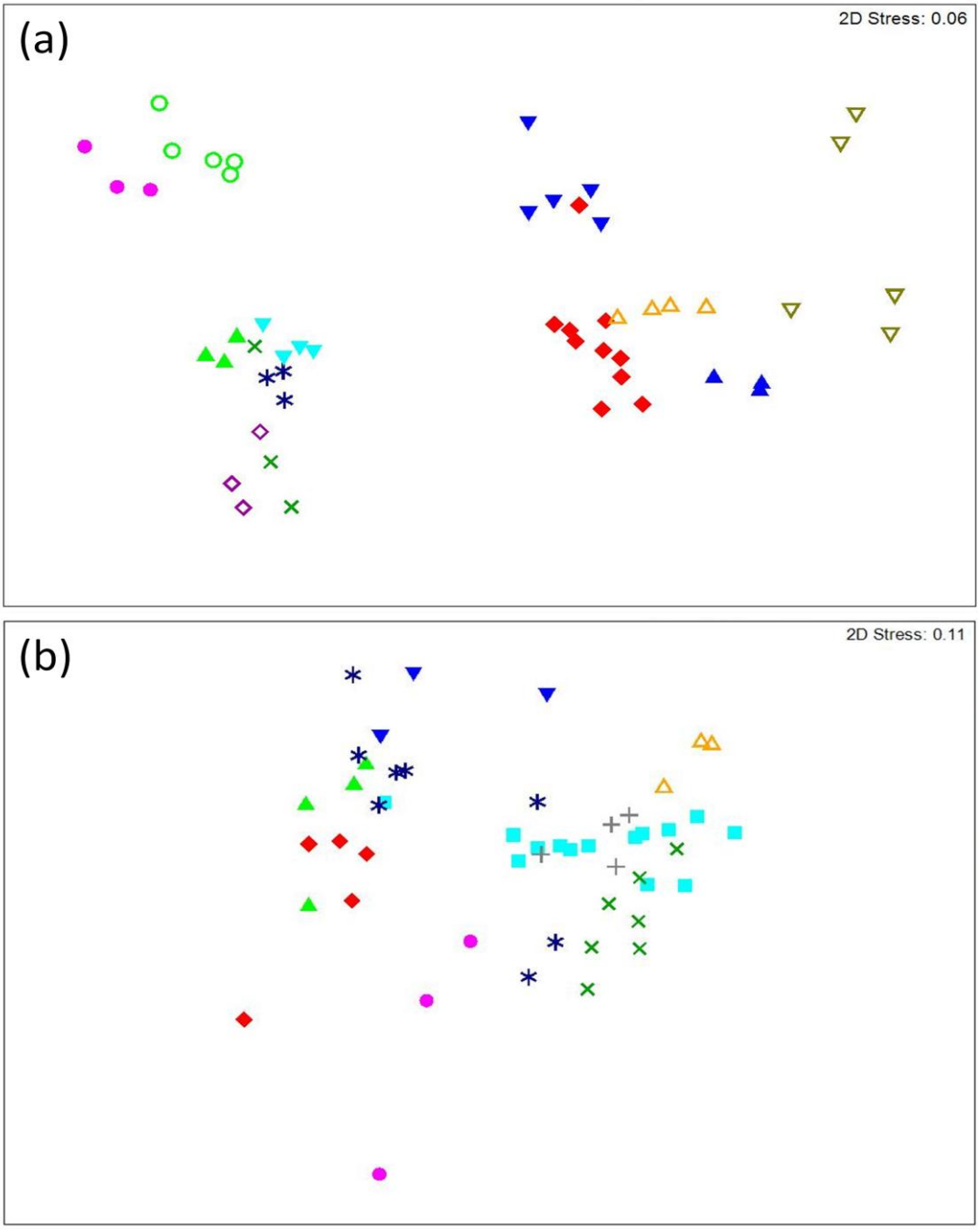

Figure 4.5. MDS plots showing differentiation between selected individuals during the mating season; (a) spur secretion; (b) cloacal secretion. Each symbol represents a different individual within each plot. Stress = 0.06 (spur), 0.11 (cloaca).

Supporting Information

Table 4.S1 (continued over page). Compounds identified in short-beaked echidna cloacal swabs, spur and cloaca wax secretions, and retained for statistical analyses. Compounds are listed in order of their Kovats' retention indices (KI).

Cloacal swab	**KI**[a]	**Cloaca, spur wax secretion**	**KI**
Trimethylamine	-	Methyl tetradecenoate	1710
3-Methylbutanal	652	Methyl *n*-tetradecanoate[b]	1723
Acetic acid	659	Methyl *n*-pentadecanoate[b]	1824
3-Methylbutanol	680	7-Tetradecenoic acid (TMS)	1833
Propanoic acid	716	*n*-Tetradecanoic acid (TMS)	1845
Dimethyldisulfide	725	*n*-Pentadecenoic acid (TMS)	1907
Isobutanoic acid	750	Methyl *n*-hexadecenoate[b]	1908
n-Butanoic acid	779	Methyl *n*-hexadecanoate[b]	1930
3-Methylbutanoic acid	832	*n*-Pentadecanoic acid (TMS)	1943
2-Methylbutanoic acid	840	(*9Z*)-9-Hexadecenoic acid (TMS)	2022
n-Pentanoic acid	886	11-Hexadecenoic acid (TMS)	2032
Dimethylsulfone	897	*n*-Hexadecanoic acid (TMS)	2042
Dimethyltrisulfide	950	Methyl (*9Z,12Z*)-9,12-	2096
Phenol	960	octadecadienoate[b]	
n-Octanal	985	Methyl (*9Z*)-9-octadecenoate[b]	2102
n-Hexanoic acid	994	Methyl 11-octadecenoate 1	2108
1-Methyl-2-pyrrolidinone	1039	Methyl 11-octadecenoate 2	2114
Cresol	1077	Methyl *n*-octadecanoate[b]	2128
n-Nonanal	1103	(*9Z,12Z*)-9,12-Octadecadienoic	2132
Octyl acetate	1158	acid (TMS)	
Benzoic acid	1184	(*9Z*)-9-Octadecenoic acid (TMS)	2213
Benzothiazole	1200	11-Octadecenoic acid 1 (TMS)	2219
n-Decanal	1202	11-Octadecenoic acid 2 (TMS)	2223
Benzeneacetic acid	1274	13-Octadecenoic acid (TMS)	2231
n-Undecanal	1301	*n*-Octadecanoic acid (TMS)	2240
n-Dodecanal	1403	*n*-Icosadienoic acid (TMS)	2407
n-Tridecanal	1506	11-Icosenoic acid (TMS)	2411
Diethyltoluamide	1555	Unknown (*m/z* 215, 331) (TMS)	2416
Diphenylamine	1610	13-Icosenoic acid (TMS)	2419
Methyl n-tetradecanoate[b]	1723	15-Icosenoic acid (TMS)	2430
n-Tetradecanoic acid	1764	*n*-Icosanoic acid (TMS)	2437
Ethyl *n*-tetradecanoate	1791	13-Docosenoic acid (TMS)	2610
Methyl *n*-pentadecanoate[b]	1824	15-Docosenoic acid (TMS)	2619
Ethyl pentadecanoate, branched	1836	17-Docosenoic acid (TMS)	2629
Ethyl 13-methyltetradecanoate	1853	*n*-Docosanoic acid (TMS)	2634
Ethyl 12-methyltetradecanoate	1861	15-Tetracosenoic acid (TMS)	2810
Ethyl *n*-pentadecanoate	1890	19-Tetracosenoic acid (TMS)	2828
Methyl *n*-hexadecenoate[b]	1908	*n*-Tetracosanoic acid (TMS)	2832
Ethyl hexadecanoate, branched	1930	Cholesta-2,4-diene[b]	2848
Methyl *n*-hexadecanoate	1930	Cholesta-3,5-diene[b]	2911
(*9Z*)-9-Hexadecenoic acid[b]	1944	17-Hexacosenoic acid (TMS)	3011

Ethyl 14-methylpentadecanoate	1954	19-Hexacosenoic acid (TMS)	3018
Ethyl (*9Z*)-9-hexadecenoate	1970	*n*-Hexacosanoic acid (TMS)	3032
n-Hexadecanoic acid	1977	Coprostenol (TMS)	3137
Ethyl *n*-hexadecanoate	1992	Cholesterol (TMS)	3151
Juvabione	2021	Cholestanol (TMS)	3162
Ethyl heptadecanoate, branched	2036	Desmosterol (TMS)	3190
Ethyl 15-methylhexadecanoate	2055	Desmostanol (TMS)[c]	3202
Ethyl 14-methylhexadecanoate	2064	19-Octacosenoic acid (TMS)	3210
Ethyl *n*-heptadecanoate	2094	21-Octacosenoic acid (TMS)	3218
Methyl (*9Z,12Z*)-9,12-octadecadienoate[b]	2096	*n*-Octacosanoic acid (TMS)	3229
		Cholest-4-en-3-one (TMS)	3274
Methyl (*9Z*)-9-octadecenoate[b]	2102	21-Triacontenoic acid (TMS)	3410
Methyl *n*-octadecanoate[b]	2128	*n*-Triacontanoic acid (TMS)	3426
Ethyl octadecanoate, branched	2132	22-Hentriacontenoic acid (TMS)	3508
(*9Z,12Z*)-9,12-Octadecadienoic acid	2139	23-Dotriacontenoic acid (TMS)	3612
Ethyl (*9Z,12Z*)-9,12-octadecadienoate	2162	24-Tritriacontenoic acid (TMS)	3714
		25-Tetratriacontenoic acid (TMS)	3811
Ethyl (*9Z*)-9-octadecenoate	2168		
n-Octadecanoic acid	2173		
Ethyl *n*-octadecanoate	2195		
Ethyl *n*-nonadecanoate	2295		
Ethyl *n*-icosanoate	2397		
Ethyl *n*-henicosanoate	2498		
Ethyl *n*-docosanoate	2598		
Ethyl 15-tetracosenoate	2774		
Ethyl *n*-tetracosanoate	2797		
Cholesta-2,4-diene[b]	2848		
Cholesta-3,5-diene[b]	2911		
Ethyl 17-hexacosenoate	2975		

[a]Calculated Kovats' retention indices and identification criteria from Harris *et al.* (2012).

[b]Compounds identified using both types of analyses (n = 9).

[c]Previously reported as 'unknown sterol (*m/z* 255, 345)', for identification details see Harris, R.L., Davies, N.W., and Nicol, S.C. (2013). Identification of desmostanol as a novel vertebrate sterol in short-beaked echidna secretions. *Aust. Mammal.* **35**, 255-258.

Table 4.S2. Results of PERMANOVAs comparing chemical profiles of odour samples collected from adult male and female echidnas, including interaction terms involving 'Year'. Some interaction terms could not be tested due to the unbalanced PERMANOVA design (Anderson *et al.* 2008). All wax secretion samples were analysed using the Bruker mass spectrometer. Significant results in bold type.

Sample	Term	df	SS	MS	Pseudo-*F*	*P* (perm)
Cloacal swab	Mass Spec.	1	34459	34459	26.16	<**0.001**
	Residuals	324	4.27E5	1317.2	-	-
Bruker mass spec.	Year	1	8823.4	8823.4	6.89	<**0.001**
	Sex	1	5352	5352	2.20	**0.04**
	Season	2	8214	4107	3.81	<**0.001**
	Animal ID (Sex)	47	66383	1412.4	1.56	<**0.001**
	Year*Sex	1	632.8	632.8	0.69	0.66
	Sex*Season	2	547.2	273.6	0.33	0.63
	Residuals	90	81544	906.0	-	-
Varian mass spec.	Year	1	12246	12246	9.50	<**0.001**
	Sex	1	11053	11053	3.59	<**0.01**
	Season	2	8670.9	4335.5	3.52	<**0.001**
	Animal ID (Sex)	32	46494	1452.9	1.22	**0.05**
	Year*Sex	1	3742	3742	3.14	<**0.01**
	Year*Season	2	2596.1	1298	1.09	0.35
	Sex*Season	2	4082.3	2041.1	1.71	0.07
	Residuals	139	19047	1190.5	-	-
Wax secretion	Gland type	1	13839	13839	63.07	<**0.001**
	Residuals	445	97642	219.42	-	-
Spur	Year	2	6188.8	3094.4	15.68	<**0.001**
	Sex	1	21309	21309	35.92	<**0.001**
	Season	2	4301.9	2150.9	18.75	<**0.001**
	Animal ID (Sex)	67	14854	221.7	2.62	<**0.001**
	Year*Sex	2	483.42	214.46	2.86	<**0.01**
	Year*Season	4	857.8	651.43	2.53	<**0.001**
	Sex*Season	2	1302.9	651.43	7.69	<**0.001**
	Year*Sex*Season	4	300.82	75.21	0.89	0.57
	Residuals	194	16418	84.627	-	-
Cloaca	Year	2	5214.2	2607.1	12.35	<**0.001**
	Sex	1	1675.8	1675.8	3.95	<**0.001**
	Season	2	1389.3	694.8	5.12	<**0.001**
	Animal ID (Sex)	42	9199.5	219.0	1.91	<**0.001**
	Year*Sex	1	178.4	178.4	1.56	0.13
	Year*Season	4	663.6	165.9	1.45	0.09
	Sex*Season	2	366.0	183.0	1.59	0.08
	Residuals	113	12938	114.5	-	-

Table 4.S3. Summary of pair-wise comparisons of spur wax secretions by PERMANOVA. Tests conducted for each pair of levels ('Pairs') within a given 'Factor'. For example, first data line: compares male and female spur secretions within the hibernating season; fourth data line: compares hibernating and breeding season samples of spur secretion from males. Significant results in bold type.

Factor	**Pairs**	**t**	**P (perm)**
Hibernating (H)	M-F	2.33	**<0.001**
Breeding (B)	M-F	7.31	**<0.001**
Non-breeding (NB)	M-F	2.83	**<0.001**
Males (M)	H-B	4.65	**<0.001**
	B-NB	6.63	**<0.001**
	H-NB	1.75	**<0.01**
Females (F)	H-B	1.54	**0.04**
	B-NB	2.11	**<0.001**
	H-NB	0.84	0.61

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Chapter 5:

The scent of attraction: male echidnas are attracted to female odour during the mating season

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Abstract

Males of many species use olfactory cues to locate potential mates and assess which females are both available and receptive to mating. Olfaction seems to have an important function in mate attraction in short-beaked echidnas, *Tachyglossus aculeatus*, but has not been confirmed by robust behavioural trials. We tested responses of wild male echidnas to female odour during the breeding and non-breeding seasons using camera traps in the field. More males were attracted to female odour during the breeding season than during the non-breeding season. Investigatory behaviours including sniffing, nose poking and tongue flicking were only directed towards odour from mating females and not towards non-reproductive females or controls. Our quantitative results support the widely-held view that male echidnas use olfactory cues to locate females in reproductive condition during the mating season.

Introduction

In sexually reproducing species, individual fitness can be enhanced by the ability to assess the reproductive state of conspecifics (Johansson and Jones 2007; Thomas 2011; Shine and Mason 2012). Solitary and seasonally-breeding animals also need to find dispersed conspecifics during a narrow window of opportunity, making chemical signals potentially more suitable for communication than other signal modes (Swaisgood *et al.* 2002; Wyatt 2003). In mammals, chemical compounds may contain information on reproductive condition and receptivity to mating, for example via changes in scent marking behaviour (Molteno *et al.* 1998; Ferkin *et al.* 2004) or signal composition (Rasmussen *et al.* 1997; Mozūraitis *et al.* 2012). Males of several species use odour to locate females (Laurie 1982; Molteno *et al.* 1998) and assess female reproductive status (Swaisgood *et al.* 2002; Ferkin *et al.* 2004; Thomas 2011). Therefore, the ability to detect and discriminate between females based on olfactory cues can allow males to minimise search costs associated with pursuing unreceptive females (Swaisgood *et al.* 2002; Johansson and Jones 2007; Thomas 2011). However, olfactory signals are sometimes combined with visual, auditory or tactile signals, making it difficult to determine if males respond to chemical cues alone (Candolin 2003; Scordato and Drea 2007). Repeatable behavioural bioassays are useful for determining whether individuals can detect and respond differently to chemical differences (e.g. sex, seasonal differences), while controlling for other cues (Wyatt 2003; Thomas 2011).

We used field trials to test whether male short-beaked echidnas (*Tachyglossus aculeatus*; 'echidna') use scent to locate females. The echidna is a usually solitary monotreme (Nicol *et al.* 2011), distributed throughout Australia and coastal New Guinea (Augee 2008). Olfactory signals are thought to be important for attracting and locating mates during the breeding season (June-September; Griffiths 1978). Males appear attracted to female scent (Boisvert and Grisham 1988; Rismiller 1992) and mating behaviour in captivity is characterised by males following females (Boisvert and Grisham 1988; Ferguson and Turner 2013). Female echidnas do not breed every year, so many males compete for access to a limited number of females in reproductive condition (Nicol and Morrow 2012). In wild populations, competition results in the formation of mating aggregations of one female with one

or more males (Morrow *et al.* 2009), or even 'trains' of up to 10 males following a female in single file (Griffiths 1978; Rismiller 1992). In the Tasmanian subspecies, *T. a. setosus*, male echidnas may use female olfactory cues in the absence of visual or auditory cues to locate hibernating females (Harris *et al.* 2012; Chapter 2), and even mate within a female's hibernaculum (Morrow and Nicol 2009). Non-reproductive females are sexually mature but do not breed in a given year, and usually hibernate through the mating season (Nicol and Morrow 2012), undisturbed by males (unpublished obs.; Chapter 6).

Although olfaction appears important for male echidnas to locate females, no robust behaviour trials have been conducted to test whether males are attracted to female scent. Males on Kangaroo Island were attracted to hessian bags that had contained females (Rismiller 1992), but other cues could have been used since bags were left close to where females were previously found in mating trains. Unlike scent trials in captivity, field trials have the advantage of integrating an animal's ability to detect, localise and discriminate odour in the response. We used experimental field trials, while controlling for non-scent cues, to test the responses of male echidnas to female odour during the breeding and non-breeding seasons. We hypothesised that male echidnas would be attracted to reproductive female odour, with the strongest response being observed during the mating season.

Materials and methods

Study site

We studied a wild population of echidnas on a grazing property in the Tasmanian southern midlands, approximately 55 km north of Hobart (42°28'S, 142°14'E) (details see Nicol *et al.* 2011). Selected individuals were fitted with radiofrequency transmitters for serial sampling (Nicol *et al.* 2011), GPS loggers (CatTrack, Perthold Engineering, SC, USA) and colour-coded straws for visual identification of individual echidnas in camera footage (Morrow and Nicol 2012).

Pillowcase trials

These preliminary experiments were designed to validate the results of the study by Rismiller (1992), which used hessian bags to test responses of male echidnas to

female whole body odour, except we controlled for non-scent cues by decoupling the scents from the mating group both temporally and spatially. We compared male responses to scent from reproductive females during the breeding and non-breeding seasons, and responses to scent from non-reproductive females and 'control' pillowcases during the non-breeding season. Females found in mating aggregations were placed inside a cotton pillowcase for 2-4 hours (mean ± standard deviation; 2.79 ± 0.69, $n = 7$) before being released at their place of capture; the male(s) had always dispersed by this time. The pillowcase and a camera were then positioned at least 100 m from where the female was captured (usually ~500 m). Cameras (Scoutguard SG550, SG560, HuntingCamOnline, Gadsden, SC, USA) and pillowcases were placed in locations commonly used by echidna mating aggregations, such as inside stumps, basal tree hollows, hollow logs or windrows (Morrow *et al.* 2009). Each trial ran for 3-7 nights, using odour from different individual females, and trials were in different locations within the study area. Two identical pillowcases were used for all scent trials during the mating season and were deliberately not washed between trials. Hence the scent from several females accumulated and contributed to an overall 'female' scent, which maximised the likelihood of attracting a male and confirming the results of Rismiller (1992). We tested male responses to odour collected from mating females during the mating season ($n = 7$ trials) and during the non-breeding season (October; $n = 3$). Although the pillowcases had not been stored frozen prior to the non-breeding season trials, they still smelt strongly of echidna. After completing these trials, the pillowcases were washed with hot water and detergent to remove the remaining scent. Standard sensitivity settings were used for cameras, which recorded 30 sec videos when triggered, with a 20 sec delay between videos. Memory cards were downloaded every 3-7 days and batteries were replaced when necessary. Video footage was examined using Windows Media Player software and visit duration calculated using the video time stamps.

We also conducted pillowcase trials during the non-breeding season (November-December) using scent from non-breeding females ($n = 6$) and 'control' pillowcases ($n = 7$). Non-breeding female scent was collected from females that had not shown signs of mating behaviour during the 2012 breeding season. A clean 'control' pillowcase, which had not come into contact with echidnas, was used to test if

animals were attracted to the pillowcase or to human scent rather than the scent of another echidna. Each trial during the non-breeding season lasted seven nights, using the same pillowcases repeatedly but a new location for each scent trial (i.e. pillowcase 'A' for all non-breeding female trials, pillowcase 'B' for all control trials). The results from two pillowcase trials (one non-breeding female trial, one control trial) were excluded due to camera failures.

Cloacal swab sample collection

In the second, more robust set of trials, we focussed on male responses to female cloacal odour using camera traps. Female echidnas have been observed scent marking or 'cloaca dragging' in the wild (Beard *et al.* 1992) and in captivity (Dobroruka 1960; Boisvert and Grisham 1988). The 'waxy' secretion from cloacal scent glands is chemically complex and possibly contains information on sex or reproductive status (Harris *et al.* 2012; Chapter 2). We collected 64 cloacal swabs from 44 captures of 15 individual female echidnas in mating aggregations (in close proximity to one or more males) between June and September 2012. Cloacal swabs were collected while the females were under light inhalation anaesthesia (4% isoflurane in oxygen) by wiping the cloaca with a section of glass fibre filter paper (Advantec, Japan, ø 47 mm) held with surgical forceps. Swabs were stored in individual glass vials on ice while in the field and then at -20 °C to preserve volatile scent components until they were used for scent trials (Harris *et al.* 2012; Chapter 2). Swabs were randomly selected for each trial and each swab was only used once.

Cloacal swab trials

Male responses to female cloacal odour were recorded by 4 camera traps set up approximately 100 m apart along a 400 m transect. The camera trap transect area was chosen because it had several overlapping male and female home ranges and for ease of access and central location within the study site. The habitat immediately surrounding each camera trap was consistent (open, dry vegetation). Each camera trap consisted of 2 infrared, motion-activated cameras directed at a single female cloacal swab housed inside a perforated 5 mL plastic container and placed in locations commonly used by echidna mating aggregations (similar to pillowcase trials). We used 2 cameras to maximise the probability of detecting animals and to collect behaviour footage from different angles. Cameras were attached to branches

or wooden stakes approximately 1 m above the ground at an approximately 90° angle to each other. Plastic containers containing scents were attached to branches using cable ties about 30 cm above ground level and 1-2 m in front of the cameras. Camera traps with empty plastic containers were established 2 weeks before scent trials so they were not novel objects in the environment. Scent trials (n = 28 over 4 camera trap sites) were conducted over 7 weeks between July and September 2012 until mating activity ceased and repeated for 11 weeks during the non-breeding season (October–December 2012). Trials ran for 3-7 nights, and cameras were left unbaited with empty plastic containers for 3 or 4 nights between most trials. A new female swab was used for each trial. Plastic containers were cleaned with hot water and detergent between trials.

Statistical analyses

We calculated the number of echidnas detected per night to allow for variations in trial duration. Chi-squared tests were carried out using Statistica software. We used Yates corrections to adjust for the small number of responses. Student's *t*-tests were used to compare means. Results are reported as means and standard deviations.

