Genetic Influences on Parental Care in *Nicrophorus vespilloides.*

Submitted by Chloe. J. Bird, to the University of Exeter as a thesis for the Degree of Doctor of Philosophy in Biological Sciences, July 2010.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signed: Chloe. J. Bird

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ABSTRACT

The burying beetle (*Nicrophorus vespilloides*) has unusually highly developed parental care; parents prepare and maintain a food resource (thereby providing indirect parental care), feed through direct provisioning by regurgitation, and protect their larvae. Parental care is highly variable and can be uniparental female care, uniparental male care, or biparental. There are genetic components to the parenting behaviour of the burying beetle, the amount of direct and indirect care given, and the size of the brood are heritable and therefore genetic traits.

In this thesis I have focused on two candidate genes that I predicted would influence parental care behaviour. The first is *foraging*, which has been shown to influence a range of social and reproductive behaviours in other insect species. Using QRTPCR and pharmacological manipulations I have investigated the role of *Nvfor* in adult and juvenile burying beetles. The second gene is *inotocin,* the insect orthologue of oxytocin. Oxytocin has been shown to influence social behaviour as well as many behaviours associated with reproduction in vertebrates and invertebrates, however the effects of inotocin have not yet been investigated in insects. I have used pharmacological manipulations to investigate the role of inotocin in parental behaviour in female burying beetles.

Collectively my results demonstrate the central role of *Nvfor* in the control of direct parental care and the association with major behavioural changes in both adult and larval burying beetles. I have also demonstrated the possible involvement of oxytocin in the control of aggression towards conspecific larvae. These insights suggest the controlling mechanism for the behavioural changes seen in burying beetles is complex and involves interactions between many genes. Combined with previous research on these genes, it is clear they are key components in the evolution of sociality. Finally, my research indicates the power of the candidate gene approach, and suggests additional components of the related pathways that could be investigated.

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During the research contributing to this thesis I was supported by a studentship from The University of Exeter.

All of the chapters presented in this thesis were written by Chloe J. Bird, with comments and editing from A. Moore.

Chapter 3: I developed the injection protocol in collaboration with Amy Simpson, who also ran some of the observation experiments that are included in my data set.

CHAPTER 1

INTRODUCTION – A GENE FOR PARENTAL CARE

The goal of this thesis

The goal of this thesis is to use a candidate gene approach to examine the potential roles of two genes influencing the expression of parental care. The two genes are orthologues of the *foraging* gene, a cGMP-dependent protein kinase that has been shown to be involved in the development of behaviour in several insect species, and inotocin, the insect orthologue of oxytocin, which is involved in social bonding in vertebrates. I will examine the effects of these genes in the burying beetle *Nicrophorus vespilloides*, an insect with unusually well-developed parental care. I will relate my findings to the existing research on burying beetle behaviour, the role of these genes in the expression of other behaviours in other animals, and the wider questions of the evolution and control of social behaviour in insects.

Sociality

Social behaviour in animals has fascinated people for centuries, particularly the more elaborately organised and highly structured eusocial insect species such as termites, ants, wasps and honey bees. How such highly developed social systems evolved and are maintained over time is a complex puzzle (Wilson 1971, Choe and Crespi 1997). Eusociality is a rare phenomenon, occurring in only five orders of insect; the majority of eusocial species fall into two orders: Hymenoptera and Isoptera. However, there are many levels of social behaviour which do not fit the classification of eusocial (Wilson 1971). Studying these systems can shed some light on how eusociality evolved (Wilson 1971).

Levels of organisation

There are several different definitions of the various levels of social organisation, all are broadly similar. For this thesis I will use the levels of organisation as outlined by Wilson in *The Insect Societies* (1971). Wilson defines eusociality by the presence of three traits

- overlap of adult generations,
- reproductive division of labor, and
- Cooperative care of young.

Degrees of sociality that do not qualify as eusocial are classified as presocial. Presociality describes any system where the degree of interaction between parents is more than just sexual, but there is no overlap of generations, or division of labour, or cooperative care. That is, presocial animals can display some degree of communal living, cooperative care of young or primitive reproductive division of labor, but they do not display *all* of the three essential traits of eusocial animals. As one might expect, presocial behavior is much more

common than complete eusociality (Costa 2006). It is possible to further categorize types of presocial behaviour as they apply to insects as follows (Costa and Fitzgerald 1996):

Solitary: No parent/offspring interactions and no significant interactions between parents beyond mating.

Subsocial: Adults care for their own nymphs/larvae for some period of time.

Communal: members of the same generation use the same composite nest without cooperating in brood care.

Quasisocial: members of the same generation use the same composite nest and also cooperate in brood care.

Semisocial: As in Quasisocial, but there is also reproductive division of labour; a worker caste care for the young of the reproductive caste

Burying beetles do not fit exclusively into any of these categories, but subsocial is probably the most accurate descriptor for most species and populations. If the breeding resources are sufficiently large burying beetles will breed communally (Eggert and Sakaluk 2000). However, the typical state is one or both parents caring for their young (Eggert *et al.* 1998, Muller *et al.* 1998).

Insects as a model

Insect systems are used as models for many reasons, not least because a colony of thousands of individuals takes up a relatively small area and are reasonably easy to raise under laboratory conditions. Beyond simple convenience, insects are useful models for studying social behaviour because of the sheer number of species and the diversity of their behaviour means that the range of behaviours seen across the species covers any social system, from asocial species that only interact long enough to mate, through all the varying degrees of presociality all the way to complex eusocial systems. (Wilson 1971, Costa 2006)

Evolution of sociality

For anything to evolve, the trait must fit three criteria:

- There must be variation in the trait.
- There must be selection on that variation.

- There must be a genetic component so that the trait is heritable.

(Endler 1986)

Most studies of insect social behaviour have focused on heritability and selection on variable traits, rather than the genetic component. Recently, the technological advances in molecular biology have allowed studies into the genetic component of the behaviour. This aspect of the field is still very much in its infancy and there is still very little integration between the quantification of the behaviours and genetics behind it. By studying insects with differing degrees of sociality it is possible to find common features of behaviour, and subsequently the underlying controlling mechanisms of the behaviours. Identifying these shared behaviours and the genes underlying them, makes it possible to investigate how the functions of the genes have been conserved across species and how they have been adapted to specific tasks. Investigating the extent of conservation of function and any adaptations of the roles, across many species with different levels of sociality can illuminate some of the evolutionary paths to eusociality

Overview of genetics of social behaviour

There is a large body of work on the genetics of social behaviour, below; I discuss some of the key aspects of this research.

Quantitative studies

***Apis mellifera*:** Selection lines in honey bees (*Apis mellifera*) have demonstrated that within relatively few generations it is possible to generate lines which show extremes of the variation of a range of behaviours. In honey bees the workers change their primary task as they age, broadly speaking this is divided between young bees performing in-nest tasks and older bees leave the nest to perform out-nest behaviours. There is genetic variability in pollen foraging (compared to foraging for various other things), and selection lines created to have high and low pollen foraging showed the expected differences in pollen foraging, but also differences in the age at the onset of foraging (Calderone and Page 1988). When placed in mixed colonies there was spatial heterogeneity within the nest and the workers from the two lines differed in the in-nest tasks they performed (Calderone and Page 1988). Further research on these selection lines showed that when workers were fostered into different colonies it affected their behaviour (Calderone and Page 1992); low pollen individuals in a high pollen colony collected more pollen than low individuals in a low colony. Similarly high individuals in a low colony collected less pollen than high individuals in a high colony. This demonstrated that though the traits were heritable and

could be selected on, there was still a large environmental influence on the foraging strategies of workers, and that some aspect of the social environment induced different foraging behaviour than the genotype would suggest (Calderone and Page 1992). It was also found that several traits could be selected on; brood quantity, population size, number of pollen foragers, proportion of pollen foragers and diurnal foraging pattern (Page and Fondrk 1995), all of these behaviours are likely candidates for traits that led to the development of sociality and the elaboration of the social system in honey bees. These experiments show that social behaviour in honey bees is heritable and can evolve further.

Nicrophorus vespilloides: Most relevant to my work, Walling *et al.* (2008) showed that there are genetic components to the parenting behaviour of *N. vespilloides*. In burying beetles parental care is divided between males and females, with both sexes specialising to certain tasks. These tasks are divided into two categories; direct care, which is the feeding of partially digested carrion to the larvae by parent(s), and indirect care, which consists of parent(s) cleaning the carcass of mould and bacteria, maintaining the crypt and guarding against competitors. By measuring phenotypic and genetic variation and covariation within and between sexes Walling *et al* (2008) showed that when providing direct parental care males are more phenotypically variable than females, but they also had a lower mean amount of direct care. There was no difference in variation of indirect care or family size. Within a sex, phenotypic correlations were low, but genetic correlations varied in both strength and direction. These differences in genetic covariation were suggested by Walling *et al* (2008) to provide lines of least evolutionary resistance toward division of labour by male and female parents. The existence of this underlying genetic architecture to parental care in the burying beetle establishes a solid base of evidence to begin investigating the underlying mechanisms of the genetic control of parental care.

Molecular studies

More recently, developments in technology for molecular biology have allowed researchers to identify and study candidate genes underlying social behaviour in insects (Boake *et al.* 2002, Robinson and Ben-Shahar 2002, Toth and Robinson 2007, Robinson *et al.* 2008). To date, all the investigated taxa have been eusocial species of hymenoptera.

Apis mellifera: The first studies on the genes controlling social behaviour in insects were in the honey bee. Using *period* as a candidate gene, selected because of the multiple effects on behaviour in *Drosophila melanogaster,* Toma *et al* (2000) found that changes in mRNA

levels of *period* are associated with changes in behaviour as worker bees age. Young adult workers perform tasks without daily rhythms, but older workers forage with strong daily rhythms. Young workers that were induced to forage by changes in social environment were found to have elevated levels of *period* mRNA to a similar level to normal age foraging workers (Toma *et al.* 2000).

The *foraging* gene was also found to play an important role in the control of changes in behaviour in honey bees. The *foraging* gene was also selected due to the effects it has on Drosophila behaviour, the honey bee orthologue is known as *Amfor*, and it is more highly expressed in older workers who have transitioned from in-hive behaviour to out-hive behaviour. Pharmacological manipulations showed that precocious foraging could be induced at an earlier age by increasing the activity of the gene product of *Amfor* (Ben-Shahar *et al.* 2002, Ben-Shahar *et al.* 2003).

In subsequent studies using microarrays, genome-wide expression in brains of individual nurses and foragers were compared (Whitfield *et al.* 2003, Whitfield *et al.* 2006). The cDNAs used in the microarrays represented approximately 40% of the genes in the honey bee genome. Patterns of expression were similar between young and old nurses, and young and old foragers, and overall, expression patterns were different between foragers and nurses. Of the genes that were identified as predictive of behaviour, 17 had strong sequence matches to functionally annotated genes from *Drosophila melanogaster* (Whitfield *et al.* 2003). Further characterisation of the honey bee genome allowed identification of more genes through microarray analyses, resulting in a list of 100 predictive genes for behavioural changes (Whitfield *et al.* 2006).

Following from the work by Paige *et al* (1995, 1998), Quantitative Trait Loci (QTLs) were identified, as being linked to various social and hive behaviours, these QTLs have been confirmed and mapped to locate and clone candidate genes, many of which were orthologs for Drosophila genes (Hunt *et al.* 2007a). The combination of data from the microarray experiments and the QTL mapping allows testable hypotheses to be developed for which genes and gene networks are controlling social behaviours in honey bees and the possibility of investigating these genes and gene networks in other social species.

Polistes metricus: Following from the work on honey bees, recent studies of the paper wasp *Polistes metricus* have also revealed several genes linked with different social

behaviours (Toth *et al.* 2007, Toth *et al.* 2009). There are four primary castes in the species studied by Toth *et al*. (2007): queen, foundress, worker and gyne. Foundresses are females who start new colonies in the spring; they perform both reproductive and maternal caring behaviour. Successful foundresses become queens once they have reared their first generation of workers, queens only perform reproductive behaviour. The workers take over the caring roles, provisioning the younger broods; the workers show little, if any reproductive behaviour. Gynes are late-season offspring; they show no reproductive or caring behaviour. After mating the gynes overwinter and become foundresses in the following spring (Toth *et al*. 2007).

By using 454 sequencing Toth *et al*. (2007) created nearly 400 000 cDNA reads, resulting in robust identification of 3017 genes, of which 32 were matched as orthologs to genes related to behaviour in honey bees. 28 of these genes were then compared for expression differences in the brains of four behavioural castes; queen, foundress, worker and gyne. 17 of these genes showed significant differences in expression between the behavioural groups, there were marked differences between each group, and temporal changes in brain gene expression as individuals shift from foundress to queen status. Since these genes were chosen from the ~3000 identified genes based on their association with social behaviour in honey bees, it is, perhaps, unsurprising that so many were significantly different between groups (Toth *et al*. 2007).

Ants: Two species of ant; *Pogonomyrmex barbatus* and *Pheidole pallidula* show differences in expression of the orthologs to *foraging* linked with behavioural differences. Both studies used the candidate gene approach, having identified *foraging* as a potentially behaviourally significant gene from previous studies. Having cloned the orthologs, expression assays of brain tissue showed significant differences between different castes and hence, different behavioural states (Ingram *et al.* 2005, Lucas and Sokolowski 2009).

***Pogonomyrmex barbatus*:** The red harvester ant, *Pogonomyrmex barbatus,* has similar patterns of behaviour as honey bees; older individuals take foraging and out-of-nest roles and younger individuals stay within the nest providing care and nest maintenance. Expression of *Pbfor* was found to be lower in foraging ants than in any other caste of worker, showing the inverse association of behaviour and expression as is seen in honey bees. (Ingram *et al*. 2005).

Pheidole pallidula: In *Pheidole pallidula,* two different worker castes were compared; major workers are large and primarily act in defence of the nest, minor workers are smaller and generally act as foragers. *ppfor* is expressed at higher levels in major workers (Lucas and Sokolowski 2009). Expression also changed in response to environmental stimuli; when presented with a large food source *ppfor* expression dropped in both castes, and when presented with an ant alien to the colony *ppfor* expression increased in both castes, though in all experiments expression in major workers was still higher than minors. As in the honey bee candidate gene studies, pharmacological manipulations induced behaviour similar to behaviour associated with high expression of *ppfor* (Lucas and Sokolowski 2009).

Candidate gene approach

A candidate gene is a nominated gene, known to have an effect on a phenotype in one or more species, hypothesised to affect a similar phenotype in another organism (Fitzpatrick *et al.* 2005). Decisions on which gene is a suitable candidate are based upon published data from previously studied species, often this involves model species such as Drosophila, though with the recent advances in sequencing technology, the number of species with a completely sequenced genome has increased dramatically. For example, the adaptive colour polymorphisms of the rock pocket mouse *Chaetodipus intermedius* arise from changes in the gene Mc1r, which was first known to affect pigmentation in the mouse *Mus musculus* (Robbins *et al*. 1993, Nachman *et al.* 2003). In the candidate gene approach, once the gene has been chosen, it is cloned, sequenced, measurements of expression (mRNA and protein assays) can be made to ascertain if there is a link with the phenotype. In the case of genes affecting behaviour, the gene expression can be manipulated through various methods, such as RNAi or pharmacological interference, and any changes in behaviour as a consequence of the manipulation can link the gene's function to the behaviour as causative, rather than correlative.

The candidate gene approach is not without drawbacks, with the current level of knowledge about gene sequences it is hard to predict what may be a conserved region to target for cloning, similarly, PCR using primers designed using few sequences from often distantly related species can frequently be unsuccessful. Even once a gene has been cloned sequenced and identified as the target gene, it is possible that the gene plays no discernable role in the trait under investigation. By this point a large amount of time and money has been invested in investigating the gene in question. This is then subject to the "bottom

drawer effect", where research with negative or inconclusive results is not published, thus biasing the knowledge base. For example, if there are several positive studies about a gene influencing a trait, researchers will be inclined to pursue this gene to investigate its involvement in a similar trait in another species. This gene may have been investigated in many other species, but the results from these studies were negative or inconclusive and not published. If the results from all attempts to investigate a candidate gene were published, other researchers might take a more cautious view of the candidate gene approach. In addition to this, the reliance of the candidate gene on a prori knowledge will precipitate several investigations of the same gene in other species at the expense of genes with unknown functions and roles. By focusing attention on single genes there is a danger of overlooking other unknown genes which may play a more central role in the trait under investigation (Zhu and Zhao 2007).

I have attempted to investigate several candidate genes, many of my attempts to clone the genes were unsuccessful, details of the genes and the primers used can be found in appendix i.

Recent developments in molecular biology have led to an increase in understanding of how some eusocial systems are controlled. Some genes have large effects on the behaviour of the insects across species, looking for conserved genes and mechanisms in different eusocial species can suggest common factors that were selected upon to generate the complex social systems we see today. By taking the information generated by eusocial studies about these genes and researching their effects in other insect species with differing degrees of sociality, it is possible to pick out genes, mechanisms and systems that have been conserved over time and adapted to generate the array of social systems we see now.

In this thesis I have focused on two genes. The first is *foraging*, which has been shown to influence a range of social and reproductive behaviours in other insect species (Sokolowski and Hansell 1983, de Belle *et al.* 1989, Osborne *et al.* 1997, Ben-Shahar 2005, Ingram *et al*. 2005, Toth *et al.* 2007, Garabagi *et al.* 2008, Lucas and Sokolowski 2009).The second is inotocin*,* the insect orthologue of oxytocin, the effects of which has not yet been investigated in insects, but in other invertebrates as well as vertebrates it has been shown to influence social behaviour as well as many behaviours associated with reproduction and social bonding (Rubin *et al.* 1983, Fahrbach *et al.* 1984, Numan 1988, Reich 1992, Van Kesteren *et al.* 1992, Oumi *et al.* 1994, Van Kesteren *et al.* 1995, Van Kesteren *et al.* 1996,

Goodson and Bass 2000, Keverne and Curley 2004, Stafflinger *et al.* 2008, Goodson *et al.* 2009, Tobin *et al.* 2010).

The *foraging* gene

The gene *foraging (for)* encodes a guanosine 3′,5′-monophosphate (cGMP) dependant protein kinase (PKG) (Osborne *et al*. 1997). PKGs are a family of serine/threonine protein kinases (Lincoln *et al.* 2001), which activate other enzymes through phosphorylation (Francis and Corbin 1994). The structure, functional domains and mode of action of PKG has been reviewed by Francis *et al* (1994) in depth. PKG is involved in a large number of signalling systems, which have been better characterised in vertebrate systems, than in invertebrate systems (Lohmann *et al.* 1997). Many of the signalling systems that involve PKG are neurological. PKG influences neurotransmission by regulating Ca^{2+} (Lohmann *et al.* 1997). Effects in Knockout mice include diminished vestibule-ocular reflex, enhanced fear and diminished nociception (Aley *et al.* 1998, Lewin and Walters 1999, Schmidtko *et al.* 2003, Schlossmann *et al.* 2005).

The expression and role of the *foraging* gene has been studied in several invertebrate species. All the known behavioural effects are associated with feeding, sociality and parental care. The gene was first identified in *Drosophila melanogaster,* where allelic variation of *for* was found to be responsible for the two naturally occurring behavioural phenotypes rover and sitter (Osborne *et al.* 1997). Subsequent investigation in *Caenorhabditis elegans* showed that *egl-4,* the orthologue to *for*, had similar effects on movement behaviour (Fujiwara *et al*. 2002), *egl-4* has also been found to affect olfactory function, resting behaviour and satiation response (L'Etoile *et al.* 2002, You *et al.* 2008, Ghosh and Emmons 2010, Lee *et al*. 2010). The orthologues of the *foraging* gene have been studied in honey bees, paper wasps, the western corn rootworm and two species of ant. In all of these species the orthologues of the *foraging* gene have been found to influence aspects of food seeking behaviour (Ben-Shahar 2005, Ingram *et al*. 2005, Toth *et al*. 2007, Garabagi *et al*. 2008, Lucas and Sokolowski 2009) and in the social species, some aspects of social behaviour such as nest defence and nursing behaviour (Ben-Shahar 2005, Ingram *et al*. 2005, Lucas and Sokolowski 2009).

foraging* in *Drosophila melanogaster: One of the first, and most complete, studies of *for* is found in *D. melanogaster*. Natural allelic variation in *D. melanogaster* leads to two different foraging strategies in larvae Rover and Sitter (Sokolowski 1980b, Sokolowski and

Hansell 1983, Sokolowski *et al.* 1984). There are two alleles; for^R and for^s. for^R is dominant over for^s, and larvae with one or more copies of for^R have the Rover behavioural phenotype; they cover a larger range when on nutrient media compared to Sitters (homozygous for for^s). There is no difference in general activity (behavioural) levels, only in the foraging strategy (de Belle *et al*. 1989).

The identification of *for* as encoding PKG (Osborne *et al*. 1997) opened up the opportunity to investigate physiological and molecular genetic basis of this gene in *D. melanogaster*. Osborne *et al* (1997) used Northern blot analysis to quantify RNA levels and protein immunoblot analysis and affinity chromatography to quantify amounts of PKG and levels of PKG activity. They found that Rovers have higher PKG activity than Sitters. Following on from this, a causative link between *for* expression and PKG activity and then behaviour was established using mutations of the gene to diminish the gene's function and transgenic strains (Osborne *et al.* 1997). Mutations were created by inserting additional copies of Rover cDNA into a normally Sitter genome. The strains with reduced gene function behaved like the natural Sitter strains, and had reduced PKG activity. The transgenic larvae had higher PKG activity and exhibited behaviour associated with higher *for* expression; behaviour usually observed in Rover flies (Osborne *et al*. 1997).

PKG and *for* expression also influence adult behaviour in *D. melanogaster* (Pereira and Sokolowski 1993). Similar differences in behaviour are seen in adults, though the effects of the allelic differences are mediated by feeding (Pereira and Sokolowski 1993). Allelic variation of *for* in *D. melanogaster* also has an effect on the habituation-like response modification in escape reflex pathways (Engel *et al.* 2000). Mery *et al* (2007) found that the allelic variation of *for* affects olfactory learning in *D. melanogaster.* They tested associative olfactory learning in an assay that tests the ability to associate an odour with mechanical shock. Rover flies that were homozygous for for^R had better short-term but poorer long-term memory than sitters (homozygous for for^S). A mutant strain was also used to determine if the difference was due to *for* or some other factor, the mutant sitter (for^{S2}) has reduce PKG levels and sitter-like behaviour (Osborne *et al.* 1997). The mutant strain was also tested in the same assays as the natural strains, and the for^{S2} strain showed learning behaviour indistinguishable from the natural sitter strain. The for^{S2} and for^R strains are isogenic except for the *for* locus, so the differences in learning and memory can be identified as specific to *for*.

cGMP dependant protein kinase in *Caenorhabditis elegans*: The a cGMP dependant protein kinase orthologue of the *foraging* gene in *C. elegans* is known as *egglaying defective 4* (*egl-4*). This gene regulates multiple developmental and behavioural processes. Mutations in *egl-4* affect the behavioural phenotype of foraging behaviour, in particular the style and duration of movement while feeding (Fujiwara *et al.* 2002). The proportions of time spent Roaming (high speed and low turn rate) and Dwelling (low speed and high turn rate) are different in wild type compared to various *egl-4* mutants. Mutations that decreased PKG signalling led to an increase in time spent performing Roaming behaviour (Fujiwara *et al*. 2002, Tan and Tang 2006). While the gene's involvement in control of the behaviour is consistent, the pattern of expression is opposite that observed for *D. melanogaster*, assuming that Rover and Roaming are homologous behaviours.

There are other effects of *egl-4* in *C. elegans*; long term exposure to an attractive odour leads to *C. elegans* ignoring that odour (L'Etoile *et al*. 2002), this acclimatisation is mediated by *egl-4*, which acts downstream of the primary sensory induction to reduce the response to the odour stimulus (L'Etoile *et al*. 2002, Lee *et al*. 2010). *egl-4* also promotes quiescence by acting downstream to acetylcholine in motor neurones (Ghosh and Emmons 2010), it also is involved in satiation responses, by acting downstream to insulin (You *et al*. 2008).

The range of behaviours and the extent of the influence that *egl-4* has in *C. elegans* suggests that further investigation of the role of the *foraging* gene and its orthologues could reveal a greater range of behaviours affected in other taxa.

The *foraging* gene in *Diabrotica virgifera virgifera* L: The western corn rootworm (*Diabrotica virgifera virgifera L.*) is a pest species in North America, and the larvae cause millions of dollars of damage every year. Larvae of this species are obligate feeders of corn root. Females oviposit in corn fields in the autumn, the eggs diapause over winter and the larvae emerge in the spring just as the new crops are being planted. Since the early 20th century crop rotation has been the main control method because if a non-corn crop is present in spring, the larvae cannot feed. In the early 1990s crop rotation was starting to lose effectiveness, the reduction in effectiveness arose because of the emergence of a new behavioural phenotype in the western corn rootworm. Females had begun laying eggs in non-corn fields, and because those fields would be rotated back to corn in the spring the larvae would have an appropriate food source. The exact conditions that lead to this

behaviour being selected for are not clear, although high population densities, changes in corn phenology and common rotation between corn and soybean may have all been contributing factors.

Part of the emerging problem with western corn rootworm reflects a recently arisen variant associated with changes in PKG expression that circumvents crop rotation (Garabagi *et al*. 2008). Individuals who displayed the variant behaviour had higher levels of the Diabrotica orthologue to *foraging* (*Dvfor*) expression than the normal individuals. This change in expression appears to result in an entirely different oviposition strategy. Normal female adults will lay eggs in the soil of a corn crop, which then incubate over winter and hatch in the spring, where the larvae attack the roots of the new crop of corn. Crop rotation between soybean and corn has worked as a pest control strategy because fields with corn crops one year have soy crops the next, so any larvae emerging from eggs that were laid in a corn field are presented with unsuitable roots (soy). The variant behaviour in adult females results in oviposition in soy fields, which are then rotated to corn in the spring, presenting the larvae with the correct target roots (Garabagi *et al*. 2008).

