THE UNIVERSITY OF HULL

Comparative chemical ecology, behaviour, and evolutionary genetics of *Acanthodactlylus boskianus* (Squamata: Lacertidae)

A PhD Thesis

Comparative Chemical Ecology, Behaviour and Evolution

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By

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Abstract

Acanthodactylus boskianus is a common lizard species frequently occurring in different habitats throughout Egypt. Both males and females have well developed epidermal femoral glands. This species is territorial and males acquire dominance hierarchies in captivity. The current work included three different techniques to study the importance of femoral gland secretions in communication and signal evolution of *A. boskianus*. These are behaviour, chemical and DNA analyses techniques.

Behavioural bioassays in different experiments showed that the femoral gland secretions are used in communication between the lizards. Communication includes possible roles in mate choice, agonistic behaviour between potential competitor males, and chemical trailing of scent pheromones. These behavioural results reflect the chemical results which showed the chemical variability between male ages, sexes, and allopatric populations. Chemical analysis of the secretions resulted in the identification of natural compounds not previously reported in reptiles, glycerolmonoethers and monoglycerides.

The secretions seem to be used as scent pheromones, which are involved in signal evolution processes resulting in divergence of the chemical fingerprints of the gland secretion between allopatric populations.

CHAPTER 1

INTRODUCTION, HISTORICAL REVIEW, AND AIM OF THE WORK

1. INTRODUCTION AND HISTORICAL REVIEW

1.1. Evolution and skin adaptation

Reptiles are considered as one of the classes under the phylum Chordata which emerged 550 million years ago. Fossil and comparative anatomical data confirm diversity of skin, but the main function as an interface separating between external and internal environment remains unchanged (Chuong et al., 2002). The skin is composed of two main layers; epidermis and dermis, unlike lower animals, vertebrates have a multilayered epidermis (Bereiter-Hahn et al., 1986).

Today the reptiles are represented by four orders: Crocodilia (alligators and crocodiles), Chelonia (tortoises and turtles), Squamata (lizards and snakes), and Rhynchocephalia (represented by one surviving species only, the tutara, Sphenodon). The reptiles were the first fully terrestrial vertebrates, and could solve the problem of reproduction in the absence of an aquatic environment. Complete removal from water has severe consequences and new demands on the skin structure are needed for the terrestrial environment. The formation of a barrier against transcutaneous water loss is needed to avoid dessication of the reptilian animal, which are mostly living in dry habitats. An efficient tool to protect the reptiles was to form lipidic membrane-like layers in the keratinized skin epidermis (Landmann et al., 1981). Another problem faced the reptiles, which is the mechanical friction to which the skin will be subjected on the surface of the earth. This problem has been solved by the fact that their epidermis is supported by both types of keratins; hard β-keratin and pliable α-keratin, and the dermis is supported by bony plates (osteoderms) (Maderson, 1985). This strength of both epidermis and dermis give the skin a very strong rigid structure which might need some flexibility for the animal to be able to move freely in its environment. This flexibility is found by the formation of the scales or the scutes which are folding of the dermo-epidermal boundary, the scales are connected with hinge regions which are supported only by α-keratin (Khannoon, 2004, Maderson, 1985). The dermis of vertebrates contains mainly fibrous collagen, secreted extracellularly by fibroblasts. There are always a relatively loosely-packed superficial dermis, and a much denser-packed deep dermis. The latter is attached to muscle fascia by subcutaneous connective tissue, the amount of which varies among body regions and species.

Epidermal renewal is a unique process occurs as a result of the presence of stratified layers of the epidermis and the presence of α- and β-keratins. In other vertebrates, epidermis is renewed by exfoliation from the surface and this necessitates the continuous cell proliferation and differentiation. In squamates, the epidermal cell division and its differentiation give rise to a new complete epidermal generation beneath the old one prior to sloughing. During sloughing, the old outer generation is shed and the subjacent new inner generation plays as the body surface (Landmann et al., 1981). The skin of squamates is playing another role in the colour change, which is important for matching the environment as a camouflage or in the communication between the animals. This happens by the movement of melanosomes in the melanophore dendrites in response to melanophore-stimulating hormone (MSH) secreted by the pituitary gland (Sawyer et al., 1983). Colour change is very obvious in lizards such as agamids, anolids and highly noticeable in chamaeleons.

1.2. Skin Glands

The reptilian skin is usually described as dry skin, which means that it is devoid of glands. Occasionally there are integumental glands which usually appear around the anal area. These glands might be named femoral glands or pre-anal glands, particularly in squamates (lizards and snakes), occuring on the femur or the posterior part of the abdomen, successively. These glands are epidermal in origin and give their secretions to the external environment. Generally there are two types of epidermal glandular specializations in the lizard epidermis (Maderson, 1967, Maderson, 1968). The first type is the generation glands, which are subdivided into “escutcheon scales” and β-glands. This type is represented by some patches of scales on the posterior abdominal or femoral regions of the body which show some concavities on the outer scale surface. Exposure of gland materials to the environment occurs during sloughing when the outer layer is lost (Maderson, 1968). The second type of gland is the femoral or pre-anal glands. Histological, these femoral or pre-anal glands might be follicular or tubular and open to the exterior by a well defined pore (Cole, 1966a). This type of glands is independent of the skin activity during the sloughing cycle. During the breeding season the stratum germinativum is active producing secretory cells which go in a series of stages to end with the secretory materials released to the external environment (El-Shershaby et al., 2006, Khannoon, 2004). (Cole, 1966b) provided the first histological description of the femoral glands in *Crotaphytus collaris*. He demonstrated that these glands are composed of branching tubes and tubules. Pre-anal glands of two gekkonid lizards, *Gekko gecko* and *Hemidactylus bowringii* were described by Chiu and Maderson (1975). The former gecko glands were tubulo-acinar, and the later one glands were simple tubular. Later on, more studies described these glands histologically. Chauhan (1986) indicated that the pre-anal glands of *H. flaviviridis* are tubulo-acinar. Khannoon (2004) studied the glands of five different lizards, two agamids, two lacertids and one gekkonid. These were of two types; tubule-acinar in lacertids and gekkonids and branched tubular in agamids. Antoniazzi et al., (1994) and Jared et al., (1999) studied the epidermal glands of the amphisbaenian *Amphisbaena alba* and proposed a pheromonal role of these glands.

1.3. Vomeronasal organ (VNO) and Pheromones perception

The Vomeronasal organ is a nasal chemosensory structure found in most terrestrial vertebrates. The organ was discovered for the first time in mammals by Ludvig Jacobson (1783–1843) and was the first time for the organ to be described. A translation of his original article has been made (Jacobson et al., 1998). The organ he discovered was renamed organon vomeronasale (Jacobsoni) by *DerAnatomische Gesellschaft* in 1895. Jacobson assumed that the organ was secretory in nature but he suggested that it could also be a sensory organ. This organ is now recognised as a chemosensory organ for pheromones. The vomeronasal organ (VNO) is embryologically derived from the olfactory placode, and is similar to the olfactory organ morphologically and physiologically (Halpern, 1992).

In squamata the VNO is located in the palate below the nasal cavity. Stimuli are collected by the tongue and transported back to the vomeronasal ducts. These narrow access ducts open into grooves in the roof of the mouth and the forked tongue tips are drawn back over them, transferring the stimulus molecules which then pass up the ducts into the VNO. A spherical “mushroom body” covered by a ciliated epithelium is occupying the lumen of the organ. In contrast to the main olfactory system, the vomeronasal system is most sensitive to non-volatile molecules (Burghardt, 1980, Halpern, 1987). Inhalation is not enough to deliver the non volatile molecules to vomeronasal sensory receptors. Some mechanisms such as Flehmen in mammals (Melesedhospital and Hart, 1985) and tongue flicking in snakes and lizards (Graves and Halpern, 1989, Halpern and Kubie, 1980) are used to actively transfer these non-volatile chemicals. Experimental study of vomeronasal system function showed that destruction of the olfactory bulbs abolishes all sexual behaviour in male hamsters (*Mesocricetus auratus*), and that deafferentiation of the vomeronasal organ also causes severe sexual deficits. At that time no link was made between the vomeronasal system and the detection of pheromones (Powers and Winans, 1975). That association was later uncovered and the possible relationship was discussed in an influential review paper by (Wysocki, 1979), who concluded that the vomeronasal system could mediate responses to pheromones. The results which showed that removal of the vomeronasal organ does interfere with pheromone responses in some species lead several researchers to accept the hypothesis that the system is specialized for pheromone detection (Belluscio et al., 1999, Buck, 2000, Dulac, 1997).

1.4. Tongue flicking mechanism

Tongue flicking is the behaviour most commonly associated with vomeronasal organ use in squamates. The lizard and snake tongue flicking is involved in transferring chemicals from the external environment to the vomeronasal organ (Graves and Halpern, 1989). Involvement of the vomeronasal organ in the behaviour was expressed by changes in tongue flicking rate relative to baseline levels or responses to a control chemicals (Burghardt, 1980, Graves and Halpern, 1989, Simon, 1983).

The Squamate tongue is an important tool for vomerolfaction (Halpern, 1992). Forked tongues in squamates are playing obvious roles in chemosensory sampling. Tropotaxis is a mechanism that depends on the comparison of concentrations of chemical samples on the right and left tines of the tongue. Forked tongues are enhancing scent trailing and location of prey and conspecific individuals using tropotaxis (Schwenk, 1994). Forking differs among genera within families of squamates, but differences among species within genera are small. In iguanian families forking is rudimentary, but highly developed in anguimorphan and lacertiform (including the family lacertidae) families (Cooper, 1996). Forking is believed to enhance vomerolfactory scent-trailing. Tongue-flick rates in response to cotton swabs bearing chemical stimuli are used to assess experimentally responses by squamates to biological important stimuli. Measuring the tongue-flick rates is used in comparative studies of response to prey chemicals, and pheromones (Burghardt, 1980).

1.5. Gas Chromatography-Mass Spectroscopy (GC-MS)

Gas chromatography (GC) is a standard method of analysis for volatile chemicals. GC has very high resolution, and more than a hundred compounds can be separated in one run. Amongst other advantages of using GC, is that the operation of a GC is simple, flexible, fast, and represents a relatively inexpensive method. The flame ionization detector (FID) is the standard detector used with GC, is sensitive and universal, and small amounts of a compound (~10-100 picograms) is enough for all the chemicals to be detected. Retention indices (relative retention times in GC compared to a hydrocarbon standard) are reproducible between instruments and they can be used as a valuable identification tool between different groups of researchers. GC can be carried out with capillary (0.2-0.32 mm i.d) or megabore (0.53 mm and larger) open tubular columns, which are used to separate nanogram to microgram quantities of materials. The other standard method is using larger capacity packed columns (~2-5 mm i.d.) for separating micrograms to milligrams per run.

Separation in GC takes place through a number of mechanisms. Solutes (analytes) are partitioned between an inert carrier gas, which is known as the mobile phase and it sweeps the analyte vapour through the column, and another phase (stationary phase), usually a liquid, which is coated on the inner surface of the column. Depending on the volatility and the interaction between the functional groups of the analyte and the liquid phase, the retention of the analyte will be specific for every compound. Temperature is the driving force in GC where the columns are heated in the range of 40-325◦C (depending on the column), the analytes move into the vapour phase as the temperature is increased and move along the column. The components of the GC consist of a valve to control the flow of clean carrier gas, an injection port, an oven (temperature controlled), the column containing the stationary phase, and detector. Retention indices is the retention times of compounds on a stationary phase relative to defined standards. Retention indices can be reproduced with fidelity on any other GC equipped with the same type of column. Retention indices can be used for the identification of the same compound with later work. Using linear alkanes as reference standards, the Kovats retention index system, is common.

$$\boldsymbol{I = 100y + 100(z - y) \times (\log t'_{r(x)} - \log t'_{r(y)} / \log t'_{r(z)} - \log t'_{r(y)})}$$

Where t'_r is the adjusted retention time (the measured retention time minus the retention time of a non-retained compound), x is the analyte, y is a linear alkane with y carbons eluting before x and z is a linear alkane with z carbon atoms eluting after x. Thus, the retention index of any compound x on any type of column and stationary phase can be calculated (Heath and Dueben, 1998).

A compound or a mixture of compounds are ionized and then broken into fragments, the ions are separated on the basis of mass/charge ratio, and then the relative abundance of each ion is recorded as a spectrum. This is simplified description of the principle of mass spectrometry (MS). If the compounds under study are known, the mass spectrometry will be an analytical tool whose sensitivity and specificity are unsurpassed, and then combining mass spectrometry to gas chromatography is used routinely. GC-MS and liquid chromatography-mass spectrometry (LC-MS) are common types of coupled instruments. If the compounds under study are unknown compounds whose structures are unknown but that is related to known compounds. In this case mass spectrometry provides needed structural information, but the MS complement information could be obtained from other spectroscopic methods such as nuclear magnetic resonance (NMR) used for pure compounds (Webster et al., 1998). Polar N-H and O-H groups on which give hydrogen bonding may be converted to relatively nonpolar groups on a relatively nonvolatile compound. The resultant product may be less polar, thus more volatile, allowing analysis by gas chromatography. Bulky, nonpolar silyl groups are often used for this purpose.

1.6. Pheromones and animal communication

Animal communication has always been an interest for ethologists and behavioural ecologists. Animals use signals in different ways to communicate. A lot of examples could be observed transferring information used by sexual partners, competitors, and predators. Some signals are common to all people and can recognize them such as song of birds, roaring of deer, and mobbing of foxes by crows. Communication is an interaction taking place between an individual acting as a sender who delivers information to another individual that acts as a receiver, the later receives the information and make a decision. There are modes of communication corresponding to the use of sound, light, chemical, or electric signals (Théry and Heeb, 2008). Animal chemoreceptors have evolved and are involved in great diversity of types of chemical communication. Hormones are the molecules acting inside an organism and allowing the communication between different organs. On the other hand, pheromones are the molecules allowing communication among conspecifics. Those detected between species are allelochemicals (Wyatt, 2006) . The pheromones can be produced by excreting glands or by organs involved in digestion or reproduction. Endocrine glands discharge hormones in the blood, while exocrine glands secrete pheromones or allelochemicals (Théry and Heeb, 2008). Depending on the role and function of the pheromones there are different types of pheromones, aggregation pheromones, alarm pheromones, sex pheromones, trail pheromones, and others. Scent marks are used for territorial marking. The boundary marks may advertise the sexual maturity and identity of the subordinates (Théry and Heeb, 2008).

1.7. Chemical studies on the epidermal glands

Chemical analysis was undertaken on the integument and integumental glands of squamates including some work on the femoral glands of lizards. Mason et al., (1987) analysed the skin lipids of the garter snakes using GC-MS. They found clear differences between sexes and also between the breeding and non-breeding seasons. The femoral gland secretions of the green iguana *Iguana iguana* were studied using Thin-layer chromatograms of samples extracted by chloroform-methanol (Weldon et al., 1990). Sterols, free fatty acids, steryl esters, phosphatidylethanolamine and lysophophatidylcholine were found. Analysis by GC-MS showed the presence of fatty acids (C14-C24), cholestan-3-ol and cholest-5-en-3-ol in the secretions of two age classes. Electrophoretic and proton NMR studies on the femoral gland secretions of the desert iguana *Dipsosaurus dorsalis* indicated that the secretions are composed of approximately 80% proteins and 20% lipid material (Alberts, 1989). The author concluded that there were individual differences in the polyacrylamide gel banding patterns of the femoral gland proteins. Also he used reflectance spectroscopy and revealed that the secretions were strongly absorbing the longwave ultraviolet light, the information which he used to conclude that the secretions can be found in the environment by this property. Two years later, he compared the femoral gland secretions of 16 lizard species using polyacrylamide gel electrophoresis (Alberts, 1991). The results were implying that the closely related species have secretions that are more similar than more distantly related species. New ketodienes were reported from integumental lipids of the guam brown tree snake, *Boiga irregularis*. This was a significant step towards the identification of pheromones that could be useful to control that snake which caused extinction of some fauna in Guam (Murata et al., 1991). Seasonal variation of the lipids in femoral gland secretions of male green iguanas *Iguana iguana* was found (Alberts et al., 1992) which showed that during the breeding season the secretion deposits are more detectable. Detailed chemical analysis was done on the precloacal gland secretions of *Liolaemus* lizards (Escobar et al., 2001). Using GC-MS to analyse the secretions of 20 species, the authors found that 49 compounds constituting the secretions, these are n-alkanes, long chain carboxylic acids, and steroids. In addition, they found different chemical patterns at the intra- and interspecific levels. Lopez and Martin (2005b) studied the femoral gland secretions of the male spiny-footed lizards *Acanthodactylus erythrurus*. Using GC-MS, the authors found that the secretions are composed of 45 lipophilic compounds, including alcohols, steroids, carboxylic acids, esters of carboxylic acids, and minor components such as lactones, ketones, squalene, and α-tocopherol. In addition they found some differences between adult and subadult male in the composition of the secretions, where they found that the longer chain carboxylic acids are more abundant in older lizards. The same authors studied the femoral gland secretions of male lizards *Psammodromus algirus* (Martin and Lopez, 2006). They found 59 lipophilic compounds, including carboxylic acids, steroids, and few and minor components such as five alcohols, six aldehydes, squalene, α-tocopherol, ketones, and a furanone. The femoral gland secretions of the giant girdlid lizard or sungazer *Cordylus giganteus* were studied using GC-MS (Louw et al., 2007). 53 relatively involatile compounds, including alcohols, carboxylic acids, ketones, esters, and steroids were identified in both sexes. Recently, the femoral gland secretions of *Psammodromus hispanicus* was studied using GC-MS (Lopez and Martin, 2009), and only 20 lipophilic compounds where found. The major groups of compounds where, steroids, carboxylic acids, and minor components of alcohols and squalene. They also suggested that phylogenetic affinities alone might not explain the compounds found in secretions, and that environmental and social organization factors might also influence the composition of femoral secretions.

1.8. Behavioural studies on the epidermal glands

Few behavioural studies have been published on the femoral gland secretions of lizards. The first study was on the secretions of iguanid lizards (Alberts, 1993). He concluded that femoral gland secretions function in conspecific recognition and range marking. In addition he explained the interspecific differences in the secretions chemistry of desert (*Dipsosaurus dorsalis*) and green iguanas (*Iguana iguana*) by the adaptations to the diverse climatic conditions of arid desert and tropical forest environments. Lopez and Martin (2005) showed that the proportions of cholesta-5,7-dien-3-ol in femoral secretions of male Iberian wall lizards (*Podarcis hispanica*) were related to their T-cell-mediated immune response. They concluded that only males with a good immune system may allocate higher amounts of this chemical to signalling. In addition, they found that males that had scents with higher proportions of cholesta-5,7-dien-3-ol, and lower proportions of cholesterol, were selected by females. Proportions of cholesterol in the femoral gland secretions of male iberian rock lizards were found to correlate to body size (Martin and Lopez, 2007), and males were able to discriminate and respond aggressively to cholesterol stimuli presented on swabs.

1.9. Y-maze bioassay

Squamates (snakes and lizards) are well known of their ability of trailing; (Cooper, 1994, Mason, 1992). They can trail conspecifics to aggregation sites, for access to mate, and for overwintering hibernacula. A number of techniques have been used for studying this behaviour, of these the so-called y-maze attraction chambers are frequently used. In y-mazes, chemical cues are laid down on the floor of one arm the maze and the other arm is left blank or contains solvent controls. Brown and Maclean (1983) used this technique with rattle snakes *Crotalus horridus* to examine trailing to overwintering hibernacula. Neonates were able to successfully trail adult conspecifics. Juvenile prairie rattlesnakes were able to be guided back to their dens following shed skins and skin lipids in y-maze experiment. Juvenile pine snakes (*Pituophis m. melanoeucus*) were able to follow and distinguish the chemical cues from adult conspecifics (Burger, 1989). No well designed y-experiment has been done for lizards to evaluate their ability of trailing.

1.10. Phylogeography and Molecular analysis

phylogeography is a term used In earlier molecular surveys of mitochondrial (mt) DNA lineages of natural populations. Various relationships between geography and gene genealogies have been referred to be phylogeographic patterns (Avise and Wollenberg, 1997). Studying the principles and processes of geographic distribution among genealogical lineages, especially among closely related species, is included in the field of phylogeography. Populations which are historically isolated are good candidates for genetic adaptations differences, which are because of the divergent selection pressures they were subjected to without the possibility of gene flow. Vicariance and dispersal are two mechanisms account for the origins of spatially disjunct taxa (Ronquist, 1997). Vicariance can be accounted for if the related populations or taxa were separated by environmental events such as, the rise of a mountain, the breakup of continental landmass, or division of a body of water by a physical subdivision (Myers and Giller, 1988, Nelson and Platnick, 1981, Nelson and Rosen, 1981) Movement of a species away from an existing population or away from the parent organism is called dispersal. Through simply moving from one habitat patch to another, the dispersal of an individual has consequences on population genetics.

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single or few copies of DNA sample to generate thousands or millions of copies of a particular DNA sequence through several cycles of heating and cooling steps. These successive cycles allow the DNA melting (separation of double strands) and enzymatic replication of the DNA. Polymerase uses mixtures of oligonucleotides which are in the medium, usually called primers because they prime DNA synthesis. Primers are designed to be used to initiate DNA synthesis at specific places on the template. The two primers are designed to anneal close to one another. Polymerases recognize single-stranded DNA as an appropriate template and bind temporarily to this strand at a point adjacent to a double stranded stretch. In addition, Polymerase binds to deoxynucleotide triphosphates (dNTPs) which are in the medium, it catalyzes a reaction that attaches the nucleotide to the second DNA strand using the energy stored in the triple phosphate bond in the next step, the enzyme moves to the new end of the double stranded section, and then this process is repeated hundreds or thousands of times a second (Palumbi, 1996). PCR cycle consists of three steps: denaturation, annealing, and extension. Denaturation is the step where high temperature is used to stop all enzymatic reactions such as the previous extension phase. In addition, the high temperature (usually 94ºC) denatures the DNA from double to single stranded. Annealing is the second step where the temperature is lowered to allow the oligonucleotide primers to bind to the appropriate sites in the template DNA. *Tm* is the temperature at which half of the potential binding sites are thought to have primer bound to them, each primer has different *Tm.* A long primer or the primer with higher percent of GC content has a higher *Tm.* Extension is the last step which allows the enzyme to work and synthesize the target DNA sequence. The most common polymerase *Taq* polymerase works well at 72ºC, that is why it is the usual temperature selected for the extension reaction.

Swofford et al., (1996) defined inferring phylogeny as an estimation procedure that is making the best estimate of an evolutionary history based on the incomplete information contained in the data. Molecular systematic depend on the data of the present contemporary species and molecules. Phylogenetic inference methods seek to set up a basis for selecting preferred tree from a set of possible phylogenies. This basis is one of two choices, to define specific sequence of steps (algorithm) which leads to determination of a tree, or to define a criterion for comparing alternative phylogenies to one another and deciding which is better.

1.11. Studied species (Acanthodactylus boskianus)

Class: Reptilia

Order: Squamata

Infraorder: Scincomorpha

Family: Lacertidae

Subfamily: Lacertinae

Genus: *Acanthodactylus*

Acanthodactylus genus is small- to medium-sized lizards and they are the most prominent reptiles in Egypt. They inhabit deserts and semideserts. Often they are called fringed-toed lizards because of the lateral fringes on the toes enabling them to adapt for locomotion on sand (Khannoon, 2004, Baha El Din, 2006). The common species in Egypt are *A. pardalis*, *A. scutellatus*, *A. longipes*, and *A. boskianus* (Baha El Din, 2006, Schleich et al., 1996).