Results

Pillowcase trials

Three echidnas (all known males; echidnas 1B75, 2E00, 6D5B) were recorded at pillowcases with scent from a mating female (total 50 nights, breeding and non-breeding season trials combined) and none were recorded at control pillowcases (non-reproductive female scent and control scent combined, 35 and 42 nights respectively), although this was not a significant difference ($\chi^2_1 = 2.49$, $P = 0.115$ after Yates correction). One male was recorded during the breeding season (visit duration: 246 sec) and 2 males during the non-breeding season (durations: 390, 87 sec). The latency for males to approach pillowcases was less than 6 hours during the breeding season, and 4 and 7 days during the non-breeding season, but sample size was too small to test significance. All 3 males showed olfactory behaviours directed at the pillowcases, including sniffing and nose poking.

Cloacal swab trials

Seven echidnas were recorded by cameras over 592 nights, all at traps with odour from mating females and none at unbaited cameras ($\chi^2_1 = 3.92$, $P = 0.048$ after Yates correction; Fig. 5.1). More echidnas were recorded at traps with odour from mating females during the breeding season (including 3 known males, echidnas 1B75, 5115, 7C0F) than non-breeding season, although this was not significant ($\chi^2_1 = 2.89$, $P = 0.09$; Fig. 5.1). Mean visit duration tended to be longer during the breeding season (115 ± 122.5 sec, $n = 5$, range: 2-348 sec) than during the non-breeding season (24 ± 2.83 sec, $n = 2$, range: 22-26 sec) although not significantly (two-tailed *t*-test, unequal variances, $t_3 = 1.70$, $P = 0.18$). The wide variation in visit duration for the breeding season may be due to some cameras not recording animal arrival or possible inspection of scent. Tongue-flicking towards female scent was only observed during the mating season and no animals showed interest in the scent vials during the non-breeding season. The latency to record an echidna at a scent vial varied from approximately 6 hours to 6 days (mean latency during breeding season: 56.0 ± 50.7 hours, range: 4-150 hours; non-breeding season: 74.0 ± 18.0 hours, range: 56-92 hours; two-tailed *t*-test, unequal variances, $t_4 = 0.57$, $P > 0.5$). The variance in latency to approach during the breeding season is largely due to 1 male which approached after approximately 150 hours, all other males approached within 53 hours.

Discussion

Our results show that male echidnas were attracted to female odour in both field trials. Five individual males were positively identified based on the position of GPS loggers, radio-transmitters and colour-coded straws. No echidnas were recorded at 'unbaited' camera traps or 'control' pillowcases, indicating that animals were attracted to female odour and not the testing equipment. Although not significant after correcting for the small number of responses, more males tended to be attracted to odour from mating females and showed more responses such as sniffing, nose poking and tongue flicking during the breeding than non-breeding season. We therefore provide supporting evidence that male echidnas are attracted to female scent and probably use scent to locate mates. Our results also support the previous qualitative study which showed that males were attracted to hessian bags

containing female scent during the breeding season (1992). However, our trials decoupled the scent from the original female location, so males could not simply be attracted to the mating aggregation or use visual cues and confirmed that female cloacal scent is attractive to males.

The low numbers of responses observed in this study were to be expected, as detection probability was likely to be low and trials were conducted within a single field season. Although the study area was chosen for the relatively high numbers of overlapping home ranges, male home ranges may be over 100 ha, more than twice the size of females' (Nicol *et al.* 2011), hence males are thinly distributed in the environment. Male response to female scent will depend on males being close enough to detect the scent stimulus, and we have never observed more than four males in a mating aggregation in 18 years at this study site (Morrow *et al.* 2009). These observations could be a function of population density, which is difficult to estimate in a cryptic species such as the echidna (Nicol *et al.* 2011). We believe the number of responses and strength of the results would benefit from another season of field data. Clearly, trials conducted in captivity are also necessary for more specific hypothesis testing; such trials were attempted in this study but not successful (Appendix II).

Male response to female odour varies with oestrous cycle stage in many mammals (Swaisgood *et al.* 2002; Ferkin *et al.* 2004; Bagley *et al.* 2006; Mozūraitis *et al.* 2012); however plasma oestradiol in female echidnas is undetectable using standard RIA techniques (Dean 2000; Jones and Nicol unpublished data), making investigating the oestrous cycle difficult. Progesterone profiles and urogenital cytology have successfully been used to confirm pregnancy and show that non-reproductive females do not cycle during the mating season, but neither technique could be used to measure an oestrous cycle (Morrow 2013). Female Tasmanian echidnas appear to be induced ovulators (Morrow and Nicol 2009) during the early part of the mating season, so proximity to male odour could be important for reproduction (which could also be tested using behavioural trials). Mating aggregations may last for 14-44 days in *T. a. multiaculeatus* (Rismiller 1992; Rismiller and McKelvey 1996, 2000) and female Tasmanian echidnas attract males and show mating activity over several weeks, including during pregnancy (Morrow

2013). Gas chromatography-mass spectrometry analyses show there is no detectable change in female chemical profiles between fertilisation and pregnancy (unpublished data; Chapter 6); therefore we believe our approach of using scent from any females found in mating aggregations is valid.

Male reproductive success can depend on the ability to locate females and assess reproductive status, so males should show heightened interest in the scent of females in breeding condition (Scordato and Drea 2007; Thomas 2011), which was observed in this study. As in other species, male echidnas may increase their reproductive success by being sensitive to sex-specific differences in scent gland secretions (Harris *et al.* 2012; Chapter 2; Harris *et al.* in press; Chapter 4), show preferences for odour from females in breeding condition and be capable of locating these females using odour (even within female hibernacula, Morrow and Nicol 2009), thereby reducing time and effort wasted searching for non-receptive females or other males (Johansson and Jones 2007; Thomas 2011). All-male mating groups have been observed in our study population, but these could be the result of males remaining together after the female has left (Morrow *et al.* 2009) or males being attracted to female odour present on other males. Males could also detect and hone in on male scent, which has not been tested. However, if this was the case then we might expect to see far more all-male groups than are currently observed.

Male echidnas were attracted to odour from mating females even outside the breeding season, suggesting that the male response is not dependent on male reproductive condition, since testes volume and testosterone concentrations are basal by October-November in Tasmanian echidnas (Morrow 2013), when our non-breeding season trials occurred. Males could have been attracted to odour from mating females because it was unexpected, as breeding females in our population are usually confined to nursery burrows by late August (Morrow and Nicol 2012). Male-female pairs have occasionally been found outside of the breeding season, but males were non-reproductive (G. Morrow pers. comm.; unpublished data) and these groups may have been coincidental, as males and females have overlapping home ranges (Nicol *et al.* 2011).

Males were recorded investigating odour from mating females over one month after the scent was formed, despite the scent not being stored frozen. This suggests the attractive component persisted despite the storage conditions. A possible explanation is that the release of attractive chemical signals is influenced by microbial action (e.g. Goodwin *et al.* 2012), which was enhanced by not being frozen. Scent longevity is likely to be important in solitary animals, where scents may be investigated irregularly by conspecifics and need to be effective over large distances (Alberts 1992). Echidna cloacal secretions are rich in non-volatile compounds that may prolong the life of more volatile scent components (Harris *et al.* 2012; Chapter 2) and vary between sexes, seasons and individuals (Harris *et al.* in press; Chapter 4). Tongue-flicking and licking behaviour suggest that non-volatiles are important components of female chemical signals and require direct contact for detection, as in other vertebrate species (Swaisgood *et al.* 2002; Scordato and Drea 2007; Shine and Mason 2012). Chemicals including non-volatiles may also contain more specific information on genetic identity (Parott *et al.* 2007; Charpentier *et al.* 2008).

Our results are consistent with the hypothesis that male echidnas use scent to locate females. Male ability to detect, locate and discriminate between females is likely to be under strong selection, particularly in solitary, widely dispersed animals (Murphy 1998; Swaisgood *et al.* 2002; Johansson and Jones 2007). In mammals with low sexual size dimorphism (including echidnas, see Nicol *et al.* 2011), males are unable to monopolise females and instead engage in scramble competition, where males which are best able to detect and locate females, often by scent, increase their mating opportunities (Johansson and Jones 2007; Lane *et al.* 2009; Thomas 2011). Male ability to locate females may also be sexually selected in echidnas, but genetic information is required to confirm this hypothesis.

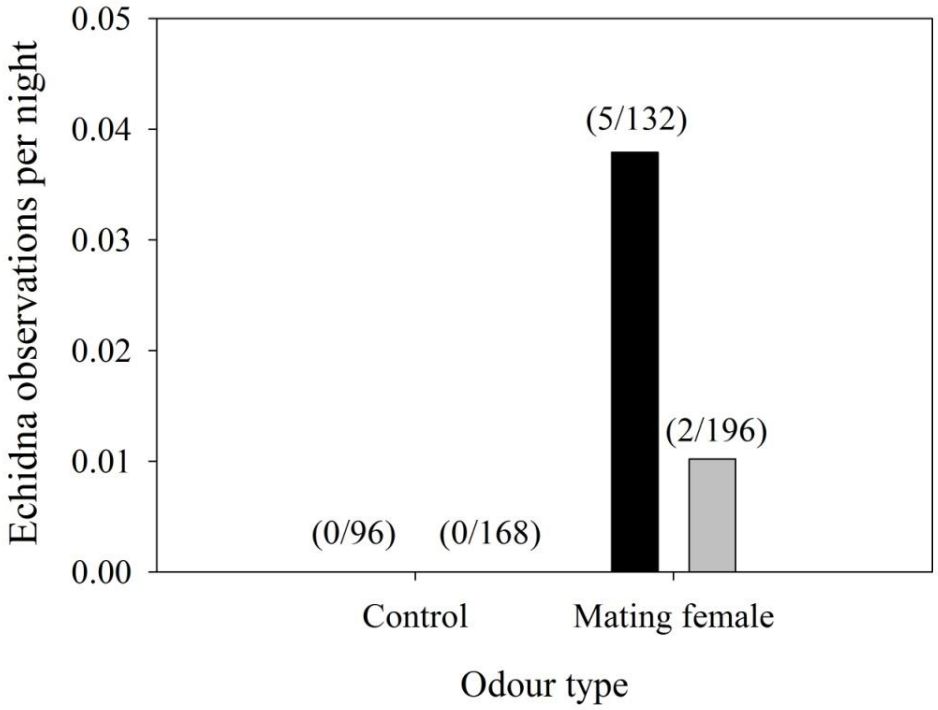

Fig. 5.1. Number of echidnas recorded per night during the breeding (black) and non-breeding (grey) seasons in 2012 in response to control (unbaited) or female cloacal swabs. Parentheses: number of echidnas recorded per number of nights.

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SECTION III:

CHEMICAL SIGNALS AND SEXUAL CONFLICT

Chapter 6:

Does odour signal female reproductive status in an egg-laying mammal, *Tachyglossus aculeatus*? Implications for breeding behaviour and sexual conflict

Chapter 6: Does odour signal female reproductive status in an egg-laying mammal, *Tachyglossus aculeatus*? Implications for breeding behaviour and sexual conflict

Abstract

In seasonally breeding animals, mate-attracting signals often vary temporally and contain information on female reproductive status, so males are attracted at the most appropriate time of year. Some vertebrates also hibernate or undergo winter torpor, but effect of the down-regulation of metabolic processes on chemical signals has only been investigated in reptiles and amphibians. Some animals even show an overlap between winter torpor and reproduction, despite these physiological states traditionally being considered mutually exclusive. We investigated mating activity and female olfactory cues in the Tasmanian short-beaked echidna (*Tachyglossus aculeatus setosus*), a monotreme which shows asynchrony in the timing of hibernation and reproduction between the sexes, female promiscuity and sexual conflict over mating. We combined chemical analyses of cloacal secretions with data from external temperature loggers, urogenital smears and behavioural observations from a wild population over a three year period, to investigate the effects of hibernation, body condition and reproductive status on odour and mating activity in females. There were no detectable differences in volatile and nonvolatile scent composition between reproductive and non-reproductive females prior to or during hibernation, despite reproductive females being disturbed by males and being in better body condition. Duration of hibernation, photoperiod and ambient temperature changes did not seem to be related to changes in chemical signals that might attract males to hibernating females, but males could use odour intensity, individual-specific differences or variations between hibernating and euthermic females to locate particular females. During the breeding season, reproductive females showed mating activity before, during, and after fertilization, but no concurrent changes in chemical signal composition. Female fertility appears concealed from males, which may function to encourage multi-male mating and

confuse paternity among males. Our study provides new insights into the potential influences of chemical cues on reproductive behaviour, particularly in situations of sexual conflict.

Introduction

Communication is vitally important for coordinating reproduction in many sexually-reproducing vertebrates (Andersson 1994; Bradbury and Vehrencamp 2011). However, signals can be costly to produce (Salvador *et al.* 1996; Gosling *et al.* 2000; Zala *et al.* 2004), increase mortality risk via predation (Andersson 1994; Zuk and Kolluru 1998; Hedrick 2000), or attract unwanted attention from conspecifics (Johansson and Jones 2007; Thomas 2011; Uhrig *et al.* 2012). Therefore sexual signals (e.g. plumage, colouration, behaviour, pheromones) tend to vary seasonally with reproductive state, so potential mates are not attracted at inappropriate times (Andersson 1994; Johansson and Jones 2007; Uhrig *et al.* 2012).

Seasonality in mate-attracting signals and reproductive physiology might be particularly strong in animals which undergo periods of torpor or hibernation, as they have a reduced active period during which locating mates, courtship and reproduction can occur. Along with depressing metabolic rate and body temperature (Geiser 2004; Geiser 2011), torpor and hibernation down-regulate ordinary behaviour patterns (Wimsatt 1969), endocrine system and reproductive organ function (Hudson and Wang 1979), and since the size and activity of scent glands are under endocrine control (Ebling 1977), probably also inhibit signalling activity. In most hibernating species, mating follows a period of euthermia which allows time for gonadal development, spermatogenesis and scent gland development (Dark 2005). However, hibernation and mating activity are closely timed or even overlap in some vertebrates (Thomas *et al.* 1979; Boyles *et al.* 2006; Shine 2012), suggesting that signal production may occur even when body temperature and metabolic rate are reduced. For example, in red-sided garter snakes *Thamnophis sirtalis parietalis*, significant pheromone synthesis occurs during winter torpor (Parker and Mason 2009), so newly emerged, torpid females are highly attractive to males (Mason *et al.* 1989; Uhrig *et al.* 2012). However, garter snakes (LeMaster and Mason 2002) and many hibernating mammals, such as marmots and ground squirrels (Exner *et al.* 2003; Blumstein *et al.* 2004), may overwinter in groups, so could use visual cues to locate potential mates once they have rewarmed, in addition to odour cues. Hibernation and mating also overlap in the usually solitary echidna

(Morrow and Nicol 2009), but the effects of hibernation on chemical signals in mammals have not been directly investigated.

The short-beaked echidna (*Tachyglossus aculeatus*) is the most common extant species of monotreme and is found throughout mainland Australia, Tasmania and parts of New Guinea (Griffiths 1978; Augee 2008). Tasmanian echidnas (*T. a. setosus*) are highly seasonal (Nicol and Andersen 2007; Nicol and Morrow 2012) and there is asynchrony in the timing of hibernation and reproduction between the sexes (Morrow and Nicol 2009; Nicol and Morrow 2012). Both males and females hibernate prior to the winter breeding season (June-September), and during hibernation, body temperature may fall to within 1 °C of substrate temperature (Nicol and Andersen 2002; Morrow *et al.* 2009). Both sexes are promiscuous (Morrow *et al.* 2009; Morrow and Nicol 2009), and a skewed operational sex ratio (Nicol and Morrow 2012) means there is intense intra-male competition for access to a limited number of reproductive females (Morrow *et al.* 2009). Males emerge from hibernation before females, and are able to locate and mate with females that are still hibernating (Morrow and Nicol 2009). In the absence of other cues, males seem to use odour to locate females, implying that females produce an attractive odour during hibernation (Harris *et al.* 2012; Chapter 2). Furthermore, non-reproductive females (sexually mature but not breeding in a given year) tend to hibernate throughout the mating season and emerge in mid-September (Nicol and Andersen 2002; Nicol and Morrow 2012), usually undisturbed by males (Harris unpublished obs., but see Morrow 2013), suggesting that males discriminate between females via scent. Progesterone profiles and urogenital cytology suggest that only reproductive females (Morrow 2013). However, reproductive females also continue to attract males and mate during pregnancy (Morrow 2013), so males appear unable to distinguish between pregnant and non-pregnant females.

To understand why female echidnas are attractive to males, even when hibernating or pregnant, we used a combination of organic chemical analyses, behavioural observations, urogenital smears (to confirm that mating had occurred, via presence of spermatozoa) and external temperature loggers to investigate variations in female chemical signals during hibernation and the mating season in a free-living

population. We concentrated our analyses on the volatile and nonvolatile components of cloacal scent gland secretions, since (1) females scent-mark using their cloaca (Dobroruka 1960; Boisvert and Grisham 1988; Beard *et al.* 1992), (2) cloacal secretion varies between sexes and seasons (Harris *et al.* in prep), and (3) males are attracted to female odour (Chapter 5; Rismiller 1992). Although we have previously shown that female odour differs between hibernation and the mating season (Harris *et al.* in press; Chapter 4), it is unclear whether odour composition and attractiveness changes during late-hibernation, and what cues (e.g. temperature, photoperiod) might trigger such a change. Female echidnas are in better body condition (heavier) in reproductive than non-reproductive years (Nicol and Morrow 2012; Sprent *et al.* 2012), and body condition or reproductive status could also be advertised by chemical signals. No previous studies seem to have directly quantified changes in female chemical profiles during hibernation or pregnancy in a mammal. We predicted that (1) female chemical profiles would change during hibernation, (2) reproductive and non-reproductive female profiles would differ, thereby allowing males to locate reproductive females; and (3) females should show changes in chemical profiles before, during and after fertilisation.

Materials and methods

Study site and animals

Fieldwork was conducted in the southern midlands of Tasmania (42° 28’ S, 142° 14’ E) between January 2010 and September 2012, encompassing three hibernating and mating seasons. Details of the site, fieldwork procedures and animal identification are detailed elsewhere (Nicol *et al.* 2011; Harris *et al.* 2012; Chapter 2). Briefly, up to 14 sexually mature female echidnas were fitted with RF tracking transmitters at any time to allow serial sampling and behaviour recording (total n = 18 individuals). All individuals are fitted with passive implantable transponders (PIT) tags on first capture for identification. Radio-tracked animals were monitored weekly and sampled approximately monthly, including during hibernation, and sampled up to three times per week during the mating season (June – September) when reproductive females were found in mating aggregations. Disturbance by us does not seem to have any negative effect on reproductive success, as closely-monitored individuals successfully raised young and showed mating activity at

similar times to those not disturbed as frequently (e.g. see Morrow and Nicol 2012). Additional females (n = 17) were sampled opportunistically after being found walking around the property or in close proximity to radio-tracked males (e.g. in a mating aggregation). Animals were captured by hand and samples (e.g. urogenital smears to confirm mating, odorant and blood samples) were collected while the animal was under anaesthetic (4 % isoflurane in oxygen). Animals were weighed and then returned to their place of capture after recovering from the anaesthetic.

Odorant sample collection

Two types of odorant samples were collected from adult females over the study period: (1) cloacal swabs (N = 209 samples from 35 individuals), and (2) cloacal wax secretion (N = 113, n = 28). Details of odorant sample collection protocols are described elsewhere (Harris *et al.* 2012; Chapter 2). All samples were stored in individual glass vials at -20 °C for a maximum of two years before being analysed by combined gas chromatography-mass spectrometry.

Recovery of sperm from female reproductive tract

Spermatozoa were recovered from the female reproductive tract by collecting a urogenital smear (Morrow and Nicol 2009). Sperm collected immediately after mating have at least four curves along their length; after four to five days sperm have fewer curves and begin to fragment (Morrow and Nicol 2009). Presence and condition of spermatozoa were used to confirm recent mating activity.

Temperature loggers

External temperature loggers (i-Button, DS1992 L, Maxim Integrated Products Inc., Sunnyvale, California) were attached to radio-transmitters using two-part epoxy glue. Loggers were pre-programmed to record temperature (resolution ± 0.5 °C) every hour and could be downloaded in the field. Loggers recorded events such as immergence and emergence from hibernation, periodic arousals, males entering female hibernacula, female entry into nursery burrows and egg-laying (Nicol and Andersen 2002; Nicol and Andersen 2006; Morrow and Nicol 2009). External temperature records were available for ten additional females (14 echidna years of data) collected in 2007-2009, which were included in our calculations of

immergence and emergence from hibernation (total years of data: $n = 20$ reproductive, $n = 10$ non-reproductive; $N = 20$ individuals; maximum six years of data from a single individual). Female body temperature (T_b) was also measured by gently advancing a thermocouple probe approximately 3 cm into the cloaca while the animal was under light inhalation anaesthesia.

Estimation of timing of fertilisation and egg-laying

Date of egg-laying was determined from i-Button temperature data: egg-laying coincides with a distinct trough or peak in otherwise stable temperatures recorded while female echidnas were in nursery burrows (Morrow and Nicol 2012). Gestation in Tasmanian echidnas is approximately 21 euthermic days (Nicol and Morrow 2012), hence the date of fertilisation could be estimated once date of egg-laying was known. This estimate was supported by the presence of fresh sperm in the female's reproductive tract within two days of the estimated date of fertilisation.

Camera traps

Motion-activated cameras (Scoutguard SG550, HuntingCamOnline, Gadsden, SC, USA; Reconyx PC800, Holmen, WI, USA) were set up outside female hibernacula prior to each mating season. Females were selected for monitoring with cameras based on their relative body condition prior to entering hibernation and breeding activity in the previous year (where known). Non-reproductive females were not routinely monitored using cameras, but their hibernacula were checked at least fortnightly for signs of male disturbance. Cameras were in continuous operation, collecting videos and still photos, and used an infra-red flash at night. Time stamps on recorded footage confirmed timing of events recorded by external temperature loggers.

Chemical analyses of odorant samples

Cloacal swabs were analysed by thermal desorption followed by combined GC-MS using a Varian CP-3800 benchtop gas chromatograph (Varian, Palo Alto, CA, USA) and 1200L triple-quadrupole mass spectrometer (Varian, Palo Alto, CA, USA) or Bruker 300-MS TQ mass spectrometer (Bruker, Preston, Victoria, Australia) (details see Harris *et al.* 2012; Chapter 2). The Varian instrument was replaced by the Bruker instrument during through the study, but all temperature programs and

scanning conditions were held constant. Cloacal wax secretion samples were analysed by GC-MS after being extracted in chloroform and derivatised to form TMS ethers (Harris *et al.* 2012; Chapter 2). All wax secretion GC-MS analyses were performed using the same Varian GC and Bruker MS instruments described above. Thermal desorption of cloacal swabs involves minimal interference with the sample (McLean *et al.* 2012) and was primarily used to describe changes in relatively volatile compounds, while analysis of derivatised wax secretions was used to describe changes in relatively nonvolatile scent components. Relative abundance data were calculated for compounds occurring in >5 % of all samples analysed (cloacal swabs n = 68 compounds; cloacal wax n = 56 compounds), using Varian instrument software (MS Data Review version 6.41). Quantified scent components included short and long-chain fatty acids, sterols, methyl and ethyl esters of fatty acids, phenolics, terpenoids, aldehydes and sulphur and nitrogen-containing compounds (Harris *et al.* in prep; Chapter 4). The automated results were manually checked for accuracy and corrected when necessary. Artefacts and contaminants were excluded from statistical analyses.

Statistical analyses

There is significant individual plasticity among females in our population regarding the timing of important events such as final immergence or emergence from hibernation and mating activity. For example, during the period of maximum mating activity (July), reproductive females may be in deep hibernation or euthermic, alone or in mating groups, and pregnant or not pregnant. Therefore we categorised odorant samples according to individual behaviour and physiology at the time of sampling (e.g. mating, alone, hibernating, euthermic), rather than by selecting *a priori* 'seasons' (although our categories approximately correspond with the timing of the mating and hibernating seasons). The Tasmanian echidna's reproductive cycle approximately corresponds with the calendar year, with weaning of young in Tasmania being completed by the end of January (Morrow *et al.* 2009; Morrow and Nicol 2012). Therefore females found in mating aggregations with males in a given year were classified as 'reproductive', while 'non-reproductive' females showed no mating activity and hibernated through the mating season. Some females were monitored for multiple years, giving a total of 18 reproductive and nine non-reproductive years of odorant sample data. The maximum number of

samples collected from a single individual was 26 cloacal swabs and 20 wax secretions.