The expression pattern fits the patterns seen in *D. melanogaster*, where individuals with high expression of the *foraging* gene also have a larger range and travel further, though the western corn rootworm adds the possibility of links with reproductive strategy as well as foraging strategy.

The *foraging* gene in *Apis mellifera*: The honey bee (*Apis mellifera*) is by far the most studied of the eusocial insects with regard to the *foraging* gene. Honey bees have fully developed eusociality, with different age and experienced bees performing different tasks within the hive. The organisation of the division of labour in honey bees is well documented; as the adult worker bee ages it changes primary task, the tasks they perform and the progression between roles is well documented: The first 7-10 days are spent as a Nurse, tending to the brood and the queen, for the next week or so they perform other in-hive tasks and then finally shifting to foraging for the last 1-3 weeks of their life (Ben-Shahar and Robinson 2001).

The switch from in-hive behaviours to out-of-hive behaviours is dependant on a time dependant switch to foraging/out-nest behaviour, and this is linked with a change in expression level of *Amfor*, the honey bee orthologue of the *foraging* gene. Younger bees

that stay in the nest and care/do nest maintenance have lower expression of *Amfor* compared to older bees that have left the nest to forage. This switch is not usually reversible except for in extreme circumstances such as the entire in-nest adult population dying. Further studies on the role and effects of *Amfor* have shown that it is a causative relationship. Positive phototaxis is a predominant feature of the behaviours associated with foraging bees (Menzel and Greggers 1985, Ben-Shahar *et al*. 2003). Pharmacological manipulation using 3-Br-cGMP to increase the activity of the product of *Amfor* induced the positive phototaxis earlier than is usually observed (Ben-Shahar *et al*. 2002, Ben-Shahar *et al*. 2003).

The *foraging* gene in Ants: The role of the *foraging* gene has been investigated in two species of ant *Pogonomyrmex barbatus* and *Pheidole pallidula*.

***Pogonomyrmex barbatus*;** The harvester ant (*Pogonomyrmex barbatus*) is another eusocial insect that shows a correlation between *Pbfor* expression levels and changes in behaviour; however, compared to honey bees, the correlation is reversed. In *P.barbatus* older individuals take foraging and out-nest roles and younger individuals stay within the nest providing care and nest maintenance. However, young ants have higher expression of *Pbfor* than older individuals. Nonetheless, the link between *Pbfor* and changes in social role and behaviour remains (Ingram *et al*. 2005).

***Pheidole pallidula*:** In a different ant species, *Pheidole pallidula*, there are also links between *ppfor* and social/foraging behaviours. In this species there are two types of worker ant, majors and minors. Major ants are larger and their role primarily is to defend the nest, minor ants are smaller and mostly forage. The roles are somewhat flexible, mostly with regard to major ants joining foraging activity if there is a particularly large food source.

There are two differences in PKG activity between the two worker groups (Lucas and Sokolowski 2009). First, major ants have higher PKG activity than minors, second it is also expressed in different patterns in their brains. A foraging stimulus (i.e., a large food source) lowers PKG activity in both castes, and an alien intruder increases PKG activity in both castes. Pharmacological manipulations similar to those in bees demonstrated that it is a causative relationship, as pharmacologically increased PKG activity increased the level of response to intruders and decreased the response to food stimulus (Lucas and Sokolowski 2009).

The *foraging* gene in *Polistes metricus*: The paper wasp *Polistes metricus* is slightly lower on the scale of sociality from the true eusocial insects, with cooperative breeding and care of the young but greater flexibility between castes (Wilson 1971). As such it is often described as primitively eusocial. In *P. metricus* there are four primary castes: queen, foundress, worker and gyne. Foundresses are females that start new colonies in the spring; they perform both reproductive and maternal caring behaviour. Successful foundresses become queens once they have reared the first generation of workers, once queens they only perform reproductive behaviour. The workers take over the caring roles, provisioning the younger broods; the workers show little, if any reproductive behaviour. Gynes are late-season offspring; they show no reproductive or caring behaviour. After mating, the gynes overwinter and become foundresses in the following spring.

Expression studies using 454 (next generation sequencing) and microarrays in each of these castes have shown that foundress and worker brain profiles are more similar to each other than to the other groups. Identifying specific candidate loci to examine reveals specific patterns of expression associated with specific genes. For *Pmforaging*, castes with the highest levels of social interaction and caring behaviour also have the highest levels of expression of *Pmforaging*. (Toth *et al*. 2007, Toth *et al*. 2009)

Oxytocin

Oxytocin and oxytocin-like-hormones are key in the control of various behaviours in both vertebrates and invertebrates (Rubin *et al.* 1983, Fahrbach *et al.* 1984, Numan 1988, Reich 1992, Van Kesteren *et al.* 1992, Oumi *et al.* 1994, Van Kesteren *et al.* 1995, Van Kesteren *et al.* 1996, Goodson and Bass 2000, Keverne and Curley 2004, Stafflinger *et al.* 2008, Goodson *et al.* 2009, Tobin *et al.* 2010). The effects of oxytocin and oxytocin-like-hormones are wide ranging, but the effects fall within the broad category of reproductive and social behaviours.

Oxytocin and vasopressin are nonapeptides; one of the oldest families of neuropeptides (Insel 2010). The structure of vasotocin/vasopressin is well conserved across species, there is some variation at peptides 3 and 7, but there is strong conservation of the amino acid sequences that are proposed to be involved in peptide binding (Goodson and Bass 2001). The nonapeptide lineage is represented in almost every vertebrate taxon, as well as several invertebrate taxa. The peptides vary slightly in form and name, but can be grouped into two types; Arginine vasotocin (arginine vasopressin in mammals) and oxytocin-like

peptides (isotocin in fish, mesotocin in non-eutherian tetrapods, and oxytocin in eutherian mammals) (Insel 2010. The structure of vasotocin/vasopressin is well conserved across all vertebrates, there is some variation at position 3 and position 7, but there is strong conservation of the amino acid sequences that are proposed to be involved in peptide binding (Goodson and Bass 2001). In finches (*Taeniopygia guttata*), mesotocin influences flock size and interference with a mesotocin antagonist reduces social behaviour, such as flock formation (Goodson *et al.* 2009). In the plainfin midshipman (*Porichthys notatus*) grunting is an important aspect of reproductive behaviour. Isotocin influences grunting in females, whereas arginine vasotocin, not isotocin, regulates grunting in males (Goodson and Bass 2000). In the mollusc *Lymnaea stagnalis* lys-conopressin influences male copulatory behaviour through selective expression in neuronal and gonadal cells (Van Kesteren *et al.* 1992, Van Kesteren *et al.* 1995, Van Kesteren *et al.* 1996). In rats, maternal behaviour is initiated after giving birth (Numan 1988) Adult virgin females avoid or attack pups, but when they were injected with oestrogen and oxytocin they developed full maternal behaviour, including nest building and attempting to nurse the pups (Pedersen *et al.* 1982, Rubin *et al.* 1983, Fahrbach *et al*. 1984).

Recently a gene coding for an oxytocin/vasopressin like peptide, christened inotocin, was identified, along with a gene for the receptor, in the genome of the red flour beetle *Tribolium castaneum* (Stafflinger *et al*. 2008). The genes for inotocin and inotocin receptor were also identified in the parasitic wasp *Nasonia vitripennis* but not in any other insects with a completely sequenced genome. However the genes were also identified in *Daphnia pulex*. The big question is does this peptide play a similar role to oxytocin or other oxytocin-like-hormones? If it does, then it would show a remarkable level of conservation of function.

In this thesis

I will investigate the role of two genes on the control of parental care in the burying beetle *Nicrophorus vespilloides*. I will relate this to the existing research on burying beetle behaviour and the wider questions of the evolution and control of social behaviour in insects. I have chosen *N. vespilloides* as a study species because it shows a particularly strong behavioural phenotype, with large changes in behaviour. In addition to this, past research on *N. vespilloides* has indicated a genetic component to their behaviour (Walling *et al.* 2008)

Burying beetle natural history

Nicrophorus vespilloides has unusually highly developed parental care for an insect species (Eggert and Muller 1997). Both parents can provide care and if they are acting in a biparental condition, will co-operate for the duration of breeding. Yet before and after they are particularly anti-social. Outside of breeding situations, adult *N. vespilloides* will kill and eat anything that is small enough for them to take, often soft invertebrates such as slugs and insect larvae, including those of the same species (Eggert and Muller 1997). Adults will mate away from any carcass suitable to breed upon, but same sex beetles will often fight, sometimes to the death (Otronen 1988). When there is a suitable carcass to breed on, a pair of adult beetles will mate and prepare the carcass, often fending off competing adult beetles and other carrion eating invertebrates (Eggert and Muller 1997). Preparation of the carcass takes many hours and requires a lot of activity from whatever adults are present. First, the beetles will move the carcass to a suitable location then remove any fur, scales or feathers. They then bury the carcass in the soil, balling the carcass up and creating a small hollow around it. The female beetle lays her eggs in the soil surrounding the burial site, then returns to the carcass, where both parents will continue to prepare, guard and clean it until the larvae arrive (Scott 1998).

Larvae arrive on the carcass around 50-60 hours after the eggs were laid, upon arrival the larvae locate a hole that the parents have chewed through the skin of the carcass, and parents will start feeding the larvae (Oldekop *et al.* 2007). For the first 24 hours on the carcass the larvae are incapable of feeding themselves, relying entirely on food from the parents. Larvae beg to be fed and the parents respond by regurgitating partially digested food into the larva's mouth (Eggert *et al.* 1998). Although the larvae are only reliant on this level of care for the first 24 hours, they will continue to beg to be fed for several days, though once capable of self-feeding the larvae do this in addition to receiving food from the parents (Eggert *et al.* 1998, Smiseth *et al.* 2003). The larvae take approximately 6 days from arrival on the carcass to mature to the final larval instar (Lock *et al.* 2004). At this point the majority of the carcass has been consumed and the level of care from the parents is waning dramatically. Once the larvae are in their final instar they begin to disperse away from the carcass site to locate a suitable place to pupate, the parents have virtually stopped all care, and over the next 24 hours they revert entirely to their pre-breeding behaviour, eating anything that is small and soft enough to kill, including any of their own offspring who happen to still be in the vicinity (Trumbo 1997, Eggert *et al.* 1998, Scott 1998). Larvae will wander without eating or further growth for one to two weeks, approximately

24h before pupation they bury down into the soil to pupate. After another one to two weeks, they emerge as adults (Lock *et al*. 2004).

The timing and changes in larval behaviour is very closely linked with parental behaviour. The eggs hatch 8-12 hours after parents start accepting larvae (Oldekop *et al*. 2007), the peak of begging behaviour coincides with the peak in direct caring behaviour (Trumbo 1997, Oldekop *et al*. 2007) and the larvae disperse within 24 hours after the parents have left the brood (Trumbo 1991, Jenkins *et al.* 2000, Smiseth *et al.* 2003, Lock *et al.* 2004), maximising the amount of care and protection that the larvae can receive from the parents, whilst avoiding the risk of cannibalism from post-caring parents. This behaviour is tied closely with the parental care cycle, and has been shown to have co-evolved in terms of the timing of peak care and peak begging (Lock *et al.* 2004). It is also possible that some of the same genes are involved in the control of larval behaviour, as studies in *Drosophila* have shown, one gene can influence foraging behaviour in adults and larvae, as well as pre-pupation behaviour (Pereira and Sokolowski 1993, Sameoto and Miller 1968, Ringo and Wood 1983, Sokolowski *et al.* 1984, Sokolowski and Hansell 1983, Wong *et al.* 1985).

The level of parental care exhibited by burying beetles is very unusual in insects, especially outside of the eusocial species. In addition to this, the male aspect of care as seen in the burying beetles is very rare (Eggert and Muller 1997)

Previous work on burying beetles

As described above, there has been a large amount of work studying the natural history of the burying beetles (Eggert and Muller 1997, Scott 1998), however more recently there have been several studies on the controlling mechanisms of the provision of parental care.

Juvenile hormone (JH) titres are strongly associated with the developmental changes and changes in behaviour in insects (Trumbo 1997). In burying beetles JH titres peak upon discovery of a suitable carcass for breeding, and it seems to stimulate ovarian and testicular development, JH also peaks a second time in females around the time that larvae arrive (Scott and Panaitof 2004). However attempts to use methoprene or JH III to simulate high JH titres have proven to affect oviposition and aggression, but not to have an effect on adult parenting behaviour (Trumbo and Robinson 2004, Scott 2006b). As there is no proof

of JH causing parental behaviour, JH could be part of the process of enabling reproduction. JH also plays a role in behaviour seen in larvae; high levels of JH increase the amount of time spent begging. However, high levels of JH also reduced body size in the larvae (Crook *et al.* 2008).

Photoperiod plays a role in the timing of parental care, light burst, prolonged nights and prolonged days can affect when parents switch to caring behaviour relative to carcass discovery/egg laying. However, again, how exactly this works is unclear. Given there is usually such precise timing of the behavioural shifts it seems reasonable to suppose that some circadian or peripheral clock is involved (Oldekop *et al*. 2007).

Walling *et al* (2008) demonstrated that there are genetic components to the parenting behaviour of *N. vespilloides*. Levels of care from uniparental beetles (beetles that have had their partner removed) are heritable. In both males and females, the amount of direct and indirect care given, and the size of the brood were found to be heritable traits. This shows that there is an underlying genetic architecture to parental care in burying beetles. The exact nature and extent of the architecture is unknown, but it is likely to involve many genes in a large network, from direct response to stimuli such as photoperiod, (Oldekop *et al*. 2007) to ovarian/testicular development (Crook *et al.* 2008), to the commencement, continuation and end of parental care as well as the division of time between different parenting tasks (Walling *et al.* 2008).

Burying beetles and understanding evolution of sociality

There are many behavioural studies of insects with various levels of sociality, but the recent developments in molecular biology have allowed new avenues to be pursued. The entire genome of the honey bee has been sequenced, allowing researchers to investigate many questions about the genetic/molecular control of social behaviours, research into the genetic control of behaviour in other eusocial and primitively eusocial species has begun to suggest common mechanisms, and systems that have been selected upon several separate times to produce similar behavioural systems (Robinson and Ben-Shahar 2002, Toth and Robinson 2007, Robinson *et al*. 2008).

The burying beetle provides a useful starting point for research into the molecular/genetic control of behaviour. Compared to many other semi-social species there has been a fairly large amount of research on their behaviour. Most importantly for my research, the recent

work by Walling *et al* (2008) shows that there is a genetic component in the control of parental care in burying beetles.

The common descent of a trait, homology, is the concept that a trait is conserved across species with some derivations from the ancestral trait (Purves *et al.* 2001). In the context of the evolution of sociality the ancestral state is asociality, and as social behaviours start to evolve, the roles of ancestral genes are adapted to create and regulate the new behaviours. Although eusociality has evolved independently several times (Wilson 1971), the same genes may have been adapted to control the social behaviour.
The concept of homology provides a framework to investigate the genetics of a trait based on the knowledge of a gene affecting a similar trait in another species; this is known as the candidate gene approach.

My thesis consists of six chapters. Following this introduction and brief review, chapter 2 examines expression of the burying beetle orthologue to *foraging, Nvfor* in caring and non-caring beetles. Chapter 3 follows up chapter two by providing a pharmacological investigation of the effects of *Nvfor* on caring beetles. Chapter 4 examines the changes in expression of *Nvfor* in larvae throughout development and with association to significant behavioural changes. Chapter 5 investigates the role of the inotocin in the onset of parental care. And finally, chapter 6 provides a summary of my findings and a discussion of the implications of them.

CHAPTER 2

THE *FORAGING* GENE - GENE EXPRESSION IN ADULT BEETLES IN *Nicrophorus vespilloides*

INTRODUCTION

Genetics of parental care

Parental care is a relatively rare trait, despite occurring in a wide range of taxa (Clutton-Brock 1991). Parental care is an important evolutionary innovation as it is one of the first steps along the sociality continuum. In the categories of sociality described by Wilson (1971), subsociality is the first level of care above solitary. Solitary insects have no parent/offspring interactions and no significant interactions between parents beyond mating. Subsocial insects care for their own nymphs/larvae for some period of time. The provision of any level of care is a larger investment of time and resources than simply laying eggs. As parental investment increases, the necessity for cooperation between carers increases, leading to cooperation between siblings (communal and quasisocial), which can lead to the development of reproductive and worker castes (semisocial). The most derived and complex level of sociality is eusociality, which has overlapping generations, reproductive and worker castes, and cooperative care of the young (Wilson 1971).

Among insects, the eusocial species are the most studied with regards to social structure and behaviour, however there are many presocial species with varying types and degrees of social behaviour, particularly parental care. By studying these presocial social species we can gain insight into how social behaviour has evolved. Most studies have focused on selection on various traits associated with social behaviour, demonstrating that these traits are heritable and evolvable. But to fully understand evolution of these traits we need to know about the genetics underlying the behaviours. Recent developments in gene sequencing and analysis technology have rapidly advanced this field and it has become possible to identify specific genes and how the influence behaviour

Using a quantitative genetic approach, Walling *et al* (2008) found that in burying beetles there are genetic components to the extent of care provided by parents. Burying beetles usually cooperate as a breeding pair to raise their brood. The caring workload is shared but each sex specialises in certain tasks. The caring tasks can be divided into two categories; direct care and indirect care. Direct care is the feeding of partially digested carrion to the larvae, indirect care consists of cleaning the carcass and maintaining and guarding the nest (Smiseth *et al*. 2003, Lock *et al*. 2004, Smiseth and Moore 2004a, Smiseth *et al.* 2006, Walling *et al*. 2008). Males tend to provide more indirect care and females provide the majority of direct care. If the female is removed, males adapt their behaviour to provide

more direct care, but if the male is removed females will continue to provide the same proportions of direct and indirect care (Smiseth *et al*. 2006). Walling *et al* (2008) showed that under uniparental conditions, males are more phenotypically variable in their provision of direct care than females, and had a lower mean amount of direct care. There was no difference in variation of indirect care or family size. Within a sex, phenotypic correlations were low, but genetic correlations varied in both strength and direction. Walling *et al*. (2008) interpreted these results as showing that there is an underlying genetic architecture to parental care in burying beetles. This establishes a solid base of evidence to begin investigating the underlying mechanisms of the genetic control of parental care.

Quantitative genetic investigations suggest the involvement of genes in a trait, but direct evidence for the nature of genetic influences is not provided. An alternative, but also complementary approach is to investigate the molecular genetic basis of a trait (Boake *et al.* 2002, Thomas and Klaper 2004). There are a number of potential methods for identifying the molecular basis of a trait, but one that is particularly useful in identifying specific genes underlying variation in behaviour is to hypothesise about and examine candidate genes (Fitzpatrick *et al.* 2005).Therefore, as a compliment to the previous quantitative genetic studies of parental behaviour in this beetle, I decided to do a candidate gene study to investigate genes that influence the expression of parental care in burying beetles.

Selecting candidate genes

Previous studies of honey bee and paper wasp behaviour have shown that many genes have significant differences in expression during different behavioural tasks (Ben-Shahar *et al*. 2002, Ben-Shahar *et al*. 2003, Toth *et al*. 2007). Of these genes one in particular has been shown to play a role in controlling behaviour in multiple insect species across several orders: *foraging.* Homologues of this gene are found across many different taxa, providing a good candidate for a gene to be co-opted for various functions (Fitzpatrick and Sokolowski 2004).

The *Foraging* gene

As discussed in chapter 1, the *foraging* gene plays a role in many behaviours in insects. The *foraging* gene and its orthologues are linked to behavioural changes in several invertebrate species. All the known behavioural effects are associated with feeding, sociality, reproductive strategy and parental care (Sokolowski and Hansell 1983, de Belle

et al. 1989, Osborne *et al.* 1997, Ben-Shahar 2005, Ingram *et al.* 2005, Toth *et al*. 2007, Garabagi *et al.* 2008, Lucas and Sokolowski 2009).

In both adult and larval *Drosophila, for* has been shown to control foraging range, as well as pupation site in larvae (Sokolowski and Hansell 1983, de Belle and Sokolowski 1987, de Belle *et al.* 1989, Osborne *et al*. 1997). In *Caenorhabditis elegans egl-4* (the orthologue of *for*) also controls the foraging range, as well as aspects of olfactory function, resting behaviour and satiation response (Fujiwara *et al.* 2002, L'Etoile *et al.* 2002, You *et al.* 2008, Ghosh and Emmons 2010, Lee *et al.* 2010). In the western corn rootworm *Diabrotica virgifera virgifera* L.*,* allelic variation of *Dvfor* effects oviposition site selection (Garabagi *et al.* 2008). In eusocial species the role of the *foraging* gene has developed to play a role in controlling some of the more elaborate behaviours relating to foraging and reproduction in a social system. Changes in expression levels of the *foraging* gene have been shown to influence behavioural changes in eusocial insects: honey bees, paper wasps and two species of ant (Ben-Shahar *et al*. 2002, Ben-Shahar *et al*. 2003, Ingram *et al.* 2005, Toth *et al.* 2007, Lucas and Sokolowski 2009).

In addition to the evidence of involvement in social behaviour in other insects, the product of the *foraging* gene is easily and reliably manipulated. The *foraging* gene encodes a cGMP dependant protein kinase (PKG), activity of PKG is increased by administering cGMP or a more stable analog such as 8-Br-cGMP (Osborne *et al.* 1997, Ben-Shahar *et al*. 2003).

Orthologues of the *foraging* gene play a role in controlling social behaviour across these 7 invertebrate species, despite many millions of years of evolution separating them. Links with reproductive strategy and changes in foraging behaviour suggest that this gene could also be involved in the control and development of similar behaviour in other invertebrate species.

While it is clear that the various orthologues of the *foraging* gene play a role in the expression of various behaviours, it is important to note that the effect of changes in expression levels or allelic variation resulting in different basal expression levels differs across these species. Some of the expression/behaviour patterns are completely reversed, as in honey bees and harvester ants; the two species have fairly comparable behaviour but

in the honey bee the in-nest workers have low expression levels, whereas in the harvester ant the in-nest workers have high expression levels.

The studies in *D. melanogaster* and *C. elegans* show that there is a link between the *foraging* gene and foraging behaviour (Sokolowski 1980b, Sokolowski and Hansell 1983, Sokolowski *et al.* 1984, Fujiwara *et al*. 2002, L'Etoile *et al*. 2002, You *et al.* 2008, Ghosh and Emmons 2010, Lee *et al.* 2010). The variants of *Diabrotica* shows that *Dvfor* is also involved in foraging behaviour in non-social invertebrates (Garabagi *et al.* 2008) and suggests that the *foraging* gene and its orthologues don't just influence feeding behaviour, but it also has some influence over reproductive behaviour. This involvement is well conserved, which suggests that the function of the *foraging* gene is key to controlling some basic behaviours and has been adapted to control some of the more elaborate behaviours observed in other insect species.

The eusocial species that have been studied show links between the *foraging* gene and food acquisition and provisioning. Honey bees and harvester ants have changes in expression of the *foraging* gene associated with changes in foraging and provisioning behaviour (Ben-Shahar 2005, Ingram *et al.* 2005). In another ant species, *Pheidole pallidula, ppfor* is associated with foraging behaviour and nest defence; in the caste that primarily forages *ppfor* is expressed at a lower level compared to the caste that primarily defends the nest (Lucas and Sokolowski 2009). Levels of *ppfor* dropped in response to foraging opportunities and increased in response to threats to the nest.

Paper wasps have differences in *Pmforaging* expression associated with different castes with different foraging and provisioning roles; castes that perform the majority of caring and foraging roles have higher expression of *Pmforaging* than the other castes (Toth *et al.* 2007).

The *foraging* gene as a candidate gene in the social behaviour of a burying beetle

Given the evidence for a genetic component to parental care in burying beetles, I consider the *N. vespilloides* orthologue for the *foraging* gene (*Nvfor*) to be a good candidate gene for investigation into the genetic control of parental care in burying beetles. Caring behaviour in *N. vespilloides* follows a very predictable timeline, beginning with the discovery of a suitable carcass and a mate (Oldekop *et al*. 2007). Preparation of the carcass

and egg laying is followed by care for the larvae and the breeding round ends with dispersal away from the carcass and larvae (which are also dispersing from the carcass to pupate) and a full reversion to non-caring behaviour (Oldekop *et al*. 2007). The start of caring behaviour occurs 8-12 hours before larvae arrive on the carcass, (Oldekop *et al*. 2007) and under our laboratory conditions (16:8 L;D, 23°C) persists for 5-6 days. The peak in caring behaviour is between 12 and 36 hours after larvae arrive (Smiseth *et al*. 2003).

Direct and indirect care

The care provided by the parents can be divided into two broad categories; direct and indirect care (Walling *et al.* 2008). Direct care is the immediate interaction between parent and offspring; most often this is regurgitating partially digested carrion for the begging larvae. Indirect care describes all the other caring activities such as cleaning the carcass, maintaining the crypt and guarding against predators and competitors (Smiseth *et al*. 2003, Lock *et al*. 2004, Smiseth and Moore 2004a, Smiseth *et al*. 2006, Walling *et al*. 2008).

Males and females

Care in burying beetles can be uniparental (male or female) or biparental within the same species and individuals can switch between breeding attempts (Eggert *et al*. 1998, Muller *et al.* 1998). However, under uniparental conditions, Walling *et al.* (2008) have shown that male and female parents divide their time differently between direct and indirect care. This difference is also seen in biparental situations where the female parent spends more time in direct care, and the male spends more time in indirect care (Smiseth *et al*. 2006). However if one beetle is removed the remaining parent's activities can change to accommodate the missing parent's work. This manifests differently in the sexes, females do not change their behaviour upon removal of the male, but males do change theirs if the female is removed. After the removal of the female, males spend more time in direct care, suggesting that direct care is more important to the success of the brood. This appears to be the case, as broods with shortened durations of care have reduced success (Eggert *et al.* 1998). It is also an important consideration when comparing male and female behaviour and gene expression levels, if males are capable of modulating their behaviour, will their genes be expressed differently than females?