Acanthodactylus boskianus (Daudin, 1802)

Synonyms: *Lacerta boskiana* Daudin, 1802

Lacerta aspera Audouin, 1829

Acanthodactylus boskianus var. *asper* Lataste, 1885

Acanthodactylus boskianus asper Boulenger, 1920

Common name: Bosc's fringe-toed lizard

1.11.1. *Identification*

One of the largest species of the genus, with male average snout-vent length (SVL) at 71.3mm and the maximum found being 79mm, females reach up to 67mm SVL. Dorsals (dorsal scales) are strongly keeled, particularly at the posterior back. Ventrals are less than 13 in straight longitudinal rows. General coloration is different between different populations; darkish or silvery grey with seven brownish dorsal longitudinal lines. The tail base of the male is strongly thickened. Hemipenis is with two lobes. The scales of the upper surface of the tail are similar to the lower surface (Schleich et al., 1996). Femoral pores are distributed on both femurs; 19-26 on each side, number of these pores varies between individuals (Khannoon, 2004).

1.11.2. ***Ecology and social behaviour***

A boskianus is living in arid regions with clay ground or open sand. The habitat usually has low or high plants (herbaceous or bushes) or rocks to be used as a cover. Usually this species is excavates burrows with one or more entrances in solidified sand. Appear in April, hibernates from October to March. Males are territorial, advertising with a slow tail wave after each locomotion, showing the bright red lower tail surface. Captive males establish a hierarchy (Schleich et al., 1996). During rival attach, the stronger male takes a high stance with spreading his gular region while approaching his rival. This species is eating a variety of insect preys; grasshoppers, coleoptera, ants, flies, tenebrionid larvae, butterflies and moths, spiders, and a young *Tropiocolotes tripolitanus* gecko (Schleich et al., 1996). The common predators of *A. boskianus* are *Chamaeleo chamaeleon* (Mosauer, 1934), *Varanus griseus*, *Malpolon moilensis*, scorpions, *Galeodes*, and *Cerastes cerastes*. The reproductive period starts on April and extends until June. Courtship starts when the male approaches the female with an arched neck, probing her with his tongue. The male then probes the female’s tail base, cloacal region and flank. The female lays eggs two weeks after mating, eggs are burried in 8 cm deep hole into moist ground. Clutches of eggs might be 2-7 eggs per clutch, incubated at 28ºC for 89-100 days.

1.11.3. *Geographic Range*

The range of the species extends from Iran to Mauritania. It ranges in Africa, from the Mediterranean, Atlantic coast, south to north Nigeria and Mali, Sudan and Abyssinia. In Asia, it extends from Iraq, Iran, Turkey, and Arabian Peninsula. Arnold et al., (2007) suggested that Arabian and North Africa populations are paraphyletic. *A boskianus* is the most widely distributed species in Egypt. Within Egypt, the species shows a great deal of variation. Almost distributed all over the Egyptian land; Nile Delta, lower Nile Valley, Coastal North Sinai, South Sinai, Eastern and Western Deserts, and Western Oasis (Baha El Din, 2006).

1.12. Hypotheses and Aim of the Work

The skin is a well known barrier that helps avoiding water loss in reptiles. It is also assumed to be a source of some communication tools such as colour change and odour. Very little is known about the skin as a pheromonal source in squamates (Garstka and Crews, 1981). Some squamates have femoral or pre-anal glands, which are giving exocrine secretions to the external environment. These glands might be present in both sexes or just in males. Little information is known about the chemistry and behavioural role of these glands.

We selected one species of the lacertid lizards to be used as a model in our study, this is *Acanthodactylus boskianus*. A number of criteria made the choice of *A. boskianus* to be an ideal test organism, these criteria included;

1- Male and females of *A. boskianus* have well developed femoral glands which are used as a tool in the identification of the species.
2- *A. boskianus* is territorial and acquires hierarchies in captivity which makes it a good example lizard to study in the lab.
3- Common and widespread in Egypt makes the species easy to obtain and to allow access to more than one population.
4- *A. boskianus* is distributed in biogeographical different habitats, and as such enables both, to study the environmental and the phylogenetic aspects of this species.

The key hypotheses of the thesis is that the femoral gland secretions are playing an important role in controlling the behaviours of *A. boskianus*, and that chemicals that stem from it could be used in communication. As an exocrine gland, femoral gland secretions could contain large numbers and quantities of chemical components, and these components needed to be identified to clarify the chemical identity of the species and examine if it has a peculiar, species specific or even population specific chemical fingerprint. If the femoral glands occur in both sexes, and if the suggestion that it plays a role in communication is proved positive, then the possibility of finding chemical sexual variation is high. This leads to another question, are these glands used in mate choice? If the species studied have different biogeographic habitats then the femoral gland secretions could reflect this distribution, and could reflect signal evolution between populations which are not interbreeding. The last resulting question of this therefore is, whether the chemical composition of these glands has phylogenetic implications.

The techniques which will be used in the thesis for behavioural experiments are TFs using cotton swab on wooden applicator and Y-maze for testing the ability of the lizards to trail the femoral gland secretions.

Ethical Note

Collection of lizards was approved by University of Hull. Collection was done in March 2007 by a noosing traps safe to the lizards and do not destroy the niches of the lizards. The Falcon export company (Trade Registration No. 101027 and Export Registration No. 62445) responsible for collecting lizards, is approved by Ministry of state for environmental affairs, Egyptian environmental affairs agency (MSEA – EEAA). Animals were provided with food and water before transfer to airport. Transfer of lizards from Egypt, Cairo airport to Heathrow airport, UK was done in contact with Defra and according to the animal welfare. Animals were transported in calico bags and in an aerated wooden boxes 150cm× 50cm× 20cm. Transfer took only one day; Animals sent from Egypt on Egyptair and received the same day at Heathrow airport. Jalsa clearing agency finished the paper work within 2 hours and then the animals were transferred to Hull, where they kept in cages and provided with mealworms and water. Animals are not controlled under CITES and therefore did not require any UK CITES permits for import (UK CITES Management Authority, Wildlife Licensing & Registration Service). Behavioural experiments were approved by Hull University, Department of Biology. During the experiment each animal was kept in its own cage and there was no need to mark the animal. The trials did not affect the lizards at all. Lizards were kept for another experiment.

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Chapter 2

Paper 1

Sexual differences in behavioural response to femoral gland pheromones of *Acanthodactylus boskianus* (Squamata: Lacertidae)

(submitted to The Herpetological Journal)

Sexual differences in behavioural response to femoral gland pheromones of *Acanthodactylus boskianus* (Squamata: Lacertidae)

KEYWORDS

Acanthodactylus boskianus; chemical communication; squamata; pheromones; Aggression; Sexual behaviour

Running title: Intersexual behaviour towards lizard pheromones

ABSTRACT

Femoral glands of lizards are holocrine structures that produce semivolatile compounds used by lizards in intraspecific communication. Here we show that the femoral gland secretions of a lacertid lizard, *Acanthodactylus boskianus*, contain semivolatile chemicals that elicit sex specific responses in conspecifics. These glands exist in both males and female *A. boskianus*. For the first time for squamates, we report on the responses of both sexes to femoral gland secretions of both sexes. Both males and females increased their tongue flick rate towards male secretions, with males also showing an increase in aggression towards the secretions of other males. Some females were aggressive towards male secretions. Female secretions only elicited elevated tongue flick frequencies in males and did not elicit any aggressive behaviour. Our results show that males and females respond differently to lipids of femoral gland secretions. As a result of male-male competition and mate choice, these secretions appear to play an important role in male territorial behaviour as well as in sex recognition, which might reduce the cost of fighting. This work opens future opportunities to test the role of chemical cues in mate choice and dominance hierarchies in lizards, and to use the secretion fractions in a behavioural assay to help eventually identify these pheromones.

INTRODUCTION

Squamate reptiles sense the chemical stimuli that carry information about several biological properties such as sex pheromonal communication, and prey and predator interaction (Cooper, 1994). Squamates have the most highly developed vomeronasal system of all vertebrates (Halpern, 1992; Cooper, 1994) and use tongue flicking to perceive odours such as pheromones. Pheromonal communication is important in many species of lizards (Mason, 1992; Cooper, 1996; Martin & Lopez, 2000). In lizards, pheromones are important in the discrimination between familiar and unfamiliar individuals (Cooper, 1996), territory marking (Alberts, 1992), sexual recognition (Cooper *et al.*, 1996), and self-recognition (Graves & Halpern, 1991).

Femoral pores are epidermal structures located on the ventral surface of the thigh and/or in the pre-cloacal region of many squamates. They are openings to glands that produce holocrine secretions (Mason, 1992; Alberts, 1993). The secretory activity of these glands varies seasonally and increases during the part of the year in which lizards are sexually active. In some lizards, males have more well-developed glands than females, and in some other lizards only males have these glands (Vanwyk, 1990; Alberts, 1993; Khannoon, 2004). In most populations of *A. boskianus* both sexes have similarly developed glands. Studies have shown that such glandular secretions consist of both lipids and proteins (Mason & Gutzke, 1990). The major compounds involved in chemical communication in squamates are lipids (LeMaster & Mason, 2001). Some studies have investigated the chemical composition of femoral glands (Escobar *et al.*, 2001; Lopez & Martin, 2005; Louw *et al.*, 2007), and others focused on their behavioural effects (Martin & Lopez, 2007). Here we provide a complete study on the behavioural response of both male and female *Acanthodactylus boskianus* towards femoral gland secretions of both sexes tested against a control.

A. boskianus is one of the largest species of the genus, average SVL (Snout-Vent Length) of males is 71.3 mm, and 6.7 mm for female (Schleich *et al.*, 1996). Femoral glands are on both femurs (Fig. 1), femoral pores are 19-26 on one side, no sexual differences exist in number of these pores. Chemical study on the epidermal gland secretions of *A. boskianus* showed that the lipidic part of the gland consists of semivolatile compounds: alcohols, steroids, carboxylic acids, alkanes, amides, aldehydes, carboxylic acid esters, and squalene (Khannoon et al., submitted, chapter 3). These compounds showed quantitative sexual and age differences. There is little information generally in lizards, and no information on *A. boskianus*, regarding the femoral glands role. Are these exocrine glands playing any role in the behaviour of those lizards? And if so, can one sex identify the other using these gland secretions? The present study characterizes behavioural responses towards these secretion components. We hypothesize that males and females can distinguish between the same sex and different sex secretions.

MATERIALS AND METHODS

Adults of *A. boskianus* (up to 79 mm SVL) were collected in Balteem at the Northern coast of Egypt in March (2007), before the beginning of the breeding season, and transported to the Sensory and Chemical Ecology laboratory at Hull University, United Kingdom. The lizards were individually housed in lidless transparent plastic cages (56 cm × 39 cm × 28.5cm; length, width, and height). Each terrarium was provided with rocks for shelter, furnished with sand and a gravel substrate, provided with tree branches, and given access to water ad libitum. Temperature ranged from 22 degrees Celsius (night) to 32 (noon). Gradual temperature change was established by using a timer-controlled heating and ventilation system. A natural photoperiod of 12:12 hrs was simulated using fluorescent lights controlled by timers. UV lamps were used to compensate for a lack of exposure to sunlight. Spot lamps were provided for basking. Lizards were fed with calcium-enriched mealworms and occasionally with crickets.

We used 30 adult individuals, (14 males and 16 females) in the experiments. Males were matched for size (SVL ± 1 mm) and weight (± 0.4 g). All individuals used in the experiment were isolated in cages 10-15 days prior to commencement of the experiments. Only the experimenter had access to the animals and animals quickly grew accustomed to his presence. To collect secretions from femoral glands, males and females were placed ventral side up and all pores were pressed gently with forceps resulting in the extrusion of glandular secretions. The semisolid secretions (8mg) from each individual were dissolved in 500 µl Dichloromethane (DCM) in glass vials if they were used on the same day. Alternatively, they were sealed and kept in a freezer at -18˚C until use. Dissolving in dichloromethane should result in the extraction of lipids from the secretions, and the volume was enough to cover and dissolve all the secretions in the vials and to be spread over an adequate surface of the cotton to be detected by the animal.

For the trials the extract of dissolved secretions (chemical stimuli) from each lizard was spread on the cotton tip (1.5cm) of a wooden applicator (40cm). The solvent was evaporated, in all trials including the control, using Nitrogen gas flow. We used a new swab for each trial. The trials were conducted in May and June 2007 (breeding season), between 09:00am and 02:00pm, a time when the lizards are generally active in the field as well as in captivity. When the lizard was active and moving in its cage, the experimenter approached and presented the swab in a position 1.5-2 cm in front of the lizard's snout. Once the first tongue extrusion (Tongue Flick, TF) was observed, the TFs towards the cotton swab were counted for a period of 60 seconds, starting with the first TF. All trials were video recorded using a digital camera (Sony, DCR-TRV480E) in a fixed position so as not to disturb the animals.

DCM served as a control (it was used as the solvent for the secretions) to gauge baseline TF rates. Responses of both sexes were recorded. Each male was subjected to three trials: (1) DCM, (2) Stimulus from a different male, and (3) Stimulus from a female. Each female was subjected to three trials: (1) DCM, (2) Stimulus from a different female, and (3) Stimulus from a male. If the animal perceived a threat and ran away the trial was cancelled immediately and the animal was not tested again that day. The trials were conducted in a random order to overcome the effect of time of day on differences in behavioural effects. The animal was tested only once a day using a random stimulus.

Analysis of behavioural data

Videotapes were analyzed by recording, during each trial, the following events:

(1) Number of TFs toward the cotton tip.

(2) Biting behaviour towards the cotton swab or the wooden applicator as a measure of aggressive response to the secretions.

For statistical analysis we used non-parametric tests (Wilcoxon signed-rank test; Fisher exact test, Mann-Whitney U test) to compare tongue flick rates and aggressive behaviours towards female and male secretions.

Collection of lizards was approved by University of Hull. Collection was done in March 2007. During the experiment each animal was kept in its own cage and there was thus no need to mark test subjects. Lizards were kept for another experiment.

RESULTS

Male response

All of the 14 males tested immediately approached and tongue flicked the cotton swab carrying femoral secretions or the control solvent. Tongue flick rate (Fig. 2) was significantly elevated for male secretions when compared to control stimuli (*P*<0.05, Wilcoxon Signed-Rank test). Nine males bit the cotton swab aggressively when confronted with male secretions (Table 1), whereas no biting occurred when exposed to the control swabs (*P*<0.05, Fisher's Exact test). Tongue flicking also increased significantly over baseline control level when exposed to female secretions (*P*<0.05, Wilcoxon Signed-Rank test). However, no bites were directed towards the swab bearing female secretions. This was also the case for response towards control swabs (*P*=1.0, Fisher's Exact test). Males showed no difference in tongue flicking frequency between male stimuli and female stimuli (*P*= 0.37, Mann-Whitney U-test).

Female response

All 16 females tested immediately approached and tongue flicked the cotton swab carrying the femoral secretions or control stimuli (Fig. 3). Tongue rate flick was significantly elevated towards the male secretions when compared to the control (*P*<0.05, Wilcoxon Signed-Rank test). However, aggression was not significantly different for the male stimulus and the control stimulus (*P*=0.101, Fisher's Exact test). Four females bit the cotton swab carrying the male secretions, but 12 females only responded with tongue flicks. Tongue flicking rate was not elevated towards the female secretions when compared to control stimuli (*P*=0.49, Wilcoxon Signed-Rank test), and there was no evidence of biting directed towards female secretions or control stimuli (*P*=1.0, Fisher Exact test).

DISCUSSION

Femoral gland secretions are deposited on the substrate either passively as lizards move through their home range, or actively as they lizards sometimes drag their thighs on the rocks. In squamates, the number of tongue-flicks is correlated with the ability to discriminate odours using the vomeronasal organ (Halpern, 1992; Cooper, 1994). Our results show that female and male *A. boskianus* were able to differentiate between femoral secretions and the DCM control, which means that these femoral secretions contain chemical signals. The absence of similar interest towards control DCM treatments demonstrates that *A. boskianus* lizards were not responding to a novel subject. The few tongue flicks directed to DCM is just the normal behavior of identifying the presented object to the lizard, and it can be considered as the baseline of TFs which is almost constant with all animals tested.

There was no significant difference between the number of TF’s made by males towards male or female secretions. However, the aggressive behaviour of males towards male secretions and the lack of any aggression towards female secretions demonstrate that males use compounds present in femoral gland secretions to discriminate between sexes. This can be explained by the fact that there are different concentrations and different bouquets of chemicals between male and female (Khannoon et al., submitted, chapter 3). Some alcohols (hexacosanol, tetracosanol, and octacosanol), steroids (cholesterol and dehydrocholesterol), and acids (hexadecanoic, octadecanoic, and octadecenoic acids) showed high abundance correlation to male secretions. Females also have the ability to discriminate between the sexes using information conferred by the secretions, as indicated by the high TF frequencies elicited by male secretions versus female secretions. Our findings confirm the conclusions of earlier work that concluded the ability of lizards in different families to discriminate between sexes: Gerrhosauridae (Cooper & Trauth, 1992), and Cordylidae (Cooper et al., 1996).

Lizards exhibit social dominance systems and use pheromones as social signals (Mason, 1992). Intra-sexual aggression in lizards is preceded by chemical recognition (Cooper & Vitt, 1987). In our study, males responded to male secretions with a high rate of TFs and with aggressive biting. Each male was separated for 10 days to two weeks prior to being exposed to such signals. The aggression of males towards other males can be explained by the tendency of the resident male to defend its territory. In nature, at first, visual estimation of size occurs. But, between similarly sized males, chemoreception could allow an individual to quickly assess a rival's fighting ability (Martin & Lopez, 2007). In accord with that, our study revealed that some males showed no aggression (biting) towards the male stimulus. This lack of aggression might be explained by the suggestion that the receiver can chemically assess the strength and relative fighting ability of the donor through the odours (characteristic bouquet of chemicals), thus avoiding costly aggressive interaction.

We hypothesize that olfactory stimuli may play a major role in *A. boskianus*. In our study, the chemical stimulus on its own was sufficient to trigger behavioural responses in the lizards. The response of males towards female secretions is an indication that sex discrimination in *A. boskianus* may be based on chemical cues. The high TF frequencies and lack of aggression towards females also suggests that the interest of males in females is predominantly sexual in nature. The high frequency of tongue flicking by male indicates interest of the sexually deprived male in the female odour. Whether the male simply detects the female's presence or whether it responds to female courtship signals, which may be present in the secretions and which may indicate female receptiveness requires further study.

Females *A. boskianus* can discriminate between male and female femoral secretions. Unexpectedly, some females showed little (insignificant compared to males) aggression towards male femoral secretions, suggesting that females may have not been receptive/ready for mating or that they may have perceived signal odours interpreted as being aggressive. Females showed no interest in female secretions. Discrimination of individual characteristics of conspecifics such as body size (Shine *et al.*, 2003), reproductive history (Shine *et al.*, 2000), and sex (Mason, 1992), may require few TFs. It could be argued that, compared to male-male and female-male relationships, female-female relationship elicit little aggression because there is little competition between females.

In conclusion, we show that the femoral gland secretions of *A. boskianus* trigger aggression of male toward other males and different response by different sexes. Because male can identify other male secretions, leaving secretions on the ground probably announces the dominant male in its territory. This suggests an important role in territorial marking, dominance, and possibly discrimination of physical condition. The ability of one sex to discriminate the other one' secretions suggest the use of these secretions in sex discrimination. The results also raise questions about the possibility of sex specificity of the signal compounds and whether these chemical signals can be used as reproductive signals. Only once the odour fingerprints are identified and the biological activities of the individual components are tested can such questions can be answered.

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Figures and Tables

Table 1. Behavioural response (biting) of male and female *A. boskianus* towards cotton swabs carrying femoral gland extract of conspecific male and female individuals. DCM is the control. *P* values of Fisher Exact Test are shown.

	Biting toward stimulus	Biting toward control	Significance level
Response of male toward male	9/14	0/14	P=0.0006
Response of male toward female	0/14	0/14	P=1.0
Response of female toward male	4/16	0/16	P=0.101
Response of female toward female	0/16	0/16	P=1.0

Figure 1. Picture showing the ventral surface of male *A. boskianus*, femoral pores can be seen on both femurs.

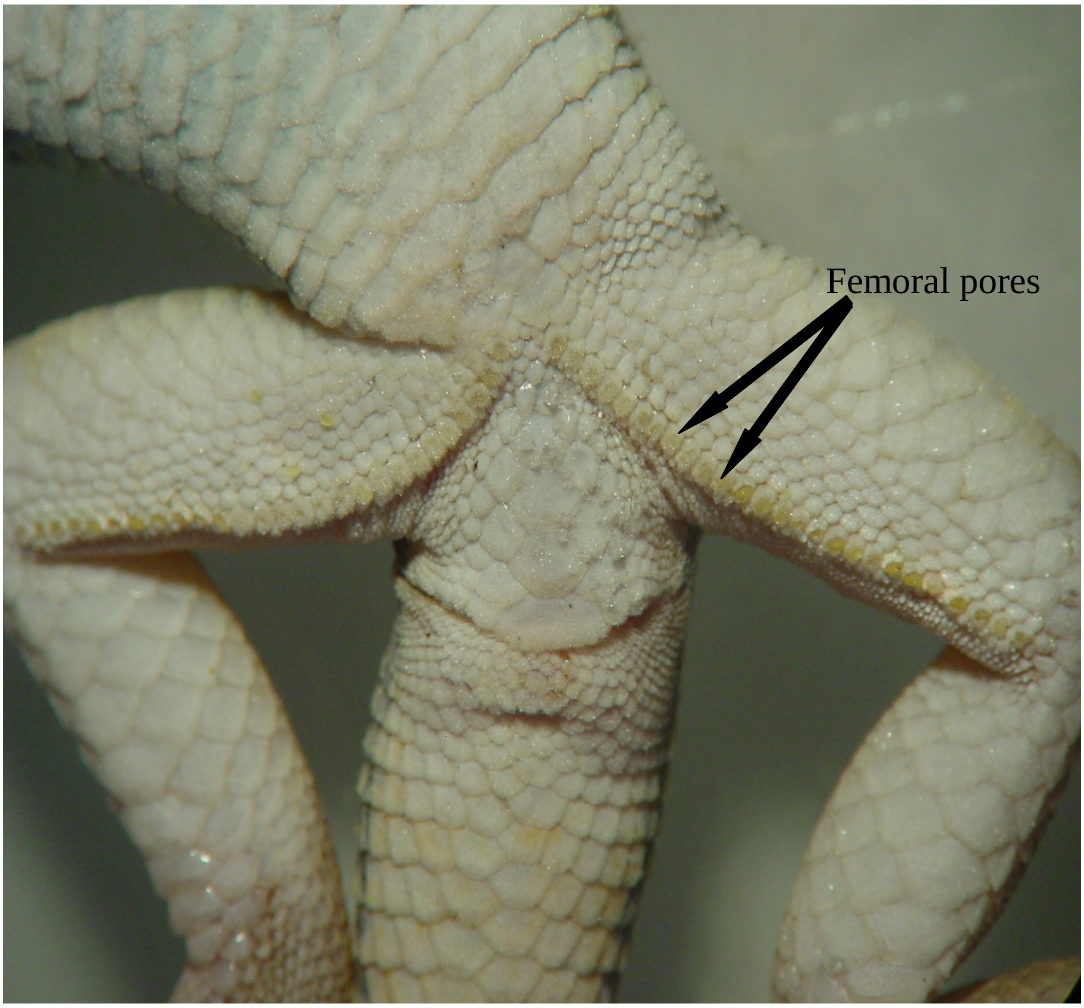


Figure 2. Mean numbers of tongue-flicks (± SE) directed by male A. boskianus over a minute period towards cotton swabs carrying femoral gland secretions extract of conspecific males and females, and towards a dichloromethane control swab.