Variations in female chemical profiles were examined using mixed model permutational multivariate analysis of variance (PERMANOVA; Anderson 2001). This non-parametric technique makes fewer assumptions about the nature and quality of multivariate data than parametric methods such as principle components analysis or MANOVA (Anderson 2001), but interpretation is similar. Cloacal swab and wax secretion samples were analysed as two separate datasets, since they were chemically analysed using different methods (i.e. thermal desorption vs. derivatisation), so could not be directly compared. Chemical relative abundances for each dataset were square-root transformed and converted into Bray-Curtis matrices prior to the distance-based PERMANOVA analyses. The overall detectable composition of cloacal swab samples varies significantly between mass spectrometers (but has no effect on main factors such as sex or seasonal differences, see Harris *et al.* in press; Chapter 4). Since some females were monitored over multiple years, and to adjust for any confounding effects, we first included 'Year' as a fixed factor. We also included 'Animal ID' as a random factor after fitting all other main effects. Due to sample size restrictions, we have not directly tested the effect of mass spectrometer in our cloacal swab PERMANOVAs, but the effect of 'Year' should approximate differences between mass spectrometers (since all samples analysed within a given year were analysed by the same instrument). We also excluded interactions involving Year or Animal ID as the data were too sparse to test them reliably (Anderson *et al.* 2008). PERMANOVA *P*-values were obtained using 9999 permutations of residuals under a reduced model with Type I (sequential) sums of squares (Anderson *et al.* 2008). PERMANOVAs were generated in Primer version 6 (Clark and Warwick 2001) with the PERMANOVA add-on package (Anderson *et al.* 2008). Significance was set at $\alpha < 0.05$ for all statistical tests. Reported values are means with standard deviations unless specified.

Hibernation and female body condition

Samples collected from reproductive and non-reproductive females prior to and during hibernation were divided into categories for the PERMANOVA analysis:

'pre-hibernation' (active, euthermic, $T_b \approx 32$ °C, prior to entry into hibernation, usually January – March), 'mid-hibernation' (in deep hibernation for approximately two months, T_b <28 °C, usually April – May), and 'late hibernation' (in deep hibernation during the mating season, T_b <28 °C, June – July). 'Hibernation stage' and 'BreedYear' (reproductive or non-reproductive year) were included as fixed factors in the PERMANOVA models. Animal ID was nested within BreedYear as most females were only sampled in a reproductive or non-reproductive year and not both. Females may re-enter hibernation after fertilisation (Morrow and Nicol 2009; Nicol and Morrow 2012), so samples collected from hibernating, pregnant females were excluded from analyses. Long-term mean body masses were calculated for females with at least three years of adult body mass data. Body mass at the time of sampling was converted to a percentage of the long-term mean (mass%; Nicol and Morrow 2012; Sprent *et al.* 2012) as an indicator of body condition.

Female chemical profiles, hibernation and mating activity during the breeding season

Differences in chemical profiles between reproductive females which were torpid (T_b <28 °C) or euthermic, and in mating aggregations or alone during the mating season were compared by including female temperature (torpid or euthermic) and activity (aggregation or alone) as fixed factors in the PERMANOVA models. This analysis also includes data from opportunistically sampled females found in mating aggregations (n = 15).

Chemical profiles during pregnancy

Differences in female chemical profiles between fertilisation and up to egg-laying were compared by categorising samples according to gestation stage: 'fertilisation' (± 2 days); 'mid-gestation' (3-11 days post-fertilisation); 'late gestation' (12-20 days post-fertilisation, or just prior to entry into a nursery burrow, whichever occurred first); and 'nursery' (female in a plugged nursery burrow, egg either in pouch or detected via ultrasound *in utero*; for methods see Morrow (2013)). Some females also showed mating activity and subsequently re-entered hibernation up to several weeks prior to fertilisation (range: 5-41 days, n = 5); samples from these

females were categorised as 'pre-fertilisation'. 'Gestation stage' was a fixed factor in the PERMANOVA models.

Results

Female body condition, chemical signals and timing of entry into hibernation

Reproductive females were in better body condition (mass%) than non-reproductive females prior to entering hibernation (January – March; reproductive females mean ± standard deviation: 116.7 ± 9.6 %, $n = 17$; non-reproductive females: 104.6 ± 9.2 %, $n = 8$; two-tailed *t*-test for unequal variances, $t_{1,14} = 3.04$, $P < 0.01$) and during the breeding season (June – September; reproductive females: 104.4 ± 7.2 %, $n = 19$; non-reproductive females: 95.7 ± 9.1 %, $n = 7$; two-tailed *t*-test for unequal variances, $t_{1,9} = 2.27$, $P < 0.05$). There was no significant effect of reproductive status on the timing of entering hibernation (range: 17 February – 16 April, $N = 30$; reproductive females: 19 March ± 11.1 days, $n = 20$; non-reproductive females: 15 March ± 16.8 days, $n = 10$; *t*-test for unequal variances, $t_{1,13} = 0.59$, $P > 0.5$; Figure 6.1). Cloacal swab and cloacal wax composition varied significantly between different hibernation stages (Table 6.1). Pair-wise tests showed secretion composition changed significantly when females entered hibernation (i.e. 'pre-hibernation' was different from 'mid' and 'late' hibernation; all $P < 0.05$, except for cloacal wax secretion comparison between 'pre-' and 'mid-hibernation' were not significantly different, $P = 0.06$), but there were no significant changes during hibernation (i.e. 'mid' and 'late' hibernation were not different; all $P > 0.1$). There was no difference in secretion composition between reproductive and non-reproductive females prior to or during hibernation, including during the mating season when males may enter reproductive female hibernacula (Table 6.1).

Emergence from hibernation and males entering female hibernacula

All camera traps set up over hibernating reproductive females in the 2010-2012 breeding seasons recorded a male entering the female's hibernaculum ($n = 6$). Cameras also recorded males entering two female hibernacula in the 2008 – 2009 seasons. Male entry into female hibernacula coincided with a distinct, abrupt increase in temperature recorded by loggers attached to the female's transmitter (Figure 6.1 inset). This temperature pattern was used to calculate when a male

entered female hibernacula for 12 additional reproductive females with temperature data but not monitored with cameras (2008-2012). Reproductive females hibernated for 108 ± 17.5 days (range: 70-154 days, *n* = 20) before being disturbed by a male entering their hibernaculum (mean date: 3 July ± 16.1 days (range: 4 June – 31 July, *n* = 20). The mean number of days since the female's last periodic arousal to when a male entered the hibernaculum was 10.1 ± 5.2 (range: 1-25 days, *n* = 20). Mean recorded temperature in the 48 hours prior to a male entering female hibernacula was 7.25 ± 1.22 °C (range: 4.96-9.38 °C; *N* = 960 temperature readings, *n* = 20 individuals). Cameras also recorded males outside a reproductive female's hibernaculum, but not entering, on several occasions in the weeks prior to a male actually entering her hibernaculum (echidna 2957, Figure 6.1a). Digging activity was not observed outside the hibernacula of non-reproductive females, and males were never recorded entering hibernacula of non-reproductive females, except for one female where cameras recorded individual males on several occasions, although they did not enter the female's hibernation chamber (echidna 6865, 2010 and 2011; Figure 6.1b). Non-reproductive females hibernated for 184 ± 24.0 days (range: 137-217 days) and emerged on 15 September ± 13.7 days (range: 31 August – 10 October, *n* = 10). Minimum external temperature for non-reproductive females was 5.1 ± 1.79 °C (range: 2.1-9.2 °C, *n* = 10) recorded on 22 July ± 5.9 days (range: 11-31 July, *n* = 10).

Chemical profiles, body temperature and mating activity during the mating season

A total of 86 mating aggregations, comprising one female in close proximity to one or more males, were found over the three mating seasons between 2010 and 2012 (mean date: 30 July ± 20.1 days; range: 12 June – 6 September). The female was accessible in 69 mating aggregations, and females were torpid (T_b <28 °C) in 15 (22 %) of these aggregations. Volatile and nonvolatile sample composition varied significantly between torpid and euthermic reproductive females during the mating season (Table 6.2). There were no differences in chemical profiles between females which were alone or in mating aggregations.

Chemical signals and mating activity during pregnancy

Fertilisation occurred on 20 July ± 15.7 days (range: 26 June – 18 August, $n = 16$); egg-laying occurred on 11 August ± 14.9 days (range: 17 July – 7 September, $n = 16$). All reproductive females monitored after fertilisation were found in mating aggregations during pregnancy ($N = 16$, $n = 11$ individuals; Figure 6.2). One female (echidna 4815 in 2011) was found in a mating aggregation with two males just two days prior to egg-laying, although sperm were not recovered from her reproductive tract. Nine females mated up to 14 days after estimated fertilisation. There were no significant changes in volatile or nonvolatile scent components between females at the time of fertilisation compared with females which showed mating activity several weeks prior to fertilisation, nor during different stages of pregnancy or at egg-laying (Table 6.3).

Discussion

We observed several differences between females of different reproductive states, consistent with previous studies: in breeding years, females were in better body condition (Nicol and Morrow 2012; Sprent *et al.* 2012) and were disturbed by males entering their hibernacula (Morrow and Nicol 2009; Nicol and Morrow 2012), while non-reproductive females were generally not disturbed by males and hibernated throughout the breeding season (Nicol and Andersen 2002; Nicol and Morrow 2012). Although reproductive females probably have developed follicles prior to entering hibernation (Morrow 2013), we found no detectable differences in odour profiles between reproductive and non-reproductive females, and odour profiles did not change significantly during hibernation. This result is surprising, since male echidnas seem to discriminate between females and only enter reproductive female hibernacula, and we have previously suggested that females may produce an attractant while hibernating (Harris *et al.* 2012; Chapter 2). Our results contrast with the limited number of other studies on amphibians and reptiles which describe qualitative and quantitative increases in female pheromone production in response to prolonged exposure to low temperatures (reviewed by Parker and Mason 2009). This difference is probably a reflection of fundamental differences between hibernation in endotherms and low temperature dormancy in ectotherms, but there are no similar published mammalian studies for comparison.

We also found no detectable changes in female odour profiles prior to or at the time of fertilization, or during pregnancy. Females continued to attract males and mate during pregnancy (consistent with Morrow *et al.* 2009; Morrow and Nicol 2009), suggesting female fertility (e.g. timing of ovulation, pregnancy) is undisclosed to males, which probably has several important influences on male and female mating behaviour.

During hibernation, body temperature and metabolic rate decreases and non-essential body functions shut down (Hudson and Wang 1979; Dark 2005; Geiser 2011). In echidnas, chemical signal production also appears to decrease during hibernation, since female chemical profiles vary depending on T_b, both sexes produce less cloacal wax secretion during hibernation (Harris *et al.* in press, Chapter 4), and secretion composition differed depending on whether animals were euthermic or active (this study, Harris *et al.* in press; Chapter 4). The duration of hibernation varied widely between individual reproductive females (range: 70-154 days), as did the date that males entered reproductive female hibernacula (range: 3 June – 31 July). Collectively, these results suggest photoperiod, prolonged exposure to low temperatures and metabolic changes during hibernation do not cause a change in female odour which attracts males. Alternatively, the timing of males entering female hibernacula may be driven by when female scent accumulates to a threshold level of intensity or attractiveness which males can detect. For example, in parasitic burrowing wasps, males may be attracted to pre-emergent females because small amounts of female pheromone become 'concentrated' within the enclosed airspace around the submerged female (Schöne and Tengö 1981). In echidnas, odour production could occur passively during hibernation, for example via microbial activity (Alberts 1992). Although several mammalian species show overlap between hibernation and reproduction (e.g. torpor during pregnancy; see Geiser and Brigham, 2012), very few show overlap between mating and hibernation (but see review by Boyles *et al.* 2006). For example, in little brown bats *Myotis lucifugus*, males mate with torpid females, and females may store sperm during hibernation (Thomas *et al.* 1979). Unfortunately, the role of olfactory signals has not been investigated in these and other hibernating mammals, so the broader influence of hibernation on chemical signals, and their potential role in mating activity during hibernation, remains unclear. Differences between euthermic and

hibernating female echidnas also raise the possibility that males can distinguish between these females, and could even prefer to mate with hibernating females as they may be less able to resist forced copulations (e.g. Shine *et al.* 2005). Many females in our study population become pregnant prior to their final emergence from hibernation (Morrow and Nicol 2009), so a male may increase his reproductive success (paternity) by preferring hibernating females which have not already mated. This hypothesis could be tested using behavioural bioassays conducted on captive animals.

In many vertebrates, odour advertises female body condition and males prefer scent from heavier females or those more likely to be receptive in the near future (e.g. reptiles (Cooper and Pèrez-Mellado 2002; LeMaster and Mason 2002; Shine *et al.* 2003; Bryant *et al.* 2011), mammals (Swaisgood *et al.* 2002; Ferkin *et al.* 2004; Fernández-Vargas *et al.* 2008)). In the absence of clear differences in odour between reproductive and non-reproductive females, male echidnas could use sex-specific differences in volatile and nonvolatile components to locate hibernating females (Harris *et al.* in press; Chapter 4), then variations in signal intensity to assess female reproductive status, which was not measured in this study. This suggestion is supported by behavioural observations: male echidnas do not simply enter every female hibernaculum they find, as males were recorded outside the hibernacula of non-reproductive females (this study; Morrow 2013), and outside the hibernaculum of a reproductive female several times in the weeks prior to a male actually entering. Signal intensity could vary between females differing in body condition because reproductive females have more fat reserves, and fat-derived scent components (e.g. fatty acids, sterols, which made up the majority of cloacal secretions), may also be more abundant in their scent gland secretions, allowing males to discriminate between females. Signal intensity (or absolute amount) of pheromone compounds is important for conspecific response to female odour in insects (e.g. see Carlson *et al.*, 1984; Carrière and McNeil, 1990; Iyengar *et al.*, 2001; reviewed by Johansson and Jones 2007) although there are few examples in vertebrates, perhaps because variations in the *proportion* of individual compounds may be more important (and easier to demonstrate) than absolute amount of chemical secretions (but see Kopena *et al.* 2011). Male-female interactions prior to entering hibernation could also be important for assessing female readiness to

breed: males could use individual-specific odour cues (this study; Harris *et al.* in press, Chapter 4) to later recognise and target hibernating females in reproductive condition. Male thirteen-lined ground squirrels *Spermophilus tridecemlineatus* assess female reproductive status and prioritise later courtship behaviour towards oestrous females, potentially requiring significant cognitive ability (Schwagmeyer 1995; Schwagmeyer *et al.* 1998). Echidnas show a high degree of home-range fidelity (Nicol *et al.* 2011), so males could use individual recognition and prior information on the reproductive status and home range of neighbouring females to locate potential mates.

Female echidnas continued to attract males and mate up to two-thirds of the way through pregnancy, consistent with Morrow (2013). Females of several species show extended sexual receptivity during pregnancy, including primates: Assamese macaques *Macaca assamensis* (Fürtbauer *et al.* 2011), long-tailed macaques *Macaca fascicularis* (Engelhardt *et al.* 2007), Hanuman langurs *Semnopithecus entellus* (Heistermann *et al.* 2001); rodents: water voles *Arvicola terrestris* (Jeppsson 1986), alpine marmots *Marmota marmota* (Exner *et al.* 2003), laboratory mice *Mus musculus* (Huck *et al.* 1982); and carnivores: lions *Panthera leo* (Packer and Pusey 1983). Since fertilisation is not possible, polyandrous mating during non-fertile stages might serve functions other than reproduction (Fürtbauer *et al.* 2011), or that male ability to assess female reproductive status is imperfect. There are several explanations for female promiscuity, including to avoid sexual harassment (Morrow 2013), guard against male infertility, to increase material benefits, paternal care, genetic diversity of offspring, genetic compatibility or litter size, enable female choice via sperm competition, or increase paternity uncertainty as a counter-strategy to male infanticide (reviewed by Wolff and Macdonald 2004). However, many of these explanations (e.g. sperm competition, guarding against male infertility) do not account for multi-male mating during pregnancy when paternity has already been determined. Although it has previously been suggested that pregnant female echidnas may 'trade-up' and mate with a male of higher genetic quality (Morrow and Nicol 2009), females do not abort their pregnancy despite mating with additional males (Morrow 2013). Males provide no paternal care and females do not gain benefits such as territories or nuptial gifts from mating (Morrow 2013). One possible explanation for female promiscuity during pregnancy

in echidnas is to confuse paternity as a counter-strategy to possible male infanticide (Harris and Nicol, in press, Appendix I). Males of many species use mating history as a proxy for likelihood of paternity and are less likely to kill offspring belonging to females that the male has previously mated with (Hrdy 1979; Ebensperger 1998; Fürtbauer *et al.* 2011). We suggest that female echidnas may benefit from multi-male mating, including during pregnancy, by confusing paternity among males (Ebensperger 1998; Wolff and Macdonald 2004), thereby decreasing the number of males which may damage her nursery burrow and potentially kill her offspring.

In primates, females can encourage males into mating during pregnancy using behavioural, auditory, or visual cues (Nunn 1999; van Schaik *et al.* 2004; Stumpf *et al.* 2011), apparently decoupled from, or at least not closely influenced by, reproductive physiology. In these examples, males are often suggested to use olfactory cues as more reliable indicators of impending ovulation (Cerda-Molina *et al.* 2006; Engelhardt *et al.* 2007; Higham *et al.* 2009; Fürtbauer *et al.* 2011). Changes in female odour cues in relation to continued mating activity during non-conceptive cycles have been described (Murray *et al.* 1985; Cerda-Molina *et al.* 2006), but these used male copulatory behaviour as proxies for female odour and did not attempt to describe qualitative and quantitative variations in volatile and nonvolatile odour composition.

If chemical signals reliably signal reproductive physiology, why, then, do female echidnas show no changes in chemical profiles before, during or after fertilisation, and into late pregnancy? Direct, causal links between reproductive hormones and odour cues are difficult to demonstrate, but oestrogen increases scent-marking behaviour (Hudson *et al.* 1990) and female attractiveness (Mendonça and Crews 2001; Ferkin *et al.* 2004; Mason and Parker 2010), even inducing female sex pheromone production in male conspecifics (Parker and Mason 2012). Conversely, progesterone associated with pregnancy decreases scent-marking behaviour and attractiveness (Johnston 1980; Molteno *et al.* 1998). Female Tasmanian echidnas appear to be induced ovulators (Morrow and Nicol 2009), at least during the early part of the mating season when males enter female hibernacula, although oestrogen levels are below standard detection thresholds (Dean 2000), making investigating the oestrous cycle difficult. Detailed studies on olfactory communication (including

chemical analyses) in induced ovulators are limited, but one well-studied species is the gray short-tailed opossum (*Monodelphis domestica*), where exposure to male pheromones is important for stimulating female sexual maturation and ovulation (Harder and Jackson 2010). However, changes in female chemical cues around fertilisation do not seem to have been characterised. Behavioural tests are needed to test physiological and behavioural responses of female echidnas to male scent.

It is possible that changes in female profiles were not detected because of a lack of statistical power or because an important chemical was excluded from our analyses. However, we detected several consistent differences in volatile and nonvolatile secretion components associated with year, individual identity (which might be expected to be particularly subtle) and differences in female activity (euthermic or torpid). Furthermore, male echidnas were attracted to females regardless of reproductive state, since many females showed mating activity several weeks prior to estimated fertilization, and males were attracted to pregnant females despite elevated progesterone concentrations during pregnancy (Nicol *et al.* 2005; Morrow 2013), collectively suggesting that if there are changes associated with fertilisation and pregnancy, male echidnas may also have difficulty detecting them. Otherwise, male mating activity should have been concentrated to the estimated time of fertilisation.

Our results have several broader implications for understanding mating behaviour and sexual conflict in echidnas. There were no differences in chemical profiles between euthermic females which were alone or with males during the mating season, suggesting females do not control when males are attracted to them. Although hibernating females do not appear to 'actively' attract males, they could indirectly select for males which are best able to locate them in their hibernacula, which may offer genetic benefits to their offspring (Wiley and Poston 1996). Males may also benefit from mating with hibernating females, and use odour intensity rather than composition to 'target' females in reproductive condition. However, females may re-enter hibernation during pregnancy (Morrow 2013) and more information is needed to determine whether males which enter female hibernacula gain an advantage over other males. Females may also be constrained in their ability to avoid males after entering a nursery burrow, as physiological processes involved

with producing an attractive female-specific odour may also be related to egg incubation or lactation, or simply due to sex differences, which cannot simply be 'switched off'. Nonetheless, females may benefit from mating with multiple males by confusing paternity, or reducing male harassment. Overall, we suggest that female odour cues influence both male and female mating behaviour, but behavioural tests must be used to test specific hypotheses.

Table 6.1. Results of PERMANOVAs comparing chemical profiles of odorants collected from reproductive and non-reproductive adult female echidnas during different stages of hibernation. 'BreedYear' indicates whether the female was reproductive or non-reproductive in a given calendar year. Significant results in bold type. Sample sizes: cloacal swab (n = 98), cloacal wax (n = 36).

Sample	**Term**	**df**	**SS**	**MS**	**Pseudo-*F***	***P* (perm)**
Cloaca swab	Year	2	12802	6401	5.05	**0.0001**
	Hibernation	2	6365	3182	2.89	**0.0006**
	BreedYear	1	2252	2252	1.21	0.2879
	Animal ID(BreedYear)	20	27432	1372	1.31	**0.0207**
	Hibernation*BreedYear	2	2016	1008	0.96	0.4874
	Residuals	70	73555	1051	-	-
Cloaca wax secretion	Year	2	424	212	2.20	**0.0081**
	Hibernation	2	559	279	4.19	**0.0002**
	BreedYear	1	75.9	76	0.56	0.8485
	Animal ID(BreedYear)	11	1347	122	2.15	**0.0001**
	Hibernation*BreedYear	2	135	67	1.18	0.2957
	Residuals	17	967	57	-	-

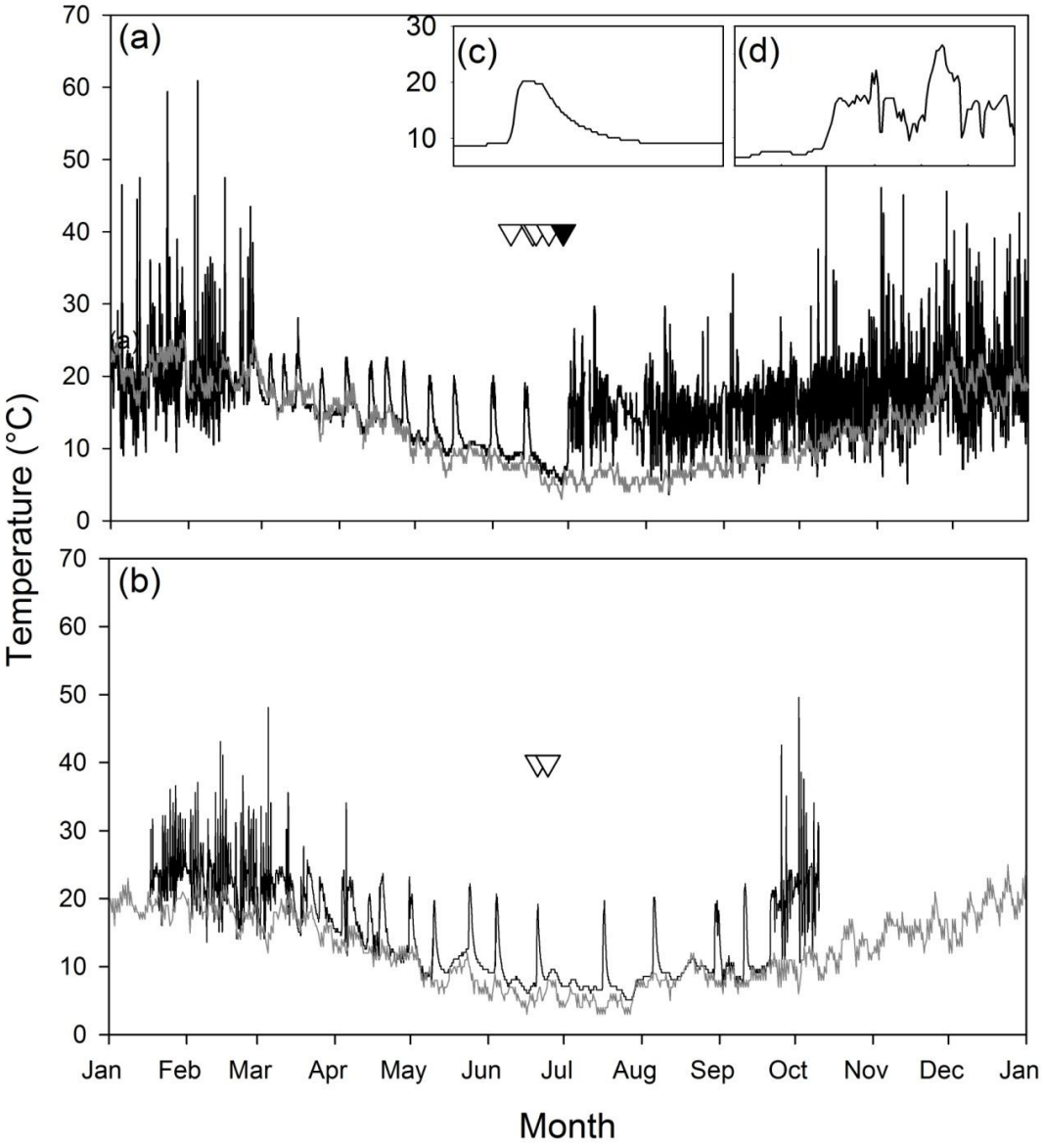

Figure 6.1. Temperature records from external temperature loggers attached to (a) reproductive female (echidna 2957) in 2012 and (b) non-reproductive female (echidna 6865) in 2011. Symbols: ▽ males recorded by cameras outside female hibernacula but did not enter; ▼ males entered the female's hibernaculum (reproductive female only). Grey lines: soil temperature (Bureau of Meteorology data). Inset: detail showing difference in temperature changes during a normal periodic arousal (c) and when male enters female hibernacula (d) over a seven-day period.