Development of parenting

To make the gene expression assays reliable it was important to ensure that the life history of all the individuals in the experiment were as similar as possible in all respects but the

behavioural states of interest. I selected three points biologically and behaviourally most significant in a breeding cycle: Virgins, individuals at the peak of care and individuals where care had clearly ceased (when larvae and parents were post dispersal from the brood). Ovarian maturation only completes after the discovery of a carcass suitable for breeding (Trumbo and Robinson 2004, Scott *et al.* 2005), and a naïve individual will kill any larvae it encounters (Scott 1990, Trumbo 1990b, a, Scott 1994) . The peak of care represents the largest difference from the non-breeding behaviour. It also coincides with the peak in juvenile hormone (JH) titres 24 hours after larvae arrive on the carcass. The combination of the most dramatic difference in behaviour and the peak of JH titres suggest that there might be other differences in biological molecules (proteins, hormones, gene expression and gene products) at the same time. Post-dispersal behaviour is indistinguishable from the behaviour of virgin beetles, though there are physiological differences, stored sperm and developed ovaries in females (Eggert and Müller 2000, Scott *et al*. 2005), and depleted resources such as fat stores for both sexes. The development of parental care followed by a full reversion to non-caring behaviour is markedly different from the linear progression of behavioural changes that are found in other social species such as honey bees and harvester ants (Ben-Shahar 2005, Ingram *et al*. 2005, Toth *et al*. 2007)

Through this targeted approach I will test the hypothesis that *Nvfor* is linked with the changes in behaviour seen during breeding in burying beetles. In this chapter I test the hypothesis that differences in expression are associated with differences in behaviour.

METHODS

Beetle collection and husbandry

Beetle collection: I collected beetles from Devichoys woods in Cornwall (**OS map number:** 104 **Grid reference:** SW 772 376). Devichoys wood is maintained by the Cornwall wildlife trust and is a semi-natural ancient woodland covering 16 hectares. More details of the woods can be found at: http://www.cornwallwildlifetrust.org.uk/nature_reserves/map/Cornwall_Wildlife_Trust_Devichoys_Wood_nature_reserve_Penryn.htm.

In mid May 2009 I set 21 Japanese beetle traps baited with a ~10g piece of fresh salmon. The traps were hung from branches between 1 and 1.5m above the ground. I added few

centimetres of compost to the bottom of each trap, to avoid stressing the beetles and to avoid deaths before I reclaimed the traps (fig. 1). I left the traps for 7 days. I then collected the traps and any beetles I had caught. I collected 50 beetles in a 54:46 male:female sex ratio. I brought these beetles to the laboratory and removed any mites with forceps and a soft paintbrush, and used these beetles as breeding stock.

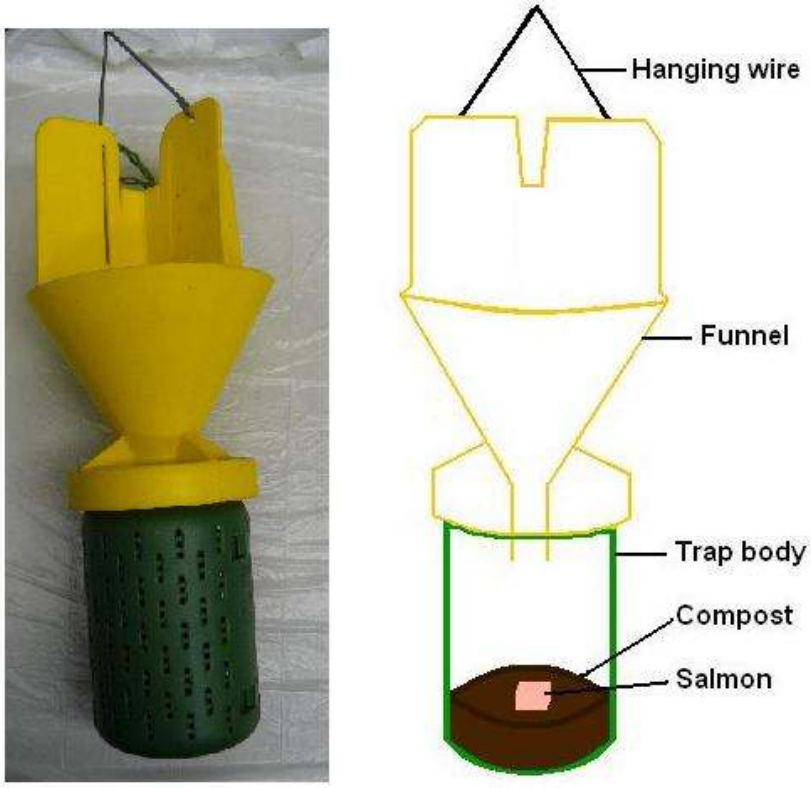

Fig. 1: a Japanese beetle trap and diagram of how the trap was prepared and baited for collection of *N. vespilloides*.

Mite removal: Burying beetles are often carriers of mites from the genus *Poecilochirus*. These mites are not normally harmful (Scott 1998), though under laboratory conditions the population of mites on the beetles can grow to an excessive size, hindering the beetles' movement. I removed these mites using forceps and a soft paintbrush immediately after beetles were brought to the laboratory from the field, and before the beetles bred.

Beetle care: After collection and mite removal the beetles were kept in individual plastic boxes measuring 4cm x 8cm x 8cm two thirds filled with damp compost (Erin multipurpose). The beetles were kept at 23°C on a 16:8 light:dark cycle and fed twice a week with 2 mealworms (livefoodsdirect) cut in half. During feeding I removed any mould

from uneaten food and checked for mites on the beetle. Infested beetles were either cleaned or removed.

Stocks: I considered beetles over 14 days post-eclosion to be of breeding age, and for breeding stock populations I randomly paired non-related, similar aged beetles and placed them in a breeding box (Perspex 11x5.5x17 cm). The box contained 1-2cm of damp compost and a mouse weighing between 18g and 25g.

After the beetles had bred, which occurred very quickly after they were placed on the carcass, I checked for eggs in the soil after 48 hours, and for the appearance of larvae after 72 hours (Oldekop *et al*. 2007). I checked for larval dispersal every morning after 7 days since the start of breeding, and once the larvae had moved away from the carcass I considered them dispersed, I picked each one out and placed them in individual pots measuring 4cm x 8cm x 8cm filled 2/3 with damp compost I labelled each box with the generation and an individual identification code. I returned the parents to their individual boxes and fed them for a week before using them to breed with a new partner.
I checked the dispersed larvae after 7 days for pupation, and after 14 days for eclosion as adult beetles. The population was purposefully outbred throughout the experiments, to avoid any effects of inbreeding.

As life history differences could have a significant impact on the expression of *foraging*. All the adult beetles used in the expression analysis were from the F1 generation; they were all kept in the same controlled temperature room and fed on the same dates.

Experimental design

These experiments were designed to test the following hypotheses:
Ho: Expression of *foraging* will not change with transitions in behaviour associated with parenting.
Ha: Expression of *foraging* will change with transitions in behaviour associated with parenting.

It is hard to predict the nature of the change in expression, because in the species that have been studied so far similar behavioural changes are associated with opposing patterns of expression. This means that the expression of *Nvfor* may start low and increase during care or start high then decrease during care.

To test this I used adult males and adult females in two separate experiments and both under uniparental conditions. In both experiments I defined three behaviourally significant time points (as described below), which correspond to those identified by Simseth *et al* (2003) as periods of no-care, full-care, and a return to no-care. In both experiments individuals at these developmental behavioural stages were collected by picking them up with forceps and placing them in a pre-labelled eppendorf tube, then immediately submerging the tube in liquid nitrogen. The tubes were left in liquid nitrogen for several minutes, and then transferred to a -80°C freezer for storage.

To ensure that the beetles were all the same age upon collection for RNA analysis, the starting times of each of the groups were staggered so the beetles in each group only differed in social behaviour and experience, not in age. The beetles were age matched to 31 days post eclosion, to within 12 hours (fig. 2). In each treatment group n=8

Virgin: virgin beetles were placed in individual breeding boxes with soil but no mouse for 96 hours then collected.

Peak care (female): Virgin beetles were mated three times with a virgin male (this has been shown to be sufficient for maximum sperm transfer and fecundity (House *et al*. 2009), and then placed alone in a breeding box with soil and a mouse carcass weighing 20±2g. They were left for 72 hours and then checked for larvae arriving on the carcass. Beetles were collected 18-24 hours after larvae arrived, the beetles were taken only during visible and active direct care, i.e. beetles were collected whilst they were feeding larvae.

Peak care (male): Virgin beetles were mated three times with a virgin female, and then both beetles were placed in a breeding box with soil and a mouse carcass weighing 20 ± 2g. They were left for 48 hours, and then the female was removed from the breeding box. After an additional 24 hours the carcasses were checked for larvae. Beetles were collected 24-36 hours after larvae arrived, the beetles were taken only during visible and active care; i.e., beetles were collected whilst they were feeding larvae

Post-care: Beetles were mated as in the peak care group and left for an additional 92 hours until the larvae had dispersed. The beetles were collected 18-24 hours after the larvae had dispersed. Care typically ceases after 74-92 h in this species (Smiseth *et al*. 2003).

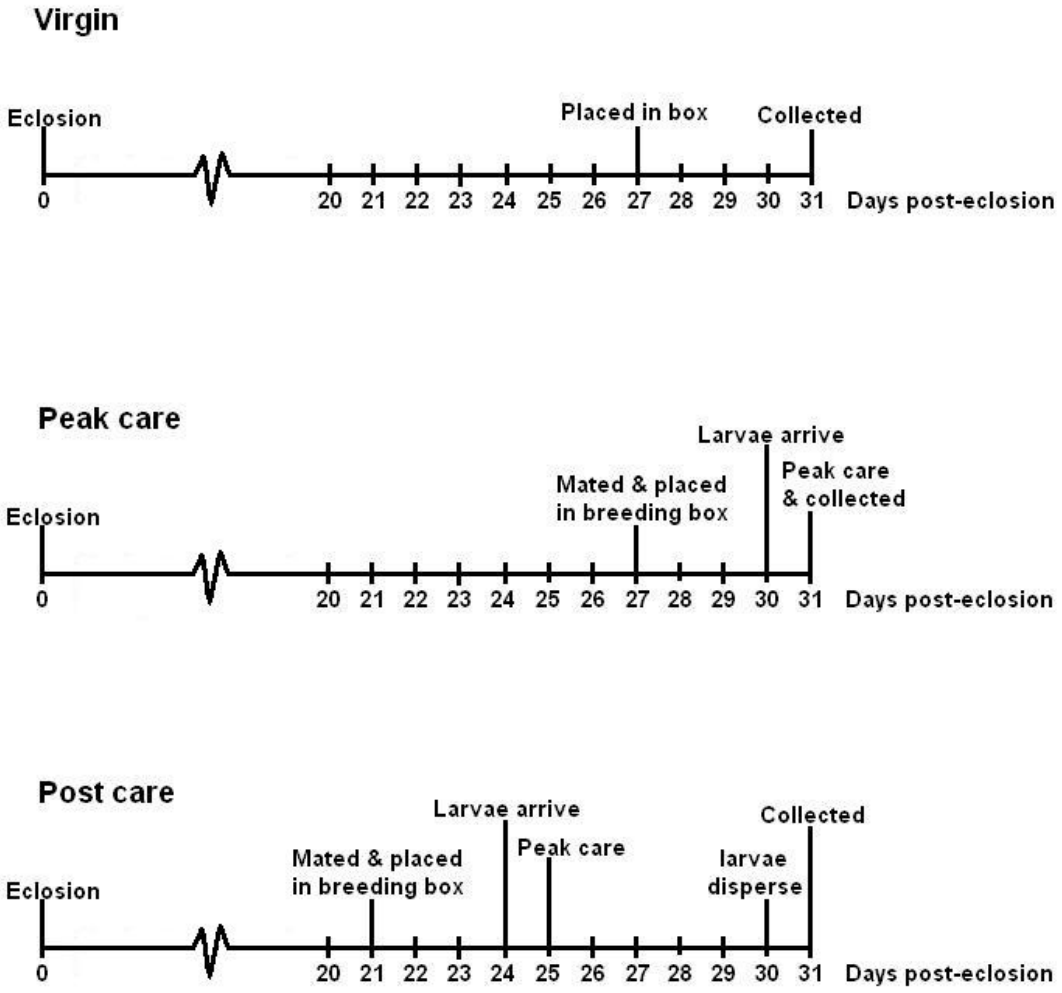

Fig. 2. Timing of mating and collection of the treatment groups to ensure a matched final age

RNA extraction, cleanup and quality

RNA extraction: I snapped the heads off the ultra frozen beetle, and placed the head in a clean 1.5ml reaction tube. I used TRIzol Plus RNA purification system (Invitrogen 12183-555) using the standard protocol to extract total RNA from the tissues. The extraction process uses a proprietary reagent (TRIzol) containing phenol and guanidine isothiocyanate, a chaotropic salt which protects the RNA from endogenous RNases (Chirgwin *et al*. 1979). After homogenising the frozen heads in the TRIzol reagent, I left the mixture to incubate for 5 minutes and then added chloroform and centrifuged the mixture to produce an aqueous phase containing the RNA and an organic phase containing the phenol. I removed the aqueous phase to a new reaction tube and added 70% ethanol, this mixture was then loaded onto a Spin Cartridge containing a silica-based membrane to which the RNA binds. A series of washes with ethanol and RNase free buffers removed any contaminating tissue, protein, lipids, salts etc (Vogelstein and Gillespie, 1979). After

washing was completed I eluted the RNA from the spin cartridge membrane with RNase free water.

Cleanup: I used DNase 1, amplification grade (Invitrogen 18068-015) to remove any DNA contamination from the samples. I used the standard protocol, where the DNase 1 is mixed with the sample and a reaction buffer, incubated at room temperature for 15 minutes. The DNase 1 was Deactivated by adding EDTA and heating the mixture to 65°C for 10 minutes.
I followed the DNase 1 treatment with a cleanup to remove any remaining solvent contamination. For the cleanup, I used the Illustra RNAspin Mini Kit (Qiagen 25-0500-70) using the standard protocol. The RNA is bound to a spin column and washed with a series of ethanol and RNase free buffers, I dried the sample through centrifugation and then eluted the RNA sample with RNase free water.

Quality and Quantity:
To check the quality of the extracted and cleaned RNA I used formaldehyde denaturing gel electrophoresis, in which the RNA sample is mixed with a loading dye and drawn through an agarose gel containing MOPS (3-(N-morpholino)propanesulfonic acid) and formaldehyde through electrophoresis. I examined the gels under ultraviolet light, clear bands indicate high quality and low contamination, smears indicate poor quality, degraded RNA or contamination of the sample.
I checked the quantity and purity of the cleaned RNA samples using the Nanovue (GE Healthcare), I ensured that every sample had A260/A280 and A260/A230 values of over 1.8. The Nanovue measures light absorbance at specific frequencies, by comparing the level of absorbance of the sample to those of pure solvent (in this case RNase free water) it is possible to calculate the concentration of various solutes in the sample. RNA absorbs ultraviolet light at 260 and 280 nm, proteins absorb ultraviolet light at 280 nm. The ratio of absorbance at 260nm to the absorbance at 280nm indicates the level of contamination by proteins, a ratio of 1.8 or higher is considered relatively free of protein contamination. Similarly, organic compounds absorb light at 230nm, so the ratio of absorbance at 260 nm to absorbance at 230 nm can indicate organic contamination. A pure sample of RNA has an A260/A230 ratio of 2 or more, a ratio of 1.8 is considered sufficiently clean.

Primer design

Primers were designed based on the partial sequence for *Nvfor* supplied by Prof. Ritchie's research group in St Andrews. I used the Primer select program from Lasergene. The sequence for the *Nvfor* primers was:

3' ATGCTGGAGGCGTGTCTGGAG 5'

5' GCTATTCTTGTAAACGCACGA 3'

These primers amplified a 100bp region of the partial sequence (fig 3).

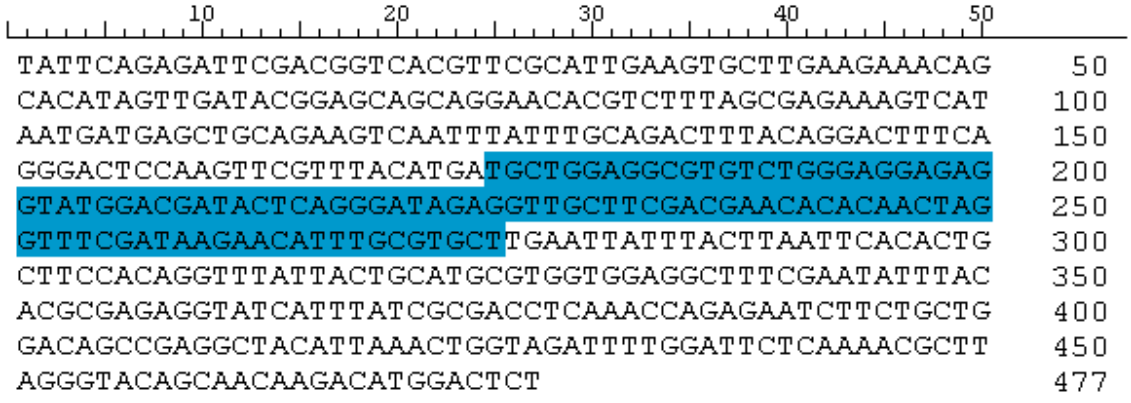

Fig 3. partial sequence of *Nvfor*, the section highlighted in blue is the region amplified by the *Nvfor* primers.

The primers for the 18S control were the QuantumRNA™ Universal 18S Internal Standard (Applied Biosystems).

Quantitative Real-Time PCR

For all the QRTPCR analyses I used QuantiTect SYBR Green RT-PCR Kit (Qiagen 204245) and the Mx3000 Real-Time PCR System (Stratagene). I tested the optimal mg^{2+} concentration and annealing temperature by using concentration and temperature gradients and found that a final concentration of 2.5mM and an annealing temperature of 50°C for 30 seconds were the optimal for efficiency and repeatability.

I ran each plate with standard curves and no template and no reverse transcriptase controls for both *foraging* and 18S to check for DNA and RNA contamination. This provided data for four samples per plate

Analysis

The data were transformed to R values, where R indicates relative level of expression of *Nvfor* compared to 18S, using the method developed by Pfaffl *et al* (2001):

$$R=\frac{E(target)^{\Delta CP(target)}}{E(reference)^{\Delta CP(reference)}}$$

$$E=10^{[-1/slope]}$$

$$\Delta CP=CP(control)-CP(sample)$$

Target is the gene of interest, in this case *Nvfor*.
Reference is the reference gene, in this case the 18S control.
Control is the control template RNA, a pool of all samples being analysed.
Sample is the specific sample of RNA being analysed
Slope is the slope of the regression line of the standard curve.

After calculating the R values for each sample, I analysed the data using a a two-way ANOVA with sex and treatment as factors, and testing the interaction between them.

RESULTS

In this experiment I found that the pattern of expression was not different between females (fig 4) and males (fig 5). There was no significant interaction between sex and stage of care ($F_{(2,42)}$ =0.20, P=0.8165). There was a significant increase in *Nvfor* expression with the expression of care Level of expression was low in virgins, increased significantly in individuals showing peak care, and returned to its low level after care had ceased ($F_{(2,42)}$ =0.20, P<0.0001). However, there was a significant difference in relative expression between males and females, male expression was consistently higher than female ($F_{(2,42)}$ =5.86, P=0.0199).

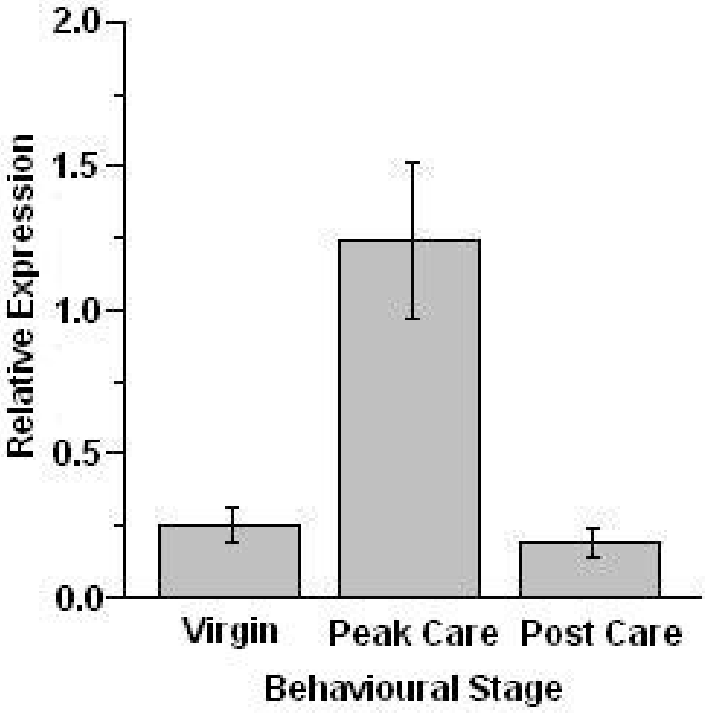

Fig. 4. Relative expression of *Nvfor* in three caring stages of female *N. vespilloides* N=8 in each group. Error bars show ± 1SE.

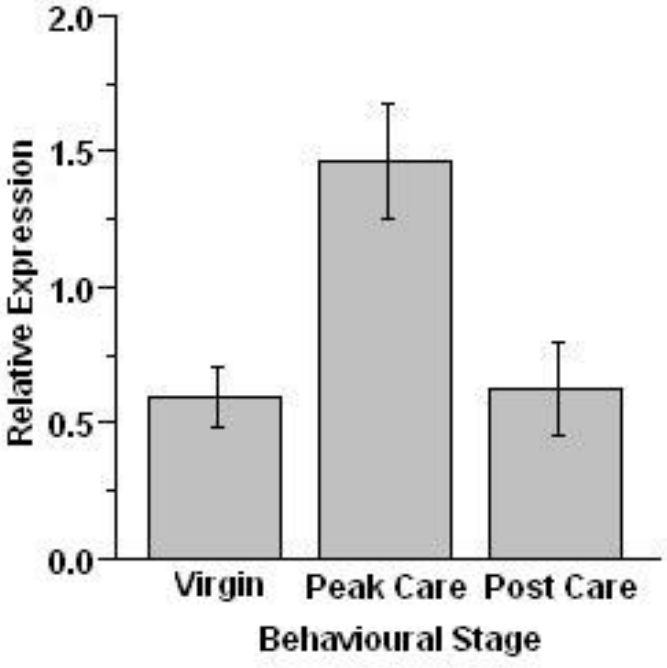

Fig. 5. Relative expression of *Nvfor* in three caring stages of male *N. vespilloides* N=8 in each group. Error bars show ± 1SE.

DISCUSSION

I have found that the expression patterns of *Nvfor* were dramatically different when different behaviours were being expressed. Relative expression of *Nvfor* was higher in males than females. However, there were no differences in the pattern of expression between males and females. The different relative expression levels between males and females may be due to the QRTPCR analyses being done at different times, and small sample sizes.

In both males and females, the peak of care had the highest expression of *Nvfor*, while virgins and post-care individuals had lower levels that are indistinguishable from each other. This is consistent with research of the *foraging* gene in other insect species, though the current research is focused on species with changes in behaviour related to development and aging. The reversal in *Nvfor* expression that I found shows that these changes in expression level in the burying beetles are not just a developmental transition, but linked with reversible behavioural changes. The discussion below compares these results to expression differences in orthologues of the *foraging* gene found in other organisms.

The role of the *foraging* gene in other species

Drosophila melanogaster* and *Caenorhabditis elegans: In both adult and larvae *D. melanogaster*, individuals with the Rover genotype are more active foragers and they cover a larger area than individuals with the Sitter genotype. Rovers have higher PKG activity than sitters (de Belle *et al*. 1989, Osborne *et al*. 1997, Engel *et al.* 2000). The opposite pattern is seen in *C. elegans*; low PKG activity increases the amount of time roaming and reduces the amount of time dwelling. Nonetheless the involvement of orthologues of the *foraging* gene in the control of these types of behaviours is persistent (Fujiwara *et al*. 2002, Tan and Tang 2006). In burying beetles, the pattern of gene expression levels and behaviour seems to match the patterns found in *C. elegans*; when the beetles are pre and post breeding they cover large distances in search of food, mates and appropriate resources for breeding, and during this time *Nvfor* expression is low.

Diabrotica virgifera virgifera: The variant behaviour in *Diabrotica virgifera virgifera* is also due to allelic variation, individuals with the variant allele have a very different oviposition strategy to those with the normal genotype. This emergent behaviour seems to

be a result of mutation in the *foraging* gene orthologue (*Dvfor1*), which results in higher expression of *Dvfor1* (Garabagi *et al*. 2008).