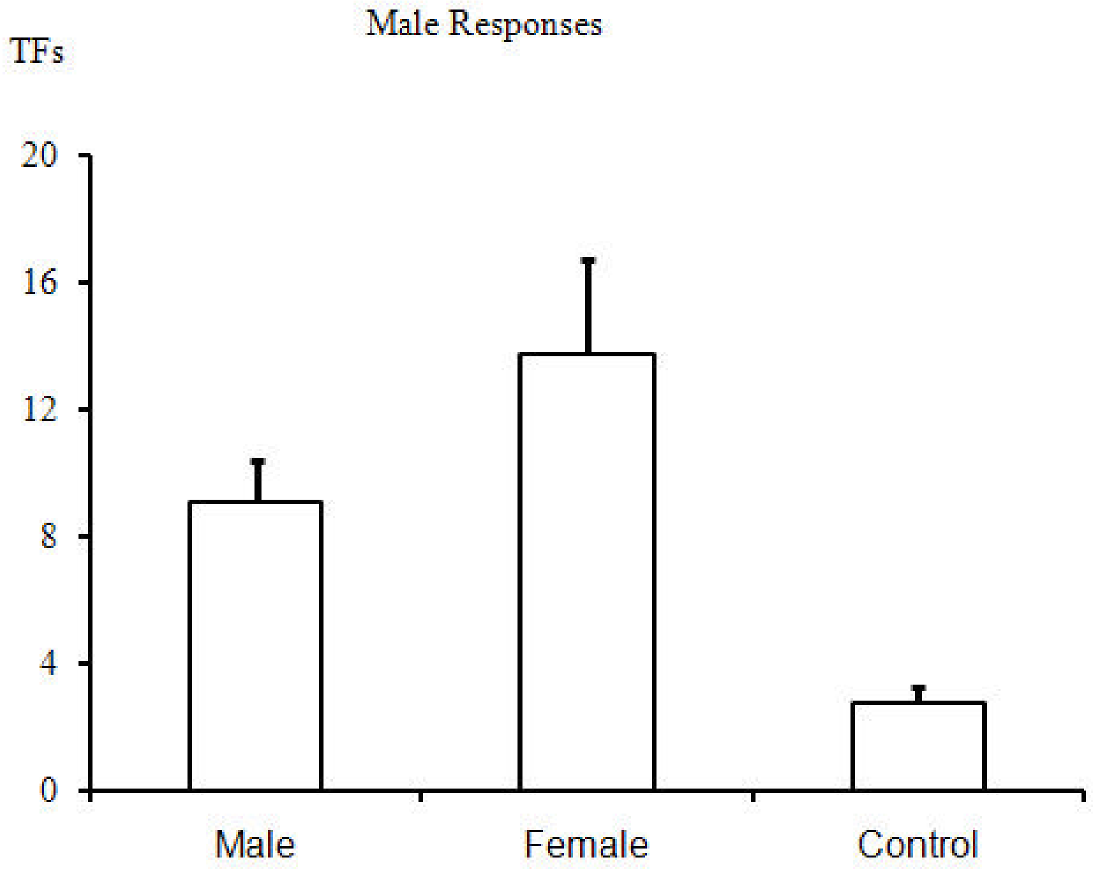
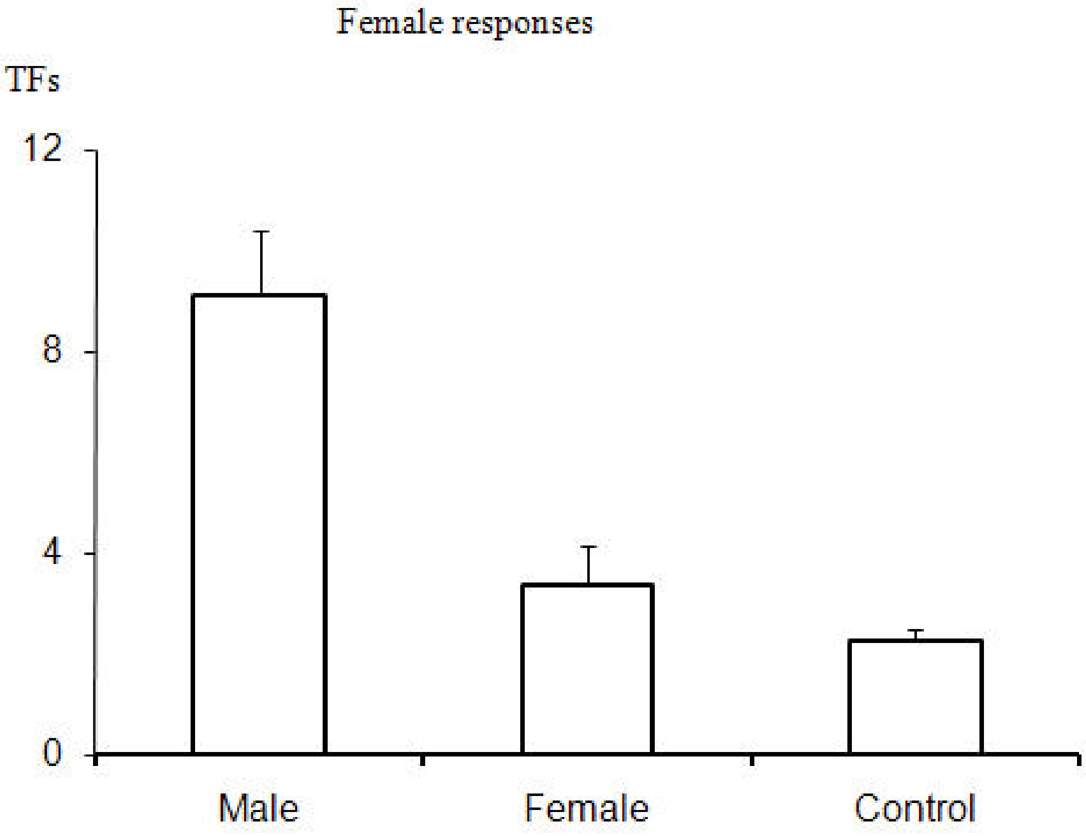


Figure 3. Mean numbers of tongue-flicks (± SE) directed by female A. boskianus over a 1 minute period towards cotton swabs carrying femoral gland secretions extract of conspecific males and females, and towards a dichloromethane control swab.



CHAPTER 3

Paper 2

New compounds, sexual differences, and age-related variations in the femoral gland secretion of the lacertid lizard *Acanthodactylus boskianus*

(Submitted to Biochemical Systematics and Ecology)

New compounds, sexual differences, and age-related variations in the femoral gland secretion of the lacertid lizard *Acanthodactylus boskianus*

Keywords

Acanthodactylus boskianus; femoral gland secretions; Glycerolmonoethers; Monoglycerides; Sexual variability; pheromones.

Abstract

Integumental gland secretions in lizards have been postulated to play a role as semiochemicals, but few studies have analysed the chemical nature of the gland secretions used in communication. We analysed the femoral gland secretions of *Acanthodactylus boskianus* using GC-MS, compared secretions of both sexes and different ages of males. For the first time in reptiles, monoglycerides of fatty acids and glycerol monoethers of long chain alcohols were identified. In addition, alcohols, steroids, carboxylic acids, alkanes, amides, aldehydes, carboxylic acid esters, and squalene occurred. Sexual differences and age correlation in the amount of all major groups of compounds occurred. These results confirm the possibility that these secretions are used as semiochemicals. This work opens opportunities to test in future the role of chemical cues in mate choice and dominance hierarchies in lizards and lays the foundation to test the activity of compounds in behavioural assays to eventually identify the pheromones involved.

Introduction

Epidermal gland secretions of reptiles can have pheromonal properties (Cooper et al., 1994; Lopez et al., 1997) and might play an important role in sexual signalling and territorial scent marking (Alberts, 1993; Mason, 1992). Chemical signals are used in intraspecific communication between lizards, e. g. in the desert iguana (Alberts, 1992; Halpern, 1992; Mason, 1992). In several species, the pheromonal activity of the femoral gland secretions has been shown (Alberts, 1993; Aragon et al., 2001; Cooper and Vitt, 1984). The secretory activity of these glands varies seasonally and increases during the period of sexual activity. In some families of squamates only males have these glands (Alberts, 1993; Cole, 1966; El-Shershaby AM et al., 2006; Khannoon, 2004; Vanwyk, 1990).

Studies of the composition of the skin chemicals originating from these glands showed that they are composed of both lipids and proteins (Mason and Gutzke, 1990; Weldon and Bagnall, 1987). The major compounds involved in chemical communication were suggested to be of lipidic nature (Bull et al., 1999; Cooper and Garstka, 1987; LeMaster and Mason, 2001; Mason and Gutzke, 1990). On the other hand, the involvement of proteins cannot be discounted, given their importance in amphibians (Toyoda et al., 2004).

Few studies have examined the lipidic part of epidermal gland secretions (Weldon et al., 2008). In *Liolaemus* lizards, *n*-alkanes, long chain carboxylic acids, and steroids were found and differences in the proportions of some compounds between different populations have been reported (Escobar et al., 2001; Escobar et al., 2003). In the lacertid lizard *Acanthodactylus erythurus*, alcohols, steroids, carboxylic acids and esters, lactones, ketones, squalene, and α-tocopherol occurred (Lopez and Martin, 2005b). The same compound classes were found in another lacertid lizard, *Lacerta monticola cyreni* (Lopez and Martin, 2005a). In the girdled lizard or sungazer, *Cordylus giganteus*, carboxylic acids, alcohols, ketones, esters, and steroids occurred in both males and females (Louw et al., 2007).

Behavioural experiments examining the femoral secretions of *A. boskianus* (Khannoon et al. submitted, chapter 2) showed a sexual difference in the responses towards the secretions, which might be related to sexual differences in their chemical composition. Our hypothesis was that the composition of the femoral secretions of *A. boskianus* might be different between sexes and probably between different male ages, and also that variability between individual males exists. Such variability may play a role in mate choice and the establishment of dominance hierarchies, or in territorial marking. We suggest that these secretions, which are passively deposited on the ground during movement or actively during dragging the hindlegs (see chapter 2), might compose a characteristic compound bouquet, typical for *A. boskianus*. In the present study we report the results of the chemical analyses of individuals of *A. boskianus* and discuss these from a chemo-ecological perspective.

Materials and Methods

Sample collection

A. boskianus individuals were captured from Balteem, Northern coast of Egypt, during April 2007 which coincides with the activity and mating season of these lizards. The animals were transferred to Hull University, UK, and kept for behavioural experiments. In order to test the effect of animal age on the chemical components of the secretions, we used 51 different males ranging from 4.80cm to 7.60 cm snout-vent length (SVL) which is reflecting the different ages of the animals (Schleich et al., 1996). For sex specificity we selected 16 adult males and 10 adult females that had the highest SVL within a very narrow range of SVL differences. Secretions were collected by gently squeezing the plugs from the femoral pores of the lizards using forceps. The secretion of each individual (8 mg) was collected directly into glass vials with Teflon-lined caps and then dissolved in 250µl of dichloromethane (DCM) (Aldrich, GC grade). The collected samples were transferred to Braunschweig, Germany at -18ºC and kept until processing for analysis. Control samples with the solvent at the same conditions of collecting the secretion were used to exclude impurities.

Derivatization

Fifty µL of the liquid secretion was placed in a 2 ml vial and the solvent was removed in a gentle stream of nitrogen at 50°C. The residue was taken up in

10 µL dichloromethane and 50 µL *N*-methyl-*N*-trimethylsilylfluoroacetamide (MSTFA) were added. The mixture was heated to 50°C for 30 – 60 minutes in a vial with a closed cap. Then the solvent and the remaining reagent were evaporated in a gentle stream of nitrogen at 50°C and the residue was taken up in 10 µL dichloromethane. Exactly 1.0 µl was injected into the gas chromatography-mass spectrometry (GC-MS) system.

GC-MS analysis

Samples were analysed using a Hewlett-Packard model 6890 gas chromatograph connected to a Hewlett-Packard model 5973 mass-selective detector equipped with a BPX-5 column: 25 m×0.22 mm i.d., 0.25 µm film thickness (SGE). The temperature program was as follows: 50°C for 5 minutes, then with 5°C/min to 320°C, 30 minutes hold time. Helium was used as carrier gas with 1 ml /min in constant flow mode. Accelerating voltage of MS was 70eV. Compounds were identified by comparison of mass spectra and retention indices of derivatized and underivatized samples with those of reference compounds.

Data Analysis

The relative amount of each component was determined as the percent of the total ion current (TIC) using its peak area. Chemicals included as variables in the statistical analysis were only those which were presented in a sufficient numbers of animals and constituted > 0.01% of TIC. The peak areas of the compounds from GC-MS data were used as variables data representing the abundance. The data were tested for normality using a One-Sample Kolmogorov-Smirnov test, and subsequently transformed (Log10) in order to achieve normality. Correlation analysis (Pearson correlation) was then carried out to determine the relationship between chemical abundance and animal size in male lizards. Principal component analysis was used to determine the chemical abundance with sex. Cluster analysis (based on Euclidean distance) was carried out, followed by analysis of similarity (ANOSIM) to determine statistical differences between male and female.

Results

The same compounds were detected in the total ion chromatogram (TIC) of both male and female *A. boskianus* secretions, but they differed quantitatively between individuals and also between the sexes. No two individuals had the same quantitative composition of the compounds. A total of more than 120 chemicals were identified in both sexes (Table 1). The identified compounds were steroids, alcohols, carboxylic acids, alkanes, amides, aldehydes, carboxylic acid esters, and squalene. These compounds were identified using standard procedures. The GC-MS of the underivatized natural extracts showed the presence of more polar compounds, which could not be identified because of their poor elution properties. Therefore the extracts were analyzed after derivatization with MSTFA to form trimethylsilyl-derivatives, thus enabling the GC-MS analysis of the more polar compounds. This method led to the identification of additional components not previously reported from lizards (Fig. 1). Monoglycerides carrying the carboxylic acid at C-1 were identified by their characteristic ions at *m/z* M-103 ($M-(CH_3)_3SiOCH_2^+$), 103 ($(CH_3)_3SiOCH_2^+$) and 205 ($(CH_3)_3SiOCH_2(CH_3)_3SiOCH^+$) (Fig. 2C). Related monoethers of glycerol connected at C-1 show a characteristic base peak at *m/z* 205 which is of low abundance in respective monoglycerides. Furthermore, an ion *m/z* M-147 ($M-(CH_3)_3SiO(CH_3)_2^{\cdot}$) is characteristic together with M-205-2H (Fig. 2D). Furthermore, 1,3-alkanediols were identified by the ions *m/z* 103 ($(CH_3)_3SiOCH_2^+$), 219 ($(CH_3)_3SiOCH_2CH_2(CH_3)_3SiOCH^+$), and M-117 (M-

$(CH_3)_3SiOCH_2CH_2^+$) (Fig. 2B). All these identifications were verified by comparison with synthetic reference compounds. The fourth class of compounds not reported from lizards before are respective glycerol ethers of the alkanediols, connected terminally at both alcohols. Their mass spectra indicated the presence of three trimethylsilyl groups. The position of the silyloxy group in the long chain is indicated by a characteristic ion, e. g. *m/z* 313 in the mass spectra shown in Fig. 2A, while the other ions can be explained as before. No synthetic reference compound existed for this ether, so that this identification can so far only be regarded as tentative.

The major compound groups present in the secretion of adult males were alcohols (50.29%) relative to other compounds, steroids (35.54%), carboxylic acids (9.37%), glycerol monoethers (2.11%), and monoglycerides (0.42%). The other classes occurred only in minor amounts. The highest individual compound found was cholesterol (31.36%). Only 29 of the identified compounds were used in the statistical analysis because the others did not occur in sufficient abundance and their occurrence or absence could not be established in every sample with confidence (Table 2). These compounds represent members of the five major compound classes mentioned above.

A quantitative analysis of the compounds allowed investigating whether the amount of a group of compounds in the glands was correlated with the size of the male lizards. This proved to be the case for the five major compound classes (Fig. 3); acids ($r= 0.36$; $P= 0.01$), alcohols ($r= 0.33$; $P= 0.02$), steroids (*r*= 0.36; *P*= 0.01), glycerol monoethers (*r*= 0.31; *P*= 0.03), and monoglycerides (*r*= 0.34; *P*= 0.02). Individual compounds showed different degrees of correlation to size (Table 3). 1-Octadecanol was the most correlated to size (*r*= 0.609; *P*<0.001). On the other hand, glycerol and hexadecenoic acid were the only compounds with insignificant negative correlation to size.

The principal cluster analysis (PCA) indicates higher abundance of all compounds in male than female lizards and analysis of similarity (ANOSIM) confirms this difference between the sexes. The five major compound classes occur in males in higher amounts than in females (Fig. 4). In addition, with the exception of glycerol, individual compounds are also more abundant in males. The Euclidean cluster analysis, using major compound classes, (Fig. 5) further indicates separation between males and females, showing a difference between males and females (*R*= 0.439; *P*> 0.001).

The PCA, using the five main chemical groups, indicates that the first 2 principal components account for 74% of the total variability in the data, with 6 principal components accounting for 90% of the variability. Therefore, higher concentrations of all chemical compounds can be considered to be highly correlated with male lizards.

Discussion

Several components of *A. boskianus* have been identified here for the first time in the femoral gland secretions of lizards. These compounds are monoglycerides and glycerol monoethers which have been found in both males and females, with intersexual and age variability. Glycerol alkyl monoethers have been rarely identified from nature, e. g. in marine sponges (Quijano et al., 1994), bacteria (Ring et al., 2006), starfish (Snyder et al., 1969), clams and mussels (Hanuš et al., 2009), octopus (Jahnke et al., 2001), or rats (Paltauf and Polheim, 1970). The unusual glycerol 1-(3-hydroxy) alkyl ethers have not been reported before form nature. The femoral gland secretions of both sexes contained the same compounds but they differed quantitatively. Some of them are similar to those found in other lizards. These are steroids, carboxylic acids, alcohols, esters, alkanes, and squalene (Alberts, 1992; Chauhan, 1986; Escobar et al., 2001; Gabirot et al., 2008; Lopez and Martin, 2005a, b; Louw et al., 2007; Mason and Gutzke, 1990; Weldon et al., 2008).

Alcohols are the most important group of femoral gland secretions and were reported earlier as major components in another *Acanthodactylus* lizard, *A. erythrurus* (Lopez and Martin, 2005a). Minor amounts were reported earlier from other species, *Lacerta monticola cyreni* (Lopez and Martin, 2005b) and *Cordylus giganteus* (Louw et al., 2007). It seems likely that the high percentage of alcohols is a potential chemical fingerprint of the *Acanthodactylus* genus. However, alcohols have been reported in extracts from the skin of *Iguana iguana* (Roberts and Lillywhite, 1980) and in the skin of several snakes (Mason, 1992), and in the precloacal gland secretions of crocodiles (Dunn JrBS et al., 1993; Garcia-Rubio et al., 2002).

(Lopez and Martin, 2005a) speculated that these alcohols may form waxy esters which can stabilize the secretions. In contrast, a more important role in the communication system of these animals may be more plausible, because of several arguments. First, there exists a high intersexual variability of the amount of the alcohols, as shown from our PCA results. Secondly, 9 of the alcohols are positively correlated to the size of the adult males, particularly 1-octadecanol. This compound was highly correlated to size in the males and showed high sexual variability, suggesting that it might potentially be an effective chemosignal in *A. boskianus.* Thirdly, the alcohols identified have very wide range of carbon atoms ranging between C_3 and C_{32}, which provides a good basis for selection for different bouquets of compounds by which individual animals can be easily discriminated. In addition, our behavioural work (Khannoon et al. submitted, chapter 2) on the responses of the lizard *A. boskianus* towards the femoral gland secretion showed high degrees of interest of females to male secretions and similarly an interest of males towards female secretions. Furthermore, males responded aggressively to male secretions, confirming the existence of a sex characteristic odour. This correlates well with the differences in the chemical compositions of the secretions presented here.

Cholesterol is of high abundance in the secretions of both sexes and is more prevalent in males than in females; its abundance is correlated to the size of the male. All lacertid lizards studied (Lopez and Martin, 2005a, b) showed the same pattern for cholesterol except *Psammodromus algirus* (Martin and Lopez, 2006), which has campesterol as the most common steroid. Escobar et al. (2003) postulated that cholesterol and the protein part of secretions functions as fixatives for semiochemicals or controlled-release carrier through helping to constitute an unreactive, apolar matrix that delivers the compounds that are true semiochemicals. However, cholesterol with its variable abundance between individuals and sexes might also play another role in communication. Mason and Gutzke (1990) reported that cholesterol provides information about the sex of an individual. In males, cholesterol levels may provide information about the condition of the male such as the dominance status or its genetic quality (Martin and Lopez 2000). Its correlation with the size of males might suggest its reflection of the strength and physical quality of a male, which are important criteria in dominance detection and mate choice. Therefore it may well serve as chemical signal.

Dehydrocholesterol (Cholesta-5,7-dien-3-ol) was present with variable amounts in the secretions and with significant differences between sexes. This compound is the precursor of Vitamin D3, which is essential in calcium metabolism. (Lopez and Martin, 2005b) stated that the relative abundance of dehydrocholesterol was related to the physical or health condition of the lizard and it therefore may play a role in female mate choice. In our study, dehydrocholesterol showed difference between sexes but not between different sizes of males. As such it might be a sex specific component by which females can recognize male scent but is unlikely to function as criteria in detecting large or dominant males.

In the present study, carboxylic acids found were ranging between C_3 and C_{24}. Hexadecanoic and octadecanoic acids were found in all individuals, while other acids were randomly distributed. Histological and ultrastructural work on the femoral glands of *A. boskianus* showed that they are holocrine in secretion (El-Shershaby AM et al., 2006; Khannoon, 2004). The presence of fatty acids (tetradecanoic, hexadecanoic, octadecanoic, and octadecenoic acids) normally present in internal tissue (Nicolaid.N, 1974), confirms the holocrine secretion of these femoral glands. Similar fatty acids were found in other studies on the skin and integumental glands of other reptiles (Weldon et al., 2008). Different to other lizard groups the lacertid lizard femoral secretion (*Liolaemus, Lacerta,* and *Acanthodactylus*) contains short chain acids (3-6 carbon atoms), probably specific for lacertids. In our study, hexadecanoic, octadecanoic, and eicosanoic acids showed high abundance in older males and they are male correlated. These long chain carboxylic acids are compounds of low volatility, which might be important for the persistence of scent markings of territorial older males. In *A. erythrurus,* adults had more abundant long chain carboxylic acids (Lopez and Martin, 2005a), which matches our results on the same genus. On the other hand, *I. iguana* showed no difference between adult and juvenile (Weldon et al., 1990).

Squalene was detected in variable amounts and with very low percentages and in variable amounts between different individuals. This terpenoid was detected earlier in different reptiles (Weldon et al., 2008).

Alkanes were detected with low abundances, but with a higher proportion in females. They could be considered as sex specific chemicals. These alkanes were not detected in the control samples so we are confident that these are chemicals from femoral secretions. (Lopez and Martin, 2005b) stated that alkanes found in *L. monticola* were contaminants, but alkanes were also detected in other lizards (Escobar et al., 2001; Escobar et al., 2003; Mason and Gutzke, 1990).

The femoral secretion is not only composed of the semi-volatile compounds discussed so far, it also contains a proteinaceous part. The chemistry of the proteinaceous part has not been studied in *A. boskianus,* but in other lizards it was shown that there are variations between different species (Alberts, 1990). He concluded that these proteins could be used in individual and sex recognition. In the secretions of *A. boskianus*, the proteins might also show individual and/or sexual variability. Additional roles for both the proteins and the lipids of the secretion might be that the proteins are reducing the evaporation rate by providing a matrix. Such a role was proved by (Humphries et al., 1999) for major urinary proteins (MUPS) which slow the release of volatiles in the urine of the house mice *Mus domesticus*.