Table 6.2. Results of PERMANOVAs comparing chemical profiles of odorants collected from reproductive female echidnas which were alone or in mating aggregations ('Mating'), and torpid or euthermic ('Temperature'). Significant results in bold type. Sample sizes: cloacal swab (n = 89), cloacal wax (n = 70).

Sample	**Term**	**df**	**SS**	**MS**	**Pseudo-*F***	***P* (perm)**
Cloaca swab	Year	2	21997	10999	6.07	**0.0001**
	Mating	1	1160	1160	1.00	0.4211
	Temperature	1	3882	3882	3.59	**0.0013**
	Animal ID	25	37535	1501	1.48	**0.0035**
	Mating*Temperature	1	1420	1420	1.40	0.1870
	Residuals	58	58764	1013	-	-
Cloaca wax secretion	Year	2	3711	1856	11.43	**0.0001**
	Mating	1	235	235	1.92	0.0641
	Temperature	1	290	290	2.53	**0.0217**
	Animal ID	20	3322	166	1.60	**0.0062**
	Mating*Temperature	1	72	72	0.69	0.6821
	Residuals	44	4577	104	-	-

Table 6.3. Results of PERMANOVAs comparing chemical profiles of odorants collected from reproductive female echidnas during different stages of gestation, ranging from prior to fertilisation through to pregnancy and egg-laying (total five categories, details in main text). Significant results in bold type. Sample sizes: cloacal swab (n = 62), cloacal wax (n = 51).

Sample	**Term**	**df**	**SS**	**MS**	**Pseudo-*F***	***P* (perm)**
Cloaca	Year	2	14245	7122	4.65	**0.0001**
swab	Gestation	4	4659	1165	1.03	0.4185
	Animal ID	10	17221	1722	1.66	**0.0033**
	Residuals	45	46641	1037	-	-
Cloaca	Year	2	2471	1236	7.35	**0.0001**
wax	Gestation	4	708	177	1.40	0.1103
secretion	Animal ID	10	1899	190	1.68	**0.0066**
	Residuals	34	3852	113	-	-

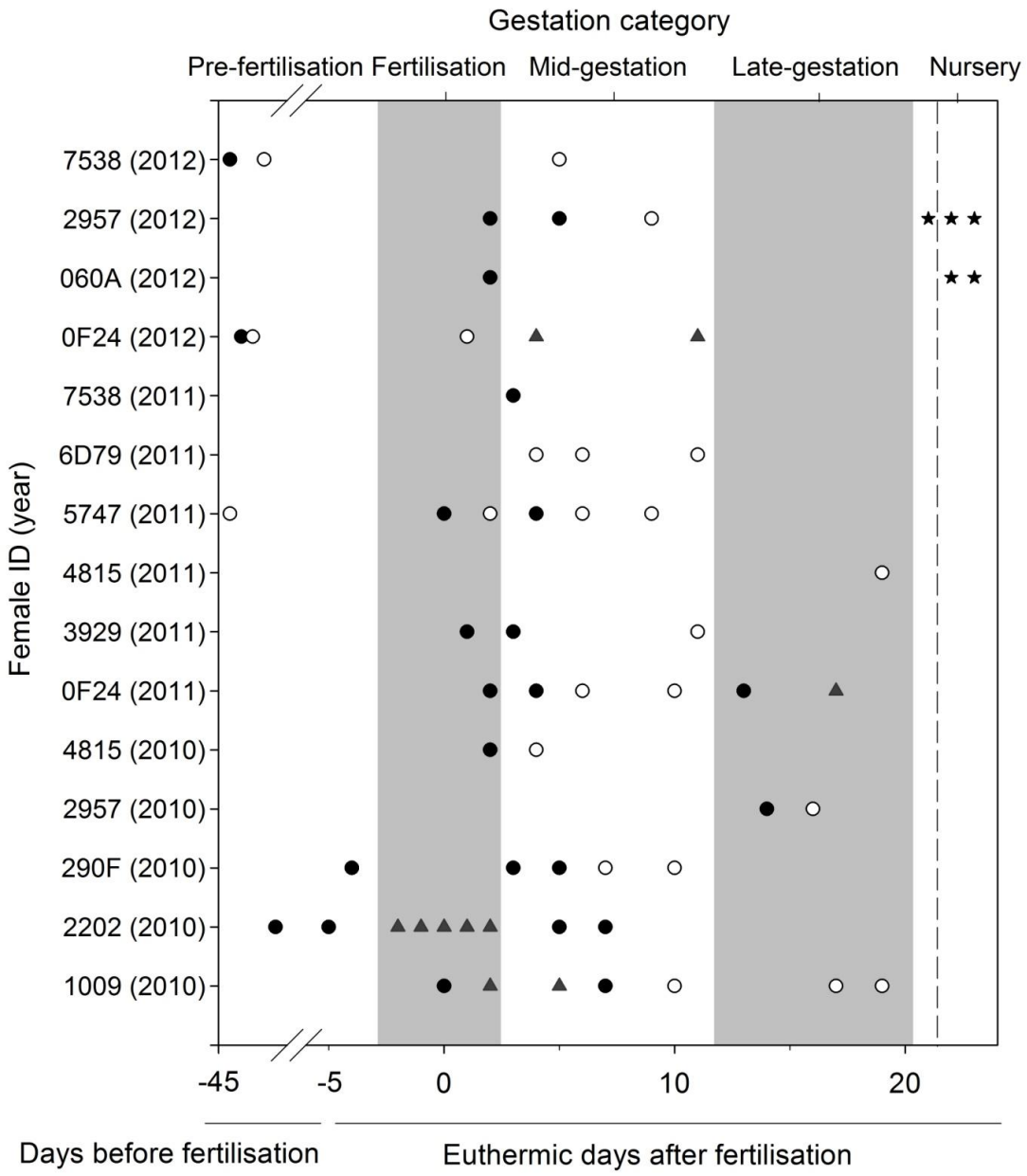

Figure 6.2. Observed mating activity relative to reproductive state (prior to fertilisation, during pregnancy and during egg incubation in nursery burrow) in female short-beaked echidnas. Shaded areas indicate gestation stage categories used for PERMANOVA analyses. Symbols: ● mating aggregation, sperm recovered; ○ mating aggregation, no new sperm recovered; ▲mating aggregation, female not accessible; ★ male digging activity outside nursery burrows (data from Harris and Nicol unpublished data). Dashed line: mean date of egg-laying (21.5 days after fertilisation; data from Morrow and Nicol 2012; Nicol and Morrow 2012). Note break in x-axis scale as females sampled more than five days prior to the time of fertilisation re-entered hibernation after mating.

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SECTION IV:

LINKING ODOUR AND GENETICS

Chapter 7:

Is chemical signal diversity related to mate choice through heterozygosity and relatedness in short-beaked echidnas?

Chapter 7: Is chemical signal diversity related to mate choice through heterozygosity and relatedness in short-beaked echidnas?

Abstract

Many vertebrate species use olfactory cues to select mates based on genetic traits such as quality (heterozygosity) or compatibility (relatedness). The short-beaked echidna (*Tachyglossus aculeatus*) appears to use chemical communication for social and sexual behaviour and may also use odour for genetic-based kin discrimination or mate choice. We combined olfactory information derived from gas chromatography-mass spectrometry with genetic information derived from microsatellite loci to investigate whether scent gland secretions contain information on genetic make-up in 61 wild male and female Tasmanian echidnas. Out of an initial 32 targeted microsatellites, only three were polymorphic and could be used in our analyses, which limits our interpretation of the results. Chemical diversity in spur and cloacal secretions varied seasonally and spur secretion complexity was greater in males than in females. Chemical diversity of spur secretion appeared related to heterozygosity and there was weak evidence for a positive relationship between chemical distance and genetic distance. Although this study provides limited insight into odour-gene covariance and genetic-based mate choice in echidnas, we have established methods which will prove useful in future studies using stronger genetic data.

Introduction

Many species show non-random mating patterns and select mates based on various morphological or behavioural traits (Andersson 1994). In resource-based mating systems, individuals may show preferences for mates which provide the best direct benefits (e.g. nuptial gifts, territories, food), potentially resulting in the evolution of elaborate indicator traits (e.g. sexual ornaments), facilitating mate choice based on these preferences (Møller and Jennions 2001; Neff and Pitcher 2005). In non-resource-based mating systems, individuals may show mate preferences without obvious direct benefits. Such individuals (usually females) may gain indirect benefits by increasing the genetic quality of their offspring, either by selecting for 'good genes' or 'compatible genes' (Trivers 1972; Mays Jr and Hill 2004; Neff and Pitcher 2005). Selection for 'good genes' usually results in females selecting males which are of the highest quality or heterozygosity and individual females show similar preferences (Brown 1997; Kempenaers 2007; Thom *et al.* 2008; Fromhage *et al.* 2009). Selection for 'compatible genes' means individual females show different preferences, since genetic compatibility (e.g. relatedness) will vary between different individuals (Tregenza and Wedell 2000; Penn 2002). Either strategy could increase offspring fitness, for example through increased heterozygosity, which increases resistance to infectious diseases (Hansson and Westerberg 2002; Reed and Frankham 2003) and avoids the deleterious effects of inbreeding (Pusey and Wolf 1996; Keller and Waller 2002).

Genetic variation may be expressed in different phenotypic traits which influence mate choice (Leclaire *et al.* 2012). Many studies have demonstrated that variations in traits such as ornaments, behaviour, colour, song and body odour are linked with 'good genes' and that females show preferences for higher quality (heterozygous) males based on these traits (reviewed by Brown 1997; Kempenaers 2007; Fromhage *et al.* 2009). The links between 'compatible genes' and morphological or behavioural traits are more difficult to demonstrate, as compatibility varies on a continuous scale between different males and females (Mays Jr and Hill 2004; Neff and Pitcher 2005).

Chemical signals are an ideal means of assessing genetic compatibility compared with other cues (Penn 2002; Mays Jr and Hill 2004), because they are closely linked to internal physiology, simultaneously advertising 'good genes' and 'compatible genes' to potential mates (Johansson and Jones 2007). Assessment of relatedness using differences in olfactory cues ('odour-gene covariance') has been described in several species via preference trials (Heth *et al.* 2003; Olsson *et al.* 2003; Roberts and Gosling 2003; Busquet and Baudoin 2005; Parott *et al.* 2007; Radwan *et al.* 2008), habituation-discrimination tests (reviewed by Todrank and Heth 2003) or by directly comparing genetic and chemical distances between individuals (Dronnet *et al.* 2006; Charpentier *et al.* 2008a; Setchell *et al.* 2011; Leclaire *et al.* 2012). Although the latter measure is useful for investigating the mechanisms of genetic kin recognition and mate choice, vertebrate odours are typically complex and a significant challenge lies in identifying the chemical compounds involved (Hurst and Beynon 2010). Recent studies have used statistical methods to attempt to identify specific chemicals important for odour-gene covariance and hence involved in kin recognition or mate choice (Boulet *et al.* 2009; Leclaire *et al.* 2012; Morelli *et al.* 2013). However, progress in non-laboratory species is limited when compared with model species such as rodents (Todrank and Heth 2003; Thom *et al.* 2008; Zhang and Zhang 2011; Raynaud *et al.* 2012).

We investigated the links between genetic quality, relatedness and chemical cues in an egg-laying mammal, the short-beaked echidna (*Tachyglossus aculeatus*; hereafter 'echidna'). Echidnas are the most common extant species of monotreme, an evolutionarily distinct mammalian order which diverged from the therian line approximately 161 million years ago (Phillips *et al.* 2009). Echidnas are usually solitary, with little sexual size dimorphism (Nicol *et al.* 2011; Nicol 2013) and neither sex possesses obvious visual ornaments. Olfaction appears important for coordinating a variety of social and sexual behaviours: scent gland secretions vary between sexes and individuals (Harris *et al.* in press; Chapter 4), so could function in individual recognition (Augee *et al.* 1978), individual spacing via communication at latrines (Sprent *et al.* 2006) and in mate attraction or courtship (Boisvert and Grisham 1988; Rismiller 1992). Olfaction may also be important for genetic-based male or female mate choice, but has not been investigated in this species.

In the Tasmanian subspecies (*T. a. setosus*), there is very little interaction between the sexes prior to mating. Both sexes hibernate for several months of the year prior to the breeding season (Nicol and Andersen 2002; Nicol and Morrow 2012) and some males locate and mate with hibernating females (Morrow and Nicol 2009), contrasting with other subspecies in more temperate areas, such as on Kangaroo Island (*T. a. multiaculeatus*), without deep hibernation and which show extended courtship prior to mating (Rismiller and Seymour 1991; Rismiller 1992; Rismiller and McKelvey 2000). Chemical communication is likely to be important in mate choice in species which have limited opportunity for interactions prior to mating (Wiley and Poston 1996). Furthermore, female echidnas appear capable of choosing between males that enter female hibernacula (Morrow 2013). Since females can only successfully raise a single offspring on average once every three years (Nicol and Morrow 2012) and males provide no parental care or resources other than sperm during mating (Morrow and Nicol 2012), females may benefit from assessing males via olfactory cues and mating with high individuals. However, any fitness benefits of 'good genes' (i.e. high heterozygosity) are unknown in this species, largely due to a lack of genetic information. Although eight polymorphic microsatellite markers have been developed for echidnas (Vanpé *et al.* 2009), these markers have low numbers of alleles per locus (average: 3.78; maximum: 8) and initial studies showed poor amplification success for some loci (Morrow 2007). Furthermore, investigating relatedness between individuals requires a larger number of microsatellites (typically >10-15) than is currently available, and selection of highly polymorphic loci (more alleles per locus) further increases power and accuracy at differentiating between individuals (Van de Casteele *et al.* 2001; Selkoe and Toonen 2006).

In this study we aimed to: (1) develop a new set of polymorphic microsatellite markers for Tasmanian echidnas; and (2) combine genetic information from these microsatellites (quality, relatedness) with gas chromatography-mass spectrometry analysis of scent gland secretions to investigate whether genetic information potentially important for mate choice (e.g. 'good genes', 'compatible genes') is advertised via olfactory cues in the echidna.

Materials and methods

Study site and animals

Odorant and genetic samples were collected from individual echidnas in a wild population located in the southern midlands of Tasmania, approximately 55 km north of Hobart (42°28'S, 142°14'E). Odorant samples were collected between January 2010 and September 2012. The majority of DNA samples were collected between 2010 and 2012, with a few additional samples collected prior to the study period. Extracted genomic DNA was available for an additional 18 individuals from previous genotyping work on our study population (Morrow 2007). Both genetic and odorant data were available for 61 adult echidnas (35 males, 26 females).

Odorant sample collection and chemical analyses

We collected wax secretion samples as described previously (Harris *et al.* 2012; Chapter 2), by gently squeezing the base of the spur, where present and the cloaca, and collecting a 1-3 mg sample of each of the resulting exudates with forceps. Forceps were cleaned with ethanol and dried between samples. Samples were stored in individual glass vials on ice for up to 8 h and then at -20 °C until they were analysed. Wax secretion samples were derivatised and analysed by gas chromatography-mass spectrometry (GC-MS) as described previously (Harris *et al.* 2012; Chapter 2). Control swabs from the animal (from the right foreleg) and blank samples were used to identify artefacts and contaminants. We identified a total of 56 compounds including C_{14}-C_{34} saturated and unsaturated fatty acids, sterols and ethyl and methyl esters of fatty acids. These compounds are present in both spur and cloacal secretions throughout the year and in both sexes (Harris *et al.* in press; Chapter 4). We calculated relative abundance for each compound as peak area divided by the sum of all quantified peaks in each sample (Harris *et al.* 2012; Chapter 2).

We calculated three diversity indices (Shannon, Simpson, Richness) to estimate chemical complexity as in previous studies (Charpentier *et al.* 2008a; Boulet *et al.* 2009; Leclaire *et al.* 2012). While Richness is simply the number of compounds detected in a given sample, the Shannon and Simpson indices also account for relative abundance. The Shannon index is more strongly affected by relatively

common compounds of intermediate abundance, whereas the Simpson index is more sensitive to more abundant compounds (McCune *et al.* 2002; Charpentier *et al.* 2008a). Alternatively, genetic characteristics including heterozygosity may be 'encoded' by particular groups of compounds rather than overall diversity (Hurst and Beynon 2010; Leclaire *et al.* 2012). Therefore we also used a principle components analysis (PCA) and retained the first two principle components (PCs; PC1 and PC2) as variables which summarize information on chemicals contributing most to variation between samples from different individuals. Any significant correlations with the genetic data can then be checked against the chemicals summarized by the different PCs, thus identifying which chemicals could contain information on genetic quality (Leclaire *et al.* 2012). To describe chemical distance, we calculated a Bray-Curtis dissimilarity index after first applying a square root transformation to the chemical relative abundance data.

DNA sample collection and genetic analyses

Blood samples (approximately 200 µL) were collected from the rostral sinus using 21 gauge needles and 1 mL plastic syringes in the field while animals were under light inhalation anaesthesia (4 % isoflurane in oxygen). Blood samples were stored on ice for up to 8 h and then at -20 °C until DNA extraction. Genomic DNA was extracted from whole blood samples (n = 89) using the UltraClean^{TM} BloodSpin Kit (MoBio Laboratories Inc, Carlsbad, CA, USA). We also extracted DNA from the buffy coat layer (layer of white blood cells present after centrifuging; n = 55) using the same kit and procedure, except that samples were incubated at 65 °C with proteinase K for an extra 10 min, and we transferred each sample to a new tube prior to adding "Solution B2" so that centrifuged red blood cells at the bottom of each tube were discarded. Genomic DNA was also extracted from muscle tissue of deceased individuals (n = 41). The total pool of DNA samples includes samples collected from echidnas found opportunistically around Tasmania (including road-kill animals; n = 25). Tissue samples were stored in individual Eppendorf tubes with 100 % ethanol at -20 °C until DNA was extracted using a proteinase K/salt precipitation method (Sunnucks and Hales 1996).

DNA samples were genotyped at 16 microsatellites distributed over six multiplexes (Table 7.1; Supplementary Material 7.S1). PCR amplifications were carried out

using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) in 25 µL reactions, each comprising 1 µL extracted DNA (2 µL for tissue-extracted DNA), 12.5 µL "master mix" (Multiplex PCR Master Mix (Qiagen, Hilden, Germany) or BioMix Red (Bioline, London, UK) used interchangeably), 2.5 µL primer mix (5 pM each primer, equal parts forward and reverse primers) and molecular grade water to bring the total volume to 25 µL. The same PCR set-up was used for all loci, using one of two temperature regimes that only differed in the annealing temperature: initial denaturation stage of 15 min at 95.0 °C, followed by 35 cycles of denaturation at 94.0 °C for 30 sec, annealing at 60.5 °C or 61.3 °C for 90 sec and extension at 72.0 °C for 60 sec, and then a final extension at 60.0 °C for 30 min. PCR efficiency was assessed using electrophoresis on 2 % agarose gels pre-stained with SYBR Gold stain (Invitrogen) to confirm successful amplification. Allele sizing was performed using GeneMapper software (Applied Biosystems). Fragments were separated based on size using an AB3730 DNA Analyser and the GS500LIZ size standard.

Twelve of the 16 loci were monomorphic and excluded from further analyses. The remaining four loci were checked for deviations from Hardy-Weinberg equilibrium (HWE) using GenAlEx version 6.5 (Peakall and Smouse 2012), evidence for linkage disequilibrium using GENEPOP version 4.2 (Raymond and Rousset 1995; Rousset 2008) and for genotyping errors and null alleles using Micro-Checker (Van Oosterhout *et al.* 2004). One locus (ECHID019) deviated significantly from HWE and contained an excess of homozygotes, potentially indicative of null alleles and therefore was excluded. Three loci showed no significant deviations from HWE or evidence for null alleles or linkage and appeared consistent with Mendelian inheritance based on comparisons of known mother-offspring pairs. Allele frequencies ranged from 0.003 to 0.833 (locus ECHID005, allele 172 frequency: 0.646, allele 176: 0.354; locus ECHID022, allele 149: 0.184; allele 153: 0.795; allele 157: 0.017; allele 165: 0.003; locus ECHID031, allele 195: 0.135; allele 201: 0.007; allele 203: 0.833; allele 205: 0.024). Due to the low number of polymorphic loci and low numbers of alleles per locus (mean ± s.d.: 3.33 ± 1.15, range: 2-4) we only included samples which had been successfully genotyped at all three loci in our statistical analyses (N = 144 from the main study population). Genotyping

scores were consistent for individuals which had been genotyped more than once (n = 9).

Genetic diversity at microsatellite loci was used as a proxy for genetic quality (Keller and Waller 2002). We calculated neutral heterozygosity (H_o; the proportion of heterozygous loci per individual) as a measure of genetic diversity (Charpentier *et al.* 2008b) using 'IRmacroN', an Excel macro (Microsoft Office 2007) written in Visual basic by W. Amos (http://www.zoo.cam.ac.uk/zoostaff/amos/#ComputerPrograms). We also calculated two other measures of heterozygosity: internal relatedness (*IR*), which estimates the relatedness of an individual's parents by weighting the extent of allele sharing at each microsatellite by allele frequency (Amos *et al.* 2001); and homozygosity by loci (*HL*), where locus contributions to the homozygosity estimate are weighted by their allelic variability (Aparicio *et al.* 2006). These measures perform differently depending on population and microsatellite characteristics (Amos *et al.* 2001; Aparicio *et al.* 2006; Charpentier *et al.* 2008a). All three measures were highly correlated (all $R^2 \geq 0.89$) and the final results did not differ between measures, so we have only presented the results for H_o. Neutral heterozygosity ranged from zero (homozygous at all three loci) to one (heterozygous at all three loci). Mean H_o was 0.37 ± 0.28 (s.d.).