It is clear that *Dvfor1* plays a role in controlling some aspect of reproductive strategy in in *D. virgifera virgifera*. The egg laying strategy in *D. virgifera virgifera* is linked to providing suitable food resources for their larvae. The feeding and direct care in the burying beetles is also focused on providing food to the larvae, so perhaps there are more shared mechanisms of control in the two species, where the burying beetles have been selected on to produce a more proximate provisioning strategy. Likely candidates for important genes in the two beetle species include genes associated with olfactory function; in *Diabrotica* the variant type beetles are attracted to soy perhaps because the presence of soy indicates that corn will be present in the spring. There are increased numbers of *Diabrotica* adults in soy crops in areas with the variant genes are present, suggesting that variant adults have lost their preference for corn (Rondon and Gray 2003, 2004) and suggesting that genes involved in recognising food are affected by the differences in *Dvfor* expression (Garabagi *et al*. 2008). In burying beetles, the adults need to find an appropriate carcass to breed on, which obviously relies on having a sensitive olfactory system. Burying beetles also recognise their breeding partner through a "breeders' badge" of cuticular hydrocarbons (Muller *et al*. 2003, Steiger *et al*. 2007). The transition from cannibalising larvae to caring for them suggests the involvement of genes involved in food recognition, as the perception of larvae changes from "food" to "not food" at the commencement of caring behaviour, and back to "food" at the end of care. Indeed it may be the case that the *foraging* gene is involved in mediating olfactory function and satiation pathways, as this has been found to be the case in *C. elegans*. *egl-4* acts in olfactory pathways to reduce the response to a long-term odour stimulus (Lee *et al*. 2010), and influences satiation responses by acting downstream to insulin (You *et al*. 2008). These may be roles of the *foraging* gene in insects that have been adapted as part of the evolution of parental care and social behaviour.

Apis mellifera: In the honey bee, *Amfor* plays a role in the changes between two broad behavioural categories, in-nest workers and out-of nest workers. The organisation of the division of labour in honey bees is well documented; as the adult worker bee ages it changes principal task, the tasks they perform and the progression between roles are well documented: The first 7-10 days are spent as a nurse, tending to the brood (including direct care involving regurgitation) and the queen, for the next week or so they perform other in-

hive tasks (cleaning, undertaking, temperature regulation, and guarding) and then finally shifting to foraging for the last 1-3 weeks of their life (Ben-Shahar *et al*. 2002). In-nest worker bees are younger and have low expression of *Amfor*, and corresponding low levels of PKG activity. Out of nest workers are older bees, which have higher expression of *Amfor* and higher levels of PKG activity. The in-nest workers have several roles within the nest; they act as nurses to the larvae and as nest cleaners. (Ben-Shahar *et al*. 2002, Ben-Shahar *et al*. 2003). This has obvious parallels with the caring behaviour shown by burying beetles, where the parents feed larvae and clean the carcass and generally maintain it as a nest, but the expression pattern found in this study is the opposite of those seen in honey bee workers.

Pogonomyrmex barbatus: The harvester ant has similar division of tasks between workers as the honey bee, but the expression patterns and PKG activity in the workers is the opposite to what is seen in bees. That is, young in-nest workers have high levels of *Pbfor* expression, and older out of nest workers have lower levels of *Pbfor* expression (Ingram *et al*. 2005). This matches what I found in the burying beetles; individuals that are caring for young and maintain the nest have higher expression of *Pbfor* than the individuals that are searching for food and not exhibiting caring behaviour. Thus, the behavioural changes parallel those of honey bees, but the pattern of expression is opposite. Harvester ant expression patterns match those I found in *N. vespilloides*, but the behavioural patterns are not as similar. This suggests that there may be some plasticity in the function of PKG, if it is part of a general pathway it can be co-opted to influence many different behavioural transitions.

Pheidole pallidula: This ant species was studied with regards to the behaviour of two different castes of workers: major and minors. Major workers' primary role is defence of the nest; they are larger and more aggressive than minor workers, whose role is primarily foraging. There is some overlap in activity between the castes if it is required (e.g., a particularly large food resource, or a large threat to the nest). Major workers have higher levels of *Ppfor* expression and PKG activity compared to minor workers. In both castes, PKG activity increases in response to an intruder and decreases in response to a food source (Lucas and Sokolowski 2009). This may relate well to burying beetle expression/behaviour patterns, caring beetles frequently have to fight off competitors and scavengers on the carcass, so an increase in *Nvfor* expression may be as a preparation for a partially defensive role. However, it is also possible to consider the carcass as a food

resource, which in the ants induces a drop in PKG activity. Given that burying beetles don't attempt to breed on every resource they find (Eggert *et al*. 1998, Scott 1998) it is reasonable to assume there is a limit to the size or quality of a food item that below it induces one response (feed) and above it induces a second (breed). The difference between the genetic control of these two responses would be an interesting avenue to pursue; that the beetles assess what is a suitable resource for breeding and some of the physiological responses have been investigated (Trumbo *et al.* 1995), but the differences in gene expression have not.

Polistes metricus: The paper wasp has ecology that is difficult to relate to the burying beetles; foundresses are capable of becoming queens, but not all foundresses do. Foundresses care for the young alongside workers, and gynes become foundresses after over-wintering and dispersing to a new nest site. However, broadly speaking, the individuals involved in care of the brood and food collection have higher expression of *Pmforaging* than the individuals that only have a reproductive role (Toth *et al*. 2007, Toth *et al*. 2009). The correlation between caring behaviour and high expression of *Pmforaging* is similar to the pattern found in burying beetles.

Prior burying beetle work

The work by Walling *et al* (2008) showed that there are genetic components to parental care in burying beetles, the differences in the strength and direction of the genetic correlations suggests that there are multiple genes involved in the control of parental care. However, Walling *et al*. (2008) also show strong genetic correlations between male and female parental care behaviours, I have demonstrated that the same pattern of changes in gene expression of *Nvfor* occurs in both males and females. In this study I have demonstrated that one of the genes involved in social and parental behaviour in other insect species is also linked to the changes in behaviour seen during burying beetle parental care. There is allelic variation in this gene in other species, including another beetle; further investigations into this gene, such as completing the sequence and identifying any alternative splicing or allelic variation of *Nvfor* in burying beetles as a source of some of the genetic variation found by Walling *et al* (2008).

Conclusion

It is clear that the expression levels of *Nvfor* change with the onset of parenting behaviour, and there is no real difference between the patterns of expression seen in males and

females. This may be induced in part by using uniparental beetles to obtain the tissue samples. It isn't unexpected to have found similar expression levels between the sexes under uniparental conditions, since their behaviour is very similar when no partner is present (Smiseth and Moore 2007) and there are shared genetic influences on male and female parenting behaviour (Walling *et al*. 2008).

This study adds to the body of work showing that the same gene is involved in the provision of care in several species of insects, and given that the social behaviour has evolved independently several times (Wilson 1971), suggests that the adaptation of the function of the *foraging* gene has also evolved several times. The research from non-social species suggests that the *foraging* gene is involved in pathways that are associated with food, particularly olfactory function, hunger recognition and foraging strategy (Sokolowski 1980b, Sokolowski *et al*. 1984, de Belle *et al.* 1989, Osborne *et al*. 1997, Fujiwara *et al*. 2002, L'Etoile *et al*. 2002, Garabagi *et al*. 2008, You *et al*. 2008, Ghosh and Emmons 2010, Lee *et al*. 2010). In the eusocial species that have been studied, the *foraging* gene seems to play a role in food-linked social behaviours, that is, foraging and provisioning of food to the young. In Burying beetles the switch in behaviour of cannibalism to care and back to cannibalism suggests that food-recognition may be a key component of the transitions between caring and non-caring.

The more recent research on the role of the *foraging* gene in insects therefore suggests that the results of older research can be interpreted in a slightly different light. The different behaviours determined by the two *for* alleles, for^S and for^R in *D. melanogaster* have been interpreted as different foraging strategies; rovers cover more area and move more than sitters, who cover fairly limited distances. However, individuals that do not move about much are also more likely to be in closer proximity to other individuals. Individuals that are heterozygous for^S also have a faster decrement in the startle reflex mediated by the giant fibre pathway, faster habituation to visual stimuli will also allow other individuals to be in closer proximity before any escape response is triggered (Engel *et al*. 2000). The close proximity to others means that there is possibly a higher probability of mating opportunities but also requires a greater tolerance of proximity to competitors. This could be seen as a very limited form of social behaviour, because in order to evolve further social systems, first there must be opportunities for social interactions.

The *foraging* gene has been shown to be associated with or influence many different types of social and parenting behaviour in several insect species (Ben-Shahar 2005, Ingram *et al.* 2005, Garabagi *et al.* 2008, Toth *et al.* 2007, Lucas and Sokolowski 2009), making it an ideal candidate gene for further investigation. Furthermore, the apparent association of *Nvfor* expression and parental care in burying beetles warrants further investigation to test whether this is a causal relationship (Eggert *et al.* 1998, Scott 1998).

CHAPTER 3

PHARMACOLOGICAL MANIPULATION OF cGMP – EFFECTS ON PARENTAL CARE IN *Nicrophorus vespilloides*

INTRODUCTION

The *foraging* gene is associated with changes in social behaviour

My previous work with *Nvfor* has shown it to be associated with the changes in parental care in *N. vespilloides* (chapter 2). Expression of *Nvfor* is significantly increased when the parent is caring for young, compared to before or after breeding. One of the advantages of using Quantitative Real Time PCR (QRTPCR) to measure gene expression levels is a number of controls are used to show that any results are genuine differences. By comparing the levels of the gene of interest to a control gene, all the readings from the samples are internally consistent. Another advantage is that it is relatively easy to replicate the experiment. Despite the advantages of QRTPCR the results that are generated are correlative, and although in this case it is a strong correlation, it is important to test whether increased levels of *Nvfor* cause the change in behaviour or if it is purely a correlation.

In several of the species studied with regards to *foraging* gene expression and PKG activity, there have been several experiments that have shown that the relationship is causal. To demonstrate causation it is necessary to manipulate the expression of the gene, or the activity of the gene product and then observe the impact of the manipulation on the behaviour of the animal.

Mutations

There are sufficient genetic information and tools available for *D. melanogaster* and *C. elegans* that it was possible to perform targeted mutations to alter the function of the gene and then observe changes in behaviour.

Drosophila melanogaster: Natural allelic variation in *D. melanogaster* leads to two different foraging strategies in larvae; Rover and Sitter. Rover is dominant over Sitter, and larvae with one or more copies of Rover cover a larger range when on nutrient media compared to homozygous Sitters. There is no difference in general activity, only in the foraging strategy (Sokolowski and Hansell 1983, Sokolowski 1985, de Belle *et al.* 1989, Osborne *et al.* 1997). Several experiments manipulating the gene have shown that the difference between the naturally occurring alleles, for^{s} and for^{R} is in the level of expression of the enzyme PKG. Strains with mutations induced on a for^{R}/for^{R} background had reduced levels of PKG expression and the same behaviour as naturally occurring Sitters

(*for*^{s} */ for*^{s}*)*. Similarly, sitter strains with induced overexpression of the gene had increased PKG activity and Rover type behaviour (Osborne *et al.* 1997).

Caenorhabditis elegans: The gene *egl-4* (*egglaying defective 4*) regulates several developmental and behavioural processes; this gene is an orthologue to *for*. *egl-4* also plays a role in controlling the response to long term exposure to an attractive odour, which leads to *C. elegans* ignoring that odour. *egl-4* moves into the nucleus and acts downstream of the primary sensory induction to reduce the response to the odour stimulus (L'Etoile *et al.* 2002, Lee *et al.* 2010). *egl-4* also promotes quiescence by acting downstream to acetylcholine in motor neurones (Ghosh and Emmons 2010), it also is involved in satiation responses, by acting downstream to insulin (You 2008). *egl-4* also plays a role in controlling foraging behaviour, the proportions of time spent roaming (high speed and low turn rate) and dwelling (low speed and high turn rate) were changed in mutants with reduced PKG signalling. Mutations that reduced PKG signalling increased the amount of time spent roaming and decreased the amount of time spent dwelling (Fujiwara *et al.* 2002).

Drugs

Manipulating genes using drugs is a fairly simple protocol, though it is really a manipulation of the gene product, rather than the gene itself. Despite the intervention having an effect further downstream from the gene, these manipulations establish whether the gene of interest has a causative relationship on the behaviour being studied. When the target gene has a known product, which has a known proximate function, there are often drugs that can interfere with the function of the gene product. In the case of the *foraging* gene, the gene product has been identified as an enzyme: cGMP dependant protein kinase (PKG). Experiments in honey bees (Ben-Shahar *et al.* 2003)and *P. pallidula* (Lucas and Sokolowski 2009) have shown that increased levels of cGMP within the animal induce higher levels of PKG activity and mimic the effects of increased expression of the *foraging* gene*,* and thus inducing any behaviour that is influenced by increased expression of the *foraging* gene

PKGs are a family of serine/threonine protein kinases (Lincoln *et al.* 2001), which activate other enzymes through phosphorylation (Francis and Corbin 1994). The structure, functional domains and mode of action of PKG has been reviewed in depth by Francis and Corbin (1994). PKG is involved in a large number of signalling systems, which have been better characterised in vertebrate systems than in invertebrate systems (Lohmann *et al.*

1997). A large number of the signalling systems that involve PKG are neurological, PKG influences neurotransmission by regulating Ca^{2+} (Lohmann *et al*. 1997). Effects in knockout mice include diminished vestibule-ocular reflex, enhanced fear and diminished nociception (Aley *et al.* 1998, Lewin and Walters 1999, Schmidtko *et al.* 2003, Schlossmann *et al.* 2005).

cGMP increases PKG activity and the associated behaviours in honey bees: Newly eclosed honey bees were fed sugar solution containing 8-Br-cGMP (a membrane permeable analogue that is relatively resistant to degradative phosphodiesterases), the bees in the control groups were fed just sugar water or sugar water mixed with 8-Br-cAMP. The bees in the cGMP treated group had elevated levels of PKG activity, similar to those seen in untreated foraging bees, bees in the cAMP group had elevated levels of PKA activity, but there was no effect on PKG activity, and bees in the sugar water control group didn't show increased levels of PKG or PKA activity. The bees treated with cGMP displayed a significant increase in precocious foraging activity, the PKA control group showed no significant change in behaviour. Usually the transition to foraging behaviour comes much later in life, so it was concluded that treatment with cGMP increases PKG activity, which in turn increases/induces foraging behaviour (Ben-Shahar *et al*. 2002, Ben-Shahar *et al.* 2003).

cGMP increases PKG activity and the associated behaviours in ants: In the ant *Pheidole pallidula,* there is a strong link between PKG activity and defensive or foraging behaviour. Worker ants are divided into two castes, majors are larger and mostly have a defensive role, are smaller and are predominantly foragers. Major ants have higher PKG activity than minor ants and a different pattern of PKG activity in the brain. Pharmacological manipulation with cGMP reduced foraging activity response to a new food source in both castes. cGMP treatment increased the defensive response to intruders in the major ants, but not in the minors (Lucas and Sokolowski 2009).

Due to the dearth of genetic tools available in *N. vespilloides*, I will investigate whether treatment with cGMP has an effect on parenting behaviour in burying beetles. I will use a similar protocol to the one developed by Ben-shahar *et al* (2003) in honey bees and used by Lucas and Sokolowski (2009) in *P. pallidula*. It is not suitable to feed the beetles cGMP; due to the pre- breeding ecology of the adults and the fact that during breeding they regurgitate food to the larvae, it would be impossible to know whether they had received

any of the intended dose, and whether they had passed any of the cGMP on to the larvae, as any change in larval behaviour could induce different behaviour from the parents (Smiseth *et al.* 2003, Suzuki 2004, Smiseth *et al.* 2007a, Smiseth and Moore 2007, Smiseth *et al.* 2007b)

METHODS

Beetle collection, husbandry and stock breeding were the same as described in chapter 2 (Methods: page 36).

Experimental beetles

All the female beetles used in this experiment were from the F1 and F2 generations. All the male beetles used in this experiment were from the F3 and F4 generations; they were all kept in the same conditions, as described in chapter 2 (Methods: page 36).
All beetles used in this experiment were 21 days post eclosion at the start of the experiment

Experimental design

The aim of these experiments was to test the hypothesis:
Ho: increasing the endogenous levels of cGMP will have no effect on the amount of care given.
Ha: increasing the endogenous levels of cGMP will increase the amount of care given.

Increasing levels of cGMP will result in increased behaviour associated with high *Nvfor* expression. Based on the results of the gene expression experiment in chapter 2, I expect that treatment with cGMP will result in an increase in caring behaviour.

I used four treatment groups:
Handling control: The beetle was picked up and held for 30 seconds, then put back onto the brood.

Ringers: The beetle was picked up and injected with 30µl of Ringers buffer solution (table 1) then put back onto the brood.

cGMP: The beetle was picked up and injected with 30µl of 8-Br cGMP (Sigma) in Ringers buffer solution (table 1) (0.5µg/µl total dose=15µg), then put back onto the brood. This dosage was calculated from the dosage used by Ben-Shahar *et al* (2003), using an estimate of volume eaten and adjusted for body size.

cAMP: The beetle was picked up and injected with 30µl of 8-Br-cAMP (Sigma) in Ringers buffer solution (table 1) (0.5µg/µl total dose=15µg), then replaced onto the brood. This dosage was calculated from the dosage used Ben-Shahar *et al* (2003) using an estimate of volume eaten and adjusted for body size.

Table 1: ingredients for beetle Ringers buffer (Holtzhausen and Nicolson 2007).

name	concentration (mmol)
NaCl	90
KCl	50
$CaCl_2$	2
$MgCl_2$	5
$NaHCO_3$	6
NaH_2PO_4	4

Injections: I held the beetle still with the head pushed down slightly, I used a 30 gauge (0.3mm) needle, which I inserted under the pronotum through the soft membrane of the joint (fig. 1). Preliminary studies showed this method of injection to have the lowest mortality (3%) and no apparent detrimental effects on the beetles' health or behaviour. I injected 30µl of liquid per injection/individual. The needle was removed and discarded between beetles.

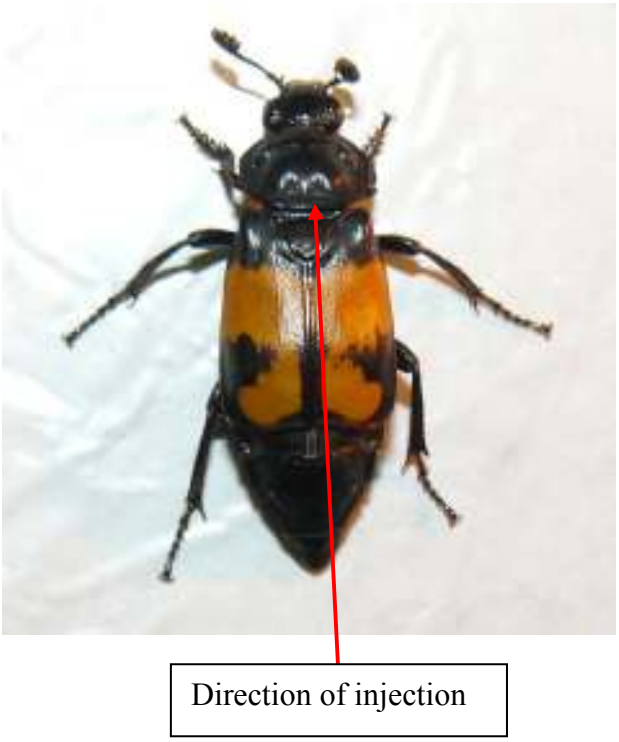

Fig. 1: injection site in *N. vespilloides*.

These four treatment groups give controls for several important issues. The handling only group controls for any effects of disturbing the beetle, as a large amount of disturbance might induce abandonment or abortion of the brood. Injecting the Ringers buffer controls for any effect of the injection, as the injection site might allow infection, or the introduction of a relatively large volume of liquid could cause metabolic or water-control issues. The cAMP in Ringers buffer is to control for any effect of a general increase in activity as a result of the increase in an important bio-signalling molecule, cAMP has been shown not to affect behaviours linked with *Amfor* expression in honey bees (Ben-Shahar *et al*. 2002, Ben-Shahar *et al*. 2003). By comparing these controls to the experimental group of cGMP in Ringers buffer I will be able to find any effect of increased cGMP whilst accounting for confounding factors of the method of treatment. I also randomly assigned the beetles to these four groups to remove any bias that could be introduced in selecting beetles for treatment.

I placed virgin pairs of age matched and unrelated beetles in a breeding box (Perspex 11x5.5x17 cm) to generate experimental beetles for the treatments above. The box contained 1-2cm of damp compost and a mouse weighing between 18g and 25g. I checked for eggs in the soil after 48 hours, and for the appearance of larvae after 72 hours. Once larvae had arrived I removed the non-experimental beetle and treated the experimental

beetle according to the assigned treatment group (above). I then left the beetle with the brood of larvae overnight.

I observed the beetles' behaviour for 20 minutes three times per day for four days.
I scored behaviour in 3 pre-defined categories to ensure that the observations were as objective as possible.

Direct care: Direct feeding of the larvae. The beetle was in or on the larvae inside the carcass. The beetle was seen responding to begging behaviour from the larvae.

Indirect care: The beetle was on the carcass but not with or responding to the larvae. The beetle was inside the crypt around the carcass.

Not caring: The beetle was in the soil away from the carcass and larvae.

I also noted if and when the beetle had killed/cannibalised larvae.

Additional experimental design

During the course of this first experiment I noticed that on the fourth day the larvae had dispersed from the carcass but it appeared that the beetles in the cGMP group were still trying to care for them. When the larvae disperse they stay as an aggregation for the first day or so, and the aggregation as a whole moves away from the carcass. By this time the parents are usually found at the opposite end of the box buried in the soil and generally not interacting in any way with the larvae. The altered behaviour in the cGMP treated beetles manifested as the parents staying on top of the aggregation as it moved around and attempting to feed the larvae. However, the larvae were no longer responding to the parent, so it was hard to score the behaviour in any meaningful way.

Because of this apparent extension of care, I adapted the experimental design to lengthen the amount of time the beetles were able to care for larvae. To extend the parental care period I transferred the treated beetles to a foster brood. This effectively increased the amount of time the beetles were able to interact with responsive larvae from 4 days (3 post treatment) to 8 days (7 post treatment). Based on my observations from the original experiment, the additional four days would be sufficient to encompass the extended duration of care.

Virgin pairs of age matched and unrelated beetles were placed in a breeding box. The box contained 1-2cm of damp compost and a mouse weighing between 18g and 25g. I checked for eggs in the soil after 48 hours, and for the appearance of larvae after 72 hours. Once larvae had arrived I removed the non-experimental beetle and treated the experimental beetle according to the assigned treatment group (above). I then left the beetle with the brood of larvae overnight.

I observed the beetles' behaviour for 20 minutes three times per day for three days, then at the end of the third day I transferred the beetle to a brood of newly hatched larvae and continued my observations for another 2 days. I scored behaviour in the same 3 pre-defined categories to ensure continuity between the two experimental designs.

I analysed the data for direct and indirect care separately, using two two-way ANOVAs with sex and treatment as the factors and testing the interaction between them.

RESULTS

First brood

Direct care

In my experiments I found that treatment with 8-Br-cGMP had an effect on the behaviour that was expressed. |In both females (fig 2) and males (fig 3) there was a significant increase in the time spent providing direct care in the cGMP treated group ($F_{(3, 169)}$=21.41, $p<0.0001$). There was a significant difference in the amount of direct care provided by males and females ($F_{(1, 169)}$=4.088, p=0.045), across the treatment groups the females provided more direct care. However there was no significant interaction between sex and treatment ($F_{(3, 169)}$=0.374, p=0.772).

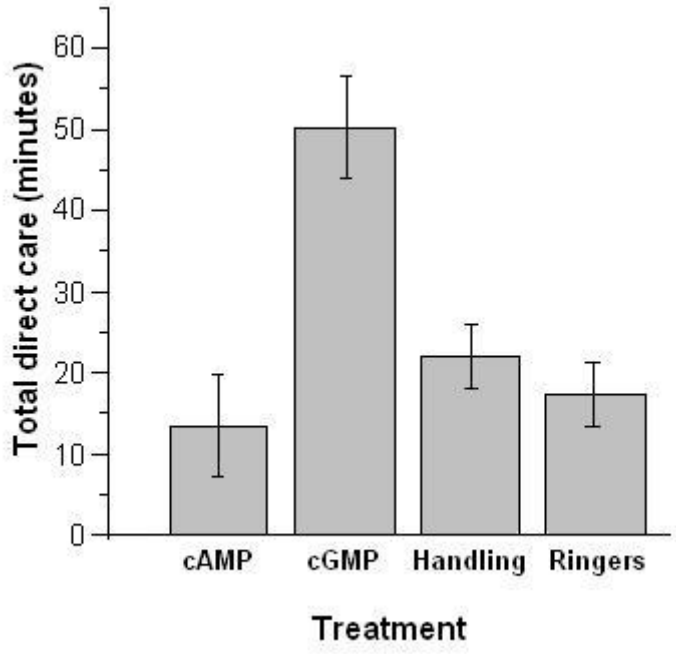

Fig. 2: Total time spent by females providing direct care to the first brood by the four treatment groups. Error bars show ± 1SE.

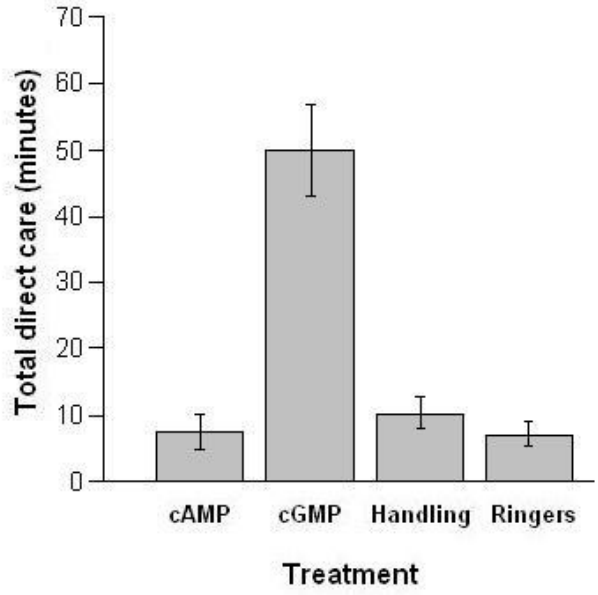

Fig. 3: Total time spent providing by males providing direct care to the first brood by the four treatment groups Error bars show ± 1SE.