In conclusion, the femoral gland secretions of *A. boskianus* contain different classes of compounds, shared by other reptiles, and some unique compounds s such as glycerol monoethers and monoglycerides. Nevertheless, it seems likely that these compounds will also be found in the secretion of other lizards if appropriate methods for their detection like silylation are performed. Almost all detected compounds showed sexual variation while some also varied with age, making them likely candidates for a role as semiochemical in *A. boskianus.* The femoral gland secretions are passively deposited on the substrate as lizards move through their home range or actively as the lizards sometimes drag their thighs on the rocks (Khannoon, personal observations), supporting their potential use in territorial marking. Behavioural experiments using fractions of these secretions or pure compounds should clarify whether they are used as pheromones. The presented chemical data confirms our behavioural work on the same species (chapter 2), which showed a sexual difference in response towards the secretions. Since the gland secretions potentially also have phylogenetic implications our study highlights the importance to further examine the chemical nature of epidermal glands. Femoral gland secretions are a potential source of chemical information about an individual. This includes amongst others its sex, health status, dominance status, and age, all of this being key elements in mate choice and territorial marking.

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Figures and Tables

Table 1. List of compounds identified in at least one sample of an individual *A. boskianus*. The gas chromatographic retention index (RI) is also given. Alcohols and acids were detected as the respective trimethylsilylated compounds, thus the RI shown is that of the respective derivative.

RI	Compound	*RI*	Compound
1058	2-Hydroxypropanoic acid	2441	Eicosanoic acid
1076	Hexanoic acid	2441	*N*,*N*-Dimethyloctadecadieneamide
1077	1,2-Dihydroxypropane	2473	Glycerol 1-hexadecyl ether
1116	Nonanal	2478	N,N-Dimethyloctadecaneamide
1216	Decanal	2500	2-Eicosenoic acid
1265	Glycerol	2500	Pentacosane
1320	2,3-Dihydroxypropanoic acid	2513	1.3-Eicosanediol
1360	Nonanoic acid	2539	Heneicosanoic acid
1400	Tetradecane	2545	1-Docosanol
1458	Decanoic acid	2568	Glycerol 1-heptadecyl ether
1490	Hydrocarbon	2580	Glycerol 1-hexadecanoate
1500	Pentadecane	2590	3-Hydroxyicosanoic acid
1507	Hexanedioic acid	2600	Hexacosane
1530	Methyl dodecanoate	2612	Docosenoic acid
1560	Hydrocarbon	2637	Docosanoic acid
1562	1-Dodecanol	2642	1-Tricosanol
1600	Hexadecane	2663	Glycerol 1-octadecyl ether
1630	Isopropyl dodecanoate	2700	Heptacosane
1645	Hydrocarbon	2709	1,3-Docosanediol
1653	Dodecanoic acid	2716	Tetracosen-1-ol
1700	Heptadecane	2741	1-Tetracosanol
1703	Hydrocarbon	2745	Glycerol 1-octadecadienoate
1725	Unknown compound	2750	Glycerol 1-octadecenoate
1731	Hydrocarbon	2760	Glycerol 1-nonadecyl ether
1755	1-Tetradecanol	2772	Glycerol 1-octadecanoate
1800	Octadecane	2792	Glycerol 1-(3-hydroxy) octadecyl ether
1811	12-Methyltridecanoic acid	2800	Octacosane
1849	Tetradecanoic acid	2814	Squalene
1900	Nonadecane	2834	Tetracosanoic acid
1910	13-Methyltetradecanoic acid	2838	1-Pentacosanol
1917	12-Methyltetradecanoic acid	2854	Glycerol 1-eicosyl ether
1933	Methyl hexadecanoate	2900	Nonacosane
1943	Hydrocarbon	2913	Hexacosen-1-ol
1947	Pentadecanoic acid	2935	1-Hexacosanol
1956	1-Hexadecanol	2948	Glycerol 1-eicosanoate

Table 1 continued

1981	14-Methylpentadecenoic acid	2977	Glycerol 1-(3-hydroxy)eicosyl ether
2000	Eicosane	3000	Triacontane
2008	14-Methylpentadecanoic acid	3024	Glycerol 1-docosenyl ether
2023	Hexadecenoic acid	3033	Heptacosanol
2044	Hexadecanoic acid	3045	Glycerol 1-docosyl ether
2100	Heneicosane	3059	Steroid M=386
2105	15-Methylhexadecanoic acid	3067	Steroid M=3880
2114	14-Methylhexadecanoic acid	3094	Steroid M=386
2116	Heptadecenoic acid	3103	Hentriacontane
2134	Methyl octadecanoate	3103	Steroid M =384
2143	Heptadecanoic acid	3116	Octacosen-1-ol
2150	1- Octadecanol	3133	1-Octacosanol
2200	Docosane	3141	Cholesterol
2213	3-Hydroxyhexadecanoic acid	3159	Cholestan-3-ol
2216	Octadecadienoic acid	3190	Cholesta-5,7-dien-3-ol
2223	Octadecenoic acid	3200	Dotriacontane
2242	Octadecanoic acid	3231	1-Nonacosanol
2267	*N*,*N*-Dimethylhexadecanamide	3247	Campesterol
2300	Tricosane	3269	Stigmasterol
2302	2-Octadecenoic acid	3300	Tritriacontane
2322	1,3-Octadecanediol	3330	1-Triacontanol
2340	Nonadecanoic acid	3332	β-Sitosterol
2348	Eicosanol	3400	Tetratriacontane
2377	Glycerol 1-pentadecyl ether	3528	1-Dotriacontanol
2387	Glycerol 1-tetradecanoate		
2398	3-Hydroxyoctadecanoic acid		
2400	Tetracosane		
2420	Eicosenoic acid		

Table 2. Compounds used as variables for the statistical analysis between sexes and male ages of male *A. boskianus* .

Σ Acids
Σ Alcohols
Σ Steroids
Σ Glycerol monoethers
Σ Monoglycerides
Cholesterol
Cholesta-5,7-dien-3-ol
Campesterol
ß-Sitosterol
Glycerol 1-eicosyl ether
Glycerol
1-Octadecanol
1-Eicosanol
1-Docosanol
1- Tricosanol
1-Tetracosanol
1-Pentacosanol
1-Hexacosanol
1-Heptacosanol
1-Octacosanol
1-Nonacosanol
1-Triacontanol
Nonanoic acid
Tetradecanoic acid
Hexadecenoic acid
Hexadecanoic acid
Heptadecenoic acid
Heptadecanoic acid
Octadecadienoic acid
Octadecenoic acid
Octadecanoic acid
Eicosenoic acid
Eicosanoic acid
Docosanoic acid

Table 3. Compounds which showed abundance significant correlation to the male size.

	Pearson correlation	**Sig. (2-tailed)**
Σ Acids	0.336	0.016
Σ Alcohols	0.390	0.005
Σ Steroids	0.390	0.005
Σ Glycerol monoethers	0.307	0.028
Σ Monoglycerides	0.383	0.006
Glycerol 1-eicosyl ether	0.357	0.010
Cholesterol	0.344	0.014
Campesterol	0.289	0.040
1-Octadecanol	0.609	>.001
1-Eicosanol	0.438	0.002
1-Tricosanol	0.369	0.011
1-Tetracosanol	0.335	0.016
1-Pentacosanol	0.330	0.018
1-Hexacosanol	0.385	0.005
1-Heptacosanol	0.303	0.031
1-Nonacosanol	0.373	0.019
1-Octacosanol	0.419	0.002
Hexadecanoic acid	0.413	0.003
Octadecadienoic acid	0.456	0.002
Octadecenoic acid	0.358	0.011

Figure 1. The structural formula of the newly recorded compounds from the femoral gland secretions of *Acanthodactylus boskianus*. Monoether of glycerol (A), monoglyceride (B), glycerol ether of alkanediol (C). Each group is shown as natural structure (top), and trimethylsilyl (TMS) derivative (-Si(CH_3)$_3$), middle and bottom.

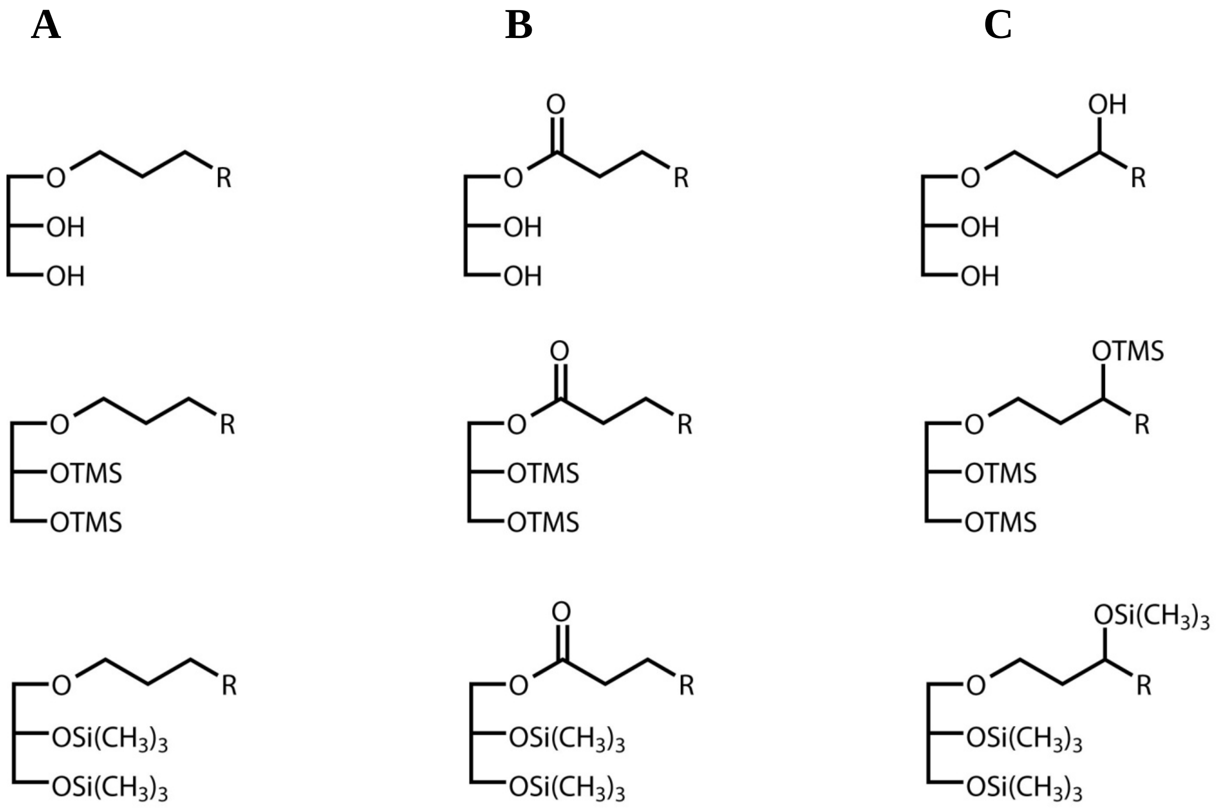


Figure 2. Mass spectra of the trimethylsilyl derivatives of compounds not reported before from lizards; (A) Glycerol ether of alkanediol (Glycerol 1-(3-hydroxy)octadecyl ether), (B) 1,3-Alkanediol (1,3-Eicosanediol), (C) Monoglyceride (Glycerol 1-octadecanoate), (D) Monoether of glycerol (Glycerol 1-eicosyl ether).

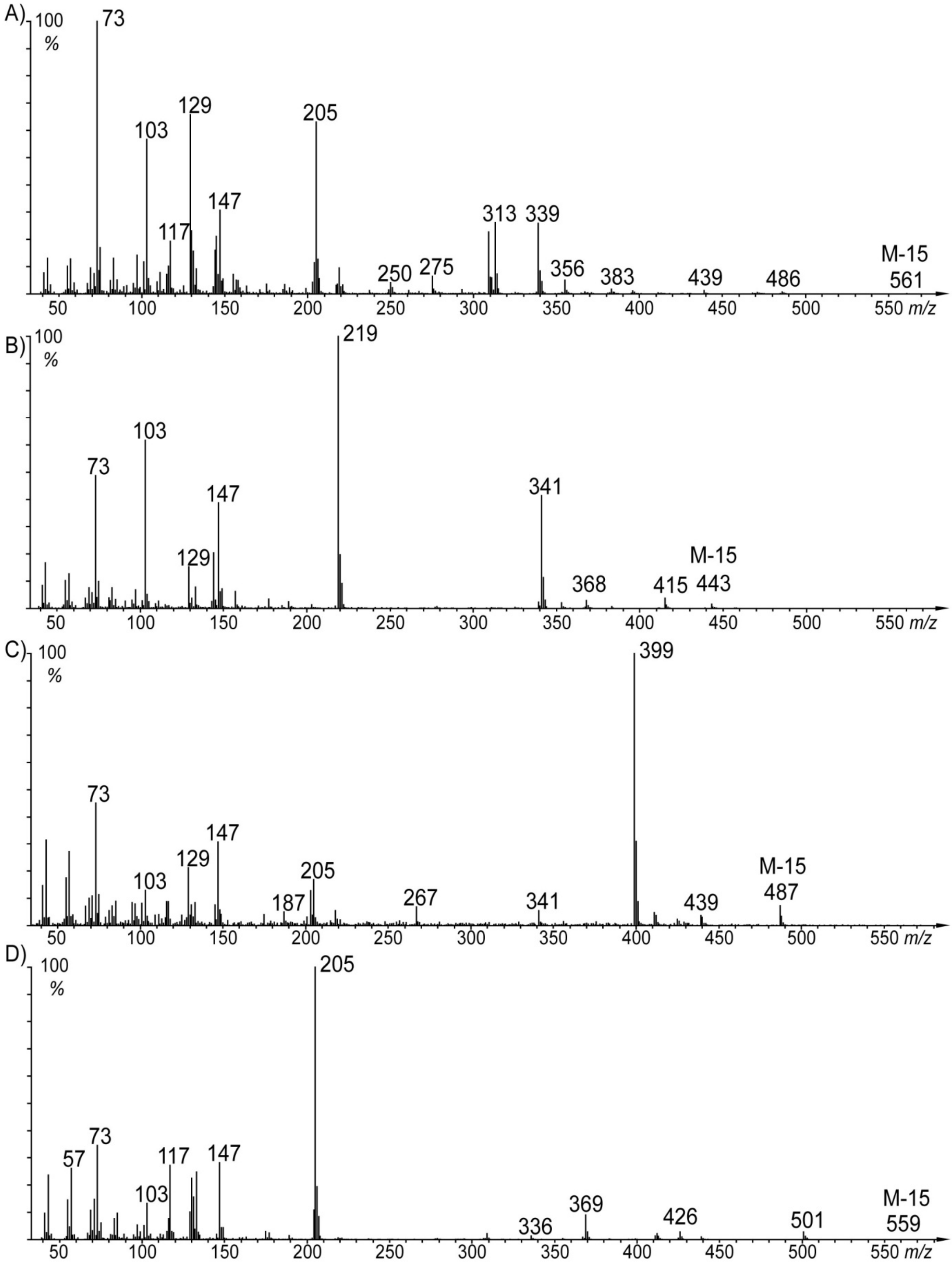
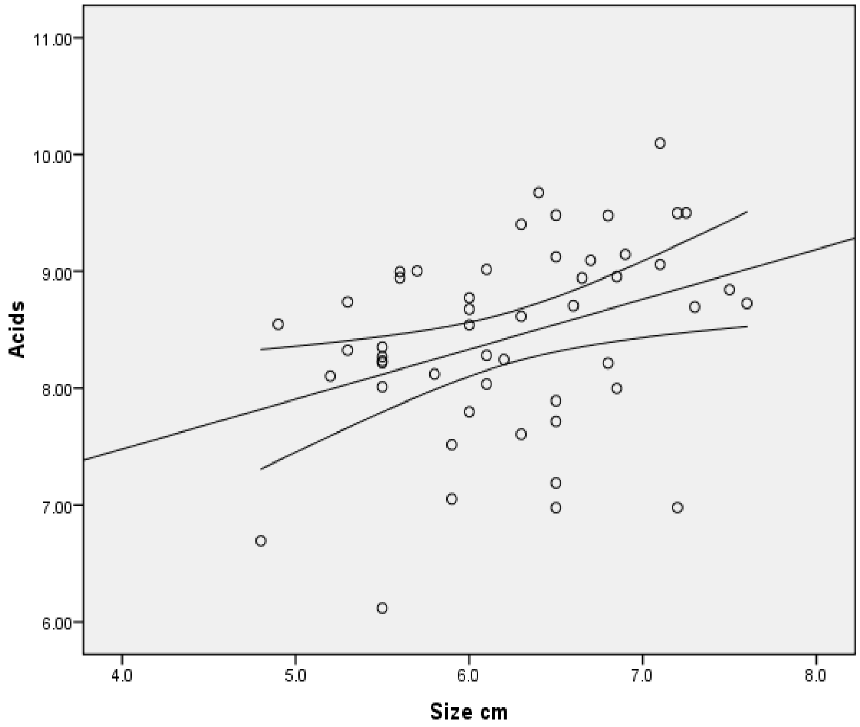
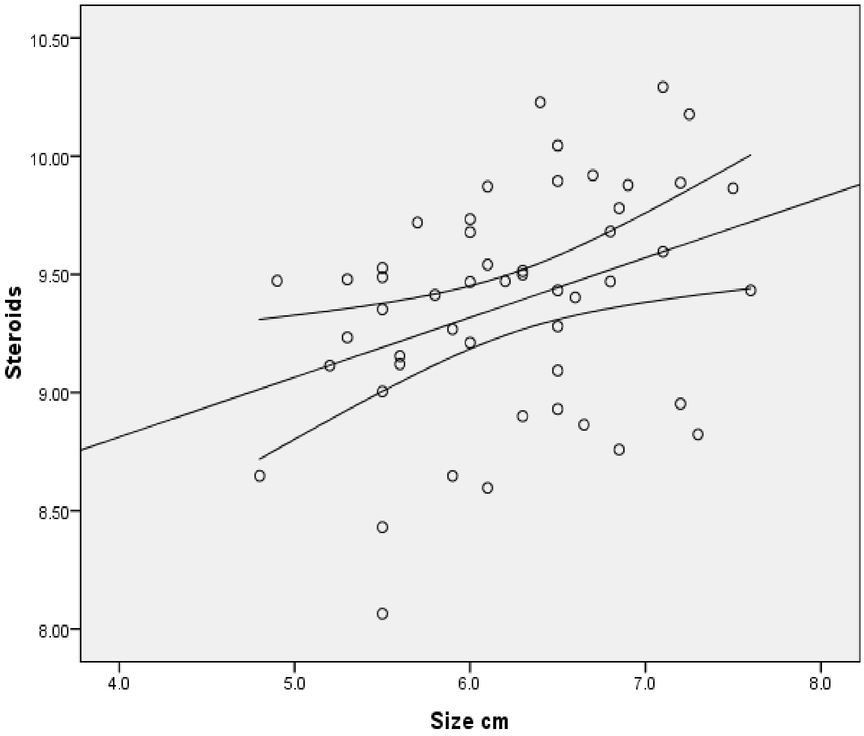


Figure 3. Correlation between compound abundance (log peak area) and size of the male lizard *A. boskianus*. The five main chemical groups (acids, steroids, alcohols, glycerolmonoethers, and monoglycerides) of the femoral gland secretions and the highest correlated compound (1-octadecanol) are shown.





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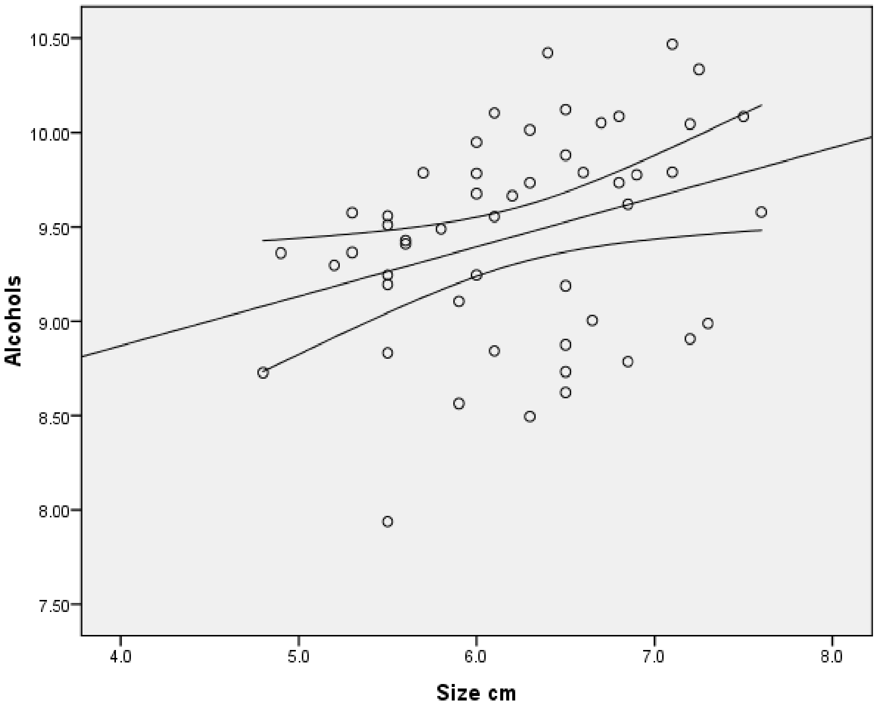

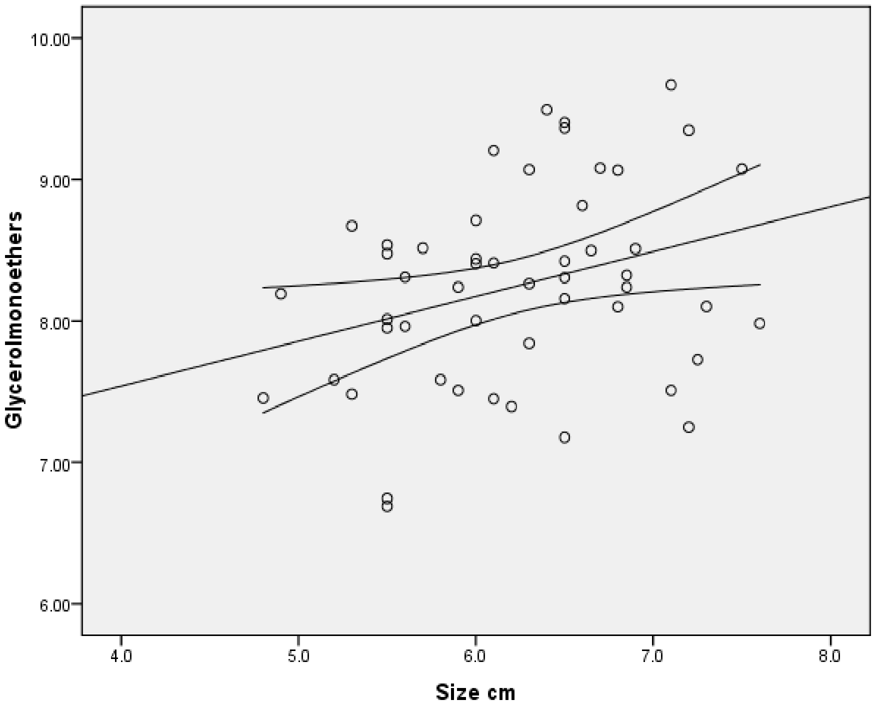

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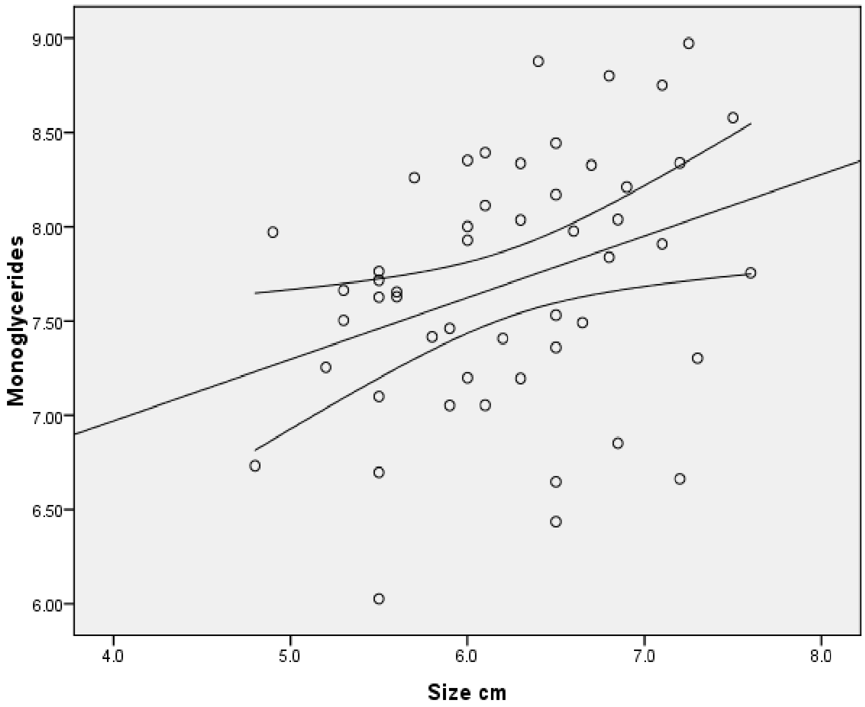