We calculated three estimates of genetic relatedness (R) between each pair of individuals: Lynch and Ritland (R_{LR}) (Lynch and Ritland 1999), Wang (R_{WANG}) (Wang 2002) and TrioML (R_{TRIOML}) (Wang 2007). The Queller-Goodnight relatedness estimator (Queller and Goodnight 1989) is widely used (Charpentier *et al.* 2008a; Boulet *et al.* 2009; Leclaire *et al.* 2012; Liu *et al.* 2013; Stephens *et al.* 2013), however we did not apply it here because it is undefined for biallelic loci (Wang 2002; Oliehock *et al.* 2006). We used three estimates because we had no prior knowledge about the genetic composition of our study population, which would otherwise be used to select the most suitable estimator (Van de Casteele *et al.* 2001). Furthermore, the moment estimators (Lynch and Ritland, Wang) do not allow for inbreeding in their relatedness calculations, whereas the likelihood estimator (TrioML) does (Wang 2011). To obtain more accurate measures of relatedness among the 61 individuals with chemical data, we calculated these

estimates using a larger dataset comprising all individuals from our study site which were successfully genotyped at all three loci (N = 144 individuals; 10,296 pairs). Estimates of genetic similarity (relatedness) varied widely between the three estimators (range: R_{LR}: -1.49-1.25; R_{WANG}: -3.07-1; R_{TRIOML}: 0 – 2; mean ± s.d.: R_{LR}: 0.006 ± 0.58; R_{WANG}: 0.052 ± 0.70; R_{TRIOML}: 0.356 ± 0.44). Positive relatedness estimates indicate two individuals are more closely related than expected, based on the entire dataset. A negative relatedness value indicates a given pair of individuals is less closely related than expected from the data and may be interpreted as essentially zero, or 'unrelated' (D. Queller, pers. comm. 25 May 2013). Values outside of the theoretical range (-1 to +1) can occur when a small number of microsatellites are used, which increases sampling errors (J. Wang, pers. comm. 20 May 2013). Relatedness estimates for the three estimators were moderately correlated (all R^2 >0.6) and calculated using COANCESTRY software (Wang 2011).

Statistical analyses

We investigated the links between genetic heterozygosity and chemical profile complexity with linear mixed effects models using the *nlme* package in R (Pinheiro *et al.* 2013). Genetic diversity (heterozygosity) was the independent variable and chemical diversity was the dependent variable. Since chemical composition varies significantly between males and females at different times of year and between individuals (Harris *et al* in press; Chapter 4), we included 'Sex' and 'Season' as fixed effects. We included 'Animal ID' as a random effect to account for multiple samples from individuals. Analyses were carried out separately for spur and cloacal secretions. Initially a full model was fitted with fixed effects of Sex, Season, H_o, the random effect of Animal ID and all two-way interaction terms (we did not test the highest-order interactions as the data were too sparse to test them reliably). Sequential model selection was conducted using the maximum likelihood method (Zuur *et al.* 2009), where fixed effects were dropped from the model when they were non-significant. All final models included Animal ID as a random effect and were refitted using REML (restricted maximum likelihood estimation). All interaction terms were non-significant and discarded from the final models unless specified.

The relationship between chemical (Bray-Curtis matrix) distance and genetic relatedness (using Lynch-Ritland, Wang, TrioML estimators) was investigated using Mantel tests with 1000 randomizations using the *ecodist* package in R (Goslee and Urban 2007). We conducted Mantel tests separately for each gland type and season (mating and non-breeding active) within male-male and female-female pairs. We did not consider relationships between chemical and genetic distances during the hibernation period. Although Mantel tests have recently been criticized (Guillot and Rousset 2013), they are routinely used to examine odour-gene covariance (Boulet *et al.* 2009; Leclaire *et al.* 2012) and computer programs implementing alternative methods have not yet been developed (Guillot and Rousset 2013). We could not use Mantel tests for male-female comparisons as the data matrices were not square. Instead we used a different type of permutation test, where we compared the observed correlation between the genetic and chemical matrices with a null distribution derived by permuting the row and column labels (1000 permutations) using a short R script (similar to Boulet *et al.* 2009; Leclaire *et al.* 2012). All statistical analyses were carried out using R software (R Core Team 2012).

Results

Principle components analysis of secretion composition

Principle components (PCs) were typically associated with the most abundant compounds in both spur and cloacal secretion datasets. In the spur secretion dataset, PC1 represented 47.2 % of the variation and was positively correlated with several saturated and unsaturated fatty acids: (9*Z*)-9-hexadecenoic acid (r = 0.21), hexadecanoic acid (r = 0.14), (*9Z*)-9-octadecenoic acid (r = 0.17) and 23-dotriacontenoic acid (r = 0.27). PC2 represented a further 31.0 % of the variation and was positively correlated with the same fatty acids as for PC1, along with (*9Z,12Z*)-9,12-octadecadienoic acid (r = 0.17), desmostanol (r = 0.13), cholesta-2,4-diene (r = 0.25) and cholesta-3,5-diene (r = 0.14). In the cloacal secretion dataset, PC1 represented 72.1 % of variation and was positively correlated with cholesta-2,4-diene (r = 0.85) and cholesta-3,5-diene (r = 0.44). PC2 represented a further 10.7 % of the variation in the dataset and was positively correlated with (*9Z,12Z*)-9,12-octadecadienoic acid (r = 0.45) and (*9Z*)-9-octadecenoic acid (r = 0.58).

Sex and seasonal variations in chemical diversity

Chemical diversity of spur secretions varied seasonally and between sexes (Figure 7.1). Spur secretion complexity was significantly higher in males than females as reflected in all three diversity indices and PC1 (Shannon: $L_1 = 14.9$, $P < 0.001$; Simpson: $L_1 = 14.5$, $P < 0.001$; Richness: $L_1 = 10.8$, $P = 0.001$; PC1: $L_1 = 13.1$, $P < 0.001$). Chemical complexity increased during the breeding season in both sexes (Shannon: $L_1 = 5.76$, $P = 0.0164$; Simpson: $L_1 = 11.5$, $P < 0.001$; Richness: $L_1 = 4.25$, $P = 0.039$; PC1: $L_1 = 30.3$, $P < 0.0001$). PC2 was related to sex and season but the interaction term was significant (sex*season, $L_1 = 21.9$, $P < 0.0001$). Cloacal secretion complexity did not vary significantly between the sexes (Shannon, Simpson, Richness, PC1; all $P > 0.2$), but PC1 and the number of compounds detected increased during the mating season (PC1: $L_1 = 8.53$, $P = 0.003$; Richness: $L_1 = 4.66$, $P = 0.031$). Chemical complexity (Shannon, Simpson indices) also tended to increase during the mating season, but not significantly (Figure 7.2). PC2 varied significantly between sexes and seasons (sex*season, $L_1 = 10.2$, $P < 0.01$).

Chemical diversity and genetic heterozygosity

There were no differences in genetic heterozygosity between males and females or different seasons (all $P > 0.1$). Chemical diversity of spur secretion appeared positively related to genetic diversity (H_o) (PC1: $L_1 = 4.97$, $P = 0.026$; Simpson: $L_1 = 3.66$, $P = 0.056$; Figure 7.3), but not for the Shannon and Richness indices (all $P > 0.1$). PC2 was significantly related to H_o but also varied between sexes (sex*H_o, $L_1 = 21.9$, $P < 0.001$). Chemical diversity of cloacal secretions, in terms of number of compounds detected and both principle components, was not significantly related to H_o (Richness, PC1, PC2; all $P > 0.5$). There was weak evidence for an interaction effect between H_o and season on the Shannon and Simpson diversity indices (Shannon: $L_1 = 4.55$, $P = 0.033$; Simpson: $L_1 = 3.79$, $P = 0.051$), suggesting heterozygosity could be reflected in chemical diversity of cloacal secretions on a seasonal basis.

Chemical distance and genetic relatedness

Chemical distance in spur and cloacal secretions did not appear related to any of the three measures of genetic relatedness (R_{TRIOML}, R_{LR}, R_{WANG}). Chemical distances in spur secretions among males tended to increase with decreasing relatedness (i.e. increasing genetic distance) during the non-breeding season (R_{WANG}, r = -0.42, P = 0.014; Figure 7.4), but this result was not significant after adjusting for multiple comparisons (Bonferroni method). Chemical distances in spur secretions did not seem related to the other measures of genetic relatedness (R_{TRIOML}, R_{LR}; all P >0.05). Chemical distances in cloacal secretions appeared related to genetic relatedness between male-female pairs during the non-breeding season (R_{TRIOML}, z = 6.13, P = 0.018), but this result was also not significant after adjusting for multiple comparisons (Supplementary Material 7.S2).

Discussion

We combined organic chemical analyses with microsatellite data to investigate odour-gene covariance and mechanisms of possible genetic-based mate choice in a terrestrial monotreme, the short-beaked echidna. Although we successfully used previously described statistical methods and our sample sizes are comparable, or even higher, than in previous studies (Boulet *et al.* 2009; Leclaire *et al.* 2012), our microsatellite markers were not polymorphic enough to address our aims. However, our results show that chemical diversity of scent gland secretions vary seasonally and between sexes, consistent with previous work demonstrating sex and seasonal differences in composition (Harris *et al.* in press; Chapter 4). Our findings provide further evidence for the potential of odour cues to contain information relevant to social and sexual behaviour in this species.

Only four microsatellites were polymorphic out of the initial 16 markers selected for multiplexing and 32 markers selected for primer development. Reasons for the low level of diversity include: inadvertent selection of monomorphic microsatellites during the primer development stage; allele drop-outs or other problems with identifying allele size fragments or amplification when using small amounts of DNA; or low genetic diversity at the population, subspecies or species level. Although microsatellite selection could be a strong contributor, we cannot confirm

this until we compare genotyping results using these microsatellites with individuals from other populations or subspecies. It is possible that at least some of the microsatellites are only monomorphic in the Tasmanian subspecies of echidna. Island populations often have lower genetic diversity than mainland populations (Frankham 1997), for example see black-footed rock wallabies *Petrogale lateralis* (Eldridge *et al.* 1999), wedge-tailed eagles *Aquila audax* (Burridge *et al.* 2013) and South Island robins *Petroica australis australis* (Boessenkool *et al.* 2007). Tasmanian echidnas seem to have slightly lower numbers of alleles per locus than some mainland subspecies (Vanpé *et al.* 2009), although this may be a function of the relatively small number of Tasmanian individuals sampled (Vanpé *et al.* unpublished data).

Although our measures of genetic diversity and relatedness are straightforward to interpret, they can be misleading when the number of microsatellites is small (Aparicio *et al.* 2006; Selkoe and Toonen 2006). For example, estimates of relatedness between individuals become particularly inaccurate when a low number of loci are used, which yields a small demonimator (D. Queller, pers. comm. 25 May 2013) and increases sampling errors (J. Wang, pers. comm. 20 May 2013). Most of the individuals genotyped in this study were relatively 'homozygous' and it is likely that with more polymorphic markers we would see increased overall heterozygosity and improved differentiation between individuals.

Odour-based advertisement of genetic information, such as quality or relatedness, could have several important functions in echidna social and sexual behaviour. Males and females have limited opportunities for interactions or courtship behaviour prior to mating, as males in our study population enter hibernation in late February, approximately four months prior to the earliest signs of mating activity (Nicol and Morrow 2012; Morrow 2013). Females could use male chemical cues to rapidly assess male quality or relatedness. When males enter female hibernacula, it appears that females must re-warm in order for mating to occur (Morrow 2013). Since females do not re-warm in response to all males which enter their hibernacula, they may be capable of exerting some choice over which males they mate with (Morrow 2013), potentially based on olfaction. Males and females may also assess genetic information prior to entering hibernation and use individual-

specific chemical cues (Harris *et al.* in press; Chapter 4) to later recognize suitable mates during the mating period.

Advertisement of genetic quality (heterozygosity) may vary seasonally, such that high quality individuals increase the complexity of their scent gland secretions during the mating period. In this study, chemical complexity (particularly that of saturated and unsaturated fatty acids) varied seasonally and in spur secretions this also varied between sexes. We have previously shown that spur and cloacal secretion composition varies seasonally and between sexes (Harris *et al.* in press; Chapter 4) and the present study provides further evidence of the potential importance of fatty acids in male spur secretion for signaling to both females and other males. Chemicals including fatty acids and their derivatives may be costly to produce (Johansson and Jones 2007), so diversity of these compounds might convey detailed information on mate quality to conspecifics (Johansson and Jones 2007; Boulet *et al.* 2010; Martín and López 2010). For example, in ring-tailed lemurs *Lemur catta*, scrotal secretions contain information on 'good genes' and 'compatible genes', but only during the mating season when signaling to females is important for mating success (Charpentier *et al.* 2008a). However (and unusually), chemical diversity *decreases* during the mating season, perhaps because males are highly stressed associated with obtaining mates, competition and aggression (Charpentier *et al.* 2008a). Our data suggest that, as in many species, chemical diversity in echidna secretions increases during the mating season, perhaps in response to increasing androgens such as testosterone which influence scent gland activity (Ebling 1977; Ferkin *et al.* 1994; Stoddart *et al.* 1994). Chemical diversity could also be influenced by stress or body condition (Zala *et al.* 2004; Martín and López 2010), thereby functioning as an honest signal of male quality to females and other males during competitive interactions (Rich and Hurst 1998; Charpentier *et al.* 2008a; Martín and López 2010). Male echidnas spend a significant amount of the mating period competing with other males for access to females (Morrow 2013) and chemical communication is probably an important component of male behaviour when in multi-male mating groups.

Despite using laboratory and statistical methods comparable to previous studies (e.g. Boulet *et al.* 2009; Leclaire *et al.* 2012), the genotyping results prevented us

from addressing the original aims of this study. However, our preliminary results suggest that scent gland secretions may contain genetic information important for mate choice. This study provides an important foundation on which to base future examinations of odour-gene covariance in echidnas and other mammalian species. Furthermore, genetic information may also be present in volatile components of scent gland secretions, which were not considered in this study because of the expected loss of statistical power associated with confounding factors (Harris *et al.* in press; Chapter 4). Alternatively, chemical secretions may contain information on genetic diversity and relatedness at the major histocompatibility complex (MHC). The MHC is a highly polymorphic gene complex important for immune function, where heterozygous individuals have an advantage over homozygous individuals (Tregenza and Wedell 2000; Penn 2002), and is involved in odour-based mate choice in several species (Eggert *et al.* 1998; Olsson *et al.* 2003; Parott *et al.* 2007; Radwan *et al.* 2008; Setchell *et al.* 2011). We recommend that a new set of highly polymorphic microsatellites be developed so the question of odour-based mate choice in the echidna can be addressed.

Table 7.1 (continued over page). Details of 16 microsatellite loci in short-beaked echidnas (*Tachyglossus aculeatus setosus*) selected for multiplexing and which showed amplification under one of two PCR conditions. Loci ECHID005, ECHID019, ECHID022 and ECHID031 were polymorphic. Shown are locus name, locus repeat motif, primer sequences (forward, *F*, and reverse, *R*), multiplex ID (*M*), fluorescent primer label (Dye), observed allele size range (base pairs), number of alleles (*k*), observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), Hardy-Weinberg equilibrium chi-squared statistics and *P*-values (HWE). Individuals genotyped (*N*) = 144. Annealing temperatures: multiplexes D – G: 60.5 °C; multiplexes H – I: 61.3 °C.

Locus	**Repeat motif**	**Primer sequences**	**M**	**Dye**	**Allele size range (bp)**	**k**	**H_o**	**H_e**	**F_{IS}**	**HWE**
ECHID001	GC	*F:* ACAAGGCAACCGTTCTCACT *R:* AAAACAGCATCTCTTGGTTGG	E	NED (yellow)	164	1	0	0	0	N/A
ECHID004	ATAG	*F*: TGCAATCTACATAGTGCCTCTTTT *R*: CTGCGAATTTGGGGATTTTA	D	FAM (blue)	246	1	0	0	0	N/A
ECHID005	CCAT	*F*: CCCAAAGGGTGTATCCATGT *R*: GTTCCCAGACCCTGTCTCAC	G	VIC (green)	172-176	2	0.472	0.457	-0.032	$\chi^2_1 = 0.150$ $P = 0.699$
ECHID008	GCCT	*F*: TCGGTGAACATCATTGAAGC *R*: GGTGGCTGTTCTCTTTCCAA	G	PET (red)	160	1	0	0	0	N/A
ECHID010	AAGCA	*F*: AAGGGCAGCAACAGAAGAAA *R*: CCGACAGTAATGGTCGTGTG	G	NED (yellow)	162	1	0	0	0	N/A
ECHID012	ATGGC	*F*: CTTCCCAAAGGAACCCAGAG *R*: AGCCTGAGCTCTCTGGACAA	D	VIC (green)	222	1	0	0	0	N/A
ECHID014	CACAA	*F*: TCCTTCTTCAAATCAGAGCACA *R*: TTCTCCCGTCCCCTACTTTT	E	VIC (green)	238	1	0	0	0	N/A
ECHID015	CCAAA	*F*: CACTTTGCCTCAGTCGAAAA *R*: GCTGAAGTTACATTTCAGAGTTTCC	E	FAM (blue)	153	1	0	0	0	N/A

Table 7.1 (continued).

Locus	Repeat motif	Primer sequences	M	Dye	Allele size range (bp)	k	H_o	H_e	F_{IS}	HWE
ECHID016	CCGGA	*F*: AGGAGAGCGGGGACCAAG *R*: ATCTCTGATGCGTCCGTAGC	H	VIC (green)	209	1	0	0	0	N/A
ECHID018	GGAGG	*F*: AGAACGTAGGCCAGGTGGTA *R*: TAAAGATCAGGCAGGGGAGA	H	NED (yellow)	150	1	0	0	0	N/A
ECHID019	GGCCA	*F*: CTGCATACATCACCCTACGC *R*: GGGACGTAGCAAGGATCG	D	NED (yellow)	227-237	3	0.408	0.509	0.198	$\chi^2_2 = 9.171$ $P = 0.027$
ECHID021	GTTGT	*F*: GTAGACCCCATAGCGTGGAG *R*: TCCTTCTTCAAATCAGAGCACA	E	PET (red)	167	1	0	0	0	N/A
ECHID022	GTTTA	*F*: TGCTTTTTCTCCTCCGTTGT *R*: AAAGACAAAAATCCCCGACA	F	NED (yellow)	149-165	4	0.354	0.334	-0.062	$\chi^2_6 = 1.881$ $P = 0.930$
ECHID027	ATTTTT	*F*: TGGAGTGTTCTCCCTTCCAG *R*: GCTGTTTCAGTCAGCATGGA	F	VIC (green)	150	1	0	0	0	N/A
ECHID030	GTTTTT	*F*: CAAGAAGCCACCTAGCTTACC *R*: TTGTTCATAAGAAAGTGATATTTCAGG	F	FAM (blue)	210	1	0	0	0	N/A
ECHID031	TCTCTG	*F*: CTTATGGCTCTCCAGGATCG *R*: TGTGTGTGTGTGTGTGTGTGAG	G	FAM (blue)	195-205	4	0.306	0.287	-0.066	$\chi^2_6 = 2.047$ $P = 0.915$

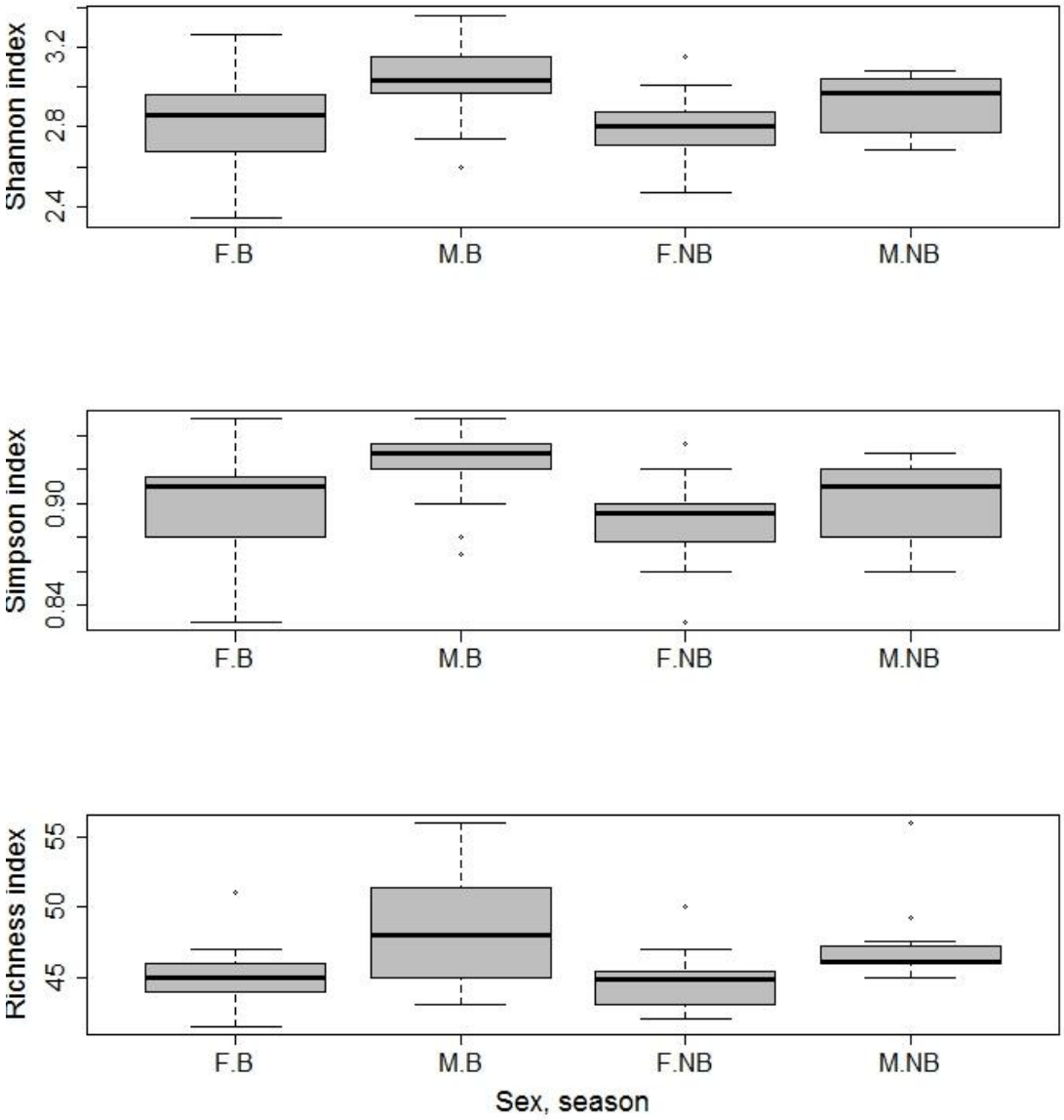

Figure 7.1. Box plots showing seasonal variations in three measures of chemical diversity (Shannon, Simpson, Richness) of spur secretion in male and female short-beaked echidnas. Labels indicate samples collected from males (M) and females (F) during the breeding (B) and non-breeding (NB) seasons.