Indirect care

I found that treatment with 8-Br-cGMP had no effect on the amount of indirect care provided by both males and females ($F_{(3, 169)}$=1.008, p=0.317) (Females, fig 4 Males, fig 5). There was no significant difference in the amount of indirect care provided by males and

females ($F_{(1, 169)}$=0.148, p=0.931), nor was there a significant interaction between sex and treatment ($F_{(3, 169)}$=1.048, p=0.373).

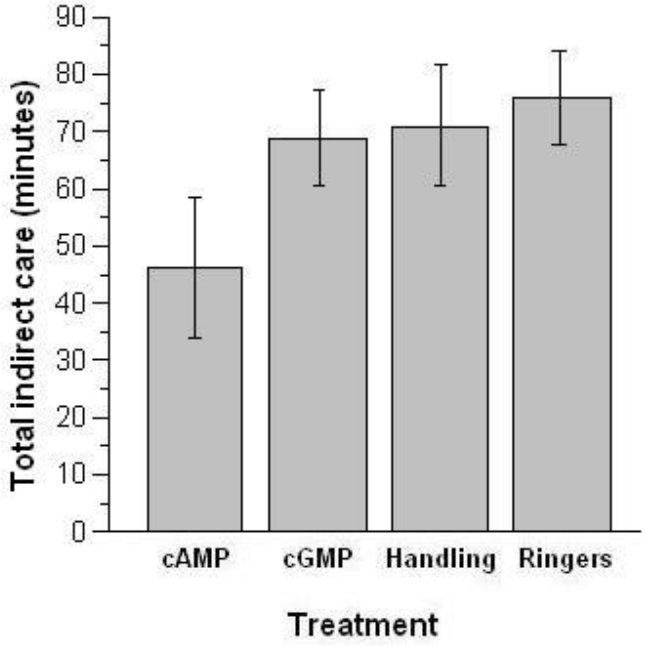

Fig. 4: Total time spent by females providing indirect care to the first brood by the four treatment groups. Error bars show ± 1SE.

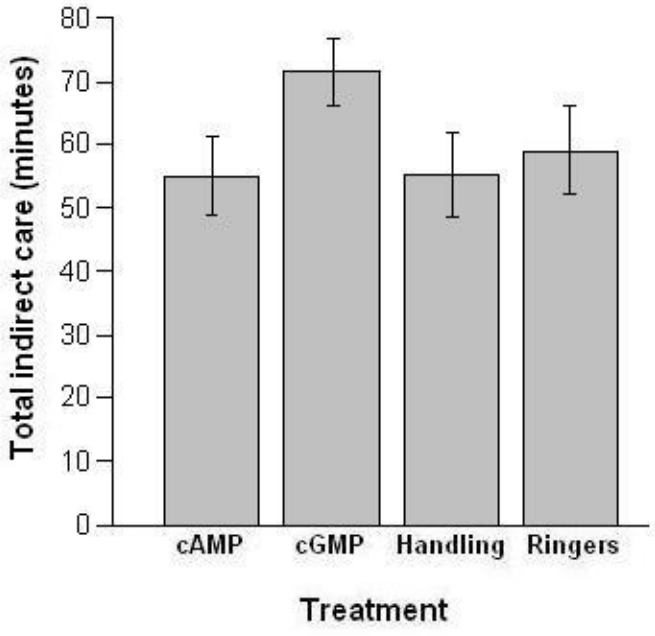

Fig. 5: Total time spent by males providing indirect care to the first brood by the four treatment groups Error bars show ± 1SE..

Second brood

Both males and females from the cGMP treated groups continued to provide direct care to the second brood, however males provided significantly more direct care than females (Wilcoxon, χ^2 =5.035, df=1, P=0.025). A further breakdown of the behaviour I observed is detailed below.

Females: After transfer to the second brood only the cGMP treated group continued to provide direct care (fig. 6, fig. 7).There was also an effect on cannibalism; treatment with cGMP prevented cannibalism of larvae in the second brood (fig. 8)

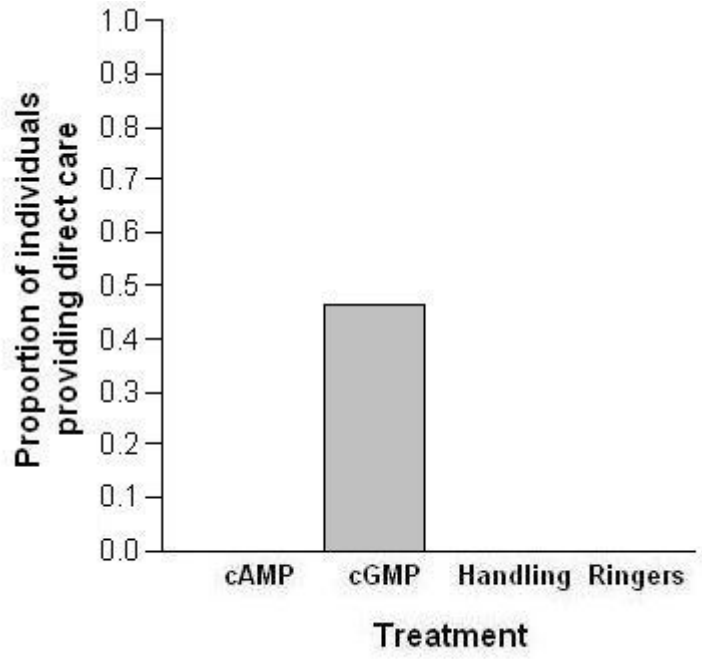

Fig. 6. Proportion of female individuals providing direct care to the second brood.

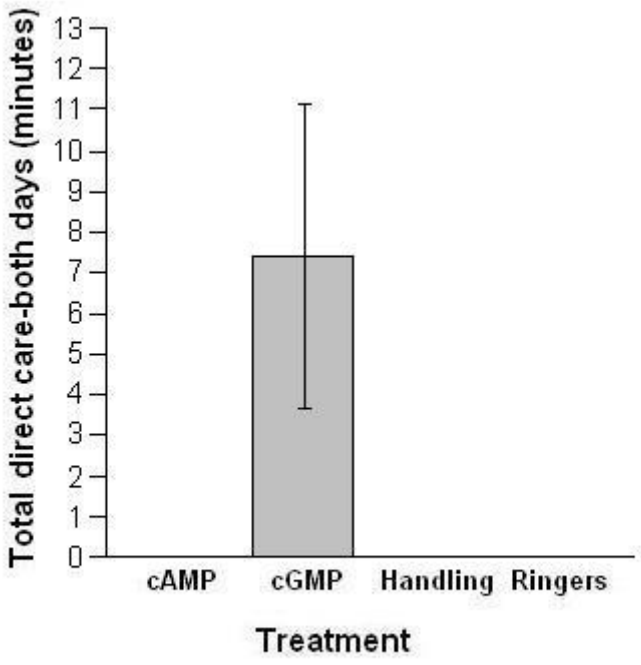

Fig. 7. Total time spent providing direct care to the second brood Error bars show ± 1SE..

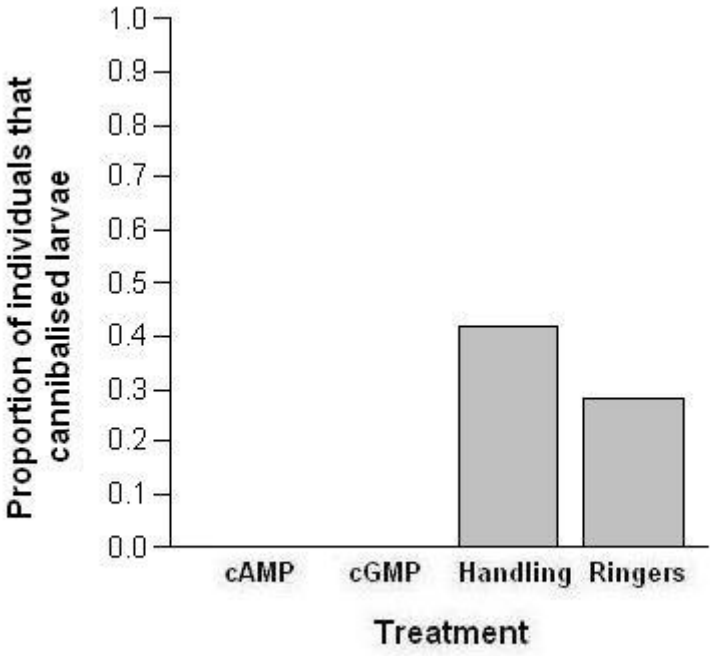

Fig. 8. Proportion of female individuals that cannibalised larvae from the second brood.

Males: Again, the same pattern was seen in males: treatment with 8-Br-cGMP continued to have an effect on the behaviour that was expressed. Only the cGMP treated group continued to provide direct care (fig. 9, fig. 10). There was also an effect on cannibalism; treatment with cGMP prevented cannibalism of larvae in the second brood (fig. 11)

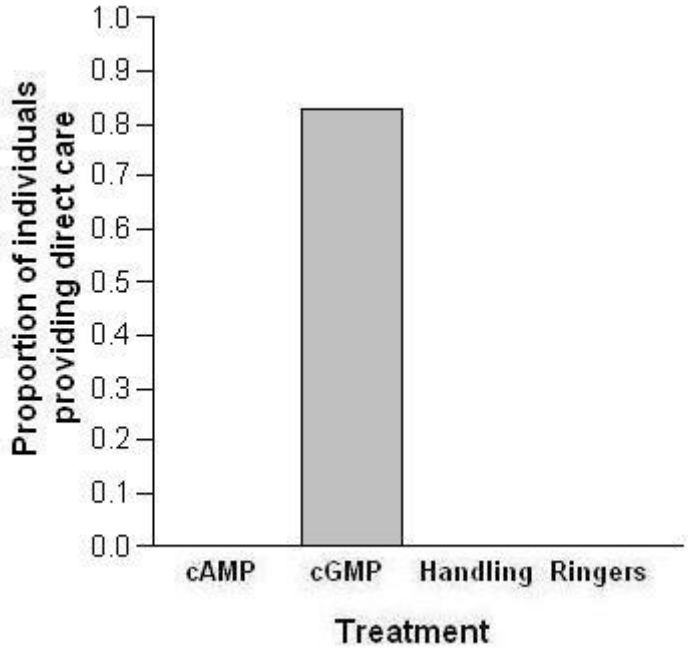

Fig. 9. Proportion of individuals providing direct care to the second brood.

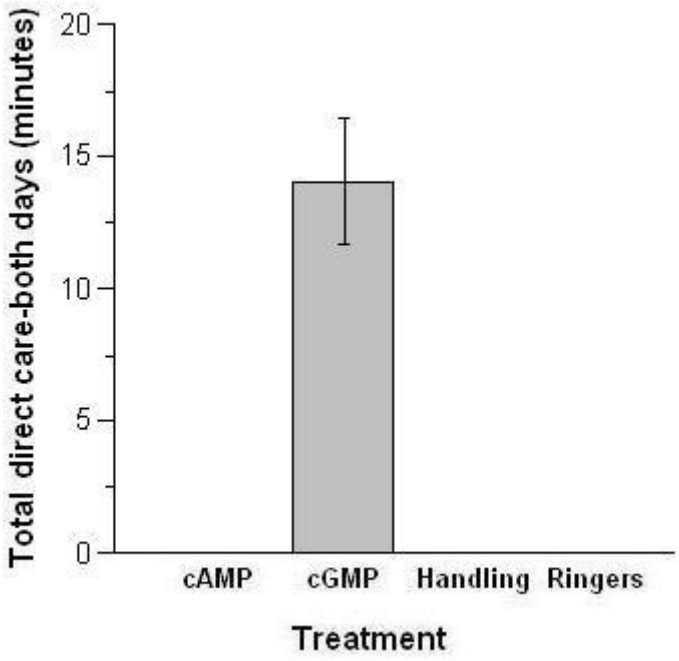

Fig. 10. Total time spent providing direct care to the second brood Error bars show ± 1SE..

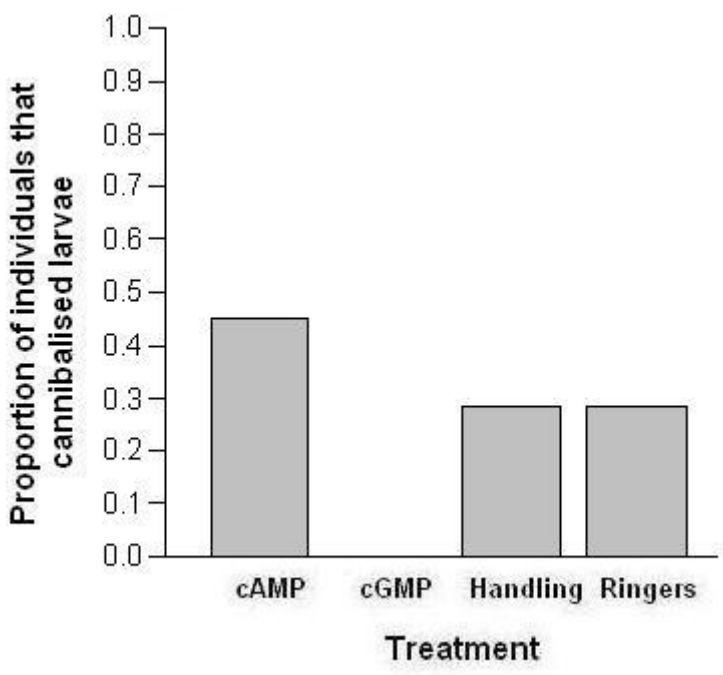

Fig. 11. Proportion of individuals that cannibalised larvae from the second brood.

DISCUSSION

This experiment shows that the relationship between *Nvfor* expression and changes in behaviour in burying beetles is not simply a correlation, but that there is a causative relationship between expression of *Nvfor* and the change in behaviour. The treatment with 8-Br-cGMP increased the amount and duration of direct parental care from both males and females, whilst none of the control groups provided extended care. cGMP treated beetles were clearly willing and able to care for a second brood of entirely unrelated larvae, well past the natural and normal end of the provision of care. In contrast, some proportion of beetles in all the control groups cannibalised the second brood or simply ignored them, whilst none of the cGMP treated beetles cannibalised young. Such a dramatic difference between the experimental group and all the control groups is a compelling illustration of the effect of *Nvfor* on parental behaviour.

cGMP had no effect on indirect care

The treatment with 8-Br-cGMP had no effect on the amount of indirect care from the parents. This is inconsistent with the findings of Walling *et al* (2008), that direct care and indirect care are evolutionarily linked, that is, evolution of an increase in one trait comes at the expense of the other. Despite the apparent inconsistency, these results are not incompatible with the findings of Walling *et al.* (2008), the role of *Nvfor* may be

downstream of the genes influencing both direct and indirect care, these results may provide some insight in to how males can modulate their behaviour according to the presence/absence of the female parent (Smiseth *et al.* 2005, Walling *et al*. 2008). Further experiments with biparental males to test whether there is a difference in *Nvfor* expression or in their response to treatment with 8-Br-cGMP would reveal whether there is an effect of the absence of the female parent, and if different molecular mechanisms influence behaviour in uniparental and biparental situations.

Males and Females

Uniparental males responded to treatment with 8-Br-cGMP in the same way to uniparental females, treatment increased the level of direct care provided to larvae as well as extending the duration of care. This isn't a surprising result because in behavioural assays, uniparental males tend to behave in similar ways to uniparental females, and my results show that females provided more direct care than males, which is consistent with previous research on male and female care (Smiseth *et al*. 2005, Walling *et al.* 2008). However, there was an unexpected effect of treatment with 8-Br-cGMP, where in the period of extended care, males provided significantly more direct care to the second brood than females.

With the addition of gene expression data and pharmacological manipulations I have shown that the similarities in behaviour are driven by similar molecular mechanisms controlling them.

Injecting cGMP Vs feeding cGMP

PKG is activated by, and dependant upon cGMP (Lohmann *et al*. 1997, Osborne *et al.* 1997), and by increasing cGMP concentrations in an animal it is possible to increase PKG activity (Ben-Shahar *et al*. 2002, Ben-Shahar *et al.* 2003, Lucas and Sokolowski 2009).

In all of the previous studies on the effect of treatment with 8-Br-cGMP it has been mixed with sugar water and fed to the subject animal, this was demonstrated to significantly increase brain PKG activity (Ben-Shahar *et al*. 2002, Ben-Shahar *et al.* 2003, Lucas and Sokolowski 2009), though the exact mechanism behind this increase in activity is not clear. Due to the natural behaviour of burying beetles, feeding 8-Br-cGMP was not appropriate as the parents regurgitate food to the offspring so it is possible that they would absorb any of the intended dose. My method of injection, though more invasive, more reliably administers the intended dosage. I have not measured PKG activity in burying beetles, but the reliance of PKG on the presence of cGMP and the established link between increased

levels of cGMP and increased PKG activity is well established (Butt *et al.* 1993, Francis and Corbin 1994, Osborne *et al.* 1997, Lewin and Walters 1999, L'Etoile *et al.* 2002, Ben-Shahar *et al.* 2003, Fitzpatrick and Sokolowski 2004, Ben-Shahar 2005, Lohmann and Walter 2005, Hofmann *et al.* 2006, Lu *et al.* 2008).

Extension of care implies a strong link

The extension of parental care beyond the normal expression is the strongest argument that the *foraging* gene is a key gene in the regulatory system for parent-offspring interactions. A single treatment over 130 hours before induces such a large behavioural shift that the 8-Br-cGMP treated beetles continued to care, well beyond what was seen in the control groups and far beyond what is necessary or normal investment in the larvae. That the entire control system could be up-regulated by a single intervention suggests that *Nvfor* is a fundamental node in the network of controlling genes, and that up-regulating this single gene causes many of the other genes to be up-regulated as a consequence, resulting in extended parental care behaviour.

These results show that it is possible to manipulate burying beetle behaviour with one simple intervention. This is entirely consistent with previous work on manipulations of PKG activity using cGMP. My method of injecting the 8-Br-cGMP rather than feeding it is effective in delivering the drug into the beetle, and gives a more accurate idea of the dosage delivered.

Apis mellifera: The first experiments to manipulate PKG activity using cGMP were in honey bees. Newly eclosed honey bees were fed sugar solution containing 8-Br-cGMP, the bees in the cGMP treated group had elevated levels of PKG activity, similar to those seen in untreated normal-aged foraging bees. The control groups did not show an increase in PKG activity. Bees treated with cGMP displayed a significant increase in precocious foraging activity, usually the change in behaviour to foraging comes much later in life, so it is safe to conclude that treatment with cGMP increases PKG activity, which in turn increases/induces foraging behaviour (Ben-Shahar *et al.* 2002, Ben-Shahar *et al*. 2003).

***Pheidole pallidula*:** Later experiments in the ant *Pheidole pallidula,* showed that there is a strong link between PKG activity and defensive or foraging behaviour. Worker ants are divided into two castes; major worker ants have higher PKG activity than minor ants and a different pattern of PKG activity within the brain. Pharmacological manipulation with 8-

Br-cGMP reduced the response to a new food source in both castes. The same treatment increased the defensive response to intruders in the major ants, but not in the minors (Lucas and Sokolowski 2009). Though the method of administering the cGMP was, through necessity, different in my experiments with the burying beetles, the resulting change in behaviour is consistent with the changes that Ben-Shahar *et al* (2003) and Lucas and Sokolowski (2009) found in the honey bees and the ants; treatment with cGMP induces behaviour associated with high levels of *Amfor* and *ppfor* (respectively) expression.

The role of the *foraging* gene is well conserved: Parental care has evolved in many different forms over many invertebrate taxa with millions of years of divergence between them. Many of the social species studied in relation to this gene were relatively closely related, as the majority of them are hymenoptera. The results of this experiment, combined with the results I have reported in chapter 2 shows that despite the many millions of years of divergent evolution between beetles and honey bees, harvester ants and paper wasps, the same gene has evolved in all species to play a similar role in regulating the timing, initiation and extent of social behaviours.

This experiment, in combination with the results from chapter 2 shows that the *foraging* gene influences the social interaction part of parental care; direct parent-offspring interaction. This is the most developed aspect of parental care, and would be expected to evolve later. This is demonstrated with the related beetles in the genus *Ptomaphila*, which also breed on carrion, the beetles prepare the carcass in a similar manner to *N. vespilloides*, but do not feed their offspring (Crisci *et al.* 1991, Archer 2000, Peck 2001, Hawkeswood and Turner 2008). Since not all aspects of parental care were affected by the treatment with cGMP, it suggests that there are multiple pathways controlling different aspects of parental care.

CHAPTER 4

THE *FORAGING* GENE – GENE EXPRESSION IN LARVAE, *Nicrophorus vespilloides*

INTRODUCTION

Previous work on the *foraging* gene in *Nicrophorus vespilloides*

In my previous work on *Nvfor* in *N. vespilloides* I have shown that gene expression changes in association with changes in parental care behaviour of adults. I then demonstrated that this is a causative link by manipulating levels of cGMP. High expression of *Nvfor* induces increased levels and duration of caring behaviour in adult beetles (chapters 2 and 3).

Larval behaviour

***Nicrophorus vespilloides* larvae:** Burying beetle larvae have very predictable and scheduled behaviour; newly hatched larvae move to the carcass and locate the crypt the parents have prepared in the carcass. The larvae beg to be fed by the adults, with a peak of begging behaviour around 24 hours after the larvae arrive on the carcass (Smiseth *et al*. 2003). They continue to beg for food, despite being able to self-feed after they are 24h old (Fetherston *et al.* 1990, Eggert *et al.* 1998). After around 6 days the larvae reach the final larval instar, they stop feeding and disperse away from the carcass (Lock *et al*. 2004). After "wandering" without eating or further growth for one to two weeks, they bury down into the soil to pupate. After another one to two weeks, they emerge as adults (Lock *et al*. 2004).

The timing and changes in larval behaviour is very closely linked with parental behaviour. The eggs hatch 8-12 hours after parents start accepting larvae (Oldekop *et al*. 2007), the peak of begging behaviour coincides with the peak in direct caring behaviour (Trumbo 1997, Oldekop *et al*. 2007) and the larvae disperse within 24 hours after the parents have left the brood (Trumbo 1991, Jenkins *et al.* 2000, Smiseth *et al.* 2003, Lock *et al.* 2004), maximising the amount of care and protection that the larvae can receive from the parents, whilst avoiding the risk of cannibalism from post-caring parents. This behaviour is tied closely with the parental care cycle, and has been shown to have co-evolved in terms of the timing of peak care and peak begging (Lock *et al.* 2004). It is also possible that some of the same genes are involved in the control of larval behaviour, as studies in *Drosophila* have shown, one gene can influence foraging behaviour in adults and larvae, as well as pre-pupation behaviour (Pereira and Sokolowski 1993, Sameoto and Miller 1968, Ringo and Wood 1983, Sokolowski *et al.* 1984, Sokolowski and Hansell 1983, Wong *et al.* 1985).

***Drosophila melanogaster* larvae:** In *D. melanogaster* there is natural allelic variation of the *foraging* gene. Rover is dominant over Sitter; the variation in this single gene has a large influence on larval behaviour. Individuals with the Rover allele cover a larger range and have higher PKG activity. Individuals that are homozygous for Sitter have a smaller range and lower PKG activity. There is no difference in general activity, only in the foraging strategy (Sokolowski and Hansell 1983, de Belle *et al.* 1989, Osborne *et al.* 1997).

There have also been several studies on how *for* influences pre-pupation behaviour in *Drosophila* larvae; Rover larvae pupate higher in the vial than Sitter larvae (Sokolowski and Hansell 1983). Larval foraging behaviours were measured in early third-instar larvae. At some time in the mid to late third instar, *Drosophila* larvae switch from food-related activities (foraging) to pre-pupation activities (wandering). This switch in motivation with respect to food can be quantified by measuring the tendency for a larva to remain on the feeding substrate (Sokolowski *et al.* 1984). Larval behaviour in the wandering phase culminates in a choice of pupation site (Wong *et al.* 1985).

***Diabrotica virgifera virgifera* L. larvae:** There has also been some research on *Dvfor*/PKG in *Diabrotica* larvae, when compared to the normal strain, the larvae of the variant strain showed similar patterns of expression of *Dvfor/*PKG as seen in the adults. Throughout development the variant individuals had higher *Dvfor* expression than the normal individuals (Garabagi *et al.* 2008). However, to date there have been no studies to test whether there is an effect of higher expression on the larva's behaviour.

Gene expression in burying beetle larvae

To make the gene expression assays reliable it is important to ensure that the life history of all the individuals in the experiment are as similar as possible, especially in this experiment as there are no prior studies to suggest what will be an important factor. Due to the nature of the experiment, the number of samples that it is possible to process is the limiting factor, and so it was important to choose the behavioural states most different to each other in order to have the strongest contrast between samples. I selected three points that seemed biologically and behaviourally most significant in larval development.

Larval behavioural changes

I selected three biologically significant time points at which I would measure *Nvfor* expression; recently hatched larvae, larvae at the peak of begging and larvae 24h post-dispersal.

Newly hatched: larvae that have yet to feed and are searching for the carcass and their parents. Their behaviour is food-oriented as they need to find the carcass quickly in order to survive.

Begging: Larvae at the peak of care are well fed, and display the strongest interactions with their parents. Although the larvae can feed independently it has been shown that they also beg for food from the parents by raising their heads while waving their legs or touching the parent (Rauter and Moore 1999, Smiseth *et al.* 2003). Larvae beg to signal hunger levels (Smiseth and Moore 2004b, Smiseth and Moore 2007, Smiseth *et al*. 2007b) and those that beg more are fed more as the parents respond to begging by adjusting the allocation of food (Smiseth and Moore 2002, 2008).