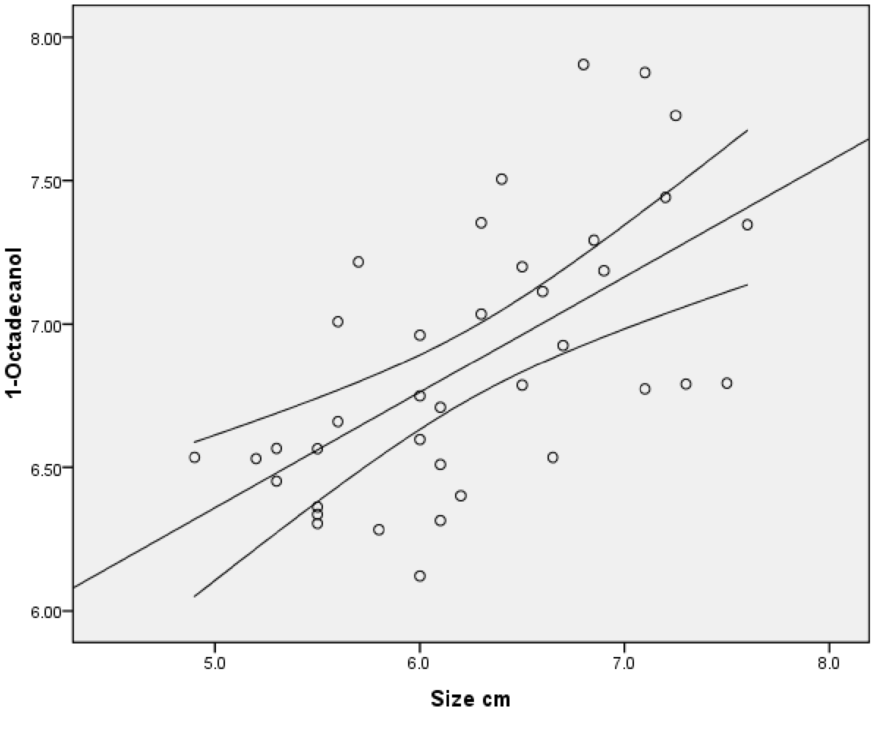


Figure 4. PCA biplot is showing the separation between males (M) and female (F) using the five chemical groups, steroids, alcohols, acids, glycerolmonoethers, and monoglycerides (A). Distribution of the five chemical groups shows higher abundance in males (B).

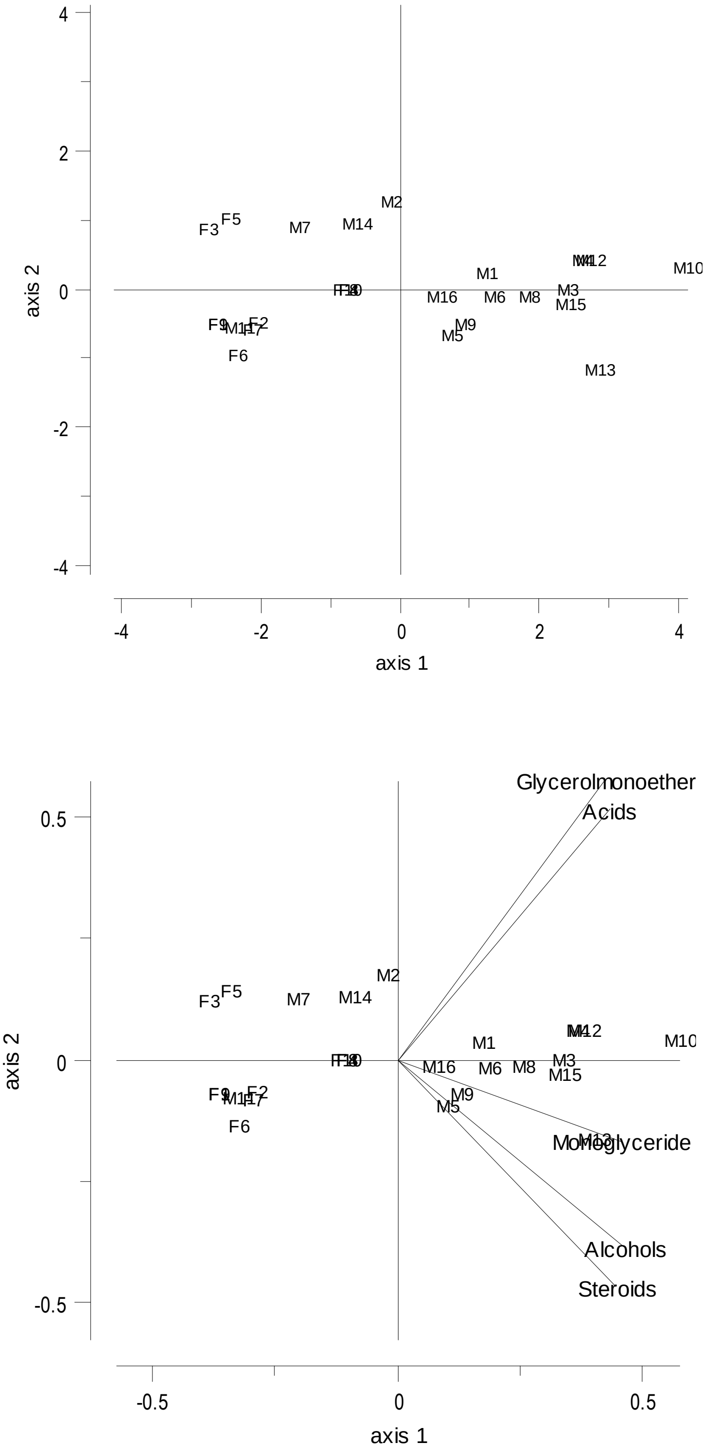
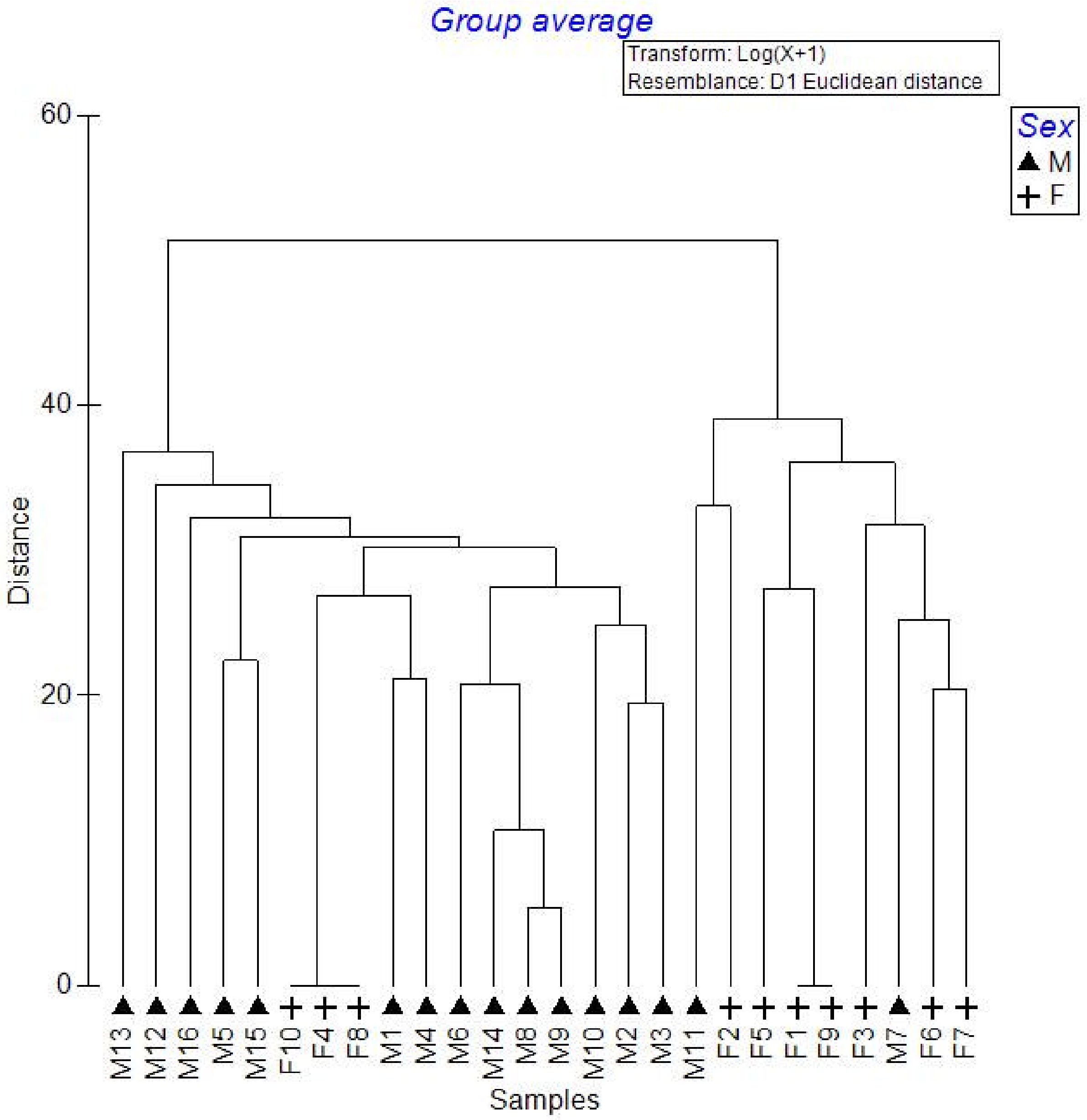


Figure 5. Cluster analysis, using the main five chemical groups, showing the difference between males and females in terms of the chemical composition of their secretions.



CHAPTER 4

Paper 3

Scent marking pheromones in lizards: Cholesterol and long chain alcohols elicit avoidance and aggression of male *Acanthodactylus boskianus* (Squamata: Lacertidae)

(Behaviour Journal)

Scent marking pheromones in lizards: Cholesterol and long chain alcohols elicit avoidance and aggression of male *Acanthodactylus boskianus* (Squamata: Lacertidae)

Keywords

Agonistic behavior, aggression, scent pheromones, femoral glands, Acanthodactylus boskianus.

Abstract

Femoral gland secretions are believed to play an important role in chemical communication and social organization of lizards. In spite of this, few studies have investigated the chemical composition and the behavioural role of these secretions. *Acanthodactylus boskianus* lizards are a good example group having these well developed glands in both sexes. We used GC-MS chemical analysis to detect the important secretion fractions and used the y-maze choice test bioassay to investigate the ability of the lizards to identify different synthetic blends of the secretions, we also reported behaviour of both lizard sexes. Based upon the GC-MS quantification data we selected representatives of the important chemical groups (steroids, alcohols, acids, alkanes) of the lizard secretions and used in the y-maze behavioural bioassay against controls. Males showed significant avoidance of cholesterol and alcohol blend, combined with agonistic behavior towards these stimuli. Females did not show any significant preference to particular odours. The data support the hypotheses that lizards use the femoral gland secretions as chemical trails and possible scent pheromones to mark territories and to establish dominance hierarchies. Cholesterol and long chain alcohols are suggested as potential scent marking pheromones in *A. boskianus*.

Introduction

Vertebrates often use chemicals as signals including urine (Dehnhard et al., 2001) and glandular secretions (Quay, 1972). Squamates (lizards and snakes) have the most highly developed vomeronasal system of all vertebrates (Halpern, 1992; Cooper, 1994), and they respond to chemical stimuli by increased tongue flicking (Cooper et al., 1996). Squamate reptiles sense chemical stimuli carrying information about several biological properties of the sender (Cooper, 1994). Despite the fact that sex recognition is often based on color signals, pheromonal communication occurs in many species of lizards (Mason, 1992; Cooper, 1996; Martin & Lopez, 2000). In lizards, pheromones are important for discrimination of familiar and unfamiliar individuals (Cooper, 1996) (Cooper, 1996), territory marking (Alberts, 1992) , sexual recognition (Cooper et al., 1996), and self-recognition (Graves & Halpern, 1991). Many lacertilian reptiles possess epidermal structures on the ventral surface of the thigh or the precloacal abdominal area. These femoral glands produce waxy secretions through external pores (Mason, 1992; Alberts, 1993). The secretory activity of these glands varies within the season and increases during the part of the year in which lizards are sexually active. In some families of squamates only males have these glands (Vanwyk, 1990; Alberts, 1993; Khannoon, 2004). Femoral gland secretions of reptiles can have pheromonal properties (Cooper et al., 1994; Lopez et al., 1997) and might play an important role in sexual signalling and territorial scent marking (Mason, 1992; Alberts, 1993). Chemical signals are used in intraspecific communication between lizards, e. g. in the desert iguana (Alberts, 1992; Halpern, 1992; Mason, 1992). In several species of lizards the pheromonal activity of the femoral gland secretions has been shown (Cooper & Vitt, 1984; Alberts, 1993; Aragon et al., 2001).

Studies have shown that femoral gland secretions are composed of both lipids and proteins (Mason & Gutzke, 1990) giving rise to the theory that the major compounds involved in chemical communication are lipid in nature (LeMaster & Mason, 2001). On the other hand, the involvement of proteins cannot be discounted, given their importance in amphibians (Toyoda et al., 2004). Few studies have examined the lipidic part of epidermal gland secretions (Weldon et al., 2008). In *Liolaemus* lizards, *n*-alkanes, long chain carboxylic acids, and steroids were found and differences in the proportions of some compounds between populations have been reported (Escobar et al., 2001; Escobar et al., 2003). In the lacertid lizard *Acanthodactylus erythurus*, alcohols, steroids, carboxylic acids, esters, lactones, ketones, squalene, and α-tocopherol occurred (Lopez & Martin, 2005b). The same classes of compounds were found in another lacertid lizard, *Lacerta monticola cyreni* (Lopez & Martin, 2005a). In the girdled lizard or sungazer, *Cordylus giganteus*, carboxylic acids, alcohols, ketones, esters, and steroids occurred in both males and females (Louw et al., 2007).

In lizards (Cooper & Vitt, 1986) and snakes (Chiszar et al., 1986; Burger, 1991), pheromonal communication was demonstrated in controlled laboratory experiments on trailing of conspecifics. Most of studies dealing with femoral gland secretions detection used the tongue flicking as the major criteria of examining bioactivity (Aragon et al., 2001; Martin & Lopez, 2007). Given that none of those studies used the choice behavior to evaluate the ability of lizards to choose between odour trails.

Behavioural experiments examining the femoral secretions of *A. boskianus* showed a sexual difference in the tongue flicking responses towards secretions (Khannoon et al., submitted, chapter 2). The composition of the femoral gland secretions of *A. boskianus* is quantitatively different between sexes and between different male ages. These secretions compose a characteristic compound bouquet, typical for *A. boskianus*. The chemical analysis of these secretions showed that they consist of alcohols, steroids, acids, alkanes, glycerolmonoethers and monoglycerides as the most abundant groups of compounds. We want to know exactly the importance of these secretion components and their potential role in communication. The variability of secretion chemistry between sexes and male ages could play important roles in mate choice and/or the establishment of dominance hierarchies, or in territorial marking.

Here we tested the following hypotheses. Since tongue flicking indicated that gland secretions play a key role in chemical communication, our study aims to demonstrate for the first time, using choice test for femoral gland secretions, whether lizards can utilize such odours to find mates. Y-maze choice test focuses on more than just measuring change in tongue flicking rate, it reflects the actual behavior towards chemical trails. Male-male aggressive interactions in vertebrates often involve chemical signaling (Wyatt, 2006), and we therefore also investigated this potential effect of the femoral gland secretions.

Materials and Methods

Samples

A. boskianus individuals were captured from Balteem, Northern coast of Egypt, during April 2008 which coincides with the activity and mating season of these lizards. The animals were transferred to Hull University, UK, and kept for experiments. We selected 18 adult males and 23 adult females that had the highest snout-vent length (SVL) within a very narrow range of SVL differences (SVL ± 3 mm). The lizards were individually housed in lidless transparent plastic cages (56 cm × 39 cm × 28.5cm; length, width, and height). Each terrarium was provided with rocks for shelter, sand and gravels as substrate, small tree branches, and water ad libitum. Temperature ranged from 22 degrees Celsius (night) to 32 (noon). Gradual temperature change was established by using a timer controlled heating and ventilation system. Natural photoperiod 12:12 hrs was simulated using fluorescent lights on timers, and UV lamps were used for compensation of the sunlight. Spot lamps were used for basking of the lizards. Lizards were fed with calcium-enriched mealworms and occasionally with crickets. The lizards were kept separately in cages for around 10 days prior to commencement of experiments. Only the experimenter had access to the animals so that they quickly grew accustomed to his presence.

Experiment 1. Chemistry

Previous work (Khannoon et al. submitted, chapter 3) using GC-MS analysis showed that the lipid part of the secretions consists of alcohols, steroids, carboxylic acids, alkanes, amides, aldehydes, carboxylic acid esters, and squalene, glycerolmonoethers and monoglycerides. Most of these compounds showed sexual and age variations in their abundance. Alcohols, steroids, and carboxylic acids were the most abundant groups of compounds in the secretions. These compounds showed higher abundance in males than in females. On the other hand, despite alkanes representing only >0.4% of the total ion current (TIC), they showed higher abundance in females than in males. In the current study, we quantified the most important individual components which fulfilled two conditions;

1- To exist in both sexes in high abundance relative to other chemicals.

2- To show sexual variations and/or male age variation

We then used synthetic compounds of in a behavioural bioassay to investigate the behaviourally active parts of the secretions.

Secretions were collected by gently squeezing the plugs from the femoral pores of the lizards (14 males and 10 females) using forceps. 8mg of secretion of each individual was collected directly into glass vials with Teflon-lined caps and then dissolved in 250µl of dichloromethane (DCM) (Aldrich, GC grade). The collected samples were kept at -18°C until processing for analysis. Control samples with the solvent at the same conditions of collecting the secretion were used to exclude impurities.

The chemicals which have been selected for the behaviour bioassay were quantified in both males and females. Quantification of these compounds was conducted by using external standards, and following the same procedures of derivatization and GC-MS. Based on the quantification results, the concentrations of these chemicals were prepared for the bioassay simulating their concentrations in the secretions. On the other hand, alkanes were not quantified because of the very low abundance.

Derivatization

Fifty µL of the liquid secretion was placed in a 2 ml vial and the solvent was removed in a gentle stream of nitrogen at 50°C. The residue was taken up in 10 µL dichloromethane and 50 µL *N*-methyl-*N*-trimethylsilylfluoroacetamide (MSTFA) were added. The mixture was heated to 50°C for 30 – 60 minutes in a vial with a closed cap. Then the solvent and the remaining reagent were evaporated in a gentle stream of nitrogen at 50°C and the residue was taken up in 10 µL dichloromethane. Exactly 1.0 µl was injected into the gas chromatography-mass spectrometry (GC-MS) system.

GC-MS analysis

Samples were analysed using a Hewlett-Packard model 6890 gas chromatograph connected to a Hewlett-Packard model 5973 mass-selective detector equipped with a BPX-5 column: 25 m×0.22 mm i.d., 0.25 µm film thickness (SGE). The temperature program was as follows: 50°C for 5 minutes, then with 5°C/min to 320°C, 30 minutes hold time. Helium was used as carrier gas with 1ml/min in constant flow mode. Accelerating voltage of MS was 70eV. Compounds were identified by comparison of mass spectra and retention indices of derivatized and underivatized samples with those of reference compounds.

Experiment 2. Behavioural bioassay

The chemicals chosen for the bioassay were obtained as following; 1-octadecanol (≥99.0%, Fluka), 1-tetracosanol (≥99%, Sigma), 1-hexacosanol (≥97.0%, Fluka), 1-octacosanol (≥99%, Sigma), cholesterol (≥99%, Sigma), dehydrocholesterol (≥98.0%, Sigma), hexadecanoic acid (≥99.0%, Fluka), octadecanoic acid (≥99.5%, Fluka), octadecenoic acid (~99%, Sigma), pentacosane (≥99.5%, Fluka), hexacosane (≥99.5%, Fluka), and heptacosane (≥99.5%, Fluka). The chemicals were dissolved in 500µl dichloromethane (DCM) which is enough volume to allow these to be spread over the gravels used in the bioassay. The chemicals were dissolved in a concentration mimicked that of an adult male’s highest concentration. As a result of quantification carried on in this experiment the highest concentrations of chemicals (µg/mg of secretions) found in adult males were; cholesterol (61.82 µg), dehydrocholesterol (4.84 µg), octadecanoic acid (9.02 µg), hexadecanoic acid (10.55 µg), eicosanoic (2.01 µg), octadecenoic (1.45 µg), tetracosanol (39.85 µg), octacosanol (32.89 µg), octadecanol (14.09 µg), and pentacosane, hexacosane, and heptacosane (<0.1µg each). The stimuli used were in a similar dilution predetermined from previous studies on tongue flicking (Khannoon et al., submitted, chapter 2) which showed that the whole secretions was functional at 8mg secretion/500µl DCM. The next step of the assay was to mix compounds of the same chemical group in one blend solution except for cholesterol and dehydrocholesterol. Cholesterol and dehydrocholesterol were tested separately because of the suggested role of each one (Lopez and Martin, 2005c). Five stimuli were used in the bioassay; three blends of alcohols, acids, and alkanes, and two individual chemicals; cholesterol and dehydrocholesterol. The stimuli were prepared by spreading the solutions of chemicals to be tested on 10mm gravels, which were used as a simulation to the actual habitat, and then evaporating the DCM solvent using a gentle stream of nitrogen gas. DCM was used as a control. Each trial was undertaken by using one of the five stimuli in one side arm against a DCM control in the other side arm of the y-maze.