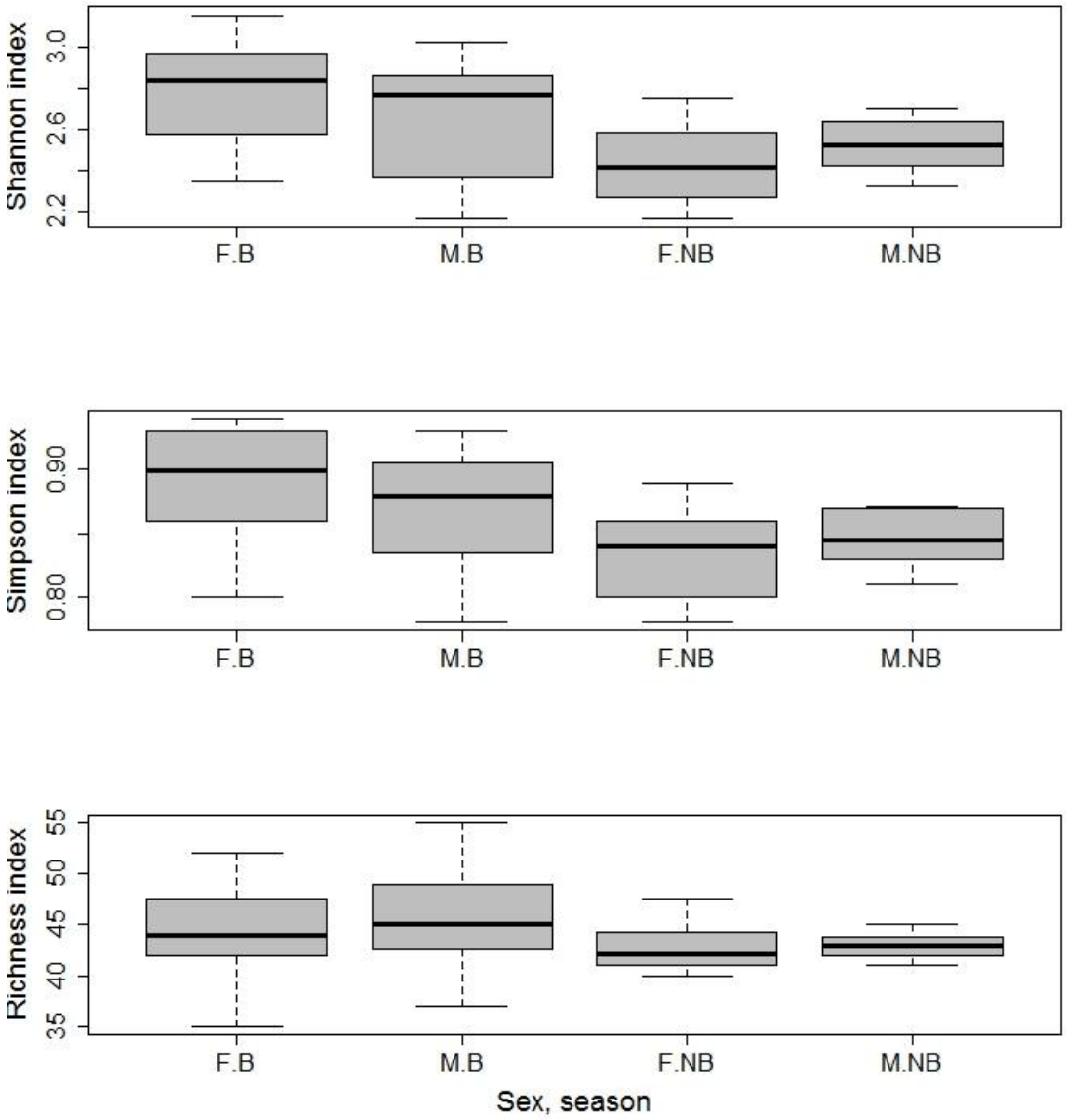

Figure 7.2. Box plots showing seasonal variations in three measures of chemical diversity (Shannon, Simpson, Richness) of cloacal secretion in male and female short-beaked echidnas. Labels indicate samples collected from males (M) and females (F) during the breeding (B) and non-breeding (NB) seasons.

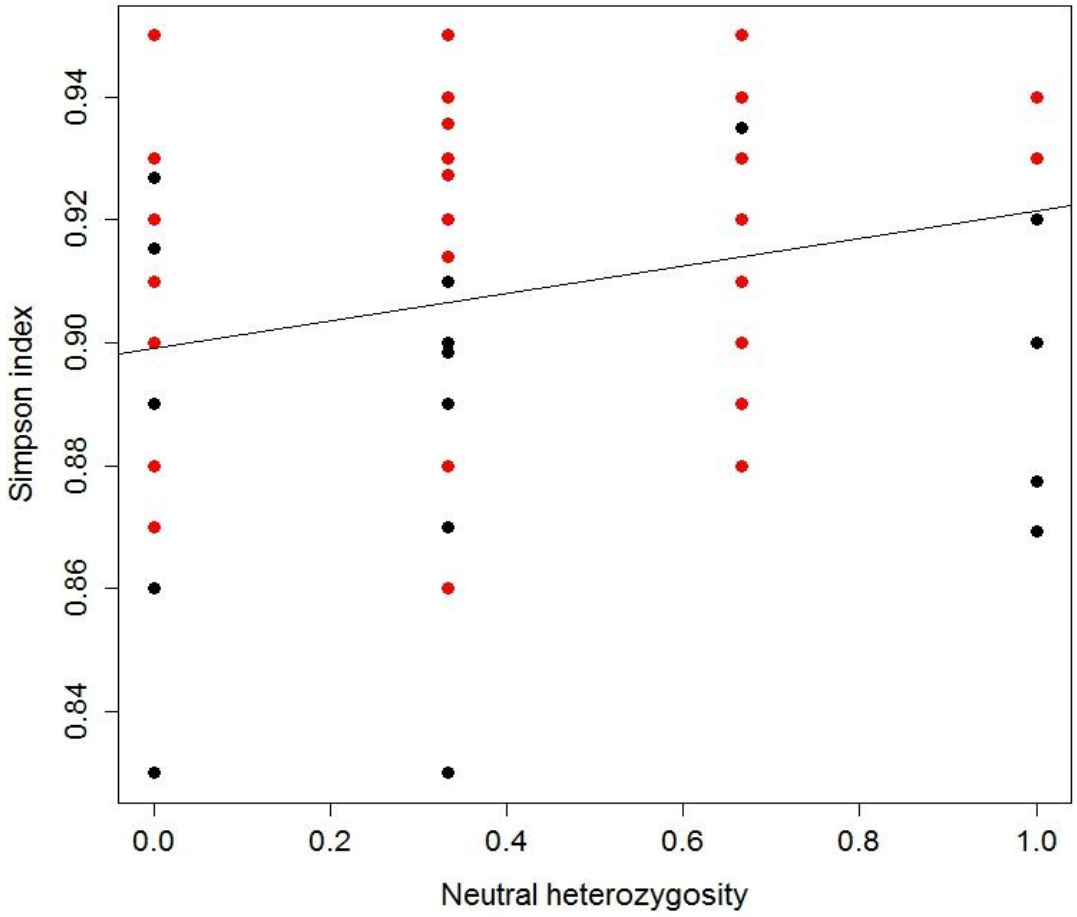

Figure 7.3. Relationship between genetic diversity (neutral heterozygosity, H_o) and chemical diversity (Simpson index) of spur secretion in male (●) and female (●) short-beaked echidnas during the non-breeding season. Adjusted $R^2 = 0.047$, $P = 0.027$. Simpson index = (number of compounds detected)/100.

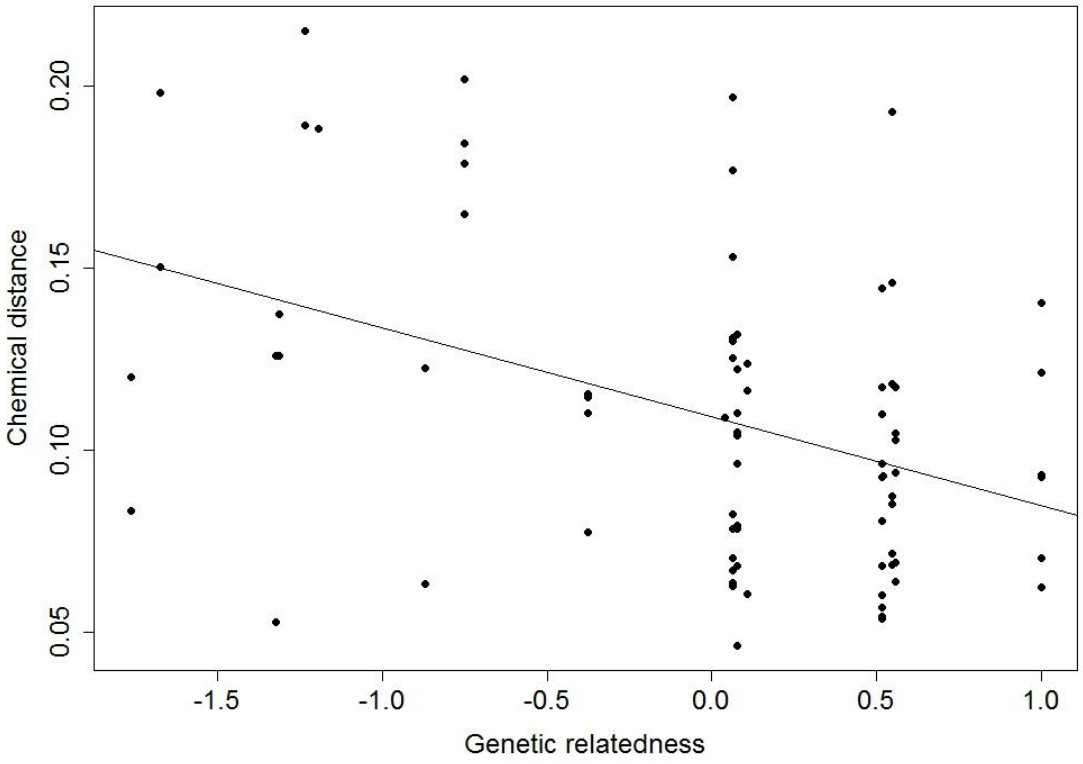

Figure 7.4. Relationship between genetic similarity (relatedness, Wang estimator) and chemical distance (Bray-Curtis distance matrix) of spur secretion among male short-beaked echidnas during the non-breeding season. Note the Y-axis is a measure of distance and the X-axis is a measure of similarity (i.e. negative slope means chemical distance increases with genetic distance). Adjusted $R^2 = 0.165$, $P < 0.001$.

Supplementary Material

7.S1. Microsatellite development and primer design

N.B. Microsatellite selection, primer design and initial genotyping were carried out by Stephen H. Kolomyjec (Biology Department, Ohio Northern University). The candidate (R. Harris) assisted with DNA extractions and PCRs, and was responsible for error checking genotyping results, rescoring and subsequent statistical analyses.

Twelve runs of short-beaked echidna genomic 454 sequencing reads were downloaded from the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra; Accession SRX000121, SRX000122). The Fasta sequence for each run was extracted and screened for perfect microsatellite repeat motifs (3-6 bp, min length 15 bp) using SciRoKo (Kofler *et al.* 2007). Out of the 885,433 Fasta files, 4,552,277 potential repeat sequences were identified, and 747 of these were represented less than 50 times in the 454 sequencing reads. We then excluded potential microsatellites which occurred more than 20 times in the sequence dataset, were within 20 bp of the ends of sequence fragments, contained interrupted repeat pairs or contained less than five repeats. Two of the remaining 34 candidate microsatellites were removed as they were complementary copies of other candidates.

Primers were designed for 32 loci using Primer 3 (http://frodo.wi.mit.edu/) and tested for amplification and polymorphism using polymerase chain reactions (PCRs) and gel electrophoresis. Initial trials with unlabeled primers were carried out using DNA from a single road-kill echidna. Nineteen loci showed amplification under one of the two PCR conditions (see Methods in main text) and 16 of these were selected for multiplexing. PCR products with minimal overlap in expected size ranges were arranged into six multiplexes (two to four loci per multiplex; see Table 1 in main text). Labeled primers were developed with fluorescent tags (FAM, VIC, NED or PET) attached at the 5’ end during synthesis (Life Technologies, Carlsbad, CA, USA).

7.S2. Summary of Mantel tests and permutation tests for MM, FF and MF pairs comparing chemical and genetic distance

We compared differences in scent gland chemical composition with genetic relatedness at different stages of the echidnas' active period. We used Mantel tests (using the *ecodist* package in R; code written by B. Holland) to compare chemical and genetic distances for male-male (MM) and female-female (FF) pairs and permutation tests (using a short R script written by B. Holland) to compare chemical and genetic distances for male-female (MF) pairs (Boulet *et al.* 2009; Leclaire *et al.* 2012). The results of each test using each of the three relatedness estimators are summarised below. 'Variable' is the gland type (spur or cloaca) and season (BR, breeding season; NB, non-breeding season). Mantel test statistics (*r;* MM, FF pairs) or permutation *z*-statistics (MF pairs) are reported. *P*-values <0.05 are in bold type, although none were significant after correcting for multiple comparisons (Bonferroni method).

MM pairs

	TrioML		**Lynch Ritland**		**Wang**	
Variable	***r***	***P***	***r***	***P***	***r***	***P***
Spur, BR	0.0407	0.785	-0.0107	0.356	-0.0913	0.138
Spur, NB	-0.0110	0.441	-0.1066	0.252	-0.4197	**0.014**
Cloaca, BR	0.06714	0.796	0.01929	0.555	-0.199	0.065
Cloaca, NB	0.1718	0.825	0.2010	0.933	0.3607	0.975

FF pairs

	TrioML		**Lynch Ritland**		**Wang**	
Variable	***r***	***P***	***r***	***P***	***r***	***P***
Spur, BR	-0.0238	0.416	0.0104	0.501	-0.075	0.308
Spur, NB	0.00987	0.526	-0.0153	0.379	0.0411	0.568
Cloaca, BR	-0.1182	0.131	-0.0688	0.237	-0.1322	0.146
Cloaca, NB	-0.189	0.128	-0.0855	0.268	0.0716	0.643

MF pairs

	TrioML		**Lynch Ritland**		**Wang**	
Variable	***z***	***P***	***z***	***P***	***z***	***P***
Spur, BR	72.79	0.773	-4.96	0.839	3.89	0.622
Spur, NB	12.98	0.979	0.5634	0.948	1.719	0.97
Cloaca, BR	26.87	0.899	0.9832	0.967	4.87	0.999
Cloaca, NB	6.126	**0.018**	1.173	0.05	1.718	0.082

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Chapter 8:

General Discussion

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Chemical communication is a major topic within the field of chemical ecology, one of the fastest-growing research areas in biology (Wyatt 2003; Müller-Schwarze 2006; Charpentier *et al.* 2012). Chemical signals are crucial for mediating a variety of social and sexual behaviours in mammals (Albone 1984; Brennan and Kendrick 2006; Petrulis 2013b). Extensive work on model study systems has illuminated many of the fundamental processes involved in the detection of pheromone signals and the neural pathways involved in triggering behavioural responses (Novotny 2003; Baum and Bakker 2013; Petrulis 2013b; Petrulis 2013a). However, non-traditional species, must also be considered to better understand the different functions of olfaction for reproduction and selective pressures acting on chemical communication systems (Müller-Schwarze 2005; Houck 2009; Drea *et al.* 2013).

I have used an integrative, multidisciplinary approach to assess the function of chemical communication for coordinating reproduction in an egg-laying mammal, the short-beaked echidna, *Tachyglossus aculeatus setosus*. This holistic approach, based on a free-living population, is a step towards overcoming several of the methodological and logistical limitations associated with 'the mammal problem' (Burger 2005; Charpentier *et al.* 2012; Drea *et al.* 2013). My research is grounded in a thorough examination of the chemical nature of echidna secretions (Chapter 2; Chapter 3), which when integrated with behavioural, genetic and physiological data (Chapters 4-7), provide links between chemical communication, reproduction and sexual selection processes. Although male and female echidnas use chemical communication to coordinate reproduction in much the same way as other seasonally breeding species (Chapter 4; Chapter 5), my results also show chemical cues are may not signal reproductive status in a reliable, predictable manner, with important implications for mating behaviour (Chapter 6). An obvious extension to this work would be the development of robust behaviour trials in both captivity and in the wild (e.g. preference tests; see Appendix II), to test responses to odours collected from wild individuals. Such trials would provide fundamental evidence on whether animals can detect and discriminate between chemical differences (or lack

thereof) identified using statistical methods (e.g. Chapter 4, Chapter 6). Furthermore, the effects of microbial action on mammalian semiochemistry (e.g. metabolism of large components into small volatiles) is a growing area of interest (e.g. Goodwin *et al.* 2012), and may be important for echidna communication. Specifically, bacterial activity may be an important contributor to possible storage effects identified in this study (Chapter 4). In this section, I integrate and synthesise my findings presented in the data chapters and summarise how my research contributes more broadly to our understanding of reproduction, behaviour, selective pressures and the evolution of chemical communication.

My work demonstrates that despite being an evolutionary 'outgroup' (Nicol 2003), echidnas show several patterns consistent with other mammals. Echidna scent gland secretions contain a range of compounds varying widely in volatility, solubility, molecular weight, functional groups and aromaticity, many of which are used in communication by other mammals and even reptiles (Chapter 2; Chapter 3; Burger 2005; Mason and Parker 2010). Species-wide similarities, even across taxa, can suggest retention of ancestral chemicals (e.g. delBarco-Trillo *et al.* 2012), or convergence on optimal chemicals to be used as signals (Wyatt 2003; Müller-Schwarze 2006; Wyatt 2010). Chemical convergence would occur because various chemical and physical properties, including toxicity, stability, biochemical pathways and cost to synthesise and secrete, make some chemicals more suitable as signals than others (Alberts 1992; Wyatt 2010). For example, fatty acids and their derivatives are common in vertebrate secretions and may function in mate assessment (Chapters 2-4; Boulet *et al*. 2010; Martín and López 2010). Mammalian semiochemicals have been described as potentially "boring" compounds (Burger 2005), perhaps because they are often common or not particularly 'novel'. In echidnas, many unusually large fatty acids were identified, along with several other obscure and even novel vertebrate compounds (e.g. juvabione, desmostanol, large sterol esters; see Chapter 2; Chapter 3). However, I did not identify the specific compounds responsible for the 'musky' odour of echidnas during the mating season (Nicol *et al.* 2004). Odours perceived by human or animal olfactory systems can in some cases be at concentrations orders of magnitude lower than the detection limit for analytical equipment (Hobbs *et al.* 1995), leading to the absence of signals despite their strong odour to humans. It is also likely that animals producing them

are even more sensitive to these odours. Although potentially difficult to develop, bioassays on specific compounds will be useful for identifying specific semiochemicals. Some compounds were excluded from statistical analyses on the basis of occurrence (Chapters 2, 4, 6), which can be problematic, since single compounds have been confirmed as active semiochemicals in mammals (Apps 2013), which is important when considering the results of Chapter 6.

Echidnas are sometimes described as primitive (Nicol 2013) and their behaviour suggested to be simple (e.g. Brattstrom 1973), but this observation is largely based on animals in unnatural conditions in captivity, and many of the behaviours described as 'missing' have since been reported (Nicol 2013). The echidna's relatively large and well-developed brain and large brain to basal metabolic rate ratio suggest their behaviour requires significant brain capacity and memory, for example in foraging, ability to adapt in a variable environment, or social behaviour (Nicol 2013). Chemical complexity in echidna secretions (this study) and complexity of olfactory structures in the brain (reviewed by Nicol 2013; Ashwell 2013) indicates a high reliance on olfaction and potentially large olfactory repertoire, which could be important for a variety of behaviours. Specifically, some reproductive behaviour may require complex processing, for example individual recognition and assessment of genetic identity, reproductive status and physiological status, in addition to the fundamental role olfaction plays in enabling conspecifics to locate mates during the breeding season.

Chemical complexity is suggested to increase with behavioural and social complexity in prosimian primates, perhaps because increased signal complexity conveys more information required for socially complex groups (delBarco-Trillo *et al.* 2011; delBarco-Trillo *et al.* 2012). However, some solitary animals may require equivalent complexity of chemical communication to more social species. For example, many of the patterns identified in this thesis, in particular chemical differences between sexes, seasons and individuals (Chapter 4) are consistent with both solitary and highly social species (e.g. Thom and Hurst 2004; Smith 2006; Burgener *et al.* 2009). Variations in multiple compounds (Chapter 2; Chapter 4), *sensu* a chemical 'mosaic' (Johnston 2003) or 'signature' (Wyatt 2010), can differ between individuals and are learnt by others for recognition (Todrank and Heth

2003; Hurst and Beynon 2004; Palagi and Dapporto 2006). Individual recognition is important for coordinating group interactions in social species where individuals may encounter conspecifics frequently (Howard and Blomquist 2005; Scordato and Drea 2007; Burgener *et al.* 2009; delBarco-Trillo *et al.* 2012), but solitary species also use individual recognition for advertising individual presence, dominance and territory ownership between widely dispersed individuals (e.g. Hurst and Beynon 2004; Nie *et al.* 2012; Soini *et al.* 2012). Regular signalling at latrines could maintain spatial distribution (Sprent *et al.* 2006) and simultaneously provide opportunities to monitor the presence and reproductive status of neighbouring individuals via individual-specific chemical profiles (Chapter 4; Chapter 6; Stewart *et al.* 2002). It is important that behaviour tests are conducted to determine whether echidnas actually use the information, such as individual identity, available in chemical secretions. Furthermore, standardised comparative studies are needed (e.g. delBarco-Trillo *et al.* 2011; delBarco-Trillo *et al.* 2012) to control for differences in analytical methods and allow robust comparisons of chemical complexity and functional differences, both among and between different taxa.

The remainder of this thesis discusses how male and female echidnas use chemical signals for reproduction and how selective pressures and conflict help to explain the pattern of echidna mating behaviours and influence evolution of communication systems. Although the 'female-calling' and 'male-response' pattern in echidnas seems at odds with the general rule that males should show greater effort in signalling, mating and courtship (Andersson 1994), it is intuitive when considering the relative costs of reproduction in both sexes. Sexual asymmetry in parental investment influences the evolution of mate-signalling and determines the different sex roles in reproductive behaviour (Trivers 1972; Phelan 1997). If advertising costs are low and search costs are high (as in chemical communication), the sex which shows greater parental investment (usually the female) should invest less in searching for mates (Greenfield 1981; Phelan 1992; Svensson 1996; Ayasse *et al.* 2001; Kokko and Wong 2007; Harari and Steinitz 2013). Therefore, female-signalling with male-searching is the dominant pattern seen in species which use chemical communication (Cardé and Baker 1984). As males compete and attempt to maximise their number of mates, there is further selection for males to be the searching sex (Kokko and Wong 2007) and to employ scent-marking or signalling

at a higher rate to advertise their quality (Heymann 2006). In echidnas, selection for these traits is consistent with the development of sexually dimorphic spur secretions (Chapter 4), which differ between individuals (Chapter 4), contain compounds which may be costly to produce (e.g. fatty acids) and advertise male quality or body condition (Chapter 2), and vary seasonally in terms of composition (Chapter 4) and complexity (Chapter 7). However, it is unclear whether male scent-marking behaviour also changes in response to increased competition (for example in multi-male mating aggregations), as might be expected if male spur secretion was a sexually selected trait (Rich and Hurst 1998; Davie *et al.* 2010).

Reproduction in Tasmanian echidnas is characterised by roving male searching behaviour and intra-sexual competition for access to widely dispersed females in reproductive condition (Morrow *et al.* 2009; Morrow and Nicol 2009; Nicol and Morrow 2012; Morrow 2013), consistent with scramble competition (Ims 1988; Murphy 1998). Females are uniformly distributed according to resources (Sprent and Nicol 2012) and the spatial and temporal distribution of reproductive females, along with low sexual size dimorphism (Nicol *et al.* 2011; Nicol 2013), mean males cannot monopolise females during the mating season (Morrow 2013). Scramble competition is common in anurans (Wells 1977), insects (Choe and Crespi 1997; Moya-Larano *et al.* 2007) and mammals, such as sciurid rodents (reviewed by Waterman 2007) and some primates (Kappeler 1997). Male fitness is primarily driven by ability to locate females and "interaction-independent sexual selection" (Murphy 1998) favours male sensory and behavioural traits rather than extravagant male traits as in female defence systems (Trivers 1972; Murphy 1998; Lane *et al.* 2009). Although selection for sensory and behavioural male traits may not be as obvious as, for example, selection for conspicuous visual ornaments, it can be just as intense as in sexually dimorphic species (Lane *et al.* 2009; Marmet *et al.* 2012). For example, in many rodents male mate-locating ability is strongly influenced by male mobility and perceptiveness, and could require sophisticated spatial memory (Gaulin and Fitzgerald 1989; Schwagmeyer 1994; Schwagmeyer 1995; Waterman 2007). Significant brain capacity in terms of spatial or olfactory memory could explain the echidna's enlarged frontal cortex (Hassiotis *et al.* 2003) and well-developed olfactory bulb (Ashwell 2006). Spatial and olfactory memory could be important for males to survey their home range and use individual-specific cues

(Chapter 4; Chapter 6) to locate and recognise potential mates, avoid females they have already mated with (Ayasse *et al.* 2001) or facilitate mate-guarding (Olsson and Shine 1998).