Wandering: Larvae that have recently dispersed have stopped feeding and have transitioned to the wandering phase. This behaviour is very different from any behaviour seen earlier in the larvae and in *D. melanogaster for* has been shown to have an effect on pre-pupation wandering in larvae (Sokolowski and Hansell 1983, Sokolowski *et al*. 1984, Wong *et al.* 1985).

Through this targeted approach I will test the hypothesis that the *foraging* gene is linked with the changes in behaviour seen larval development in burying beetles.

METHODS

I collected beetles and maintained stocks as described in chapter 2 (Methods: page 36).

Experimental design

These experiments were designed to test the following hypotheses:

Ho: Expression of *foraging* will not change with transitions in behaviour associated with larval development.

Ha: Expression of *foraging* will change with transitions in behaviour associated with larval development.

It is hard to predict the nature of the change in expression as there has been little research on the role of the *foraging* gene in larval behaviour. However, the studies in *D. melanogaster* suggest that the *forging* gene plays a role in dispersal/pre-pupation behaviour, so I expect to see a difference in expression between begging larvae and wandering larvae.

To test this, I defined three behaviourally significant time points (as described below), which correspond to those identified by Smiseth *et al* (2003). I collected all individuals at the appropriate developmental stage by picking them up with forceps, and placing them in a pre-labelled eppendorf tube, then immediately submerging the tube in liquid nitrogen. The tubes were left in liquid nitrogen for several minutes, and then transferred to a -80°C freezer for storage.

Larvae

It was very important to ensure the individuals had as few differences in life history as possible, as these would be impossible to quantify or account for in the analysis and due to the nature of the investigation, life history differences could have a significant impact on the expression of *Nvfor.* All the larvae used in the expression analysis were from the F4 generation, all the families were set up on the same date. Several pairs of beetles were set up in breeding boxes with soil and a mouse carcass weighing 20±2g. Each family was left for 72 hours then checked for eclosed larvae. Only families which had enough larvae to take 5 at each stage were used in the QPCR analysis.

Newly hatched: Larvae were collected from the soil surrounding the carcass as soon as they had hatched and before they had any opportunity to feed or be cared for by the parents (Eggert *et al.* 1998).

Begging: Larvae were collected 24-36 hours after they arrived on the carcass. Larvae were only taken when they were observed begging to be fed. Begging behaviour is quite obvious, the larvae rear up in front of the parent, waving their legs or touching the parent (Rauter and Moore 1999, Smiseth *et al*. 2003).

Wandering: Larvae were collected 24 hours after the brood had dispersed from the carcass, approximately 92 hours after larvae arrived on the carcass.

In each treatment group n=15, comprised of 3 individuals from 5 families. The families are the same between treatment groups.

RNA extraction, cleanup and quality

I used whole larvae from the newly hatched stage and just the heads from the begging and dispersed stage.

I used the same RNA extraction, cleanup quality and quantification checks as well as the same primers and protocols for the Quantitative real-time PCR, all of these methods are described in chapter 2.

Analysis

The data were transformed to R values using the method developed by Pfaffl *et al* (2001).

$$R=\frac{E(\text{target})^{\Delta CP(\text{target})}}{E(\text{reference})^{\Delta CP(\text{reference})}}$$

$$E=10^{[-1/\text{slope}]}$$

$$\Delta CP=CP(\text{control})-CP(\text{sample})$$

Target is the gene of interest, in this case *Nvfor*.
Reference is the reference gene, in this case the 18S control.
Control is the control template RNA, a pool of all samples being analysed.
Sample is the specific sample of RNA being analysed
Slope is the slope of the regression line of the standard curve.

After calculating the R values for each sample, I analysed the data using a non-parametric ANOVA (Wilcoxon test) given the non-normal distribution of data. Parametric and non-parametric tests, however, give identical results (parametric results not shown).

RESULTS

In my experiments I found that *Nvfor* expression was correlated with the developmental stage and behaviour that was expressed. There was a significant increase in *Nvfor* expression with the start of wandering behaviour ($\chi 2 = 5.7608$, df = 2, P <0.001). The level of expression was low in newly hatched and begging larvae, and increased after dispersal (fig. 1, table 1).

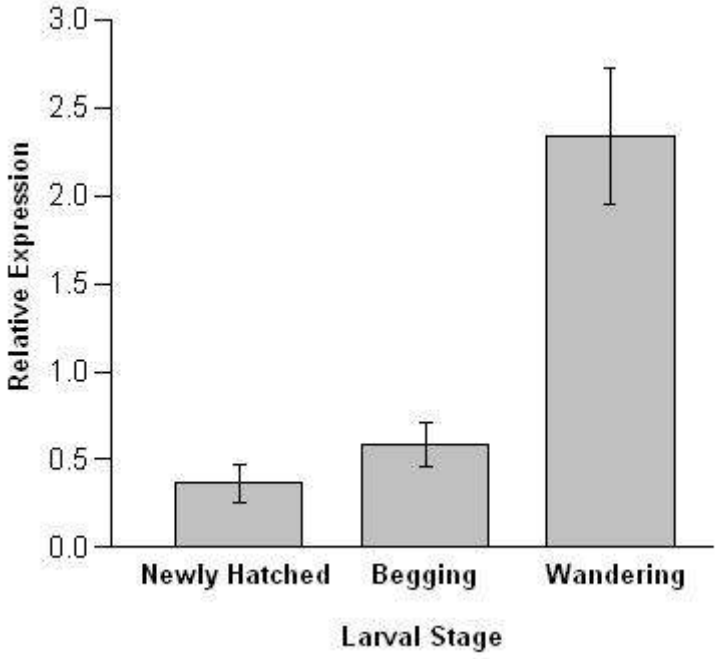

Fig. 1. Relative expression of *Nvfor* in three larval stages of N. *vespilloides* N=8 in each group. Error bars show ± 1SE..

DISCUSSION

The expression of *Nvfor* was higher in wandering larvae, after the larvae had completed feeding and dispersed from the food source. This pattern of expression is not consistent with the results of my previous work on *Nvfor* in adult burying beetles, however it corresponds with behaviour and expression patterns seen in Drosophila larvae; wandering Drosophila larvae with the Rover allele have high levels of *for* expression and move further and pupate at different heights than those that are heterozygous for Sitter.

for expression in *Drosophila* is linked with pre-pupation behaviour

In Drosophila larvae Sitter individuals have low PKG activity when they are on food and don't move around much, compared to Rover individuals, which have higher PKG activity and move over larger distances. Allelic differences in *for* in *Drosophila* larvae have also been linked with digging, response to moisture, pupation site preferences (Sameoto and Miller 1968, Ringo and Wood 1983, Sokolowski and Hansell 1983, Wong *et al.* 1985). The most relevant of these is the effect of *Nvfor* on pupation site and pre-pupation behaviour. At some time in the mid to late third instar, *Drosophila* larvae switch from foraging behaviours to pre-pupation behaviours. This switch in motivation with respect to food can be quantified by measuring the tendency for a larva to remain on the feeding substrate (Sokolowski *et al.* 1984). Larval behaviour in the wandering phase culminates in a choice of pupation site. This is very similar to the behaviour seen in burying beetle larvae, where post-dispersal wandering lasts for up to two weeks before the larva pupates.

The *foraging* gene expression in beetle larvae is linked to pre-pupation behaviour

The results from this experiment suggest a similar involvement of *Nvfor* in the wandering phase and pre-pupation behaviour in burying beetles, as seen in *Drosophila.* In the beetle larvae, the newly hatched larvae and those begging do not move particularly large distances and they are focused on foraging and feeding, whereas when they are dispersing the larvae move quickly and cover large distances, ignoring opportunities to eat.

Further research

These results indicate that *Nvfor* is involved in the control of larval behaviour, however it is clear that further research is needed to clarify the role of this gene and to investigate other parts of the controlling mechanism.

More time points: These results show that *Nvfor* expression is higher when the larvae are dispersing, a clear route for further investigation is to take expression patterns throughout the larval stage, particularly the wandering phase, to see if expression changes over the 7-10 days that larvae spend wandering, and if there is a role of *Nvfor* in the selection of a pupation site.

Pharmacological manipulations: These results are strongly suggestive of a link between *Nvfor* and larval behaviour, but as in the adults, manipulative experiments are needed to

confirm this link. However, unlike the adult beetles I have yet to find a method to treat the larvae with cGMP. When the larvae are small they are very delicate, and removal from the carcass can easily result in damage or death. The size of the larvae also makes injections difficult, as even the finest gauge needle causes a lot of damage to a small larva. Injections shortly before dispersal may be possible as the larvae are larger then, but as there is variation in the age that larvae disperse, even within a single brood, it would be hard to generate clear data. It may be possible to use DMSO (Dimethyl sulfoxide) so that cGMP is "inhaled" or absorbed (Dawson-Scully *et al.* 2010).

Conclusion

The exact role of *Nvfor* in controlling larval behaviour is not clear, though these results suggest that there is a link between expression levels of *Nvfor* and changes in larval behaviour. The definite link between *for* and larval behaviour in Drosophila, combined with the results from my previous work in adult burying beetles (chapters 2 and 3) is suggestive that this is not purely correlative.

The *foraging* gene and its orthologues are involved in several behavioural switches in insects. In social insects such as honey bees and the harvester ant, the *forging* gene is involved in the unidirectional development and changes in behaviour (Ben-Shahar *et al.* 2002, Ben-Shahar *et al.* 2003, Ingram *et al.* 2005). In contrast, in adult burying beetles the behavioural changes are reversible (chapters 2 and 3). The results of this experiment suggest that *Nvfor* and the network of genes it interacts with control similar behaviours in adults and larvae; food-related and movement behaviours, in both adults and larvae an increase in *Nvfor* is associated with a transition from feeding to not-feeding. However in adults these behaviours are adapted to provisioning food to larvae in the form of parental care, whereas in larvae the role appears to be linked to developmental changes in behaviour from feeding to pre-pupation wandering.

CHAPTER 5

THE EFFECT OF OXYTOCIN ON PARENTAL CARE BEHAVIOUR IN ADULT BEETLES, *Nicrophorus vespilloides*

INTRODUCTION

Oxytocin

Oxytocin and vasopressin are nonapeptides; one of the oldest families of neuropeptides (Insel 2010). Each has nine amino acids and are structurally very similar, only differing at peptide 3 and 8 (Stafflinger *et al.* 2008). The nonapeptide lineage is represented in almost every vertebrate taxon, as well as several invertebrate taxa. The peptides vary slightly in form and name, but can be grouped into two types; Arginine vasotocin (arginine vasopressin in mammals) and oxytocin-like peptides (isotocin in fish, mesotocin in non-eutherian tetrapods, and oxytocin in eutherian mammals) (Insel 2010). There are many examples of the evolutionary conservation of the behavioural effects of this family of peptides, collectively showing that oxytocin/vasopressin is important for social cognition and influences many aspects of behaviour with gender and steroid-dependent effects. There is a lot of interspecies and intraspecies variation in the role and function of oxytocin/vasopressin, but the general principle of a role in social behaviour is consistent (Insel 2010).

Perhaps the most well known of studies on the effects of oxytocin/vasopressin are those on the prairie and montane voles. Vole species present a large range of levels of social behaviour, from highly social, monogamous and biparental to solitary, promiscuous and uniparental. Field and laboratory studies have demonstrated that the prairie vole (*Microtus ochrogaster*) are highly social, forming enduring pair bonds between mates. In contrast, the montane vole (*Microtus montanus*) is much less affiliative, and does not form pair bonds (Winslow *et al.* 1993, Carter *et al.* 1995, Carter 1998, Young *et al.* 1998, Insel and Young 2001, Young *et al.* 2001). Treatment of female prairie voles with oxytocin increases affiliative behaviour and pair bonding, even when mating has not occurred. Similar effects of treatment with vasopressin are found in male prairie voles, treatment increases affiliative behaviour and aggression towards intruders (Williams *et al.* 1994, Cho *et al.* 1999). These effects of treatment are not seen in the montane voles (Goodson and Bass 2001). Patterns of Oxytocin receptors in the brains of prairie and montane voles differ dramatically, where prairie voles had high levels of oxytocin receptor in the prelimbic cortex, bed nucleus of the stria terminalis, nucleus accumbens, midline nuclei of the thalamus, and the lateral aspects of the amydala. These areas showed low levels of oxytocin receptor in the montane voles (Insel and Shapiro 1992). Similarly, montane voles have lower brain expression of the vasopressin receptor *V1aR* (Lim *et al.* 2004). Increased

brain expression of *V1aR* in montane voles through a viral vector enhanced partner preference, demonstrating the key role of a single gene in pre-existing genetic and neural circuits on a complex trait (Lim *et al.* 2004).

The roles of oxytocin and vasopressin in social mating systems have also been investigated in many other vertebrates (Goodson and Bass 2001). The structure of vasotocin/vasopressin is well conserved across all vertebrates, there is some variation at position 3 and position 7, but there is strong conservation of the amino acid sequences that are proposed to be involved in peptide binding (Goodson and Bass 2001). The function of Vasopressin/vasotocin varies widely across species, treatment of male colonial zebra finches (*taeniopygia* guttata) with vasotocin facilitates overt aggression (Goodson and Adkins-Regan 1999), whereas the same treatment in male territorial field sparrows (*Spizella pusilla*) inhibits aggression. (Goodson 1998). In a comprehensive review of the role of vasopressin/vasotocin in vertebrates Goodson and Bass (2001) found that vasopressin/vasotocin influences a wide range of effects on social spacing and aggression, and that these patterns are associated with independent and convergent evolution of peptide function and receptor binding. However, the exact peptide function and polarity of behavioural influence varied across species combined with large gaps in knowledge between anatomy and function making it difficult to predict function or role of vasopressin/vasotocin across species (Goodson and Bass 2001).

Although best studied in mammals, there have been investigations of the role of this hormone in social interactions in a variety of species. In the mollusc *Lymnaea stagnalis* lys-conopressin influences male copulatory behaviour through selective expression in neuronal and gonadal cells (Van Kesteren *et al.* 1992, Van Kesteren *et al.* 1995, Van Kesteren *et al.* 1996). In finches (*Taeniopygia guttata*), mesotocin influences flock size and interference with a mesotocin antagonist reduces social behaviour, such as flock formation (Goodson *et al.* 2009). In the plainfin midshipman (*Porichthys notatus*) grunting is an important aspect of reproductive behaviour. Isotocin influences grunting in females, whereas Arginine vasotocin, not isotocin, regulates grunting in males (Goodson and Bass 2000).

In rats, maternal behaviour is initiated after giving birth (Numan, 1988). Adult virgin females avoid or attack pups, but when they were injected with oestrogen and oxytocin they developed full maternal behaviour, including nest building and attempting to nurse the

pups (Pedersen *et al.* 1982, Rubin *et al.* 1983, Fahrbach *et al.* 1984). The change in behaviour in rats has a lot of similarities to the changes seen in burying beetles; non-breeding beetles are solitary and will attack and kill conspecific larvae but after finding a suitable carcass for breeding their behaviour changes to caring behaviour, which includes nest building and feeding their larvae.

Whilst it is unwise to draw conclusions from similar behaviour seen in two species that are so distantly related as burying beetles and rats, the conservation of function across taxa in oxytocin-like peptides is well established. Although so far the role and function of oxytocin-like peptides have not been demonstrated in insects, other invertebrate species have shown the connection between oxytocin-like peptides and social and reproductive behaviour.

Inotocin

The oxytocin/vasopressin-like peptide inotocin was identified over 20 years ago in the locust *Locusta migratoria* (Proux *et al.* 1987), but no similar peptide was identified in other insects. Recently, Stafflinger *et al* (2008) identified a gene coding for an identical peptide in the recently sequenced *Tribolium castaneum* genome. Quantitative RT-PCR of this gene showed that in adult *Tribolium* it is more highly expressed in the head than in other tissues. The gene was also identified in *Nasonia vitripennis* and *Daphnia pulex*, however, the genes could not be found in the genomes of any other holometabolous insect with a completely sequenced genome (to date: 12 *Drosophila* species, *Anopheles gambiae, Aedes aegypti, Bombyx mori* and *Apis mellifera*). The role of inotocin in any of these invertebrates is not clear, it has been suggested that inotocin has a role in water balance (Proux *et al.* 1987), however this is disputed by Stafflinger *et al* (2008) as expression patterns show that it is less expressed in malpighian tubes and the hindgut than it is in the head. Stafflinger *et al* (2008) suggest that inotocin may be involved in the stimulation of carbohydrate and lipid mobilisation or stimulation of the heartbeat or induction of ecdysis, but they acknowledge that this is speculation and much more research on the function of inotocin is needed. The amino acid sequence of inotocin is different from oxytocin and other oxytocin-like hormones (table 1). However in cell cultures, the receptor was activated by oxytocin, vasotocin, isotocin, Arg-conopressin and Lys-conopressin. The reactivity to inotocin was much higher than to any other oxytocin-like hormones, meaning that there is some cross-reactivity despite the differences in peptide sequence.

Table 1: Structures of vasopressin, oxytocin, and some selected vasopressin- and oxytocin-like peptides (taken from Stafflinger *et al* 2008)

Name	Peptide structure	Source	Reference
Vasopressin	**CYFQNCPRGamide**	Mammals	(Acher and Chauvet 1953, du Vigneaud *et al.* 1953a)
Lys-Vasopressin	**CYFQNCPKGamide**	Pig, some marsupials	(Chauvet *et al.* 1983)
Phenypressin	**CFFQNCPRGamide**	Some marsupials	(Chauvet *et al.* 1980)
Inotocin	**C**LIT**NCPRGamide**	*Locusta migratoria,*	(Proux *et al*. 1987) (Stafflinger *et al*. 2008)
Inotocin	**C**LIT**NCPRGamide**	*Tribolium castaneum*	(Li *et al.* 2008, Stafflinger *et al*. 2008)
Inotocin	**C**LIT**NCPRGamide**	*Nasonia vitripennis*	(Stafflinger *et al*. 2008)
Crustacean Oxytocin/vasopressin-like peptide	**C**FIT**NCPPGamid**	*Daphnia pulex*	(Stafflinger *et al*. 2008)
Vasotocin	**CY**I**QNCPRGamide**	Nonmammalian vertebrates	(Acher *et al.* 1960)
Arg-conopressin	**C**IIR**NCPRGamide**	*Conus geographicus*	(Cruz *et al.* 1987)
Lys-conopressin	**C**FIR**NCPK****Gamide**	Leech, various molluscs	(Salzet *et al.* 1993)
Oxytocin	**CY**I**QNCP**L**Gamide**	Mammals	(du Vigneaud *et al.* 1953b)
Isotocin	**CY**IS**NCP**I**Gamide**	Fish	(Acher *et al.* 1962)
Annetocin	**C**FVR**NCP**T**Gamide**	Annelids	(Oumi *et al.* 1994)
Cephalotocin	**CYF**R**NCP**I**Gamide**	*Octopus vulgaris*	(Reich 1992)
Octopressin	**C**FWTS**CP**I**Gamide**	*Octopus vulgaris*	(Takuwa-Kuroda *et al.* 2003)

Oxytocin in burying beetles

Given the wide ranging effects of oxytocin and oxytocin-like hormones in other animals, I considered inotocin to be a reasonable candidate gene to have some effect on parental care in burying beetles. This is a highly speculative experiment because the work by Stafflinger *et al* (2008) suggests that the gene encoding inotocin is not present in all insect species, though it was found in another beetle; *Tribolium castaneum*. In addition to this, if the inotocin gene is present in the burying beetle genome, it still may not play a role in social or parenting behaviour.

I attempted to use the primers developed by Stafflinger *et al* (2008) to amplify the inotocin gene from cDNA generated from total RNA extracted from pooled samples of tissue taken from all life stages and both sexes. I was unsuccessful, but this does not mean that the gene encoding inotocin is not in the burying beetle genome. A Western blot, with antibody probes designed to recognise the conserved region of the inotocin that is shared with other

oxytocin/vasopressin like peptides, may reveal the presence of inotocin or a related protein. Extensive sequencing of the *N. vespilloides* genome/transcriptome though next generation sequencing would be a far larger undertaking but it may reveal the presence of inotocin along with many other genes that may be of interest.

Pharmacological manipulations with oxytocin

Having been successful in developing the injection protocol for pharmacological manipulations, and the ready availability of oxytocin from biological chemical suppliers, and Stafflinger *et al*'s (2008) demonstration that the inotocin receptor is reactive with oxytocin, it seemed worthwhile to attempt a manipulation experiment.

METHODS

Beetle collection and husbandry

I collected beetles and maintained stocks as described in chapter 2 (Methods: page 36).

Experimental beetles

All the beetles used in the manipulation of oxytocin were from the F5 generation; they were all kept under the same conditions, as described in chapter 2 (Methods: page 36). All beetles were 21 days post-eclosion at the start of this experiment.

Experimental design

The aim of these experiments was to test the hypothesis:
Ho: increasing the endogenous levels of oxytocin will have no effect on the amount/timing of care given.
Ha: increasing the endogenous levels of oxytocin will change the amount/timing of care given.

I used three experimental treatments:
Oxytocin: the beetle was picked up and injected with 30µl of oxytocin (Sigma) in Ringers buffer solution (table 2) (0.5µg/µl, total dose =15µg), then placed onto the foster brood.

Handling control: the beetle was picked up and held for 30 seconds, then replaced onto the brood.

Ringers: the beetle was picked up and injected with 30µl of Ringers buffer solution (table 2) then replaced onto the brood.

Table 2: ingredients for beetle Ringers buffer (Holtzhausen and Nicolson 2007).

name	concentration (mmol)
NaCl	90
KCl	50
$CaCl_2$	2
$MgCl_2$	5
$NaHCO_3$	6
NaH_2PO_4	4

Injections: I held the beetle still with the head pushed down slightly, I used a 30 gauge (0.3mm) needle, which I inserted under the pronotum through the soft membrane of the joint (fig. 1). Preliminary studies showed this method of injection to have the lowest mortality (3%) and no apparent detrimental effects on the beetles' health or behaviour. I injected 30µl of liquid per injection/individual. The needle was removed and discarded between beetles.

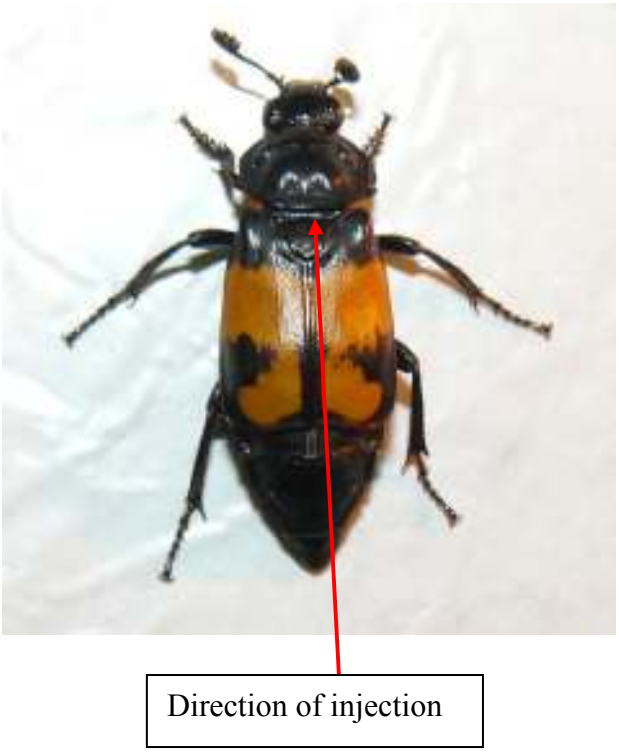

Fig. 1: injection site in *N. vespilloides*.

These three treatment groups give controls for several important issues. The handling only group controls for any effects of disturbing the beetle, as a large amount of disturbance might induce abandonment or abortion of the brood. Injecting the Ringers buffer controls

for any effect of the injection, as the injection site might allow infection, or the introduction of a relatively large volume of liquid could cause metabolic or water-control issues. By comparing these controls to the experimental group of oxytocin in Ringers buffer I will be able to find any effect of oxytocin whilst accounting for confounding factors of the method of treatment. I also randomly assigned the beetles to these three groups to remove any bias that could be introduced in selecting beetles for treatment.

I placed a pair of non-experimental beetles in a breeding box. The box contained 1-2cm of damp compost and a mouse weighing between 18g and 25g. I checked for eggs in the soil after 48 hours, and for the appearance of larvae after 72 hours. These larvae were the foster broods. 24 hours before I expected larvae to arrive in the foster broods, I placed a pair of unrelated, age-matched virgin beetles in a breeding box. The box contained 1-2cm of damp compost and a mouse weighing between 18g and 25g. Once larvae had arrived in the foster broods I removed the parent beetles and treated the experimental beetle according to the assigned treatment group (above). I also moved larvae between broods to ensure that all broods started the experiment with 15 larvae, I then placed the experimental beetle with the foster brood and left them for 30 minutes.

Behavioural observations

I observed the beetles' behaviour for 5 minutes at set time points: 30 minutes, 1 hour, 3 hours, 6 hours and 12 hours post-manipulation. During these observations I categorised their behaviour as in the cGMP manipulation experiments:

Direct care Direct feeding of the larvae. The beetle was in or on the larvae inside the carcass. The beetle was seen responding to begging behaviour from the larvae.

Indirect care: The beetle was on the carcass but not with or responding to the larvae. The beetle was inside the crypt around the carcass.

Not caring: The beetle was in the soil away from the carcass and larvae

I also noted if and when the beetle had killed larvae, I noted how many had been killed and if any new larvae had arrived since the last observation.

I analysed the data using a non-parametric ANOVA (Wilcoxon test) given the non-normal distribution of data. Parametric and non-parametric tests, however, give identical results (parametric results not shown).