We chose the y-maze choice test to determine whether the lizard could avoid or choose to follow a trail of chemical compounds. The experiments were done in a glass cage 39.5cm×98.5cm×39.5cm, divided into two parts; entrance and y-maze partitions, separated by a mobile door (10.5cm×20cm) which was controlled from the top by a long rope. This was used by the experimenter to open and close up and down, and was controlled from around two meters distance. A video monitor showed the experiment that was recorded by a Sony handycam placed 40-50cm over the top of the cage. The entrance partition (23.5cm×39.5×cm) was the part in which the lizard under test should stay for 5 minutes for relaxation before the door was opened to start the experiment towards the y-maze. The base arm of the y-maze was 17cm long, 20cm wide and 25.3cm high, and at the end of the base arm, there were two side arms (60 ^{o} angles from the base arm) of the same height and 10cm wide and 54.5cm length. The gravels carrying the stimuli and the control were placed on both sides of the base arm and continued until they diverged into the two side arms, one carrying the stimulus and the other carrying the control. The lizard had the chance to flick towards both sides once it entered the base arm, and had the chance to select and detect the favourite side. Placing the stimuli and controls in the sides of the base arm was to give the lizards the enough time for detection of the signals and to be able to detect the favourite side before going to the side arms, and this design was able to reduce a possible disadvantage of y-maze choice test.

The experiment started by transferring the animal under test into the experimental cage's entrance partition while the door was closed. The animal was allowed to accommodate for 5 minutes and then the door was opened using the rope, allowing the lizard to enter the y-maze partition. If the animal spent long time (over 15 minutes) after door was opened without entering the y-maze, the trial was cancelled and the animal transferred back to its home cage. The lizards were observed and the trials recorded by video camera. Each side arm was marked into three thirds, if the animal chose one arm and passed the line of the second third this was considered as a decision to choose that side. If the lizard returned back before the second line that was not considered as a choice. Also the behavior of the lizards toward the gravels carrying the stimuli was monitored. After each trial the experimental cage was cleaned by alcohol, the gravels were replaced and the entire y-maze was cleaned for the next trial. The location of the stimulus was swapped randomely. Each animal was tested randomly for any stimulus not more than once a day. Choice of sex with stimulus was also random.

Two reactions were recorded to evaluate the behavior of the lizards; (1) the choice from between the stimulus and the control, (2) the behavior towards the stimulus-carrying gravels. To discriminate whether tongue flicking was towards the stimulus or towards the control was difficult, because the lizards were tongue flicking frequently and it was not easy to detect towards which side they were flicking. The important response we aimed to record was any type of aggression. This was designed to examine which compound could be responsible for such aggression recorded from the response towards the femoral gland secretions.

Statistical analysis

Independent sample t-test was used to analyze the differences in the quantities of compounds produced between males and females. *G*-test was used to evaluate the response frequency in the y-maze. Test statistic G were corrected (Williams' correction).

Results

Experiment 1

The compounds detected in the total ion chromatogram (TIC) of both male and female *A. boskianus* secretions were qualitatively similar, but they differed quantitatively between the sexes (Figure 1). A total of more than 120 chemicals were identified in both sexes. The identified compounds were steroids, alcohols, carboxylic acids, alkanes, amides, aldehydes, carboxylic acid esters, glycerolmonoethers, monoglycerides and squalene. These compounds were identified using standard procedures. The extracts were analyzed after derivatization with MSTFA to form trimethylsilyl-derivatives, thus enabling the GC-MS analysis of the more polar compounds. The major compound groups, measured as relative percent to the total ion current TIC, present in the secretion were alcohols (50.29%), steroids (35.54%), carboxylic acids (9.37%), glycerol monoethers (2.11%), and monoglycerides (0.42%). The other classes occurred only in minor percents. The concentration (µg/mg of the secretions) of the selected alcohols, steroids, and carboxylic acids were significantly higher in males than in females, particularly for cholesterol and alcohols (Table 1). The highest concentrations of these chemicals in males have been mentioned in the materials and methods section, and these concentrations were used for the bioassay.

Experiment 2

Once the door separating the entrance partition from the y-maze was opened, lizards started to tongue flick and move towards the y-maze. Towards cholesterol and alcohols, males showed significant avoidance and chose the control side arm (N=15, G_{adj} (adjusted G) = 5.59, P<0.05 and N=16, G_{adj}= 4.06, P<0.05, respectively). Towards dehydrocholesterol, acids, and alkanes, males showed no significant choice (Figure 2, Table 2). Females showed no significant choice for any of the stimuli used, and only one female did bite once towards the alkanes. Males aggressive behaviour was observed through their biting towards the gravels carrying the stimuli of both cholesterol and alcohols blend. Five males bite the cholesterol flavored-gravels aggressively, four of them chose the control arm and only one chose the cholesterol arm. Three males did bite the alcohols flavored-gravels and all of them chose the control arm.

Discussion

The present results, together with the previous results on tongue flicking (Khannoon et al., submitted, chapter 2) show that *A. boskianus* lizards are able to detect femoral gland secretions. The relative differences in chemical concentration between male and female secretions supports the possible role of these chemicals in communication and the highly suggested role as scent pheromones. Cholesterol and alcohols showed very big differences between sexes which give them the possibility of being the high priority chemosignals. Using the y-maze was useful to test the ability of lizards to discriminate chemicals of conspecifics, similar to studies with snakes (Burger, 1989; Burger, 1991). The significant responses of *A. boskianus* lizards (Table 2) are responses towards the most common and sexually and/or age variable components of the femoral gland secretions detected by GC-MS. *A. boskianus* is territorial and dominant males advertise with a slow tail wave after locomotion (Schleich et al., 1996). This leads to a high likelihood of leaving secretions from the femoral glands on the ground gravels during this movement. In addition, males drag their legs on the ground leaving potentially traces of these secretions.

The present data showed that the males were avoiding cholesterol. Cholesterol is the most abundant individual component in the femoral gland secretions of *A. boskianus* (Khannoon et al., submitted, chapter 2) and other lacertid lizards (Lopez & Martin, 2005b). Cholesterol was found to be more abundant in males than in females and was prominent in older males than younger ones in the femoral secretions of *A. boskianus*. Mason & Gutzke (1990) suggested that cholesterol could provide information about the sex of an individual. In males, cholesterol levels may also provide information about the condition of the male such as the dominance status or its genetic quality (Martin & Lopez, 2000). When the territorial male *A. boskianus* moves around its territory it should leave marks for its presence, leaving traces of these secretions could be one of these means during the characteristic tail wave. When an intruder male comes into the territory it could assess the strength of the resident male through odour profiles of these secretions, and cholesterol seems to be the most important component in these odours to announce the physical strength of males.

Some males showed, strengthening the hypothesis of aggression, biting towards cholesterol stimulus, and there was significant avoidance of cholesterol side arm. This suggests a function of the secretions because chemoreception could allow an individual to quickly assess a rival's fighting ability (Martin & Lopez, 2007). The lack of aggression for some males could be explained as receiver assessing the strength of the odour's owner as superior to its own strength, thus avoiding costly aggressive interaction.

Dehydrocholesterol is the precursor of Vitamin D_3, which is essential in calcium metabolism. The abundance of dehydrocholesterol is likely to also signal the availability of vitamin D_3 and indicate a strong, dominant lizard. For males, it will be beneficial to announce high abundance of dehydrocholesterol and cholesterol to highlight its dominant status. In spite of this there was neither avoidance nor aggression towards dehydrocholesterol. It is feasible that dehydrocholesterol has no direct effect alone but is an important component in the bouquets within the secretions. Lopez & Martin (2005b) speculated that the relative abundance of dehydrocholesterol was related to the physical or health condition of the lizard and it therefore may play a role in female mate choice. Surprisingly, in our results, no female interest was shown towards dehydrocholesterol.

Alcohols could also form important chemosignals in the femoral gland secretions of *A. boskianus*, since this group of chemicals represents the highest abundant class of chemicals in the secretions. Alcohol concentrations showed sexual variation particularly the most important and highly abundant individual alcohols used in this study. In the present experiment these alcohols were used in a stimulus blend, constituting long chain C_{18}, C_{24}, C_{26}, C_{28} alcohols. Avoidance and aggression occurring in males indicates such possible role of alcohols specially since we only tested a proportion of the 22 alcohols detected in the gland secretions. Alcohols have been recorded as pheromones throughout the animal kingdom. In insects (Hoddle et al., 2009) and in mammals where nonadecan-1-ol alcohol was suggested to be a chemosignal produced by peri-anal gland of crested porcupines (Massolo et al., 2009).

Males did not show any significant choice towards acids or towards alkanes trials. Despite high abundance of acids in the secretions and significant sexual variation, they did not elicit any interest or avoidance by males. Acids represent substantial part of the lipids in the secretions and give the genus *Acanthodactylus* its specific secretion contents (Lopez & Martin, 2005a; Khannoon et al., submitted, chapter 3), and further studies are required to clarify their role.

Unlike males, the females did not show any significant responses towards cholesterol, dehydrocholesterol, alcohols, acids, and alkanes. Initially is surprising since cholesterol appears to indicate strong males that makes them attractive. There exist two possible explanations. Cholesterol might be an announcement of aggression and strength towards males (competetors) but not used for mate choice, at least not as a single compound. Mate choice and selection of partners may be entirely male driven, with female having little impact. As such females would not have evolved a preference for compounds indicating a male’s strength.

Overall, steroids and alcohols seem to be important chemosignals in *A. boskianus* lizards could be used in territorial marking and dominance hierarchies. Using scent marking to announce a dominant male’s presence in its territory would save such cost of fighting between males. One benefit of these scent markings is that they announce messages even when the animal is not present (Wyatt, 2006). Scent marks provid information to intruding animals on the status of the resident through the intrinsic characteristics of the marks or give the identity of the owner (Wyatt, 2006). Other reptiles are using cloacal gland secretions as marking signals. Prairie rattlesnakes show a rise in their heart rate when exposed to cloacal gland materials from conspecifics (Graves & Duvall, 1988). In alligators, the cloacal glands are used to mark territories during the breeding season (Evans, 1961).

Femoral glands are epidermal in origin and produce holocrine secretions (Mason, 1992; Khannoon, 2004), and it is considered as one of the skin derivatives of lizards. Skin derivatives are commonly used in communication. In snakes, integumentary-derived lipids function in mate attraction, aggregation, and trailing among conspecifics (LeMaster et al., 2001). Garter snakes *Thamnophis sirtalis parietalis* use methyl ketones, from integumental skin lipids, as pheromones for sex attractiveness (Mason et al., 1989). Males leopard gecko *Eublepharis macularis* responded towards skin-derived semiochemicals of other males with agonistic behavior (Mason & Gutzke, 1990). Maintainance of femoral gland lipid proportions in male lizards *Iberolacerta cyreni* was related to predation risk (Aragon et al., 2008).

Other chemicals which did not elicit any significant response in the present study cannot be excluded entirely from being semiochemicals or at least sharing such roles when they are in mixtures with other secretion components. Some vertebrate semiochemicals consist of dozens of compounds which can lose their signal function when separated into fractions (Duvall et al., 1986).

In conclusion, here we present a further support that the femoral gland secretions might be playing an important role in the territorial marking and in dominance hierarchies. At least cholesterol and long chain alcohols might be working as scent marking pheromones and they could elicit aggressive behavior between males. This is an initial step in understanding the individual chemicals playing as semiochemicals and to understand the mechanism of communication in lizard communities. Future work is needed to focus on the possibility that some chemicals from the femoral gland secretions could be responsible for mate choice.

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Figures and Tables

Table 1: Mean amounts (μg/mg secretions) ±SE of some selected chemicals in both sexes of *Acanthodactylus boskianus* lizards. Results of t-test which compared males and females amounts are shown as *P* value.

Compounds	Mean±SE		*P*
	Males	Females	
Cholesterol	20.39±4.50	2.99±0.64	<0.001
Dehydrocholesterol	1.03±0.36	0.04±0.005	0.001
Octadecanoic acid	3.07±0.81	0.09±0.02	<0.001
Octadecenoic acid	0.48±0.12	0.16±0.04	0.035
Hexadecanoic acid	2.69±0.80	0.54±0.06	0.005
Eicosanoic acid	0.47±0.14	0.04±0.01	0.001
1-Tetracosanol	14.41±3.12	1.53±0.41	<0.001
1-Octacosanol	7.63±2.31	0.39±0.13	<0.001
1-Octadecanol	3.96±1.17	0.40±0.09	0.004

Table 2: *G*-test results evaluate the response frequency for both males and females. Number of animals (N), adjusted test statistic *G* (after Williams' correction), and significance value are given.

	Males			Females		
Stimulus	N	G_{adj}	*P*	N	G_{adj}	*P*
Cholesterol	15	5.59	P<0.05	15	3.28	P>0.05
Dehydrocholesterol	14	2.56	P>0.05	17	0.05	P>0.05
Alcohols	16	4.06	P<0.05	15	0.06	P>0.05
Acids	17	1.45	P>0.05	21	1.17	P>0.05
Alkanes	15	0.06	P>0.05	15	0.06	P>0.05

Figure 1: GC traces of femoral gland secretions collected in dichloromethane from male and female lacertid lizard, *Acanthodactylus boskianus*.

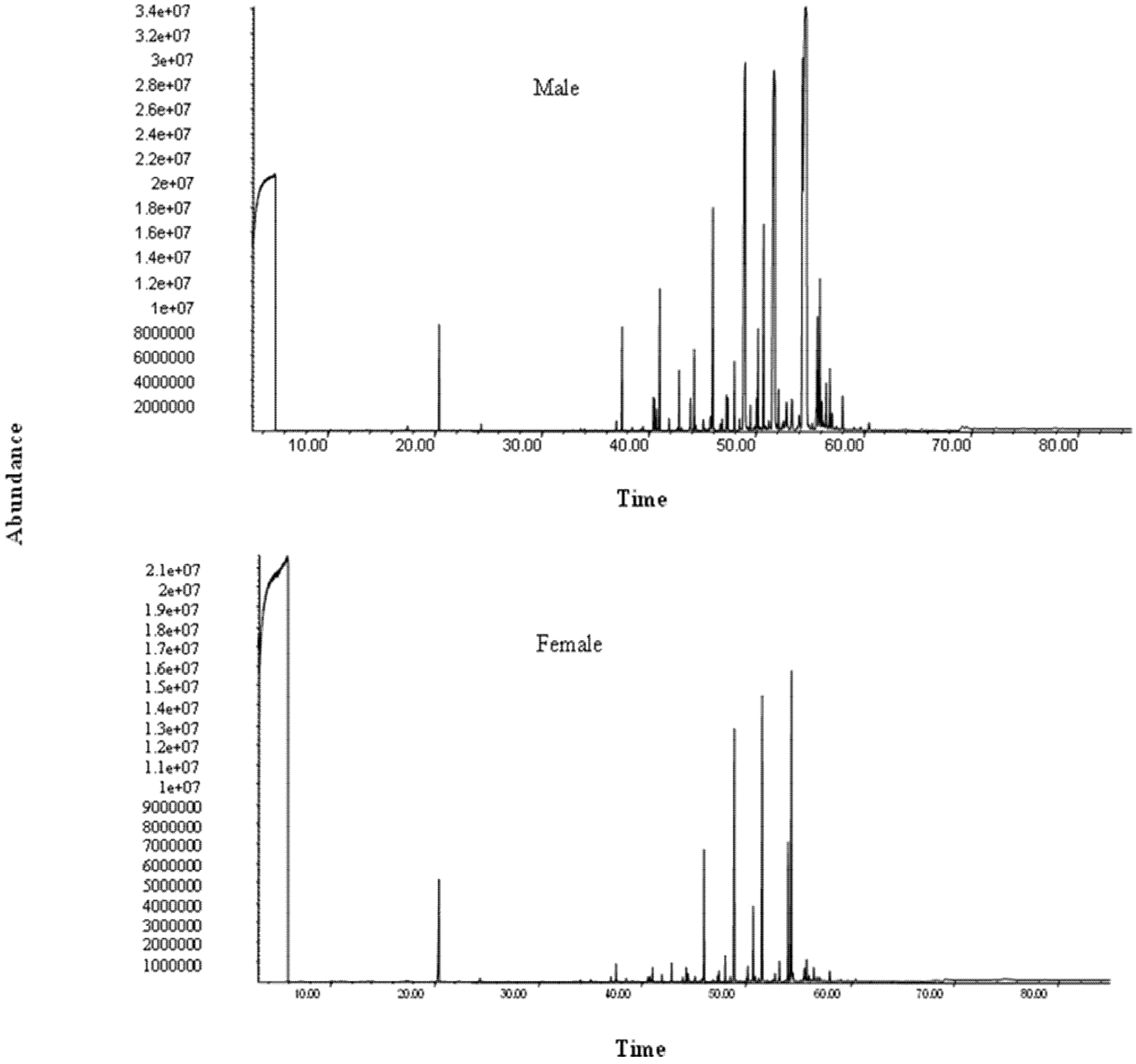
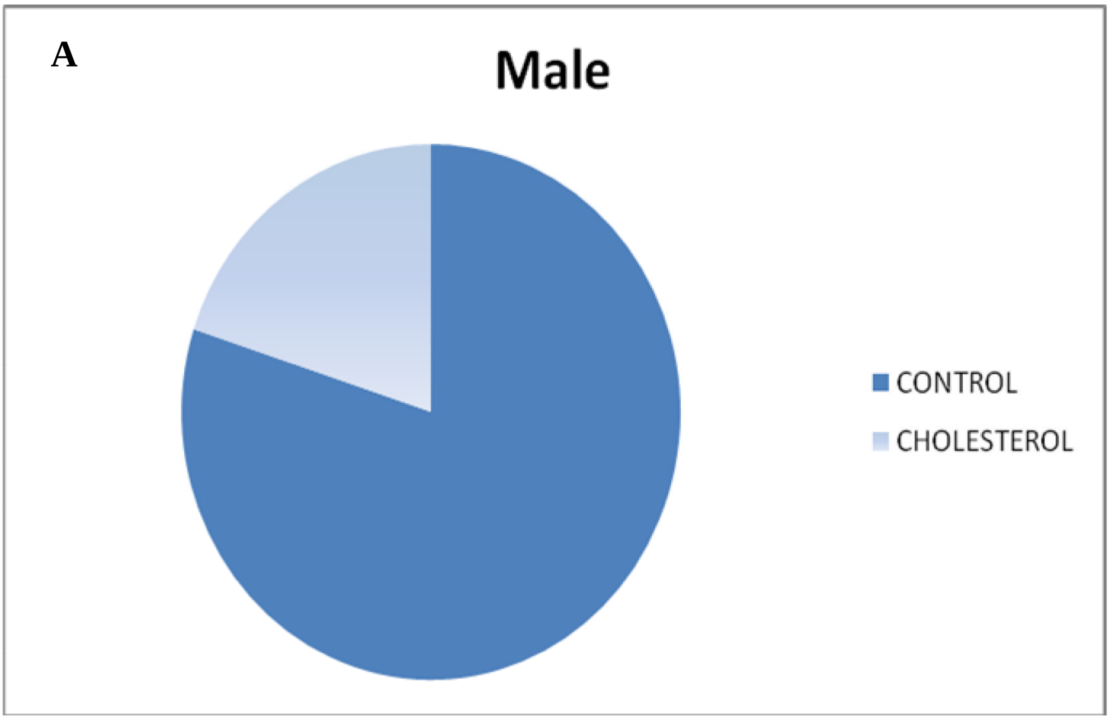
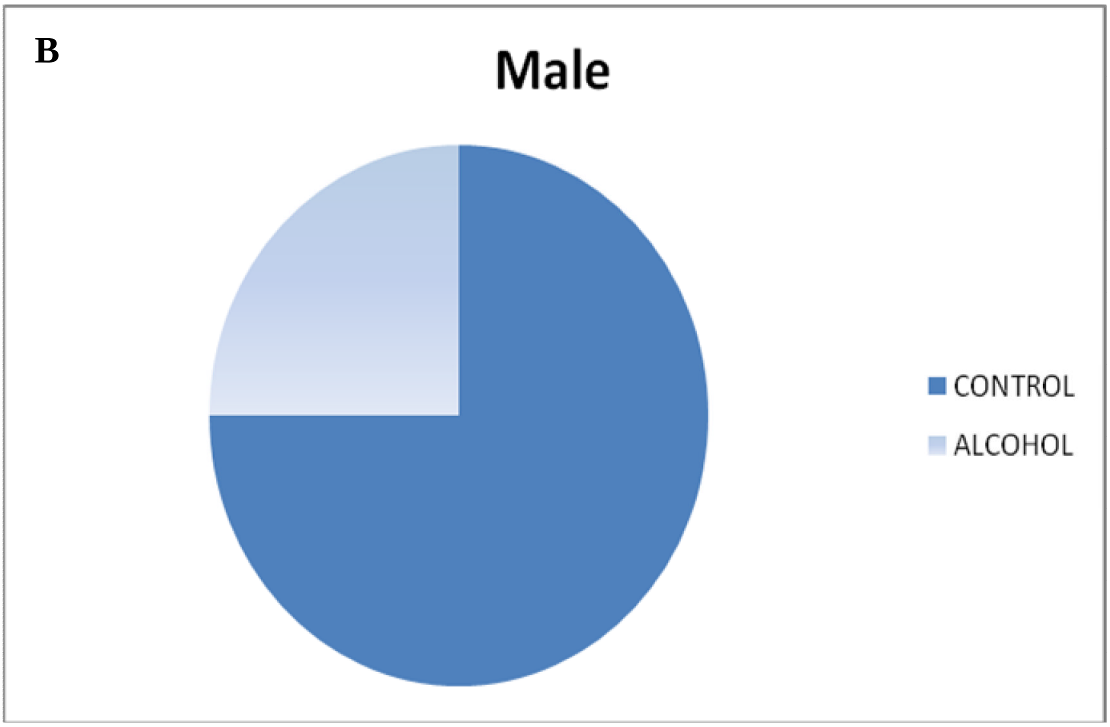


Figure 2: Choice of males towards both cholesterol (A) and alcohols blend (B) against control. Each coloured area represents the percent of male choice to a stimulus or the control.





CHAPTER 5

Paper 4

Divergence of scent pheromones in allopatric populations of *Acanthodactylus boskianus* (Squamata: Lacertidae)

(Journal of Chemical Ecology)

Divergence of scent pheromones in allopatric populations of *Acanthodactylus boskianus* (Squamata: Lacertidae)

Keywords

Acanthodactylus boskianus, Signal evolution, phylogeography, femoral gland,

Abstract

The evolutionary phenomena associated with divergence in chemical signals between populations of the same species help to understand the process of speciation. Animals detect and react to semiochemicals and pheromones used in communication. Comparison between populations of the same species that are geographically isolated from one another allows us to determine the genetic or environmental perspectives responsible for chemical differentiation. *Acanthodactylus boskianus* populations from east and west of Egypt were used as an example to compare the geographical diversity in chemical fingerprints of its femoral gland secretions and its phylogeography. Chemical analysis via GC-MS showed that the two geographically distinct population’s odour fingerprints are quantitatively different whilst sharing the same components of the secretions. Equally, phylogenetic analysis, using DNA analysis of mitochondrial genes 12S, ND4 and Cytb, showed that east and west populations of Egypt are genetically diverged. These differences suggest the existence of a geographic barrier as the main reason for genetic and chemical divergence of these lizards and imply these as an example of signal evolution.

Introduction

Chemical signals are widely used in animal communication and in many vertebrates detected by the vomeronasal system (Jacobson's organ). As highlighted in a recent review by Johansson and Jones (2007) chemical signals can play significant roles in mate choice and reproductive isolation but to date little is known about the chemical nature and the mechanisms of their biological functions. Chemical cues potentially allow for nest mate and kin recognition (Baker 2008; Carlin and Holldobler 1983) where evolutionary shifts in signalling systems have been studied intensively (Lofstedt et al. 1986).