Searching for mates can be a costly activity (Kokko and Wong 2007) and males of many species reduce search costs and increase their number of potential mates by employing efficient search strategies based on female odour signals (Murphy 1998; Johansson and Jones 2007; Edward and Chapman 2011; Thomas 2011). Male echidnas spend a significant portion of the mating season searching for females and competing with other males (Morrow 2013) and weight-loss is maximal during this period (Nicol and Morrow 2012). Males can minimise search costs and increase reproductive fitness by being sensitive to variations in female-specific chemical cues (Chapter 4; Svensson 1996), allowing males to quickly locate females (Chapter 5) and target females in reproductive condition (Chapter 6; Thomas 2011). A high degree of individual variation in sensory sensitivity is expected in populations with intense selection for ability to locate mates (Ronald *et al.* 2012). Therefore, females benefit from producing small amounts of chemical signal by indirectly selecting for high quality males which are best able to locate her quickly and precisely (Greenfield 1981; Phelan 1992; Wiley and Poston 1996). This strategy could enable females to obtain high quality mates with minimal effort, by 'filtering out' less sensitive males (Phelan 1992; Svensson 1996; Phelan 1997; Ayasse *et al.* 2001).

In addition to being sensitive to female chemical cues, males can maximise their reproductive success by increasing search effort (Schwagmeyer 1994; Murphy 1998; Waterman 2007; Lane *et al.* 2009; Marmet *et al.* 2012) and by becoming reproductively active earlier and for longer than their competitors (Murphy 1998; Ball and Ketterson 2008). Male echidnas maintain home ranges that are larger than needed only for food and shelter throughout the year (Sprent and Nicol 2012). Males are also highly mobile, covering large distances within their home range during the mating season (e.g. up to 3.4 km in 24 h, an area of 33 ha; Nicol unpublished data), which increases the number of females they may encounter. Furthermore, reproductive males in my study population emerge from hibernation as early as mid-May and as late as July (the middle of the breeding season) and males which emerge earlier tend to be found in more mating aggregations than

later-emerging males (pers. obs.). Males in good body condition may be able to emerge from hibernation early (shortening the period of energy saving) and expend more effort searching for females, thereby gaining an advantage over later-emerging males. This hypothesis could be tested by comparing reproductive success (e.g. number of matings, paternity) with search effort (e.g. date of emergence, distance covered) or male body condition, although this approach requires more detailed genetic information than is currently available (Chapter 7). Similarly, male reproductive success depends on energy reserves (e.g. body mass) in several ground squirrels and marmots, including golden-mantled ground squirrels *Spermophilus saturatus* (Barnes 1984) or *S. lateralis* (Frank 1992) and Belding's ground squirrels *Spermophilus beldingi* (French 1982). Males with inadequate fat reserves do not emerge early from hibernation and hence have limited capacity to breed in spring when females emerge while food availability remains low (reviewed by Dark 2005). Inter-annual variations in resource availability may also influence male body condition and reproductive effort in different years, thereby influencing year-to-year male reproductive tactics. Condition-dependent male reproductive tactics would potentially make signalling male body condition via sexually selected odours (Chapter 4; Chapter 7) even more informative for competitive interactions and mate choice (e.g. see Martin and Lopez 2008; Martín and López 2010).

A small investment by females (e.g. releasing minute quantities of pheromones) can be important for guiding male searching behaviour and increases the likelihood of males and females encountering each other during the mating season (Kokko and Wong 2007). However, sexual conflict and sperm competition can drive a shift in female investment, even if that investment is small initially (Kokko and Wong 2007). Intense competition between males can even make female investment in pheromone production entirely redundant (Ayasse *et al.* 2001). For example, males of several species of ground-nesting bees and wasps compete intensively for access to females and may detect and unearth pre-emergent females (Cane and Tengö 1981; Crankshaw and Matthews 1981; Schöne and Tengö 1981; Cardé and Hagaman 1984), possibly because small amounts of female pheromone gradually become 'concentrated' within the enclosed airspace around the submerged female (Schöne and Tengö 1981). In echidnas, many females are still hibernating when located by males (Morrow and Nicol 2009), hence female effort in attracting males

appears to be low. Females do not seem to control this male behaviour, as they show no changes in chemical profiles during hibernation (Chapter 6). Instead, males seem to use sex differences in chemical profiles (Chapter 4) to locate hibernating females, although it is unclear whether this female odour represents a 'signal' or 'cue'. Intra-sexual competition has driven males to emerge from hibernation as early as they are physiologically capable (Morrow 2013), without giving females the opportunity to emerge from hibernation and 'actively' signal to males. In echidna populations with less intense male-male competition and without deep hibernation, we might expect to see a seasonal change in female chemical signals which function to attract males.

From a male perspective, it may be adaptive to target hibernating females over euthermic females by using differences in odour (Chapter 6), as this behaviour provides mating opportunities without competition from other males (Morrow 2013) and hibernating females seem less likely to be pregnant. In other species, males may discriminate between females using chemical cues which derive from functions other than mating (Thomas 2011). For example, male bushcrickets (*Requena verticalis*) use age as a cue to preferentially mate with young females, thereby reducing the probability of mating with older, already mated females (Simmons *et al.* 1994). Male echidnas may use odour differences to target hibernating females (Chapter 6), to reduce the probability of mating with an already pregnant female (since more females become euthermic as the mating season progresses, but these females are often already pregnant). In red-sided garter snakes *Thamnophis sirtalis parietalis*, males prefer the scent of newly-emerged females over euthermic females, perhaps because such females are more sluggish and less able to resist forced copulations (Shine *et al.* 2005). Similar bioassays could test this idea in echidnas, but genetic information is also needed to determine how reproductive success varies between individual males using different reproductive tactics. However, mating with a hibernating female does not guarantee paternity, as females may re-enter hibernation while pregnant (Nicol and Morrow 2012) and many females show mating activity several weeks prior to the time of fertilisation (Chapter 6; Morrow 2013). Physiological constraints might prevent females from becoming pregnant from these early matings. Some species of bat also show mating

activity during hibernation (Thomas *et al.* 1979; Boyles *et al.* 2006), but chemical signalling and female choice has not been investigated.

Although echidna mating behaviour is driven by male response to female scent, female 'deception' via olfactory cues is also possible, although deception is difficult to demonstrate. Prior to my work, chemical signals were thought to be cheat-proof and reliable (Gosling and Roberts 2001; Zala *et al.* 2004; Johansson and Jones 2007) and reduce the probability of male harassment during the non-reproductive period (Johansson and Jones 2007). However, my results highlight that chemical cues do not reliably signal female reproductive status (pregnancy, timing of fertilisation) to males, probably resulting in males continuing to be attracted to pregnant females (Chapter 6). Instead, reproductive female odour may be attractive to males regardless of pregnancy, but female odour may be less sensitive to changes in ovarian hormones associated with ovulation or pregnancy than other species. Although continued mating activity and male harassment may be costly for females (Johnstone and Keller 2000), these costs could be outweighed by the potential fitness benefits of confusing paternity and reducing infanticide risk (Chapter 6; Appendix I), particularly as female echidnas only successfully raise a young on average once every three to four years (Nicol and Morrow 2012). In situations of sexual conflict, selection favours recipients skilled at discriminating genuine signals from those that are not (Seyfarth and Cheney 2003) and males are expected to be selective of females when mating is costly or compromises future mate acquisition (Kokko and Monaghan 2001; Kokko and Wong 2007; Harari and Steinitz 2013). Males may pay 'opportunity costs' by wasting time and energy locating, courting, mating with and guarding pregnant females, which decreases mating opportunities with other females (Shine 2012; Morrow 2013). Therefore, male echidnas should benefit from detecting female reproductive status (pregnancy) via olfactory cues, but such male discriminatory ability can simultaneously decrease female reproductive success by reducing her potential number of mates (Thomas 2011). These processes can result in an evolutionary arms race, with increasing subtlety in female signals and a corresponding increase in male sensory ability (Figure 8.1) (Stumpf *et al.* 2011).

The evolution of chemical signals is a product of co-evolution between the different costs and sex roles involved in signalling (Svensson 1996), sensory systems, cognitive systems, habitat choice and behaviour (Endler 1992). A shift in any of these factors can have fitness consequences, so sensory systems are condition dependent and vary between populations and situations (Endler 1993). Demographic conditions, such as density, distribution and sex ratios, influence mating systems (Kokko and Rankin 2006), patterns of sex pheromone use (Ayasse *et al.* 2001) and scent-marking strategies (Heymann 2006). Sexual selection for mate-locating ability will be especially strong when females are sparsely (spatially and temporally) distributed and difficult to detect (Ims 1988; Endler 1992; Kokko and Rankin 2006) and female distribution, in turn, depends on variations in resource distribution (Clutton-Brock 1989; Sprent and Nicol 2012). My research suggests that male and female Tasmanian echidnas use chemical signals to increase their reproductive success in different ways, even at the expense of the other sex, reflecting differences in parental investment and ecological pressures such as diet, metabolism and resource distribution. Many of my results are consistent with previous studies on mammals and other vertebrates, including secretion chemical complexity, differences between sexes, seasons, individuals and different scent glands, and male sensitivity to female odour cues. I have also shown how variations in scent (or lack thereof) have important effects on mating systems and individual reproductive behaviour. The broad scope of this thesis demonstrates the benefits of using a multi-faceted approach. With further contributions from studies on non-model species and developments in chemical, behavioural and statistical techniques, we can continue to expand the study of chemical ecology and broaden our understanding of the interrelationships and selective pressures involved in the evolution of chemical communication.

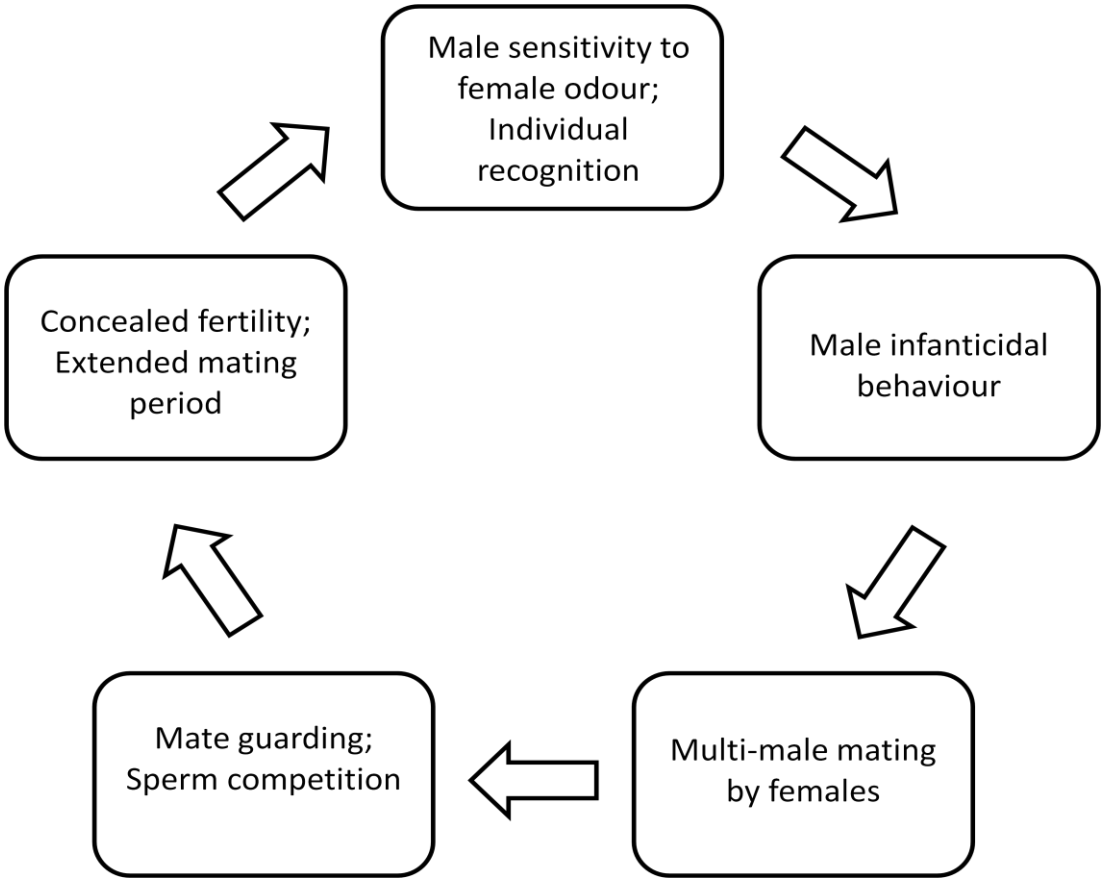

Figure 8.1. Diagram illustrating hypothetical evolutionary arms race resulting from divergent interests in reproduction in a polyandrous (or promiscuous) mating system, and the influence of olfactory cues on male and female behaviour strategies. In mating systems where offspring are vulnerable to infanticide by males (because males seek to increase their reproductive success by subsequently mating with the female), females may attempt to confuse paternity by mating with multiple males. This female behaviour can lead to sperm competition, and males may attempt to restrict multi-male mating with females by mate guarding (requiring the capacity for individual recognition). Females may attempt to overcome mate guarding by concealing fertility to encourage mating, even during pregnancy, using olfactory cues. Therefore, the duration of the mating period may increase, while decreasing the effectiveness of sperm competition and male ability to mate-guard. In turn, males suffer 'opportunity costs' by courting and mating with pregnant females. Therefore, males should benefit from being sensitive to subtle changes in female odour signals and target females in reproductive condition and when fertilisation is possible. However, reduced male confusion over paternity may increase infanticide risk, thereby decreasing female reproductive success. These effects will select for competing behavioural strategies and counter-strategies in both sexes (adapted from Stumpf *et al.* 2011).

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Appendix I:

Observations of breeding behaviour and possible infanticide in a wild population of Tasmanian echidnas (*Tachyglossus aculeatus setosus*)

Appendix I: Observations of breeding behaviour and possible infanticide in a wild population of Tasmanian echidnas (*Tachyglossus aculeatus setosus*)

Abstract

We describe field observations of Tasmanian echidna behaviour, including possible infanticide, where males damaged and entered female nursery burrows. We also present the second report of a female producing a second offspring within a single reproductive season after the loss of her first young at an early stage.

Introduction

The short-beaked echidna (*Tachyglossus aculeatus*) is the most common extant species of monotreme and has a near ubiquitous distribution throughout most of mainland Australia, Tasmania, New Guinea and several offshore islands (Griffiths 1968; Augee 2008). Despite being common, details of echidna life history, reproduction and breeding behaviour are quite patchy. Echidnas are usually solitary, cryptic, semi-fossorial animals (Griffiths 1978) and mating often occurs in shelters (Nicol *et al.* 2005; Morrow *et al.* 2009), which makes observing breeding behaviour difficult. The mating season occurs between June and September throughout Australia (Morrow *et al.* 2009) and is characterised by intense male-male competition for access to females (Griffiths 1978; Rismiller and Seymour 1991; Morrow *et al.* 2009). However, there are several differences in activity patterns, courtship and maternal behaviour between the geographic subspecies (reviewed by Morrow *et al.* 2009). For example, courtship is relatively prolonged in Kangaroo Island echidnas (*T. a. multiaculeatus*), where males follow females in 'trains' for several days or weeks prior to the formation of mating 'ruts' (Rismiller 1992; Rismiller and McKelvey 2000), while in Tasmania, courtship appears to be minimal during the early part of the breeding season, as males seek out and mate with females prior to the female's final emergence from hibernation (Morrow and Nicol 2009). Females typically produce a single young in years in which they have mated (Rismiller and McKelvey 2000; Morrow *et al.* 2009). Beard and Grigg (2000) report a single female producing a second offspring within a single mating season after the apparent loss of the first young, but it is unclear whether this behaviour also occurs in other subspecies. Females enter purpose-built nursery burrows prior to egg-laying (Griffiths 1978; Morrow and Nicol 2012), while males provide no parental care. The period of maternal burrow confinement and age at weaning differs between subspecies (reviewed by Morrow *et al.* 2009). In Tasmania, females construct and enter a single-chambered nursery burrow approximately three days prior to egg-laying and remain in the nursery until young are approximately 37 days old. The female then begins to leave the burrow on foraging trips until the young is weaned at approximately 150 days (Morrow and Nicol 2012).

Echidnas have only recently been successfully and consistently bred in captivity (Ferguson and Turner 2013). This may be due to high levels of offspring mortality during the early stages of egg incubation and lactation, as observed in our Tasmanian study population (Morrow and Nicol 2012). Females may move their nursery burrows to new locations, but offspring often do not survive (Morrow and Nicol 2012) and the causes are unclear, although one possible explanation is male harassment. Female Tasmanian echidnas are promiscuous (Morrow *et al.* 2009; Morrow and Nicol 2009) and continue to attract males and even mate during pregnancy (Morrow 2013). Pregnant females have been found with males within a few days of constructing a nursery burrow and egg-laying (Chapter 6). It is unknown whether females also continue to attract males after entering nursery burrows, as to date cameras have not been used to monitor burrows at such an early stage of maternal confinement. Here, we used camera traps, external temperature loggers and behavioural observations to intensively monitor several females during the 2012 mating season, in order to describe female behaviour and that of conspecifics around the time of nursery burrow construction and egg-laying. Specifically, we asked whether females continue to experience male harassment after burrow construction and around the time of egg-laying, and whether females consequently move to new nurseries.

Materials and methods

Field site and animals

Observations reported here were made as part of a long-term study on ecological and physiological aspects of Tasmanian echidnas (*T. a. setosus*) at our field site in the southern midlands (42°28'S, 142°14'E), located approximately 55 km north of Hobart (for details see Morrow *et al.* 2009; Nicol *et al.* 2011). Between January 1996 and December 2013, 276 individual echidnas have been captured and fitted with passive implantable PIT tags for identification. Selected individuals had radio-frequency transmitters (Holohil Systems, Ontario, Canada) glued to the spines on the lower back, allowing us to monitor mating activity and maternal behaviour up to three times per week during the mating season. Twenty-four adults (15 females, 9 males) had radio-transmitters attached during the 2012 field season. The records from external temperature loggers (Thermochron iButtons, DS1922L,

Maxim/Dallas Semiconductor, TX, USA) attached to the radio-transmitters allow accurate dating of events such as arousal from hibernation, entry into nursery burrows and timing of egg-laying (Nicol and Andersen 2002; Nicol and Andersen 2006; Morrow and Nicol 2009). Camera traps (Scoutguard SG550, HuntingCamOnline, Gadsden, SC, USA; Reconyx PC800, Holmen, WI, USA) were set up outside female hibernacula prior to the mating period (Morrow and Nicol 2009) and over nursery burrows (Morrow and Nicol 2012), but we set up cameras immediately after the female was first tracked to a plugged burrow following mating activity. The positions of radio-transmitters and colour-coded plastic tubing attached to the spines allowed us to identify individuals on camera footage. Cameras were downloaded and batteries replaced at least weekly. We checked for the presence of spermatozoa (confirming recent mating activity) in female reproductive tracts by collecting urogenital smears while the animals were under light inhalation anaesthesia (Morrow and Nicol 2009).

Results and discussion

Echidna activity at nursery burrows

During the 2012 breeding season, seven female echidnas were observed in mating aggregations and subsequently entered nursery burrows. Camera traps were set up over ten separate nurseries between 19 July and 21 September, usually within 2-3 days of burrow construction. The number of nursery burrows exceeds the number of females because one female produced two eggs within the same reproductive season and moved her nursery to different locations several times (details below); we have counted these as separate burrows. At eight nurseries, cameras recorded a combined total of 18 instances of echidna activity (excluding the mother), involving sniffing, digging and probing the soil. This activity was concentrated around the burrow entrance and above the chamber where the female was located, often resulting in substantial damage (Figure A1.1). Diggings and new ‘entrances’ were also found before the camera had been set up, indicating high levels of echidna activity in the days immediately after burrow construction. At one nursery burrow, eight instances of intense digging activity were recorded over a three-day period. One female moved her nurseries three times (details below), each time coinciding with intense digging activity and damage at the previous burrow. Known males

were recorded outside the nursery burrows of females which they had previously been found with in mating groups (*n*=3; echidnas 1B75, 5036, 6D18). The identities and sexes of the remaining animals recorded disturbing nursery burrows (*n*=15) are unknown and at least some of these could have been the same individual multiple times. It is likely that these animals were also males, as most reproductive females are confined to nursery burrows by this time of year (Morrow *et al.* 2009; Morrow and Nicol 2012) and non-reproductive females usually hibernate until mid-September (Nicol and Morrow 2012).

Echidnas other than the mother were recorded entering five nursery burrows: three times while females were inside and twice after burrows had been abandoned. Known males that had been found with the female in mating aggregations only appeared to enter nurseries after burrows had been already disturbed by other echidnas (*n*=2; echidnas 6D18, 5036). The entry of a second echidna was associated with a sudden, prolonged change in temperature recorded by the logger attached to the female's transmitter (e.g. female 060A, see Table A1.1, Figure A1.2).

Our results show females attract conspecifics and experience continued disturbance even after entering nursery burrows. Echidnas have occasionally been recorded outside nursery burrows in previous studies, including one instance of an unidentified echidna entering a nursery while the lactating female was away foraging (G. Morrow pers. comm.), but this is the first time we have set up cameras immediately after burrow construction. Female echidnas in our study population often move to a new nursery burrow early in the incubation or lactation stages and this was previously suggested to be in response to burrow collapse, flooding, or to regulate burrow temperature (Morrow and Nicol 2012). New nursery burrow entrances have also been observed (G. Morrow pers. comm.), but were thought to be caused by the female abandoning or rebuilding the nursery (Morrow and Nicol 2012). Our observations indicate that females may move nurseries in response to male harassment and damage caused to the burrow. Male interest in females is suggested to decline after mating has occurred (Rismiller and Seymour 1991; Ferguson and Turner 2013), however, females in our study population continue to attract males during pregnancy (Morrow 2013), and even after entering a nursery (this study). Males probably use sex-specific differences in olfactory cues to locate

females during the mating season (Harris *et al.* 2012) and it is possible that males continue to be attracted to female scent even after she has entered a nursery. The intensity of echidna activity at least indicates that conspecifics detect the presence of females within nursery burrows.

Second female breeding event within a single season

One female (060A) had an egg in her pouch on 23 July (confirmed by visual inspection) and lost her young approximately 12 days later, possibly as a result of repeated disturbance by males while in two separate nurseries (Table A1.1). No egg or young was found, but loss of young was confirmed when she abandoned her second nursery and re-entered hibernation. 060A emerged from hibernation and was later found with a male (echidna 5036) on 30 August, with no egg or young in her pouch (Table A1.1, Figure A1.2). A 210 g young was later found in her fourth nursery burrow in November. Based on the offspring's size and development (Morrow and Nicol 2012), we can estimate that fertilization had occurred in late August. Another female (echidna 2957) also re-entered hibernation after losing her egg (confirmed by visual inspection), and was found in further mating aggregations, but did not produce a second young in 2012.