RESULTS

I found that cannibalism was affected by treatment group, but the level of care provided did not vary between groups (Fig. 2, table 3). There was a significant increase in cannibalism in the Ringers control group, and no difference between the oxytocin group and the handling control ($\chi 2 = 8.004$, df = 2, P = 0.018).

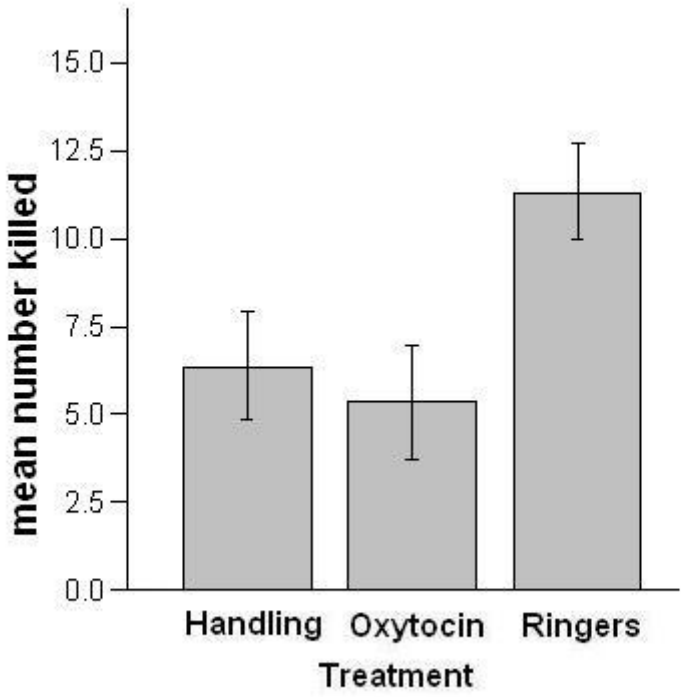

Fig. 2: Mean numbers of larvae killed out of 15 larvae, 12 hours after treatment, each beetle started with a brood of 15 larvae. Error bars show ± 1SE..

None of the groups provided direct care and there was no difference in the levels of indirect care provided ($\chi 2 = 2.873$, df = 2, P = 0.238). (Fig. 3, table 4).

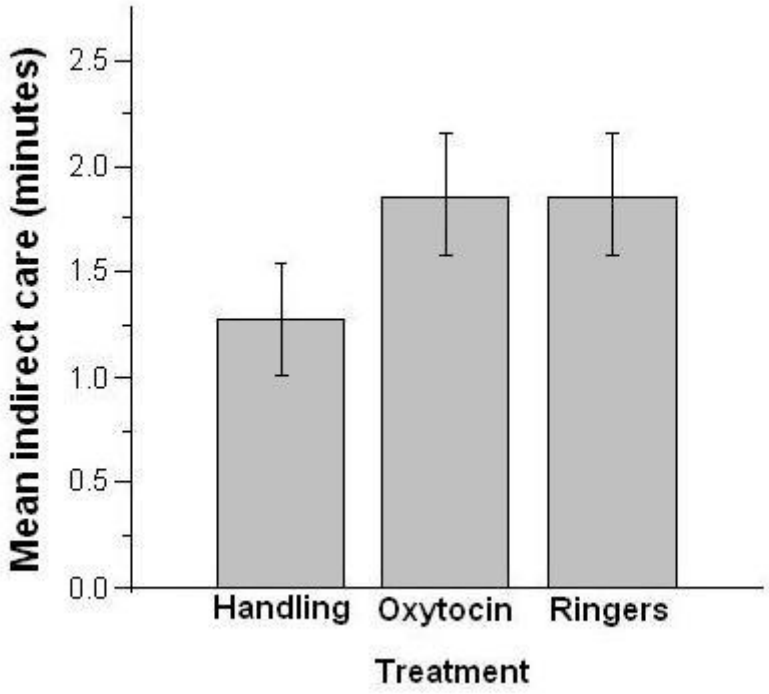

Fig. 3: mean time spent performing indirect care by each treatment group. Error bars show ± 1SE.

DISCUSSION

There was no significant effect of treatment on the amount or type of care given, in all treatment groups no time was spent giving direct care, and there was no difference in the amounts of indirect care given. There was an effect on the number of larvae cannibalised; beetles in the Ringers buffer control group killed significantly more larvae than the handling control or the oxytocin treated group.

Effects on cannibalism

These results show a clear difference between the ringers control group and the other two treatments. There are two possible explanations for the effect: Either Ringers has no effect and the handling and oxytocin treatments both reduce the normal levels of cannibalism. Or, the handling control group shows the normal levels of cannibalism and the act of injection increases cannibalism, which is counteracted by the oxytocin treatment.

The first explanation fits with previous studies, which have shown that usually a beetle will try to take over a breeding resource (Otronen 1988, Scott 2006a, Trumbo 2007). This

suggests that the high level of cannibalism seen in the Ringers group is normal. However, there is no physiological reason why handling would have an effect to reduce cannibalism when injection with Ringers doesn't, especially as injection with oxytocin had the same effect as the handling control.

The second possibility, that there is a negative effect of being injected and that oxytocin mediates this negative effect, assumes that the level of cannibalism seen in the handling control group is normal, which is inconsistent with previous research (Otronen 1988, Scott 2006a, Trumbo 2007). This explanation does not fit with my previous results of neutral effects of injection with Ringers buffer (chapter 3). Although this disparity may be due to the timing of the injections; in the cGMP manipulations, the beetles were already well into their caring phase, in this experiment the beetles were not yet caring. If this is the case the difference in response to injections between these experiments suggests that whilst the beetles are in the caring stage they are more robust to interference and can overcome or ignore the negative effects of a manipulation. This fits with their ability to cope with physiological stress that the beetles are under during parental care, and their willingness to continue to care despite injuries from defending the carcass (Otronen 1988, Trumbo and Valletta 2007, Creighton *et al.* 2009).

If the apparent effect of oxytocin that I observed is real, it suggests that it is not influential on the levels of care given, as there was no difference between treatment groups in the amount of time spent in indirect or direct care. The difference was only present in the number of larvae cannibalised. This suggests that the role of oxytocin may be to reduce aggression rather than to increase caring behaviour.

It is important to remember that the small sample size (Handling N=14, Oxytocin N=15, Ringers N=15) of this experiment may have had an influence on the results; it is possible that there is no effect of oxytocin or the effect is different to what I have observed. If these results are simply an artefact of a small sample size, the difference between the results of this experiment and the reality (whatever that may be) of the role of inotocin and the effects of oxytocin. Small sample sizes are especially vulnerable to extreme values. However, the fact that the parametric and non-parametric results were the same provides some small argument against a sample size or outlier effect.

Measuring the wrong thing/ at the wrong time

If oxytocin does have an effect on some aspect of parental behaviour, it is possible that I have been measuring the wrong component, or at the wrong time. In other species oxytocin has been shown to influence virtually every aspect of breeding; mate choice, egg laying, nest building, care giving, and parent/offspring bonding etc. This experiment only measured any effect on care giving and propensity to cannibalise, if the effect on oxytocin was on some other aspect of parenting I would not have measured or recorded it.

Wrong form of oxytocin/dosage

It is also possible that the form or dosage of oxytocin I used was not reactive in burying beetles. I used mammalian oxytocin and Stafflinger *et al* (2008) found that although the genetic and peptide sequences were different to inotocin, the inotocin receptor was still activated by mammalian oxytocin. The required dosage of oxytocin in cell culture was high ($EC_{50} > 10^{-6}$ M, roughly 10µg/ml) and the dosage I used was 15µg per beetle, it is possible that this fell below the activity threshold.

No inotocin gene

Although it is not a particularly parsimonious explanation, it is also possible that like 17 of the 20 species tested by Stafflinger *et al* (2008) burying beetles do not have the gene encoding inotocin in their genome. At some point in the evolution of those species the inotocin gene was lost from the genome: Stafflinger *et al* (2008) constructed a phylogeny and suggest that the gene encoding inotocin was lost once around 350 million years ago and again around 50 million years ago.

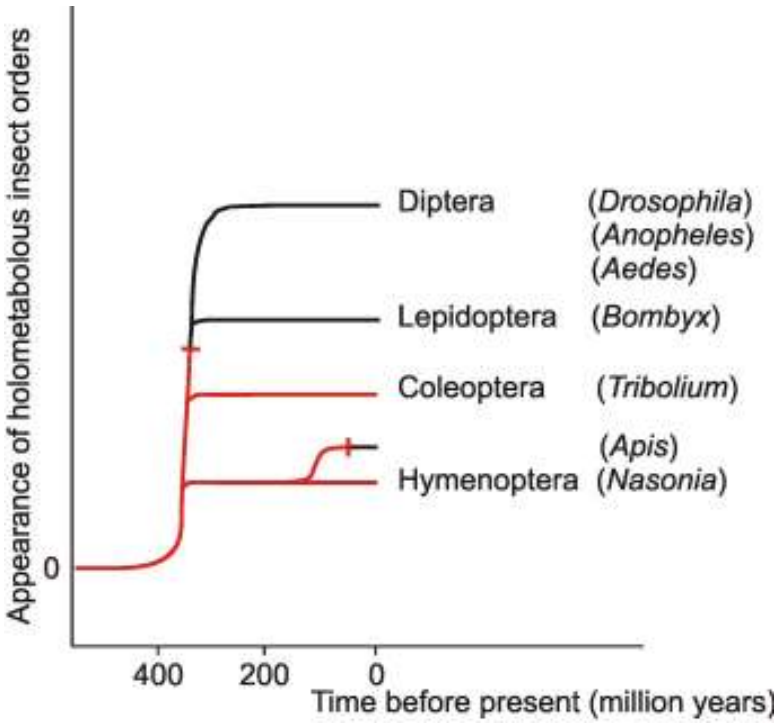

Fig. 4: Schematic representation of the appearance of the major orders of holometabolous insects and the occurrence of the inotocin hormonal system (highlighted in red). This hormonal system has been conserved only in the evolutionary lines leading to basal holometabolous insects: Coleoptera (beetles) and Hymenoptera (wasps). The inotocin system must have been abandoned at least two times during the evolution of the Holometabola (taken from Stafflinger *et al* 2008)

Given that the gene encoding inotocin has been identified in *Tribolium*, if it is the case that this gene no longer exists in burying beetles then it must be due to an independent loss of the gene. This is possible as *Tribolium* and *Nicrophorus* are distantly related beetles, sharing a common ancestor around 200 million years ago (Farrell 1998, Hunt *et al.* 2007b, Whitfield and Kjer 2008). It is entirely possible that during the time since the lineages split the lineage containing *Nicrophorus* lost the gene for inotocin, as this has happened in the hymenoptera lineage over a far shorter time-scale. A Western blot, with antibody probes designed to recognise inotocin and conserved regions that are shared with other oxytocin/vasopressin like peptides, may reveal the presence of inotocin or a related protein.

Conclusion

Negative results are always hard to interpret. Further, the small sample size makes it difficult to be confident in the soundness of the result. However, these results suggest an effect of oxytocin making non-caring beetles less aggressive towards larvae. This certainly warrants further investigation, it would be particularly informative to definitively answer whether the gene encoding inotocin is present in the burying beetle genome, and if so,

further manipulative experiments using inotocin or high doses of oxytocin would demonstrate the role of the hormone in burying beetle behaviour.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

Summary of findings

Overall I have shown that *Nvfor* plays a role in controlling aspects of parental behaviour in burying beetles, and that it is also linked to behavioural changes in larvae. I have also shown there is a possible role of inotocin in parental behaviour in adult beetles.

The work I have presented in chapters 2 and 3 demonstrates that *Nvfor* plays a role in regulating behaviour in adult burying beetles. Increased expression of *Nvfor* is linked to the switch to caring behaviour, and pharmacologically increased PKG activity increases the amount and duration of direct parental care. In chapter 4 I have shown that *Nvfor* is also associated with developmental changes in behaviour in larvae, where expression of *Nvfor* increases when larvae are dispersing. The data I have presented in chapter 5 suggests inotocin may also play a role in controlling social interactions between parents and offspring. The results were unclear, however treatment with oxytocin may have reduced the amount of cannibalism of a foster brood.

Signature of a genetic influence that affects social interactions

The *foraging* gene plays a role in the control of behaviour in several social insects, though the exact mechanism of this control remains unknown, it may have evolved several times since eusociality has evolved separately multiple times (Wilson 1971). Burying beetles are the first non-hymenopteran species with parental care to have been studied with regards to the effects of the *foraging* gene, the patterns of expression and behaviour are consistent with those seen in harvester ants (Ingram *et al*. 2005) and paper wasps (Toth *et al*. 2007). Although the *foraging* gene influences the transition from caring to foraging in honey bees the expression patterns seen in honey bees (Ben-Shahar *et al*. 2002, Ben-Shahar *et al*. 2003, Whitfield *et al*. 2003, Ben-Shahar 2005, Whitfield *et al.* 2006) are the inverse.

The only species in which the role of the *foraging* gene in influencing larval behaviour has been studied extensively are all species of *Drosophila,* where there are patterns of gene expression and PKG activity that are consistent with the results I have found in the beetle larvae. In *D. melanogaster* larvae, allelic variation of *for* leads to different expression levels of PKG. Larvae with lower levels of PKG activity wander less and pupate lower than those with higher levels of PKG activity (Sokolowski and Hansell 1983, de Belle and Sokolowski 1987, Osborne *et al*. 1997). In beetle larvae the behavioural shift from begging/feeding to wandering before pupation is correlated with an increase in *Nvfor* expression.

Despite the fact that the overall network of genes controlling social and parental behaviour remains something of a black box, it is clear that the *foraging* gene plays a central role as a node in the network of genes that influence social and parental behaviour in invertebrates.

Part of pathways

The *foraging* gene encodes PKG, which is involved in a large number of signalling systems. These signalling systems have been better characterised in vertebrate systems than in invertebrate systems (Lohmann *et al.* 1997). A large number of the signalling systems that involve PKG are neurological, PKG influences neurotransmission by regulating Ca^{2+} (Lohmann *et al.* 1997). Effects in knockout mice include diminished vestibule-ocular reflex, enhanced fear and diminished nociception (Aley *et al.* 1998, Lewin and Walters 1999, Schmidtko *et al.* 2003, Schlossmann *et al.* 2005). Research in *C. elegans* has shown that *egl-4*, the *C. elegans* orthologue of the *foraging* gene is involved in several pathways including olfactory function, learning (L'Etoile *et al*. 2002, Lee *et al.* 2006a), satiation response (You *et al*. 2008) and quiescence (Ghosh and Emmons 2010). Whitfield *et al* (2003 & 2006) and Toth *et al.* (2007 & 2009) found that there are multiple genes changing in expression in honey bees and paper wasps (respectively) in relation to changes in caring/social behaviour, several of these suggest involvement of other systems, such as insulin pathways, olfactory function, learning and neural structure (Whitfield *et al*. 2003, Whitfield *et al*. 2006, Toth *et al.* 2007, Toth *et al.* 2009).

Given the high levels of conservation of function we have seen so far in the various orthologues of the *foraging* gene, it is likely that the various orthologues of the *foraging* gene are involved in several pathways as part of a network of genes that result in the observed social and parental behaviours. The ubiquity of the effect of the *foraging* gene and its orthologues suggests it may be a key node in this pathway.

Parallel Evolution

Given that the *foraging* gene has a function in influencing similar social and parental behaviours in many unrelated species, it is possible that the *foraging* gene has been adapted several times to fit this role. Stern and Orgogozo (2008, 2009) discuss the predictability of evolution, and how genetic evolution is constrained by pre-existing gene functions and networks as well as population biology. The pre-existing role of a gene in a regulatory network may influence which genes in that network are more likely to acquire

evolutionarily relevant mutations. The non-random distribution of evolutionarily relevant mutations can result in parallel evolution, where entirely isolated populations of a species acquire the mutations in the same genes that alter gene function in the same way. For example, DDT and pyrethroid resistance has evolved in 11 insect species by mutations in the gene *para* at either the amino acid Leu^{1014} or Thr^{929} (ffrench-Constant *et al.* 1998). Even in networks where multiple genes can influence a trait, some genes are more likely to produce and evolutionary change, for example hundreds of genes regulate trichome pattern on *D. melanogaster* larvae, but only one gene has evolved to alter trichome pattern between Drosophila species (McGregor *et al.* 2007), such genes are known as hotspot genes, due to the accumulation of multiple evolutionarily relevant mutations. Gene function, structure and the role of the gene and its products in networks all affect whether particular mutations will contribute to phenotypic evolution, so for some traits, the evolutionarily relevant mutations will accumulate within a few hotspot genes Stern and Orgogozo (2008, 2009).
It is possible that the *foraging* gene is one of these hotspot genes, where due to its role in networks it is more prone to mutations and more easily adapted to new roles.

Not a gene for parental care

Although the evidence I have presented shows that *Nvfor* has a strong influence on parental care, it is not a "gene for parental care". Rather it is likely to be a node in a network of interacting genes that influence the expression of parental care in burying beetles. This is an important distinction, as the *foraging* gene is in no way predictive of parental care. For example *C. elegans* is perhaps the most studied species with regards to *foraging* gene orthologues (*egl-4*) and although *egl-4* influences many different aspects of behaviour, they do not have parental care (Daniels *et al*. 2000, Fujiwara *et al*. 2002, L'Etoile *et al*. 2002, You *et al*. 2008, Ghosh and Emmons 2010, Lee *et al*. 2010).

The role of the *foraging* gene in the network of genes controlling parental care in burying beetles does seem to be quite substantial, as evidenced by the major changes in behaviour after treatment with cGMP (chapter 3). As it is currently unclear what the other genes involved in this network are it is impossible to know the exact role of the *foraging* gene in it, though some aspects are clear: whatever controls the levels and provision of indirect care is not influenced by the *foraging* gene.

Direct and Indirect care

An important feature of the expression data and the results of the cGMP manipulations in adults is that the effects were limited to direct care, and there was no effect on indirect care. This is not consistent with the results of Walling *et al.* (2008), where direct and indirect care evolve at the expense of each other, that is, an increase in one trait results in a decrease in the other. This apparent non-involvement of *Nvfor* in the control of indirect care suggests that there are two separate systems controlling direct and indirect care. All parenting behaviour is triggered by the discovery of a suitable carcass (Oldekop *et al*. 2007), so it is possible that there is a single triggering mechanism which induces changes in separate networks of genes that control the provision of direct and indirect care.

Co-opted in social evolution

Social behaviour in insects has evolved independently several times (Wilson 1971), and in all the species studied so far, the *foraging* gene has played some role in influencing aspects of social behaviour. There is something about the ancestral role of the *foraging* gene and the genes with which it interacts that makes them "easy targets" for selection to generate and control aspects of social behaviour.

In the non-social species that have been studied, the *foraging* gene seems to influence a lot of systems associated with foraging; olfactory function (Lee *et al*. 2010), movement behaviour (Osborne *et al*. 1997) and satiation response (You *et al*. 2008). In *Diabrotica virgifera virgifera, Dvfor* plays a role in controlling oviposition behaviour (Garabagi *et al.* 2008), which may be influenced by altered food recognition systems, as the variant behaviour lays eggs in fields with soybeans. Soybeans are not a suitable host plant for larvae of this species.

In social species, the *foraging* gene plays a role in controlling aspects of social behaviour that are also food-related, foraging and provisioning the young. If, for example, satiation response is altered so that workers continue to forage for food beyond what is necessary, the surplus can be used to provision other individuals in the nest. Satiation has been shown to influence foraging behaviour in honey bees (Ben-Shahar and Robinson 2001), so it is possible that part of the systems controlling foraging behaviour in social insects are adaptations of ancestral food-related systems.

The *foraging* gene and developmental changes

Another key aspect of the work I have presented in chapter 4 is the correlation of changed expression levels of *Nvfor* with developmental changes and the associated behavioural changes in larvae. Ben-Shahar *et al* (2003) demonstrated that it is possible to manipulate PKG activity and doing so induced behaviour associated with an older developmental stage. I have demonstrated that this approach also works in burying beetles (chapter 3) and that although the change in behaviour of burying beetles is reversible and not associated with developmental changes the manipulations with cGMP do induce behaviour associated with high expression of *Nvfor*. Larval changes in behaviour are associated with development and I have shown that there is a large increase in *Nvfor* expression as larvae change from relatively sedentary feeding behaviour to non-feeding pre-pupation wandering.

Future work

From the work I have presented there are many avenues for further research on the genetics of social behaviour and parent/offspring interactions in both adult and larval burying beetles.

cGMP in larvae: The most obvious piece of research that my work leads to is pharmacological manipulations of larvae using cGMP. The pattern of expression of *Nvfor* in larvae suggests a link between increased expression and the change from begging/feeding to wandering. A similar experiment to my work on adults would show whether this is a causative link or purely correlative. There are several challenges to working with larvae as administering an injection may prove difficult due to the softness of the larva and feeding may not give a suitable dose. Another issue is identification of larvae between treatment groups, as once they have been returned to the carcass they will be indistinguishable from each other. Marking individuals through paint spots or leg clipping is not appropriate as the growth of burying beetle larvae is so fast that they will moult several times during an experiment. There has been some success in staining live mosquito larvae with dyes known to be biologically inert (Silver 2008), however whether these methods translate to beetle larvae is currently unknown.

RNA interference: Another method for verifying the role of a gene in the expression of a phenotype is RNA interference (RNAi); this can be used to knock down expression of the target gene making it possible to see any effects of reduced expression level on behaviour.

The most challenging aspect of RNAi is developing the correct sequence for the interfering RNA so that it binds to the target RNA. But once this has been done it is reasonably straightforward to administer the RNA via injection (Bucher *et al.* 2002, Tomoyasu and Denell 2004). In the case of *Nvfor,* based on the results of the cGMP manipulations, I would expect knocked down expression to reduce the amount of direct care given and decrease the duration of care, or possibly induce spontaneous abortion/abandonment of the brood.

Other candidate genes: Identifying other genes that are likely to influence burying beetle behaviour is a matter of searching through the existing literature on the genetics of insect behaviour. The volume of literature on the matter is constantly increasing. Genes that have already been identified in honey bees and paper wasps as having correlated changes in expression, they include *fax* (axonogenesis, transmission of nerve impulse), *tun* (olfactory learning) *NPF* (feeding and coordination of behavioural changes), circadian genes such as *per* and *tim*, and genes involved in insulin pathways such as *IRS* (insulin receptor substrate), *ILP2* (insulin-like peptide 2) and *InR1* (Insulin-like receptor 1) (Whitfield *et al.* 2003, Whitfield *et al.* 2006, Toth *et al.* 2007, Toth *et al.* 2009). Once a likely candidate has been chosen, it needs to be cloned, the cloned product then needs to be identified as the target gene, and finally it is possible to test for expression differences and, if possible, manipulate the gene or gene product to test for a causative effect (Fitzpatrick *et al.* 2005).

Next generation sequencing: One of the major challenges of investigating the genetics of the burying beetles has been the lack of sequence information, with the increasing ease and decreasing cost of high-throughput sequencing a major boon to future work would be to sequence the transcriptome. Once this has been done it will become much easier to identify genes by comparison to insects with sequenced genomes, and to select and investigate a large numbers of genes by selecting likely candidates from genes known to be linked with similar behaviours in other insects (Whitfield *et al.* 2003, Whitfield *et al*. 2006, Toth *et al.* 2007, Toth *et al.* 2009). Methods such as microarrays can then be used to investigate expression levels of a large number of genes at once, and to compare the expression levels across several behavioural states.

Other approaches: There are other approaches available for further studying the role of *Nvfor*. For example it has been shown that direct care is an evolvable trait (Walling *et al.* 2008), so it should be possible to create selection lines for high and low levels of direct

care. As I have established reliable protocols for testing *Nvfor* expression levels it would be straightforward to investigate if the differences in behaviour are linked with differing levels of *Nvfor* expression. Investigation of the genetics in the selection lines might also reveal whether there is allelic variation of *Nvfor* between the lines, as seen in *D. melanogaster* (Sokolowski 1980a, Sokolowski and Hansell 1983, Osborne *et al.* 1997) and *D. virgifera virgifera* (Garabagi *et al.* 2008).

Conclusions

Dramatic changes

It is becoming increasingly clear that the *foraging* gene plays an important role in influencing behaviour in invertebrates. The behavioural changes that the *foraging* gene influences are major changes in behaviour, whether they are linear developmental changes as seen in honey bees (Ben-Shahar *et al.* 2002, Ben-Shahar *et al*. 2003, Whitfield *et al.* 2003, Ben-Shahar 2005, Whitfield *et al*. 2006) or reversible changes such as those that I have investigated in burying beetles

The *foraging* gene is not a gene that is predictive of social or parental behaviour, but it is a node in a network of genes that have been adapted in some species to generate social and parental behaviours. I have shown that *Nvfor* plays an important role in the gene network controlling parental care in burying beetles, as the effects of manipulating PKG activity are dramatic changes in behaviour. My results are consistent with previous research in other insects, that have shown that the *foraging* gene plays a key role in influencing behaviour in insects (Sokolowski 1980a, Sokolowski and Hansell 1983, Osborne *et al*. 1997, Whitfield *et al*. 2003, Ben-Shahar 2005, Ingram *et al*. 2005, Whitfield *et al*. 2006, Toth *et al*. 2007, Garabagi *et al*. 2008, Lucas and Sokolowski 2009, Toth *et al*. 2009, Sokolowski 2010)

APPENDIX I

At the start of this project the intention was to clone several candidate genes, which had been identified as good targets though literature searches.
I used the following primers in touchdown PCR using cDNA generated from pooled tissues from male and female beetles, pupae and larvae.

Failed axon connections

FAX is associated with axonogenesis and has been shown to have different expression levels at different life stages in honey bees and paper wasps (Whitfield *et al.* 2003, Toth and Robinson 2007). These degenerate primers were based on published sequences from *D. melanogaster, D. virillis, A. mellifera* and *T. castaneum*.