Tongue-flicking is the quintessential squamate behaviour (Cooper 1994) and the chemical signals encountered are called vomoders and detected by the vomeronasal system (Jacobson's organ). Tongue flicking thus plays a role in delivery to Jacobson's organ that is located in the roof of the mouth or in the nasal passages. Lizards can use femoral (epidermal) glands to deposit semiochemicals on the substrate as lizards move through their home ranges, which may convey information about social status and competitive ability (Lopez and others 2002), but few studies have examined the chemical nature of the epidermal gland secretions in lizards and these showed that gland secretions consist of both lipids and proteins (Mason and Gutzke 1990; Weldon and Bagnall 1987; Weldon et al. 2008).

The major compounds involved in chemical communication are believed to be lipid in nature (Cooper and Garstka 1987; LeMaster and Mason 2001).

The chemical composition of the femoral gland secretions has been studied in a number of lizard species. In the lacertid lizard *Acanthodactylus erythurus* these include alcohols, steroids, carboxylic acids and esters, lactones, ketones, squalene, and α-tocopherol occurred (Lopez and Martin 2005). The same class of compounds were found in another lacertid lizard, *Lacerta monticola cyreni* (Lopez and Martin 2005), but with different relative proportions. In the girdled lizard or sungazer, *Cordylus giganteus*, carboxylic acids, alcohols, ketones, esters, and steroids occurred in both males and females (Louw et al. 2007). Alcohols and steroids are the most important chemical classes in femoral gland secretions, the former were reported earlier as major components in another *Acanthodactylus* lizard, *A. erythrurus* (Lopez and Martin 2005). Chemical studies on the femoral gland secretions of *A. boskianus* (Khannoon et al. submitted, chapter 3) showed sexual quantitative differences in their components.

Acanthodactylus is a genus of ground-dwelling lizards containing 38 species that are widely distributed in arid areas of southwest Asia, North Africa, and Iberia (Fonseca et al. 2009). *A. boskianus* is one of the most widely distributed species of lizards in Egypt, recorded in the eastern desert, western desert, northern Mediterranean coast, along the River Nile and in Sinai (Baha El Din 2006). Its range extends in Northern Africa, reaches the Mediterranean and Atlantic coast; south to Mali and north Nigeria, Sudan and Abyssinia. The range extends in Asia; Near East to Iraq and Arabian Peninsula (Schleich et al. 1996).

The nature and extent of communication among animals can as such be useful to answer evolutionary questions of population diversification. Because of phylogenetic inertia it may be possible to use comparative analysis of signal transmission and receiving systems to determine the phylogenetic relationships between species (Théry and Heeb 2008). Allopatric populations have signal divergence in African striped mouse (Pillay 2000), in *Drosophila serrata* (Higgie and Blows 2008), and in calling songs of Mormon crickets (Bailey et al. 2007).

The aim of the current study is to investigate whether differences exist in the composition of the femoral gland secretions between different geographic populations of *A. boskianus* that may function as chemosignals and compare these with molecular data. If the composition of the secretions has diverged i.e. through the evolution of signal traits and mating preferences, we could expect population dependent odour profiles and behavioural separation upon exposure to gland secretions and these to be manifested through genetic divergence~~.~~

Materials and Methods

Samples

A. boskianus individuals were captured in Egypt, during April 2008 and 2009 which coincides with the activity and mating season of these lizards. Samples from the selected populations of *A. boskianus* were collected and identified according to Schleich et al. (1996) and Baha El Din (2006). Populations were selected representing east and west of Egypt (Figure 1). The Sinai peninsula population represents the east of Egypt, and both the Siwa oasis (29°11'N, 25°31'E) and west of Abu Rawash (30°02'N, 30°50'E) populations representing the west of the country (Figure1). The Sinai populations are represented by two sites; Maghara (28°45'N, 33°25'E) and Sharm El Sheikh (27°51'N, 34°16'E). The animals were transferred to Hull University, UK, and kept for chemical and molecular experiments.

1. Chemical analysis of gland secretions

To avoid the effect of sexual variation we used only adult males from 3 different populations; and population 1, Sinai (N=17); population 2, Siwa (N=10); and population 3, west of Abu Rawash (N=13). Age effects were avoided by choosing individuals with snout-vent lengths (SVL) with very narrow size range (±3mm). Secretions were collected by gently squeezing the plugs from the femoral pores of the lizards using forceps. 8 mg of the secretions of each individual was collected directly into glass vials with

Teflon-lined caps and then dissolved in 250µl of dichloromethane (DCM) (Aldrich, GC grade). The collected samples were kept at -18ºC until processing for analysis. Control samples with DCM solvent at the same conditions of collecting the secretion were used.

Derivatization

Fifty µL of liquid secretion was placed in a 2 ml vial and the solvent was removed in a gentle stream of nitrogen at 50°C. The residue was taken up in 10 µL dichloromethane and 50 µL *N*-methyl-*N*-trimethylsilylfluoroacetamide (MSTFA) were added. The mixture was heated to 50°C for 30 – 60 minutes in a vial with a closed cap. Then the solvent and the remaining reagent were evaporated in a gentle stream of nitrogen at 50°C and the residue was taken up in 10 µL dichloromethane. Exactly 1.0 µl was injected into the gas chromatography-mass spectrometry (GC-MS) system.

GC-MS analysis

Samples were analysed using a Hewlett-Packard model 6890 gas chromatograph connected to a Hewlett-Packard model 5973 mass-selective detector equipped with a BPX-5 column: 25 m×0.22 mm i.d., 0.25 µm film thickness (SGE). The temperature program was as follows: 50°C for 5 minutes, then with 5°C/min to 320°C, 30 minutes hold time. Helium was used as carrier gas with 1 ml /min in constant flow mode. Accelerating voltage of MS was 70eV. Compounds were identified by comparison of mass spectra and retention indices of derivatized and underivatized samples with those of reference compounds.

2. DNA analysis

DNA extraction, amplification and sequencing

Lizard tail tips were cut, stored in 100% ethanol, and kept for DNA extraction for the three populations; population 1 (Sinai)(N=15), population 2 (Siwa) (N=14), population 3 (Abu Rawash) (N=5). *A. scutellatus* from Siwa, was used as an outgroup. Total genomic DNA was extracted using either DNeasy blood and tissue kit or hot sodium hydroxide-Tris solution (HotSHOT) technique (Truett et al. 2000). Fragments of 12S, ND4, and cyt b genes were amplified using the primers 12Sa and 12Sb for 12S rRNA gene (Kocher et al. 1989), ND4 and LEU for ND4 gene (Arévalo et al. 1994) and cyt b1 and CB3 for cyt b gene (Palumbi 1996). Amplifications were performed in 2.5 µl of 10x reaction buffer, 3 µl of 25 mM Mg^{+2}, 2.5µl of 8mM dNTP, 1µl of 10 mM of each primer, 0.2 µl Bio *Taq* DNA polymerase (500 unit), 1µl genomic DNA and 13.8µl ultrapure H_2O. Amplification started with initial cycle of denaturation at 94 ºC for 3 min, 35 cycles of; denaturation at 94 ºC for 30s, annealing at 55 ºC for 1 min, extension at 72 ºC for 3 min. A final extension was conducted at 72 ºC for 10 min. Negative controls were run for all amplifications. PCR products were sent to a commercial company (Macrogen, Korea) for purification and sequencing. Sequences for representative individuals included in this study have been deposited in Genbank (accession numbers GU225704-GU225707

Phylogenetic analyses

Sequence traces from 12S, cytb, and ND4 genes were aligned using CodonCode Aligner (version 3). Neighbor-joining and Maximum likelihood (ML) phylogenetic analyses were conducted using BioNJ (Gascuel 1997) and PhyML (Guindon and Gascuel 2003) using the general time-reversible model with a gamma distribution of rate heterogeneity and 4 rate categories. Confidence in nodes was assessed using both bootstraps and approximate likelihood ratio test (aLRT) support values.

3. Statistical analysis

The relative amount of each component analysed chromatographically was determined as the percent of the total ion current (TIC). Chemicals included as variables in the statistical analysis were only those which were presented in a most numbers of animals and constituted > 0.01% of TIC. The peak area of each chemical was used as a variable representing the abundance. The data were tested for normality using a One-Sample Kolmogorov-Smirnov test, and subsequently transformed (Log10) in order to achieve normality. Each main group of compounds was the summation of all the peak areas under its category. The five major groups of compounds (acids, alcohols, steroids, monoglycerides and glycerolmonoethers) were used as variables in ANOVA test followed by Scheffe post-hoc test to look if and which populations are chemically significantly different. Multivariate Principal Component Analysis (PCA) was used to determine the chemical separation of populations followed by Analysis of Similarity (ANOSIM) used to determine statistical differences between populations.

Results

1. Chemical composition of femoral gland secretions

A total of more than 120 chemicals were identified in all populations without qualitative differences (Table 1). The identified compounds as percents of TIC were alcohols (50.29%), steroids (35.54%), carboxylic acids (9.37%), glyceolmonoethers (2.11%), monoglycerides (0.42%), and minor percent of alkanes, amides, aldehydes, carboxylic acid esters, and squalene. The GC-MS analysis of the underivatized natural extracts showed the presence of more polar compounds, which could not be identified because of their poor elution properties. Therefore the extracts were analyzed after derivatization with MSTFA to form trimethylsilyl-derivatives, thus enabling the GC-MS analysis of the more polar compounds.

Quantitatively the populations 3 (Abu Rawash) and 2 (Siwa) are not chemically different (Figure 2 and Table 2). On the other hand both of these populations are significantly different from population 1 (Sinai). This means that the two populations from the west of Egypt populations, (2 and 3) are chemically similar, but different from the third population 1 from the east of Egypt. The principal component analysis (PCA, Figure 3) and analysis of similarity (ANOSIM) confirm these population differences. All five major compound classes occur in west population in higher amounts than in east one. The Euclidean cluster analysis, using the five major compound classes further indicates separation between east and west populations, showing a difference between them (R= 0.412; P= 0.001).

2. Molecular analysis

The aligned sequence data set of 12S (408bp), cytb (662) and ND4 (846bp) were used totalled 1916bp with 34 individuals from *A. boskianus* and two outgroup sequences. The maximum likelihood tree provides evidence of two clear lineages corresponding to east and west of Egypt (Figure 4). Clade 1 (East), which corresponds to the Sinai population, represents a well-supported geographical cluster different from clade 2 (West) represented by individuals from Siwa and Abu Rawash.

Discussion

The femoral gland secretions of lizards play an important role in chemical signalling (Alberts 1993; Aragon et al. 2001; Cooper and Vitt 1984) and our previous work on *A. boskianus* confirms the role of these chemicals, particularly alcohols and steroids, as scent pheromones that appear to be used in territorial marking (Khannoon et al. submitted, chapters 2 and 3). Similarly, we have shown that *A. boskianus* are able to discriminate conspecifics of the same or the opposite sex by differences in the chemical components of their secretions. The five main groups of compounds, alcohols, steroids, acids, glycerolmonoethers and monoglycerides detected in this study represent more than 97% of the TIC from *A. boskianus* secretions. It is very likely therefore that the chemical repertoire sampled in this present study includes the active elements responsible for these scent differences. The two clades provided by the molecular analysis are genetically diverged as well as they are chemically. In other meaning, the significant differences in the abundance of chemical components of the scent glands between the populations are parallel to the genetic divergence.

The difference in secretion component concentration between the eastern and western populations could be explained by either environmental conditions and/or genetic differences. Evolution of signals depends on different types of constraint (phylogenetic or environmental) and it is the result of trade-off between natural and sexual selection (Théry and Heeb 2008). Environmental conditions are different between Sinai and Siwa habitats. While South Sinai (population 1, eastern) represents mountainous regions up to (2,641 m) with a maximum temperature 29.7°C and 1-35 mm annual rainfall, Siwa oasis (population 2, western) lies deep in the high aridly western desert with temperatures of up to 53°C and highly infrequent and unpredictable rainfall (Goodman and Meininger 1989; Kassas 1993). The environmental and temperature variability between the two habitats might support the environmental constraint effect on the chemical composition of secretions. This environmental effect might be important if these long chain lipids with lower volatility and higher chemical stability are needed in higher temperature habitats to render volatilization and decomposition of the secretions. Signals result from multiple adaptations to the environment, and the physical properties of the environment exert important constraints on the nature and shape of signals (Théry and Heeb 2008). On the other hand, Abu Rawash (population 3, north-western) lies adjacent to Delta and Nile where the temperatures and aridity are much less than in Siwa. In spite of this, population 3 is chemically very similar to population 2 that are together in the western side of the Nile. This raises the possibility that the chemical composition reflects phylogenetic rather than environmental relationships.

In order to investigate the genetic relationship between these populations we sequenced three separate mitochondrial genes from a total of 34 individuals. The phylogenetic results (Fig 4) clearly show that the samples of *A. boskianus* fall into two geographically-restricted clades. Clade 1 represents the east (population 1, Sinai), and clade 2 represents the west, containing individuals from both Siwa (population 2) and Abu Rawash (population 3). These two clades are robust to the addition of many more samples representing populations throughout Egypt, North Africa and Arabia (Khannoon et al. unpublished data). It is likely that these groups represent a phylogeographic genetic subdivision or the species, possibly corresponding to races or subspecies, as has been reported in very many organismal systems (Avise and Wollenberg 1997; Walker and Avise 1998). There are a number of potential hypotheses to explain the situation. Allopatric lineages and the cladogenesis can be explained by in situ divergence, or vicariant lineages that meet at the river Nile and therefore represent an allopatric speciation case. To explain the divergence one can use the mechanisms suggested by Kapli et al. (2008) for *Mesalina guttulata*. According to this, the separation of the two clades took place during the late Miocene, which coincided with the flooding of the Nile, in the early Pliocene, following the uplift of the Mediterranean sea-level (Goudie 2005).

A. boskianus populations diversification into two clades east and west of the Nile could be due to this event which did not allow the two clades to come in contact again. To accept this scenario for *A. boskianus* also makes sense because *Acanthodactylus* is one of the genera, that together with *Mesalina* belongs to the *Eremidianae* tribe of the subfamily *Lacertinae*. This group was suggested to have evolved in Southwest Asia and dispersed into North Africa ~13 million years ago (mya) (Mayer and Pavilcev 2007) (Mayer and Pavlicev 2007). The invasion of this ancestor gave rise to xeric forms of *Mesalina, Acanthodactylus, Eremias, and Ophisops,* which colonized North Africa (Arnold et al. 2007; Mayer and Pavilcev 2007).

We have here demonstrated a significant genetic split between eastern and western populations of *A. boskianus*, and shown that the chemical characteristics of the femoral secretions mirror this subdivision. It is likely therefore that the time spent in isolation by these groups, with little or no gene flow between them has also lead to divergence in secretion characteristics. Although a robust test of an environmental basis for the secretion differences would require a carefully design transplantation or common-garden experiment, our current data casts doubt on this as an explanation. Samples from Abu Rawash are geographically closest to those from Siwa, and they are also found in the same phylogenetic clade, and are not chemically different. Despite these similarities the lizards inhabit very different environments with regard to temperature and rainfall.

The type of signal divergence in allopatry that we propose has been seen in other systems also. In mammals, such as the mouse *Rhabdomys* *pumilio*, population−specific communication signals and in particular olfactory cues, assortative mate choice, and pre-mating reproductive isolation have all been described as a result of divergence in allopatry (Pillay 2000). In anurans, call structure was diverged with allopatric populations (Smith et al. 2003) and calling songs in Mormon crickets (Bailey et al. 2007) and odorant signals of the house mouse (Smadja and Ganem 2008) have the same patterns. A lot of other examples have been shown in many literatures (Tregenza et al. 2002; Tregenza et al. 2000**)**

Territorial behaviour and scent marking is potentially a significant factor in population isolation in many species (Higgie and Blows 2008; Pillay 2000). Our molecular analysis shows the existence of two genetically diverged clades of these lizards in Egypt. This divergence is reflected in the equally large differences in the odour profiles of the populations suggesting that lizards could well be capable of detecting such differences. As such odour profiles in scent marking may form the basis of mating preferences, kin and nest mate recognition as well as indicators for health and immune status as known in house mice (Zala et al. 2004), ants (Carlin and Holldobler 1983) and moths (Baker 2008). Odour based population isolation is well known from insects such as moths where pheromone bouquets vary significantly between populations (Lofstedt 1993), and it is recently shown similar isolation to exist in marine invertebrates (Sutton et al. 2005). The present study showed that allopatric populations are quantitatively and not qualitatively different in their scent gland secretions.

In turnip moths *Agrostis segetum,* Löftsedt (1993) described the population dependent pheromone bouquets as genetically based but as resulting from mate choice driven selection pressures that favoured geographical differences where the compounds themselves are the same but show geographical diverse odour fingerprints.

In conclusion, we demonstrate that the inter-population chemosignal variability of *A. boskianus* most likely reflects the genetic divergence in allopatry of the populations under study. Further studies on the chemical composition of the sympatric species *A. scutellatus* and other species of the same genus might give important insights into signal evolution and speciation within this group.

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Figures and Tables

Table 1. Compounds identified (qualitatively the same in Table 1, chapter 3) in at least one sample of an individual *A. boskianus* of the 3 populations. The gas chromatographic retention index (RI) is also given. Alcohols and acids were detected as the respective trimethylsilylated compounds, thus the RI shown is that of the respective derivative.

RI	Compound	*RI*	Compound
1058	2-Hydroxypropanoic acid	2441	Eicosanoic acid
1076	Hexanoic acid	2441	*N*,*N*-Dimethyloctadecadieneamide
1077	1,2-Dihydroxypropane	2473	Glycerol 1-hexadecyl ether
1116	Nonanal	2478	N,N-Dimethyloctadecaneamide
1216	Decanal	2500	2-Eicosenoic acid
1265	Glycerol	2500	Pentacosane
1320	2,3-Dihydroxypropanoic acid	2513	1.3-Eicosanediol
1360	Nonanoic acid	2539	Heneicosanoic acid
1400	Tetradecane	2545	1-Docosanol
1458	Decanoic acid	2568	Glycerol 1-heptadecyl ether
1490	Hydrocarbon	2580	Glycerol 1-hexadecanoate
1500	Pentadecane	2590	3-Hydroxyicosanoic acid
1507	Hexanedioic acid	2600	Hexacosane
1530	Methyl dodecanoate	2612	Docosenoic acid
1560	Hydrocarbon	2637	Docosanoic acid
1562	1-Dodecanol	2642	1-Tricosanol
1600	Hexadecane	2663	Glycerol 1-octadecyl ether
1630	Isopropyl dodecanoate	2700	Heptacosane
1645	Hydrocarbon	2709	1,3-Docosanediol
1653	Dodecanoic acid	2716	Tetracosen-1-ol
1700	Heptadecane	2741	1-Tetracosanol
1703	Hydrocarbon	2745	Glycerol 1-octadecadienoate
1725	Unknown compound	2750	Glycerol 1-octadecenoate
1731	Hydrocarbon	2760	Glycerol 1-nonadecyl ether
1755	1-Tetradecanol	2772	Glycerol 1-octadecanoate
1800	Octadecane	2792	Glycerol 1-(3-hydroxy)octadecyl ether
1811	12-Methyltridecanoic acid	2800	Octacosane
1849	Tetradecanoic acid	2814	Squalene
1900	Nonadecane	2834	Tetracosanoic acid
1910	13-Methyltetradecanoic acid	2838	1-Pentacosanol
1917	12-Methyltetradecanoic acid	2854	Glycerol 1-eicosyl ether
1933	Methyl hexadecanoate	2900	Nonacosane
1943	Hydrocarbon	2913	Hexacosen-1-ol

Table 1. continued

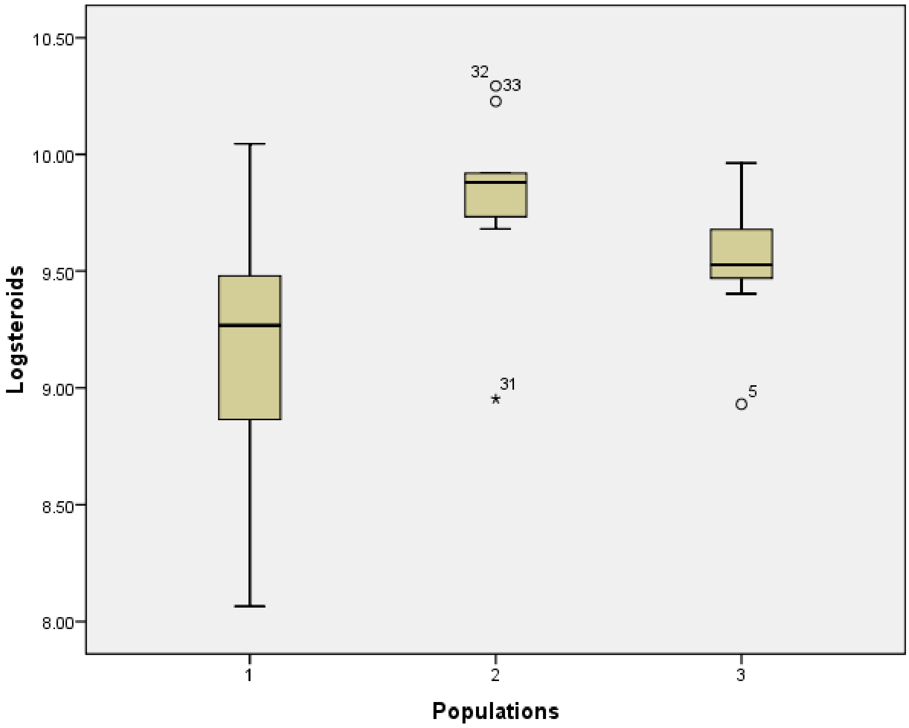
1947	Pentadecanoic acid
1956	1-Hexadecanol
1981	14-Methylpentadecenoic acid
2000	Eicosane
2008	14-Methylpentadecanoic acid
2023	Hexadecenoic acid
2044	Hexadecanoic acid
2100	Heneicosane
2105	15-Methylhexadecanoic acid
2114	14-Methylhexadecanoic acid
2116	Heptadecenoic acid
2134	Methyl octadecanoate
2143	Heptadecanoic acid
2150	1- Octadecanol
2200	Docosane
2213	3-Hydroxyhexadecanoic acid
2216	Octadecadienoic acid
2223	Octadecenoic acid
2242	Octadecanoic acid
2267	*N*,*N*-Dimethylhexadecanamide
2300	Tricosane
2302	2-Octadecenoic acid
2322	1,3-Octadecanediol
2340	Nonadecanoic acid
2348	Eicosanol
2377	Glycerol 1-pentadecyl ether
2387	Glycerol 1-tetradecanoate
2398	3-Hydroxyoctadecanoic acid
2400	Tetracosane
2420	Eicosenoic acid
2935	1-Hexacosanol
2948	Glycerol 1-eicosanoate
2977	Glycerol 1-(3-hydroxy)eicosyl ether
3000	Triacontane
3024	Glycerol 1-docosenyl ether
3033	Heptacosanol
3045	Glycerol 1-docosyl ether
3059	Steroid M=386
3067	Steroid M=388
3094	Steroid M=386
3103	Hentriacontane
3103	Steroid M =384
3116	Octacosen-1-ol
3133	1-Octacosanol
3141	Cholesterol
3159	Cholestan-3-ol
3190	Cholesta-5,7-dien-3-ol
3200	Dotriacontane
3231	1-Nonacosanol
3247	Campesterol
3269	Stigmasterol
3300	Tritriacontane
3330	1-Triacontanol
3332	β-Sitosterol
3400	Tetratriacontane
3528	1-Dotriacontanol

Table 2. Results of Scheffe test (Post-hoc test), used after Anova analysis, showing the chemical differences between the populations; Sinai (1), Siwa (2) and Abu Rawash (3).