This is only the second report of a wild female echidna producing a second offspring within a single reproductive season. Beard and Grigg (2000) reported an approximately 27-day interval between loss of the first young and re-mating, compared with ~26-day interval observed here. However, female 060A was often inaccessible after emerging from hibernation, so fertilization could have occurred before she was found on 30 August. Nevertheless, these timings are consistent with the suggested length of the echidna oestrous cycle of 33 days (Higgins *et al.* 2004). Although we did not recover sperm from 060A after the loss of her first young or see the second egg, successful re-mating must have taken place as we later found a young in her nursery burrow. Beard and Grigg (2000) state that producing a second offspring within a single season "may be more likely to occur in Queensland than in cooler parts of Australia because of the longer time available for young to develop and for the female to regain condition before the onset of a comparatively shorter, milder winter". Our observations demonstrate that female echidnas are capable of producing a second offspring, even in colder areas such as Tasmania. The loss of an

offspring prior to hatching, or shortly after, would represent a small energetic investment by the mother relative to the major energetic costs of early arousal from hibernation and time spent euthermic with minimal feeding during winter (Nicol and Andersen 2002). Females have been found near our study site with eggs in their pouches as late as October (Morrow *et al.* 2009), which could also be the result of a second mating after the loss of the first offspring. One female at Philadelphia Zoo produced three eggs over a five-month period, although no young were successfully weaned (T. Sinander unpubl. obs.).

Infanticide in echidnas?

The observations that females are capable of producing a second offspring following the loss of the first young within a single reproductive season and that males disturb and enter nursery burrows suggest the possibility of sexually-selected infanticide in echidnas. Possible infanticide behaviour has been described in captivity (T. Sinander unpubl. obs.), but not in a wild population. Infanticide (the killing of offspring by conspecifics other than the parents) has been widely documented in mammals (Hrdy 1979; Ebensperger 1998) and may increase male reproductive success, provided (1) the infanticidal male is not related to the young; (2) the mother's subsequent inter-birth interval is shortened; and (3) the male has access to the female when she becomes receptive again (Borries 1997; Ebensperger 1998). Morrow and Nicol (2012) reported that in our study population only 20% of young survived to weaning, while 60% of young died before hatching or in the first two weeks of lactation and it is possible that this is at least partly due to males disturbing females in nursery burrows. Stable nursery burrow temperatures are important for embryo growth and development during egg incubation (Morrow and Nicol 2012) and a male entering the nursery, or causing the female to move to a new nursery following disturbance, would destroy that stable thermal environment. Females which lose their offspring are more likely to breed in the following year (Borries 1997; Morrow and Nicol 2012) or even within the same season (this study). Whether this behaviour is truly sexually-selected infanticide cannot be verified without genetic information and further monitoring, thereby confirming whether potentially infanticidal males are related to the young, whether they have previously mated with the female and whether they are successful in siring subsequent offspring. The success of the captive breeding program described by

Ferguson and Turner (2013) may be partly due to females being housed alone after mating, preventing any opportunity for nursery burrow damage to occur.

Figure A1.1. Photograph of nursery burrow following 8 instances of echidna digging activity over approximately 52 hours. Note burrow entrance (top arrow) and evidence of digging activity over and surrounding area above burrow chamber (remaining arrows). Extra 'entrance' (bottom left arrow), not fully plugged, leads to incubation chamber where female is located inside and matches the location of digging activity recorded by motion-sensing camera. Photo: R. Harris, 26 July 2012.

Table A1.1. Details of mating activity, echidna digging activity recorded outside nursery burrows and second breeding event for female 060A during the 2012 breeding season. Timing of egg-laying and entry into nursery burrows is inferred from external temperature logger records. Times are based on camera trap time stamps or time direct observations were made. Dates of egg-laying and when found in mating aggregations are in bold type (indicated by stars and filled triangles in Figure A1.2, respectively).

Date	Time	Details
25 June	21:31	Male 5036 enters female 060A's hibernaculum.
28 June	10:45	Found in mating aggregation with male 5036. Sperm recovered from 060A.
17 July	-	060A constructs and enters nursery burrow #1
18 July	-	Egg-laying
19 July	11:10	Camera set up over nursery #1
20 July	00:24	Unknown echidna enters nursery #1 (Figure 1)
	00:40	Male 5036 outside nursery #1, does not enter
	17:52	Unknown echidna leaves nursery #1
23 July	10:40	Egg in pouch, no sperm recovered from 060A
24 July	17:00	060A leaves nursery #1
	17:19	Unknown echidna outside abandoned nursery #1
	23:21	Unknown echidna outside abandoned nursery #1
27 July	-	060A constructs and enters nursery burrow #2, ~320m from nursery #1
30 July	10:45	Camera set up over nursery #2
31 July – 4 August	-	Three unknown echidnas digging into nursery #2 (over five days)
4 August	16:11	060A leaves nursery #2
6 August	10:30	Burrow found open, new 'entrance', abandoned. Egg probably lost.
9 August	-	060A re-enters hibernation
21 August	04:45	Emerges from hibernation, leaves hibernaculum.
30 August	12:08	Found in mating aggregation with male 5036. No sperm recovered. No egg in pouch.
7 September	-	060A constructs and enters nursery #3, ~420m from nursery #2
9 September	-	Egg-laying
10 September	13:55	Camera set up over nursery #3
11 September	17:21	Unknown echidna digging over burrow chamber (over 2 h)
14 September	20:42	Male 5036 digging over burrow chamber
18 September	17:00	060A leaves nursery #3060A constructs and enters nursery #4,
19 September	-	~240m from nursery #3
21 September	00:27	Unknown echidna enters abandoned nursery #3
	00:36	Unknown echidna leaves abandoned nursery #3
5 November	13:25	Nursery #4 partly excavated, 210 g young inside

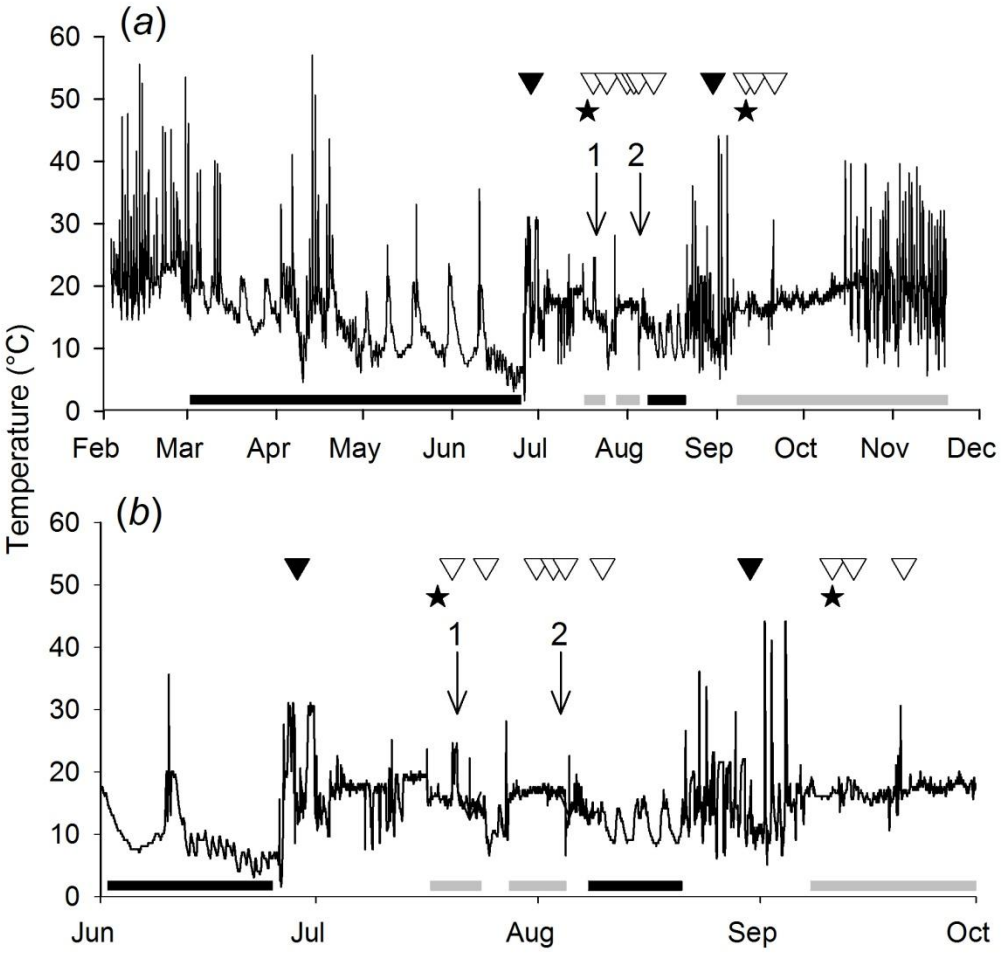

Figure A1.2. Temperature records for female 060A in 2012 (upper: February – December; lower: detail of June – October). Filled triangles: observed mating groups; open triangles: echidna activity (including males) recorded outside nursery burrow; stars: estimated dates of egg-laying. Black bars: hibernating; grey bars: in nursery burrow. Arrows in both figures: (1) echidna captured on camera entering nursery, coinciding with sharp spike in otherwise constant temperature while in nursery burrow; (2) probable loss of first egg, followed by re-entry into hibernation.

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Appendix II:

Testing behavioural responses of male echidnas to female odour using a Y-maze choice paradigm in captivity

Appendix II: Testing behavioural responses of male echidnas to female odour using a Y-maze choice paradigm in captivity

Abstract

This appendix describes the results of a series of behavioural choice tests conducted on wild male echidnas temporarily housed in captivity. These trials were unsuccessful, so have not been included in the main body of the thesis, but are important when considering the results of Chapter 6. Preference trials in captivity were conducted with the intention of developing them to test specific hypotheses directly relating to findings of earlier thesis chapters (e.g. Chapter 4, Chapter 6).

Introduction

Repeatable behaviour trials, or bioassays, are an important component of olfactory communication studies, as they provide useful starting points for testing animal responses to odour stimuli while controlling for other cues (Wyatt 2003; Thomas 2011). Behaviour tests are also essential for confirming whether animals can detect and discriminate between scents which may differ in chemical composition (for example, differing between individuals), and therefore whether animals actually use the information available in a given odour. Consistent differences in measured responses are typically interpreted as a preference for or avoidance of a particular scent, or simply evidence of discriminatory ability (Nolte and Mason 1998). Measured responses include time spent sniffing, tongue-flicking, counter-marking, flehmen behaviour, time spent in proximity to a stimulus, or longer-term physiological responses (Nolte and Mason 1998; Wyatt 2003; Charpentier *et al.* 2010). Behaviour tests may be conducted in the wild or, more commonly, in captivity, and a variety of experimental designs can be used. Trials conducted in captivity enable potentially confounding variables to be controlled, and simultaneous presentations, including Y-maze choice paradigms, control for temporal variation in motivation and thus provide a sensitive test of discriminatory ability (White *et al.* 2003). Field trials may be used when it is not practical to conduct trials in captivity (e.g. Kimball *et al.* 1998). Behaviour responses conducted in the field on wild individuals are thought to yield more 'natural' behaviours, but external variables can be more difficult to control, so both techniques have advantages and disadvantages.

Short-beaked echidnas (*Tachyglossus aculeatus*) are thought to rely on scent to locate mates during the breeding season (Johnson 1978; Russell 1985; Boisvert and Grisham 1988; Beard and Grigg 2000), but captive trials have not been conducted to confirm this idea. Field experiments conducted on wild echidnas on Kangaroo Island indicate that males are strongly attracted to the scent of hessian bags containing a female previously found in a mating group, and males were also attracted to the scent of empty hessian bags which had previously held a female for several hours (Rismiller 1992). Behavioural trials in captivity have previously been carried out on echidnas (Saunders *et al.* 1971a; Saunders *et al.* 1971b; Buchmann

and Rhodes 1978; Burke *et al.* 2002), although none examined aspects of olfaction, mate choice or reproductive behaviour.

In this small-scale study, I tested behavioural responses of wild male echidnas to scent from reproductively active females in captivity. Specifically, I used a Y-maze choice paradigm, with the intention of ultimately conducting robust preference tests using scent collected from reproductively active and inactive wild individuals, and presenting scent to individuals in captivity. I predicted that males would 'prefer' to associate with scent from females, and show the strongest response during the mating season.

Materials and methods

Study site and animals

All animals described here were part of a wild population at our field site in the Tasmanian southern midlands, located approximately 55 km north of Hobart (42°28'S, 142°14'E) (details see Nicol *et al.* 2005; Nicol *et al.* 2011). A 12 km^2 section of grazing property has been the location of ongoing physiological and behavioural investigations on the resident population of wild echidnas since 1996, and during that time over 270 individuals have been captured and tagged with passive implantable transponders (PIT tags; LifeChip, Destron-Fearing, USA) for identification. Selected individuals are fitted with radio-transmitters and monitored approximately weekly, or more frequently during the mating season (Harris *et al.* 2012; Chapter 2).

Scents

Scents consisted of cloacal swabs collected from females found in mating aggregations (in close proximity to one or more males) during the 2010 and 2011 mating seasons (June-September). Scents were collected by wiping the cloaca with a section of glass micro fibre filter paper (Advantec, Japan, ø 47 mm), while the animal was under light isoflurane anaesthetic (methods see Harris *et al.* 2012; Chapter 2). Swabs were stored in individual glass vials on ice while in the field, and then at -20 °C until they were used for scent trials. Multiple scent donor animals were used for all trials, in order to minimise any potential biases associated with

pseudoreplication of odour sources (Ramírez *et al.* 2000) such as habituation to, or preference for, scent from particular donor individuals. Scents collected during the most recent mating season were used for each scent trial. Each swab sample was used in only one scent trial.

Recipient animals and husbandry

Five male echidnas were collected from the study site in late January (n = 2) and mid-August (n = 3) in 2011 and transported to captive facilities at the University of Tasmania, Hobart. Animals were weighed in the field before and after scent trials. All males were of adult size, weighing 4.86 kg, 3.87 kg, 4.02 kg, 3.20 kg and 3.75 kg at the time they were brought into captivity and four were known to be reproductively active, having previously been observed in mating aggregations with females. A maximum of one animal was in captivity at any one time during the study period and all animals were returned to their place of capture at the study site after captive trials were completed.

Echidnas were housed in an indoor enclosure (L x W x H: 4 m x 2 m x 2.5 m) with a wooden nest box (0.7 m x 0.3 m x 0.25 m) containing dry grass bedding. The enclosure was subject to natural (outdoor) light conditions at all times. Animals were provided with food on a daily basis using a diet based on that used at Perth Zoo (A. Ferguson, pers. comm.), comprising a mixture of equal parts tinned cat food (Whiskas jellymeat) and rice pollard (CopRice rice bran), with ~0.2 mL multivitamin (Pentavite, Bayer Australia Ltd, Pymble, NSW) and water, presented as a wet slurry (total ~250 g). Fresh water was available *ad libitum*. Animals were kept in captivity for a maximum of four nights, and monitored for signs of stress (i.e. loss of body condition, inactivity) several times each day. Animals which continued to appear stressed after two nights in captivity (e.g. lost weight, little movement) were returned to their place of capture without further scent trials.

Experimental set-up and procedure

The testing apparatus was a Y-maze constructed from opaque plastic, located within the animal enclosure throughout the study period. Removable, perforated partitions were located at the ends of all three arms, and a fourth partition formed the 'start' area at the base of the maze (Figure A2.1). Partitions prevented the animal from

moving out of the Y-maze during trials, but allowed the flow of air and scent through the entire maze. In each trial, the focal male was presented with a choice between a cloacal swab from a mating female and an unused, blank paper swab ('control'). Scents were randomly allocated to each arm of the maze. Scents were held in place with clean surgical forceps and placed outside the perforated partitions at each end of the Y-maze arms. Air was blown over each scent, through the perforated partitions and into the Y-maze arms at a constant rate using small fans. Animal behaviour was monitored using a motion-triggered remote camera (NightTrakker NT50B, UWay Outdoor Products, Norcross, GA, USA) attached to the enclosure ceiling directly above the Y-maze. The camera recorded 30 sec videos when triggered, and footage was downloaded and batteries replaced after each trial. The number of visits and amount of time spent in each arm of the Y-maze was recorded. The footage was also checked for behaviours such as sniffing, tongue-flicking, scent-marking and grooming, which are indicative of olfactory interest and signalling in many species (Wyatt 2003; Müller-Schwarze 2006). Echidnas at our study site show a high degree of nocturnal behaviour (Nicol *et al.* 2004), so all trials were carried out overnight for approximately 14 hrs, over consecutive nights (maximum three trials per animal). Due to the long duration of the scent trials, there are likely to be extended periods of animal inactivity during each trial. Therefore, I did not include time the animal spent inactive in a particular Y-maze arm (e.g. asleep), as this may introduce bias not associated with olfactory interest or active searching behaviour. Males were exposed to scents from females whose home range boundaries were >2 km from that of the male, such that the two individuals were unlikely to have previously encountered each other in the wild and hence avoid potential biases in scent preference due to familiarity with donor individuals (White *et al.* 2003).

At the start of each scent trial, males were first placed in the 'start' area at the base of the Y-maze for 10 mins to acclimatize to the maze and air flow. The partition between the 'start' area and the rest of the maze was then removed and the animal was free to move about the maze until the end of the trial. Each male was allowed 24 hrs to acclimatize to captivity prior to the first scent trial and then approximately 10 hrs between subsequent scent trials. At the completion of each trial, the partitions were removed, and the animal was released from the Y-maze. The Y-

maze was cleaned with water and ethanol and allowed to air-dry. When scent trials were not in progress, all partitions were removed so that animals could freely explore the inside of the maze.

Results

Four out of the five male echidnas failed to leave the start area of the Y-maze in any of their scent trials and data from these trials were discarded. Two males showed continued signs of stress and were returned to their place of capture after two nights. All five animals lost weight during the two to four nights spent in captivity (mean 10.7 ± 6.99 % of original body mass) and none appeared to consume the food available.

One male (436C) left the start area of the Y-maze in all three scent trials conducted during the mating season (Table A2.1). On average, male 436C visited more often and spent more time in the maze arm with scent from a mating female, but was not a significant difference (number of visits: Student's t-test, two-tailed, $t = 0.277$, $P = 0.81$; visit duration: Student's t-test, two-tailed, $t = 0.067$, $P = 0.95$). The most commonly observed behaviour was climbing the maze walls, although this was more likely to be attempted escape than exploration. Other behaviours included sniffing and scratching, although these did not appear to be directed towards or in association with particular scents.

Discussion

This small-scale study sought to address two main aims: first, to provide experimental confirmation that male echidnas respond (are attracted) to female odour; and second, to assess the efficacy of preference behaviour trials in captivity. The results of this study do not indicate that a Y-maze choice paradigm would be an ideal method of investigating olfactory cues and mate choice in wild echidnas. However, there are a number of potential explanations for why this part of the study was not successful. Simply because an animal fails to respond is not evidence that it has failed to detect a stimulus (Nolte and Mason 1998). The nature of the experimental set-up and the type of odour source used may be contributing factors, however this study followed published protocols (e.g. Parott *et al.* 2007) and

constructed a Y-maze based on the size and style of published designs, including those previously used on echidnas (Saunders *et al.* 1971b; Burke *et al.* 2002). The odour sources used in trials conducted during the mating season were less than a few weeks old and had been stored frozen (similar to scents used in trials conducted in the field, see Chapter 5), so it is unlikely that scents were simply not strong enough to be detected by focal males in the Y-maze. The time of year that males were brought into captivity could have contributed to the low levels of activity observed during this study. Two males were brought into captivity in February, coinciding with the time that many males in our study population begin to enter hibernation (Nicol and Andersen 2002). Therefore, these males may have had reduced motivation to investigate female odours and reduced activity levels associated with preparing to enter hibernation.

A major contributing factor is likely to be the amount of time allowed for the animals to acclimatise to captive conditions. 'T-maze' choice paradigms have successfully been used to investigate echidna spatial memory, cognition and position habits (Saunders *et al.* 1971a; Saunders *et al.* 1971b; Burke *et al.* 2002), but the animals used in these studies had either been housed for several weeks or had been bred in captivity, and therefore would have had considerably more time to habituate to captive conditions and human proximity. For example, the echidnas used in experiments by Saunders *et al.* (1971b) had either been in captivity for more than a year or had been collected from the wild three weeks prior to experiments beginning. Wild animals initially displayed complete withdrawal behaviour, however this all but disappeared and animals fed easily after approximately three weeks (Saunders *et al.* 1971b). The echidnas used in this study were housed in captivity for 24 hours prior to beginning scent trials and for a total of four nights before being released back into the wild. It is likely that a period of at least several weeks of acclimatisation would be required prior to conducting odour choice trials using wild echidnas. Alternatively, preference trials could be carried out using scent collected from wild individuals and presented to long-term captive animals, although such trials were not practical in the present study. Although not successful here, it is important that robust behaviour tests for echidnas be developed. For example, trials could be used to test whether males differentiate between, and show preferences for, male and female odour, scent from mating aggregations, detect

female reproductive status, or recognise individuals, thereby providing important validation of whether echidnas are able to detect many of the chemical differences previously identified (e.g. Chapter 4, Chapter 6). Odour-based captive behaviour trials may be feasible if conducted on captive-bred animals or on wild echidnas after an extended period of acclimatisation to captive conditions.

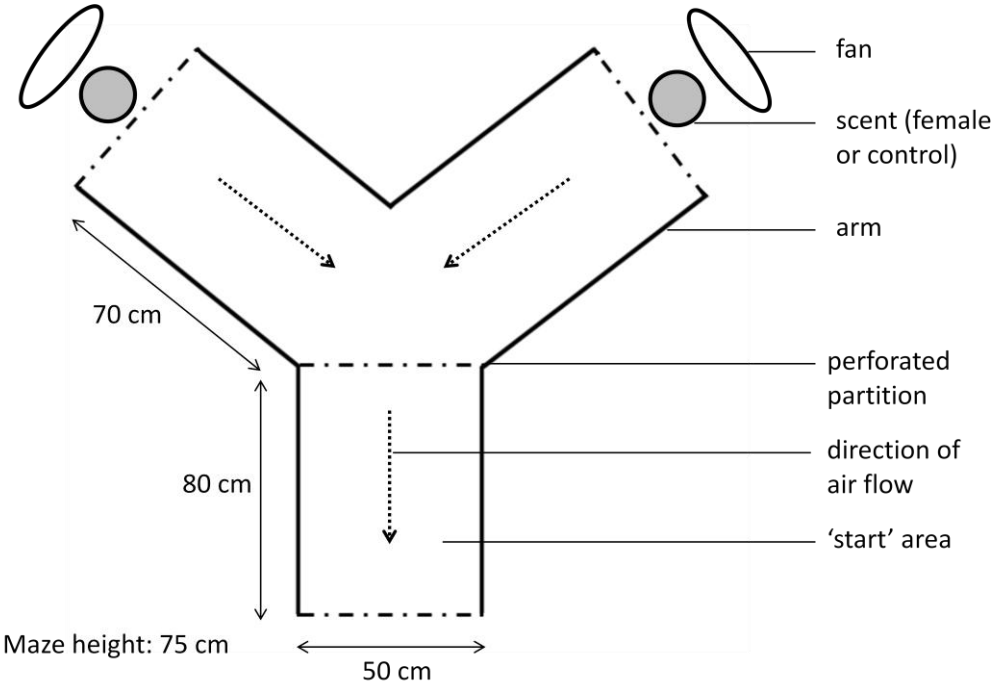

Figure A2.1. Schematic diagram of the Y-maze testing apparatus with size measurements, direction of airflow, position of removable partitions, fans, 'start' area and female scents shown.

Table A2.1. Summary of number of visits and time spent in Y-maze arm containing scent from a female ('scent') or a blank paper swab ('control') over three trials by male 436C. Trials conducted on consecutive nights over 16-19th August 2011.

	# Visits		Visit duration (sec)	
	scent	**control**	**scent**	**control**
Trial 1	3	1	240	30
Trial 2	2	4	30	250
Trial 3	3	2	75	40
Mean	2.67	2.33	115.00	106.67
Total	8	7	345	320

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