Group of compounds	Populations	(*P* value)	
	1	2	3
Steroids	1	<0.001	0.008
	2		0.098
Alcohols	1	<0.001	<0.001
	2		0.061
Acids	1	0.001	0.010
	2		0.332
Glycerolmonoethers	1	0.005	0.335
	2		0.055
Monoglycerides	1	<0.001	0.003
	2		0.347
	3		

Figure 1. Egypt map showing the locations from which the populations were collected, Sinai (1), Siwa (2) and Abu Rawash (3). Source (Google Earth).

Fig 2. Boxplots of the five chemical groups (steroids, acids, alcohols, glycerolmonoethers, and monoglycerides) expressed as log abundance for the 3 populations, 1(Sinai), 2 (Siwa) and 3 (Abu Rawash). Populations1 and 2 are different for all groups of chemicals, similarly 1 and 3 except for glycerolmonoethers.



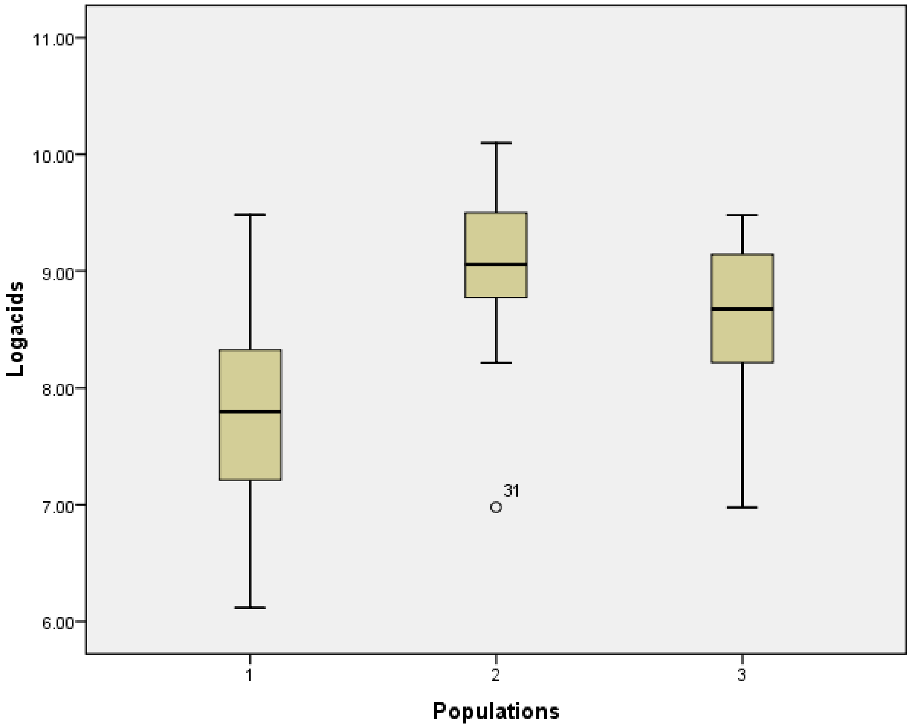
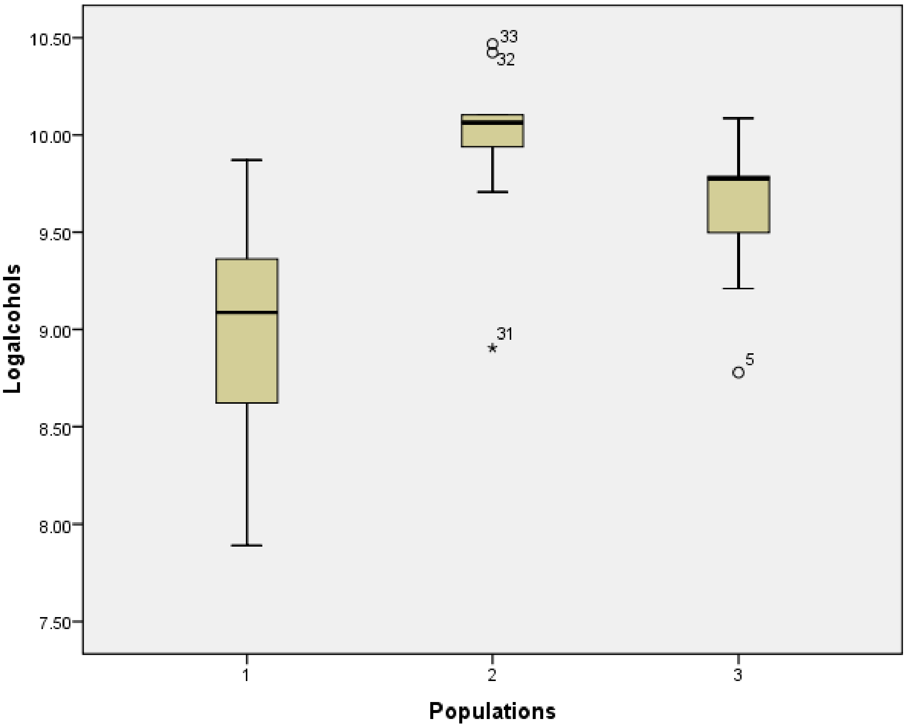


Figure 2. continued



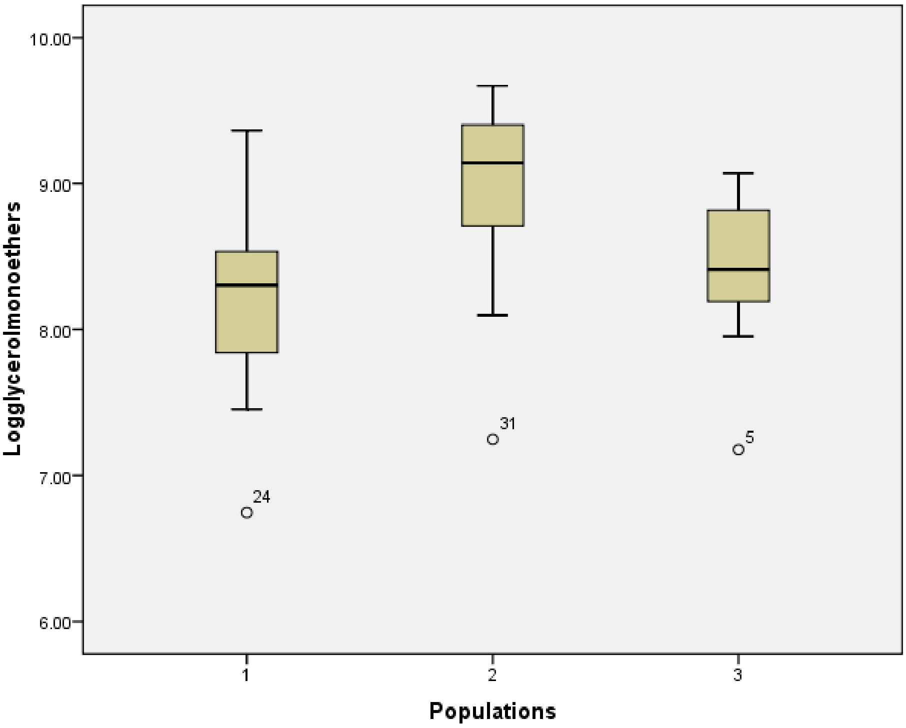


Figure 2. continued

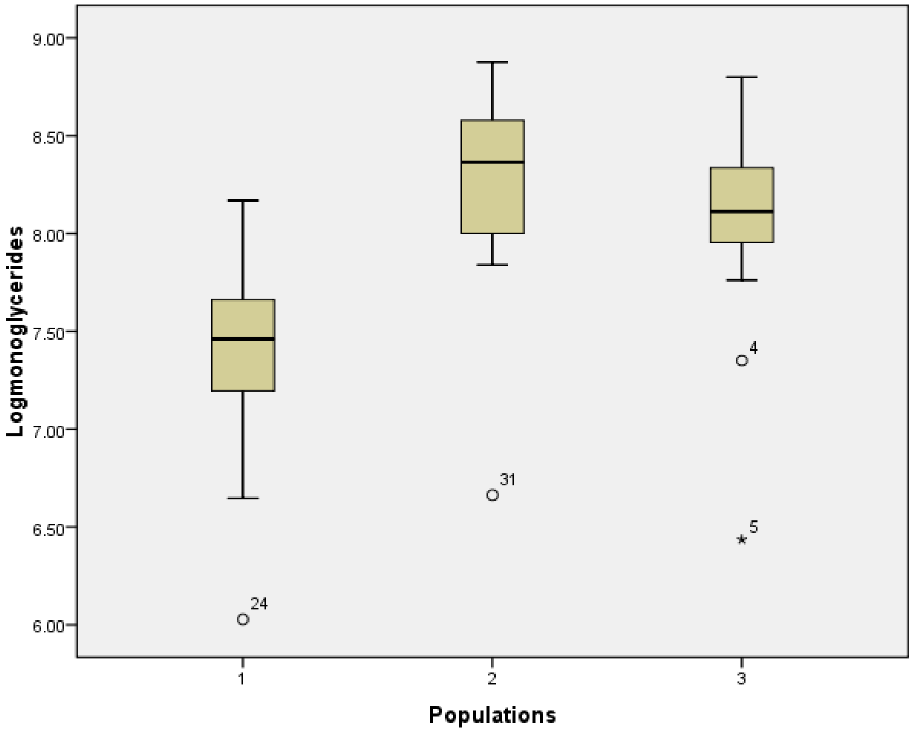


Figure 3. PCA showing the separation of the samples of Sinai population (circles) from both Siwa (triangles) and Abu Rawash (rectangles) of *A. boskianus* using the main classes of chemicals of the femoral secretions.

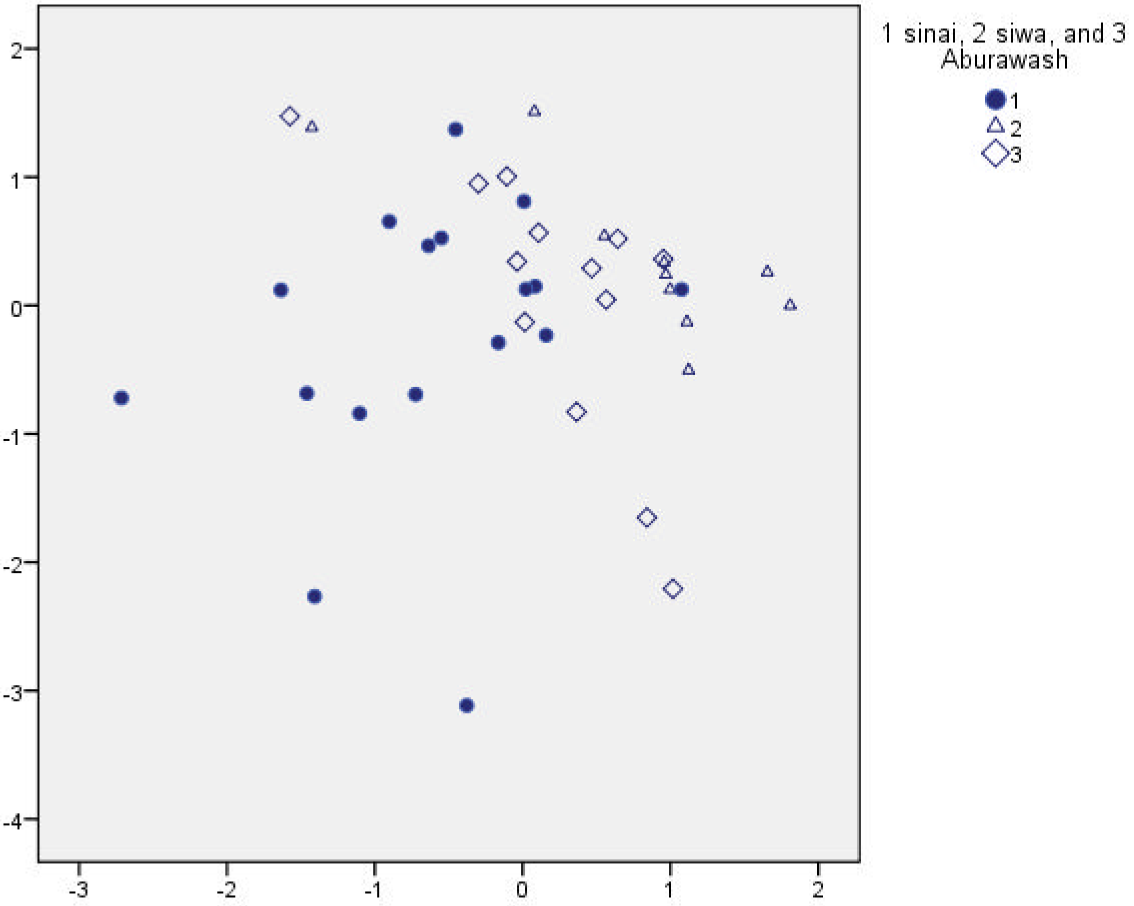
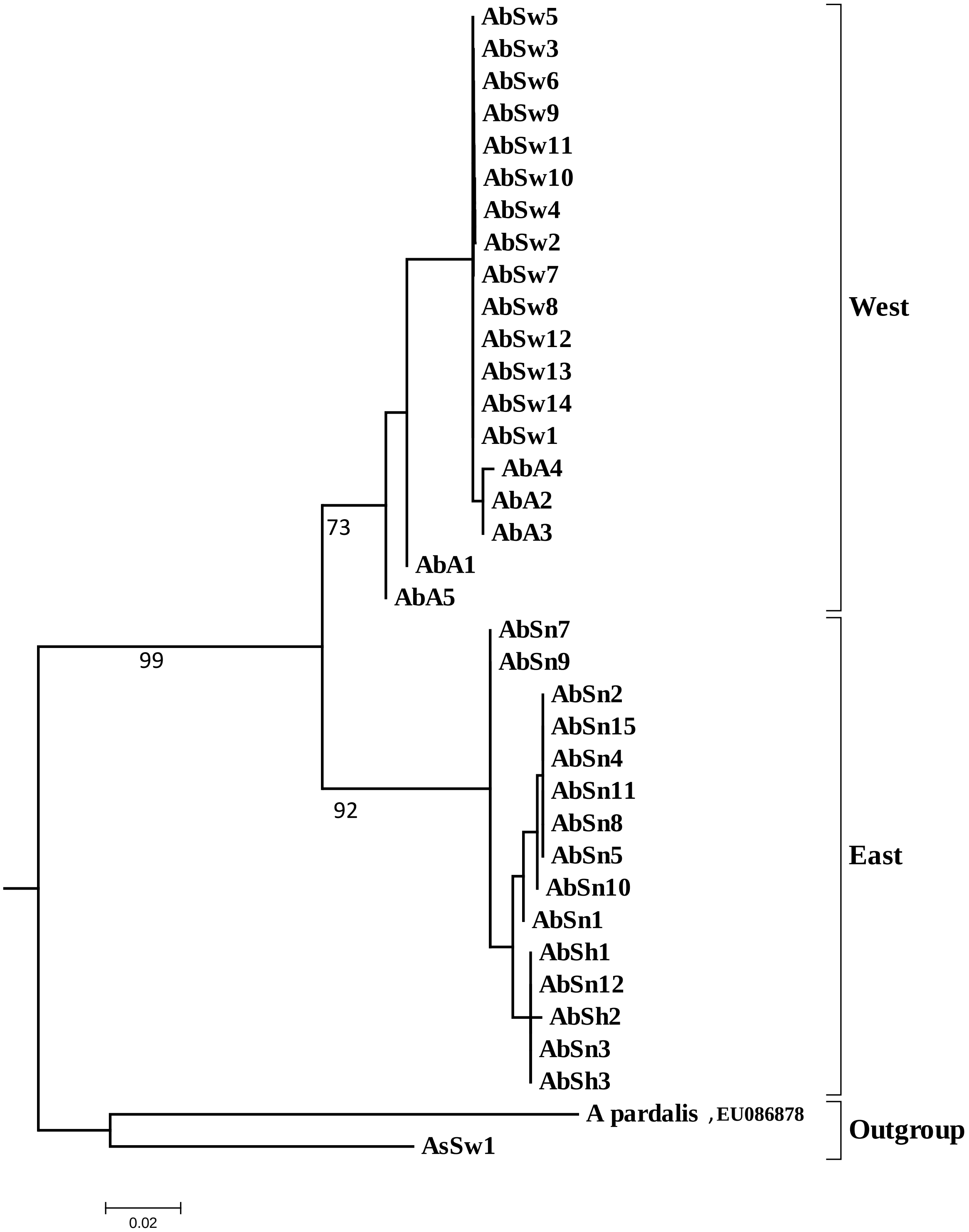


Figure 4. Phylogenetic relationships among the three populations of *Acanthodactylus boskianus*. Individual of *A. scutellatus* and genbank data of *A. pardalis* were used as an outgroup taxa. Phylogenetic analyses of maximum likelihood (ML) and neighbour joining (NJ) produced trees with the same topology. Only maximum likelihood is presented. Numbers above the branches are bootstrap values. The individuals of the three populations are given short names; *A. boskianus*, AbSn and AbSh (Sinai), AbSw (Siwa), and AbA (Abu Rawash). *A. scutellatus* outgroup was collected from siwa (AsSw).



CHAPTER 6

Discussion and Conclusions

Discussion and Conclusions

In the present work, *A. boskianus* was able to use tongue flicking to discriminate the chemicals in its environment. Tongue flicking was a useful tool for evaluating the behavioural response of lizards. Having the femoral glands in both sexes made *A. boskianus* a good candidate to be used in studying the chemistry of these glands and the behaviour of the lizards towards the secretions. Male *A. boskianus* could discriminate the sex of the secretion's owner with different responses, and showed interest towards the female's secretions and aggression towards the male's secretions. The chemistry data support the behavioural results, where the former showed sexual and age variability. This variability might be the explanation of the male behaviour. Age and sex chemical variability is potentially responsible for the aggression recorded towards the other male's secretions, where males can assess the other male's physical ability announced by the secretions. On the other hand, the sexual chemical variability is explaining the male interest to female secretions, which is reflecting the characters of the female. The female's interest in male secretion might reflect the important role of the femoral gland secretions in mate choice. These results support the possibility of using the femoral gland secretions in mate choice, and also their role in territorial marking and dominance hierarchies.

Dissolving the secretions in DCM and subsequent derivatization using MSTFA was a good technique, which resulted in the detection of some new chemicals (glycerolmonoethers and monoglycerides) (chapter 3) not previously reported from reptiles. These new compounds have not been reported in the same genus from previous work on *A. erythrurus* (Lopez and Martin, 2005a). It remains unknown whether *A. boskianus* is characterised by these new chemicals, which would potentially give it a unique chemical fingerprint, or whether other species also produce these new chemicals,. The second point is, do these new chemicals are a consequence of the environmental differences between the two species?, considering that *A. erythrurus* studied from the Iberian peninsula inhabit lower temperatures and aridity than the Egyptian *A. boskianus*. More studies should be undertaken on different species from the same genus to investigate the presence of such compounds and consequently suggest their possible role. The characteristic chemical characters derived from the femoral gland secretions of *A. boskianus* raise the importance of future studies on the epidermal glands in lizards and in reptiles generally.

The present data on both the behavioural and the chemical analysis of femoral gland secretions is leading us to a number of conclusions: First, it is highly likely that alcohols and steroids are the most important chemosignals in *A. boskianus* secretions. This conclusion is based on the high sexual and male age variabilities in the abundance of these chemicals. In addition, choice tests using a y-maze showed that they are avoided and they also elicited aggression by males. Using tongue flicking towards cotton swabs carrying the secretions elicited aggression of the males. Therefore one can suggest that alcohols and steroids (particularly cholesterol) function as important signals used between males to assess their physical abilities. This conclusion is supported by the results of choice test where the males avoided these chemicals when these were present in high concentrations. *A. boskianus* is territorial, and when it is kept in captivity males establish a dominance hierarchy (Schleich, 1996). Therefore, the aggression of lizards towards the chemical components of the secretions is signalling this hierarchy.

The carboxylic acids in the femoral glands did not evoke any responses in the mate choice tests. Nevertheless these chemicals cannot be excluded as signals, because they might be functional when they are forming a part of a mixture with other chemicals. Many semiochemicals in vertebrates consist of compounds which can lose their signal function when separated into fractions (Duvall, Muller-Schwarze & Silverstein, 1986). On the other hand, we did not test all the acids found in the secretions, but used only the most common and abundant acids. Therefore, there might be other acids in minor percentiles in the gland excretions and potentially play a role in communication. The same can be assumed for all other minor chemical constituents of the glands, which have not been studied in the behavioural bioassay.

Away from playing a direct role in communication, long chain carboxylic acids and other long chain organic chemicals found in the secretions which showed no behavioural effect, can form parts of the unreactive, apolar matrix that delivers the true semiochemicals, similar to what is known from mice where urinary proteins (MUPS) form the transport vehicle of the volatile pheromones (Humphries et al., 1999). These long chain compounds might reduce the volatility of the secretions and this is an important role to keep the functional signals as long as possible in arid environments.

In the present work, the femoral gland secretions showed quantitative variations between genetically divergent populations of *A. boskianus*. Potentially these secretions are playing a role in signal evolution which was discussed in details in chapter 5. This role gives these secretions, and subsequently the skin derivatives of lizards, phylogenetic implications. The differences in the secretion's chemistry between different lizard species were qualitative, and different species also had different species-specific chemicals, as well as interspecific variations of common compounds. On the other hand, in populations of *A. boskianus* there were only quantitative differences which are the intraspecific differences. The later differences (fingerprints) are keeping the same species chemistry but allow for allopatric populations variability. More species from the same genus are needed to be studied, and then more studies on different genera are required to get a more complete insight into the potential role of gland secretions in phylogeny. *A. boskianus* is highly distributed in Egypt and a variety of morphological differences between different populations occurs, consequently a large comparative project on the species phylogeography is needed to study its differentiation potential. Many different habitats exist in Egypt; dry desert, oases, mountainous, cultivated land, and human occupied land habitats. *A. boskianus* is living in almost all these habitats, and these potentially have enabled this species to form a large diversity of independent populations, further studies on the ecology and phylogeny of this species are required to clarify this.

In this thesis chemical and behavioural techniques and DNA analysis were used to investigate the importance of the epidermal femoral gland secretions of *A. boskianus.* The results and data discussed provide many conclusions:

1- The skin has an important role in communication and behaviour of lizards. The skin is not only protecting the lizard from the danger of desiccation and water loss, but also used in the social organisation of lizard communities through producing specific odour compounds.

2- The femoral gland secretions (particularly steroids and alcohols) are important in territorial marking and dominance hierarchies.

3- Despite this we could not detect specific components, which might be used in mate choice. One cannot exclude that some components of the femoral gland secretions might be used for this purpose.

4- Analysis of the exocrine gland secretions of squamates is a viable tool to identify natural products and chemicals distributed among different taxa.

5- Femoral gland secretions function as scent pheromones and each taxa could have a chemical fingerprint specific to it.

6- Signal diversification and evolution potentially occurs in lizards. The femoral gland as source of these chemicals reflects this and these data give the study an evolutionary perspective.

7- Such information about the behaviour of lizards in general and *A. boskianus* in particular can potentially be used as data source to protect endangered species. Understanding the pheromones and animal behaviour is an important tool to protect animals, i.e. when combating invasive species.

8- Since the gland chemicals potentially reflect a sender's physiology and its fitness/dominance position, the cues may be important in territorial behaviour, mate choice as well as detection of health. The later being a potential application for example in commercial cultures or zoos.

9- Captive breeding programs of endangered lizards requires a good understanding of the behavioural ecology of a species and its social interactions. Femoral gland secretion could hold the key for developing such programs in lizards.

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