FAX.FWD1 ARNTNAAYGNGARGARATHGC
FAX.FWD2 AAYGGNGARGARATHGCNGA

FAX.REV1 GTNGGYTCMTCNCCRAARAARAANGG
FAX.FWD2 TCRTCCCARTCNGGRAARCA

Neuropeptide F

NPF is associated with food seeking behaviour in *C. elegans* and was identified as a gene with significant role in feeding behaviour in Drosophila larvae (Lee *et al.* 2006a). In *C. elegans* NPR-1 (a receptor to the orthologue of NPF), has two isoforms responsible for regulating social or solitary feeding (Coates and de Bono 2002). These degenerate primers were designed based on published sequences from *D. melanogaster, A. gambiae* and *T. castaneum.*

AGM62: GGTTACCATCACGACATCAACG
AGM66: ACTCATTTTATTGCGGTTGAG

NPF.FWD1 GAYTTYGAYGCNAARCCNATG
NPF.FWD2 GAYMGNGGNTAYGAYTTYCANGG

NPF.REV1 TCYTTNCCYAANACNSWNGGCAT
NPF.REV2 CKNCGNCWYTTDATNARRTC

Period

Period is a circadian clock gene, changes in expression are associated with changes in behaviour in honey bees (Toma *et al.* 2000, Bloch *et al.* 2001) and mating behaviour in female Drosophila. Photoperiod has been linked with timing of behavioural changes in *N. vespilloides,* and the circadian genes have been suggested as possible candidates for involvement in this process (Oldekop *et al.* 2007). These degenerate primers were designed based on published sequences from *T. castaneum.*

deg5 59-CCCGAATTCATGGARACNYTNATGGAYGA-39
deg3 59-CCCGAATTCRTCRTARTARTCRTGRTG-39

Timeless

Like *period*, *timeless* is a circadian clock gene, which have been proposed as candidates in the control of the timing of parental care in *N. vespilloides* (Oldekop *et al.* 2007). *Tim* has also been linked with reproductive behaviour in Drosophila (Beaver *et al.* 2002, Beaver and Giebultowicz 2004). These degenerate primers were designed based on published sequences from *D. melanogaster, T. castaneum, B. mori* and *A. aegypti.*

5TimDeg3: AARGARTTYACNGTNGAYTT.
3TimDeg3: GTNACNARCCARAARAARTG.

5TimDeg6: AAYGAYTGYATHTTYAC.
3TimDeg6: KTRTGDATNACRTAYTC.

5TimDeg1: GGNMGNCAYACNATHTTYGA.
5TimDeg2: GAYTGYGGNTAYGGNACNCA.

3TimDegCG1: ARYTTNACRAARCARCA.
3TimDegend1: TCRTCYTCRTCNSWNACRTACAT

Transferrin

The primers designed to amplify *period* for produced a product approximately 800 bp long, with the following sequence:

CCCGAATTCATGGAGACGTTTATGGACGATTGCAAAGAGATGATTCAACAGA
AAACTAAAGCCACCGCTAAGATCGTTTGCATCCCAGCTAGAGACAGAATTGA
ATGCATCGAGAAGATCAAGGAACATGTAGCTGATTTCGGTATGGTGGATCCT
GAAGACATGTACGTCGCCGCTAAACTTCCAGACAGCGATTTTCAGGTTTTCGA
AGAAATCCGCACCATCGAAGAACCTGAAGCCGAGTTCAGGTATGAAGGTGTC
GCTGTCGTGCACAAAGATTTGGAGATCAACAGCGTTCAAGGCTTGAAAGGTT
TAAGATCTTGTCATACCGGAGTGGGCAGAAATGTGGGATACAAAATCCCCTT
GACCAAGTTGAAGAACATGGGCATTATTGGTAATTTAGCCGAACCCACTTTAT
CGCCACGCGAAAACGAACTAGAAGCCTTCTCCAAGCTCTTCTCTAAGGCTTG
CATCGTCGGAAAATGGTCCCCTGATGCAGACATTGACTCCAAAATGAAGAAA
CGCTTCAGCAATTTGTGCGAGCTTTGCGAGCACCCAGACAAATGCGACTACC
CAGATAACTTCTCCGGTTACGACGGTGCTTTGAGATGCTTGGCCCATAACAA
CGGTCAAATCGCCTGGACCAAGGTCATCTACGTCCGCAAGTTCTTCGGTCTT
CCTGTTGGAATAACTCCTGGTCAACCCAGCGCCGAGAACCCAGACAATTTCG
CTTACTTCTGCCCAGATGGTTCCAAGGTACCAATCACCGGAACTCCATGCAG
ATGGGCCGCTCGTCCATTAACGTTTCCATGAATTCGGGA

I searched published gene sequences using BLAST (Basic Local Alignment Search Tool, from NCBI) and identified it as transferrin, an iron transport protein. Transferrin is associated with basic immune function as well as some stress responses (Lee *et al.* 2006b). From this sequence I was unable to generate reliable primers or probes for gene expression assays, so I did not pursue this as a further avenue in my thesis research. The unsuccessful primers are listed below:

TF Fwd: TCGCCGCTAAACTTCCAGACAG

TF Rev: CATACTTCCACAGCGACAGCACG

T1F: TACGTCGCCGCTAAACTTCCAGA

T1R: TCCAGGCGATTTGACCGTTGTTA

T2F: AATTTAGCCGAACCCACTTTATC

T2R: GACGAGCGGCCCATCTGC

T3F: TCGGTATGGATCCTGAAGACA

T3R: CTCGCAAAGCTCGCACAAAT

T4F: TCGGTATGGTGGATTCCTGAAGAC
T4R: AGGCGATTTGACCGTTGTTATGG

T5F: TTTGCATCCCAGCTAGAGACAGAA
T5R: AGAACTTGCGGACGTAGATGACCT

Inotocin and Inotocin receptor,

In chapter 5 I investigate the role of the insect oxytocin orthologue, inotocin. I attempted to clone the *N. vespilloides* orthologue of inotocin using the following primers, the non-degenerate primers are taken from the work by Stafflinger *et al* (2008), the degenerate primers were developed using Inotocin and other Oxytocin orthologue sequences. The degenerate primers were used in every possible combination of forward and reverse pairs.

Inotocin
INO_FWD CAACACAACCAACTGCACC
INO_REV CAATTGCTCAAAAGTTCTTCACACAC

INO_DEG_FWD1 GGNTGYYTNATHCANAAYTGYCC
INO_DEG_FWD2 CANAAYTGYCCNSGNGGNGGNAA
INO_DEG_FWD3 TGYYTNATHCANAAYTGYCCNSGN
INO_DEG_FWD4 TGYTTYGGNCCNHVNATHTGYTG
INO_DEG_FWD5 GGNTGYYTNATHCANAAYTGYC

INO_DEG_REV1 CARCADATNCCRTYNRMNGC
INO_DEG_REV2 ATNCCRTYNRMNGCRCANCSNCC
INO_DEG_REV4 CADATNCCRTYNRMNGCRCA
INO_DEG_REV3 CCRTYNRMNGCRCANCSNCC
INO_DEG_REV5 RCADATNBDNGGNCCRAARCA
INO_DEG_REV6 RMNGCRCANCKNCCNGCNAYRCA

Inotocin receptor

INRE_FWD CCGCTAGCCCGATGTACACCCCGAAAC

INRE_REV CCGCTAGCTCAGGTGGTCGTGACGATC

INRE_DEG_FWD1 GTNYTNCCNCARYTNGCNTCGGA

INRE_DEG_FWD2 YTNWSNWSNTAYGTNYT

INRE_DEG_FWD3 GNGCNTAYGTNACNTCGT

INRE_DEG_FWD4 RARMGNGCNTAYGTNCANTCG

INRE_DEG_FWD5 CARYTNTGGGCNCANTGGGAYCC

INRE_DEG_REV1 TGYGTYTTNACNGTRTTDATYTT

INRE_DEG_REV2 TGYTTNACNGTRTTDATYTTNGC

INRE_DEG_REV3 GGRTCCCANGTNGCCCANARYTG

INRE_DEG_REV4 TADATCCANGGRTTNACRCA

INRE_DEG_REV5 RTADATCCANGGRTTNACRCA

BIBLIOGRAPHY

Acher, R., and J. Chauvet. 1953. La Structure de la vasopressin de boef. Biochimica et Biophysica Acta **12**:487-488.

Acher, R., J. Chauvet, M. T. Lenci, Morel, and J. Maetz. 1960. Presence d'une vasotocine dans la neurohypophyse de la grenouille (*Rana esculenta L.*). Biochimica et Biophysica Acta **42**:379-380.

Acher, R., M. T. Chauvet, D. Crepy, and J. Chauvet. 1962. Isolement d'une nouvelle hormone neurohypophysaire, l'isotocine, presente chez les poissons osseux. Biochimica et Biophysica Acta **58**:624-625.

Aley, K. O., G. McCarter, and J. D. Levine. 1998. Nitric oxide signaling in pain and nociceptor sensitization in the rat. Journal of Neuroscience **18**:7008-7014.

Archer, M. S. 2000. Natural history observations of the native carrion beetle, *Ptomaphila lacrymosa* Schreibers (Coleoptera: Silphidae). Proceedings of the Royal Society of Victoria **112**:133-136.

Beaver, L. M., and J. M. Giebultowicz. 2004. Regulation of copulation duration by period and timeless in *Drosophila melanogaster*. Current Biology **14**:1492-1497.

Beaver, L. M., B. O. Gvakharia, T. S. Vollintine, D. M. Hege, R. Stanewsky, and J. M. Giebultowicz. 2002. Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. Proceedings of the NationalAcademy of Sciences of the U S A **99**:2134-2139.

Ben-Shahar, Y. 2005. The foraging gene, behavioral plasticity, and honeybee division of labor. Journal of Comparative Physiology A-Neuroethology Sensory Neural and Behavioral Physiology **191**:987-994.

Ben-Shahar, Y., H. T. Leung, W. L. Pak, M. B. Sokolowski, and G. E. Robinson. 2003. cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor. Journal of Experimental Biology **206**:2507-2515.

Ben-Shahar, Y., A. Robichon, M. B. Sokolowski, and G. E. Robinson. 2002. Influence of gene action across different time scales on behavior. Science **296**:741-744.

Ben-Shahar, Y., and G. E. Robinson. 2001. Satiation differentially affects performance in a learning assay by nurse and forager honey bees. Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology **187**:891-899.

Bloch, G., D. P. Toma, and G. E. Robinson. 2001. Behavioral rhythmicity, age, division of labor and period expression in the honey bee brain. Journal of Biological Rhythms **16**:444-456.

Boake, C. R. B., S. J. Arnold, F. Breden, L. M. Meffert, M. G. Ritchie, B. J. Taylor, J. B. Wolf, and A. J. Moore. 2002. Genetic tools for studying adaptation and the evolution of behavior. American Naturalist **160**:S143-S159.

Bucher, G., J. Scholten, and M. Klingler. 2002. Parental RNAi in Tribolium (Coleoptera). Current Biology **12**:R85-R86.

Butt, E., J. Geiger, T. Jarchau, S. M. Lohmann, and U. Walter. 1993. The cGMP-dependent protein-kinase - gene, protein, and function. Neurochemical Research **18**:27-42.

Calderone, N. W., and R. E. Page. 1988. Genotypic variability in age polyethism and task specialization in the honey bee, *Apis mellifera* (Hymenoptera, Apidae). Behavioral Ecology and Sociobiology **22**:17-25.

Calderone, N. W., and R. E. Page. 1992. Effects of interactions among genotypically diverse nestmates on task specialization by foraging honey bees (*Apis mellifera*). Behavioral Ecology and Sociobiology **30**:219-226.

Carter, C. S. 1998. Neuroendocrine perspectives on social attachment and love. Psychoneuroendocrinology **23**:779-818.

Carter, C. S., A. C. Devries, and L. L. Getz. 1995. Physiological substrates of mammalian monogamy - the prairie vole model. Neuroscience and Biobehavioral Reviews **19**:303-314.

Chauvet, M. T., T. Colne, D. Hurpet, J. Chauvet, and R. Acher. 1983. A multigene family for the vasopressin-like hormones? Identification of mesotocin, lysipressin and phenypressin in Australian macropods. Biochemical and Biophysical Research Communications **116**:258-263.

Chauvet, M. T., D. Hurpet, J. Chauvet, and R. Acher. 1980. Phenypressin (Phe2-Arg8-vasopressin), a new neurohypophysial peptide found in marsupials. Nature **287**:640-642.

Cho, M. M., A. C. DeVries, J. R. Williams, and C. S. Carter. 1999. The effects of oxytocin and vasopressin on partner preferences in male and female prairie voles (*Microtus ochrogaster*). Behavioral Neuroscience **113**:1071-1079.

Choe, J. C., and B. J. Crespi. 1997. *Social Behaviour in Insects and Arachnids*. Cambridge University Press.

Clutton-Brock, T. 1991. *The Evolution of Parental Care*. Princeton University Press.

Coates, J. C., and M. de Bono. 2002. Antagonistic pathways in neurons exposed to body fluid regulate social feeding in *Caenorhabditis elegans*. Nature **419**:925-929.

Costa, J. T. 2006. *The Other Insect Societies*. Harvard University Press.

Costa, J. T., and T. D. Fitzgerald. 1996. Developments in social terminology: Semantic battles in a conceptual war. Trends in Ecology & Evolution **11**:285-289.

Creighton, J. C., N. D. Heflin, and M. C. Belk. 2009. Cost of reproduction, resource quality, and terminal investment in a burying beetle. American Naturalist **174**:673-684.

Crisci, J. V., M. M. Cigliano, J. J. Morrone, and S. Roigjunent. 1991. Historical Biogeography of Southern South-America. Systematic Zoology **40**:152-171.

Crook, T. C., T. Flatt, and P. T. Smiseth. 2008. Hormonal modulation of larval begging and growth in the burying beetle *Nicrophorus vespilloides*. Animal Behaviour **75**:71-77.

Cruz, L. J., V. Desantos, G. C. Zafaralla, C. A. Ramilo, R. Zeikus, W. R. Gray, and B. M. Olivera. 1987. Invertebrate Vasopressin Oxytocin Homologs - Characterization of peptides from *Conus geographus* and *Conus striatus* venoms. Journal of Biological Chemistry **262**:15821-15824.

Daniels, S. A., M. Ailion, J. H. Thomas, and P. Sengupta. 2000. *egl-4* acts through a transforming growth factor-beta/SMAD pathway in *Caenorhabditis elegans* to regulate multiple neuronal circuits in response to sensory cues. Genetics **156**:123-141.

Dawson-Scully, K., D. Bukvic, M. Chakaborty-Chatterjee, R. Ferreira, S. L. Milton, and M. B. Sokolowski. 2010. Controlling anoxic tolerance in adult *Drosophila* via the cGMP–PKG pathway. Journal of Experimental Biology **213**:2410-2416.

de Belle, J. S., A. J. Hilliker, and M. B. Sokolowski. 1989. Genetic localization of foraging (*for*) - a major gene for larval behavior in *Drosophila melanogaster*. Genetics **123**:157-163.

de Belle, J. S., and M. B. Sokolowski. 1987. Heredity of Rover Sitter - alternative foraging strategies of *Drosophila melanogaster* larvae. Heredity **59**:73-83.

du Vigneaud, V., H. C. Lawler, and E. A. Popenoe. 1953a. Enzymatic cleavage of glycinamide from vasopressin and a proposed structire for the pressor-antidiuretic hormone of the posterior pituitary. Journal of the American Chemical Society **75**:4880-4881.

du Vigneaud, V., C. Ressler, and S. Thrippett. 1953b. The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin. Journal of Biological Chemistry **205**:949-957.

Eggert, A.-K., and J. K. Müller. 2000. Timing of oviposition and reproductive skew in cobreeding female burying beetles (*Nicrophorus vespilloides*). Behavioural Ecology **11**:357-366.

Eggert, A., M. Reinking, and J. K. Muller. 1998. Parental care improves offspring survival and growth in burying beetles. Animal Behaviour **55**:97-107.

Eggert, A. K., and J. K. Muller. 1997. Biparental care and social evolution in burying beetles: Lessons from the larder. Pages 216-236 *The evolution of social behavior in insects and arachnids*. Cambridge University Press.

Eggert, A. K., and S. K. Sakaluk. 2000. Benefits of communal breeding in burying beetles: a field experiment. Ecological Entomology **25**:262-266.

Endler, J. A. 1986. *Natural Selection in the Wild*. Princeton University Press.

Engel, J. E., X. J. Xie, M. B. Sokolowski, and C. F. Wu. 2000. A cGMP-dependent protein kinase gene, foraging, modifies habituation-like response decrement of the giant fiber escape circuit in *Drosophila*. Learning & Memory **7**:341-352.

Fahrbach, S. E., J. I. Morrell, and D. W. Pfaff. 1984. Oxytocin induction of short latency maternal bhavior in nulliparous, estrogen-primed female rats. Hormones and behavior **18**:267-286.

Farrell, B. D. 1998. "Inordinate fondness" explained: Why are there so many beetles? Science **281**:555-559.

Fetherston, I. A., M. P. Scott, and J. F. A. Traniello. 1990. Parental care in burying beetles - The organization of male and female brood-care behavior. Ethology **85**:177-190.

ffrench-Constant, R. H., B. Pittendrigh, A. Vaughan, and N. Anthony. 1998. Why are there so few resistance-associated mutations in insecticide target genes? Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **353**:1685-1693.

Fitzpatrick, M. J., Y. Ben-Shahar, H. M. Smid, L. E. M. Vet, G. E. Robinson, and M. B. Sokolowski. 2005. Candidate genes for behavioural ecology. Trends in Ecology & Evolution **20**:96-104.

Fitzpatrick, M. J., and M. B. Sokolowski. 2004. In search of food: Exploring the evolutionary link between cGMP-dependent protein kinase (PKG) and behaviour. Integrative and Comparative Biology **44**:28-36.

Francis, S. H., and J. D. Corbin. 1994. Structure and function of cyclic nucleotide-dependent protein kinases. Annual Review of Physiology **56**:237-272.

Fujiwara, M., P. Sengupta, and S. L. McIntire. 2002. Regulation of body size and behavioral state of *C. elegans* by sensory perception and the EGL-4 cGMP-dependent protein kinase. Neuron **36**:1091-1102.

Garabagi, F., B. W. French, A. W. Schaafsma, and K. P. Pauls. 2008. Increased expression of a cGMP-dependent protein kinase in rotation-adapted western corn rootworm (*Diabrotica virgifera virgifera* L.). Insect Biochemistry and Molecular Biology **38**:697-704.

Ghosh, R., and S. W. Emmons. 2010. Calcineurin and protein kinase G regulate *C. elegans* behavioral quiescence during locomotion in liquid. Bmc Genetics **11**:9.

Goodson, J. L. 1998. Territorial aggression and dawn song are modulated by septal vasotocin and vasoactive intestinal polypeptide in male field sparrows (*Spizella pusilla*). Hormones and behavior **34**:67-77.

Goodson, J. L., and E. Adkins-Regan. 1999. Effect of intraseptal vasotocin and vasoactive intestinal polypeptide infusions on courtship song and aggression in the male zebra finch (*Taeniopygia guttata*). Journal of Neuroendocrinology **11**:19-25.

Goodson, J. L., and A. H. Bass. 2000. Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature **403**:769-772.

Goodson, J. L., and A. H. Bass. 2001. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. Brain Research Reviews **35**:246-265.

Goodson, J. L., S. E. Schrock, J. D. Klatt, D. Kabelik, and M. A. Kingsbury. 2009. Mesotocin and nonapeptide receptors promote estrildid flocking Behavior. Science **325**:862-866.

Hawkeswood, T. J., and J. R. Turner. 2008. Record of the Australian burying beetle, *Ptomaphila perlata* Kraatz, 1876 (Coleoptera: Silphidae) feeding and breeding in the dead carcass of a Swamp Wallaby (*Wallabia bicolor*, Mammalia: Macropodidae), with a review of its biology and habitat. Calodema **381**:1-5.

Hofmann, F., R. Feil, T. Kleppisch, and J. Schlossmann. 2006. Function of cGMP-dependent protein kinases as revealed by gene deletion. Physiological Reviews **86**:1-23.

Holtzhausen, W. D., and S. W. Nicolson. 2007. Beetle diuretic peptides: The response of mealworm (*Tenebrio molitor*) Malpighian tubules to synthetic peptides, and cross-

reactivity studies with a dung beetle (*Onthophagus gazella*). Journal of Insect Physiology **53**:361-369.

House, C. M., C. A. Walling, C. E. Stamper, and A. J. Moore. 2009. Females benefit from multiple mating but not multiple mates in the burying beetle *Nicrophorus vespilloides*. Journal of Evolutionary Biology **22**:1961-1966.

Hunt, G. J., G. V. Amdam, D. Schlipalius, C. Emore, N. Sardesai, C. E. Williams, O. Rueppell, E. Guzman-Novoa, M. Arechavaleta-Velasco, S. Chandra, M. K. Fondrk, M. Beye, and R. E. Page. 2007a. Behavioral genomics of honeybee foraging and nest defense. Naturwissenschaften **94**:247-267.

Hunt, T., J. Bergsten, Z. Levkanicova, A. Papadopoulou, O. S. John, R. Wild, P. M. Hammond, D. Ahrens, M. Balke, M. S. Caterino, J. Gomez-Zurita, I. Ribera, T. G. Barraclough, M. Bocakova, L. Bocak, and A. P. Vogler. 2007b. A comprehensive phylogeny of beetles reveals the evolutionary origins of a superradiation. Science **318**:1913-1916.

Ingram, K. K., P. Oefner, and D. M. Gordon. 2005. Task-specific expression of the foraging gene in harvester ants. Molecular Ecology **14**:813-818.

Insel, T. R. 2010. The challenge of translation in social neuroscience: A review of oxytocin, vasopressin, and affiliative behavior. Neuron **65**:768-779.

Insel, T. R., and L. E. Shapiro. 1992. Oxytocin receptor distribution reflects social-organization in monogamous and polygamous voles. Proceedings of the National Academy of Sciences of the U S A **89**:5981-5985.

Insel, T. R., and L. J. Young. 2001. The neurobiology of attachment. Nature Reviews Neuroscience **2**:129-136.

Jenkins, E. V., C. Morris, and S. Blackman. 2000. Delayed benefits of paternal care in the burying beetle *Nicrophorus vespilloides*. Animal Behaviour **60**:443-451.

Keverne, E. B., and J. P. Curley. 2004. Vasopressin, oxytocin and social behaviour. Current Opinion in Neurobiology **14**:777-783.

L'Etoile, N. D., C. M. Coburn, J. Eastham, A. Kistler, G. Gallegos, and C. I. Bargmann. 2002. The cyclic GMP-dependent protein kinase EGL-4 regulates olfactory adaptation in *C elegans*. Neuron **36**:1079-1089.

Lee, G. H., J. H. Bahn, and J. H. Park. 2006. Sex- and clock-controlled expression of the neuropeptide F gene in *Drosophila*. Proceedings of the National Acadademy of Science of the U S A **103**:12580-12585.

Lee, J. I., D. M. O'Halloran, J. Eastham-Anderson, B. T. Juang, J. A. Kaye, O. S. Hamilton, B. Lesch, A. Goga, and N. D. L'Etoile. 2010. Nuclear entry of a cGMP-

dependent kinase converts transient into long-lasting olfactory adaptation. Proceedings of the National Academy of Science of the U S A **107**:6016-6021.

Lee, K. S., B. Y. Kim, H. J. Kim, S. J. Seo, H. J. Yoon, Y. S. Choi, I. Kim, Y. S. Han, Y. H. Je, S. M. Lee, D. H. Kim, H. D. Sohn, and B. R. Jin. 2006b. Transferrin inhibits stress-induced apoptosis in a beetle. Free Radical Biology and Medicine **41**:1151-1161.

Lewin, M. R., and E. T. Walters. 1999. Cyclic GMP pathway is critical for inducing long-term sensitization of nociceptive sensory neurons. Nature Neuroscience **2**:18-23.

Li, B., R. Predel, S. Neupert, F. Hauser, Y. Tanaka, G. Cazzamali, M. Williamson, Y. Arakane, P. Verleyen, L. Schoofs, J. Schachtner, C. J. P. Grimmelikhuijzen, and Y. Park. 2008. Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red flour beetle *Tribolium castaneum*. Genome Research **18**:113-122.

Lim, M. M., Z. X. Wang, D. E. Olazabal, X. H. Ren, E. F. Terwilliger, and L. J. Young. 2004. Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. Nature **429**:754-757

Lincoln, T. M., N. Dey, and H. Sellak. 2001. Signal transduction in smooth muscle - Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. Journal of Applied Physiology **91**:1421-1430.

Lock, J. E., P. T. Smiseth, and A. J. Moore. 2004. Selection, inheritance, and the evolution of parent-offspring interactions. American Naturalist **164**:13-24.

Lohmann, S. M., A. B. Vaandrager, A. Smolenski, U. Walter, and H. R. DeJonge. 1997. Distinct and specific functions of cCMP-dependent protein kinases. Trends in Biochemical Sciences **22**:307-312.

Lohmann, S. M., and U. Walter. 2005. Tracking functions of cGMP-dependent protein kinases (CGK). Frontiers in Bioscience **10**:1313-1328.

Lucas, C., and M. B. Sokolowski. 2009. Molecular basis for changes in behavioral state in ant social behaviors. Proceedings of the National Acadademy of Sciences of the U S A **106**:6351-6356.

Lu, J. X., Q. Y. Bao, J. Y. Wu, H. Wang, D. Li, Y. L. Xi, S. Q. Wang, S. S. Yu, and J. Qu. 2008. CSCDB: The cAMP and cGMP signaling components database. Genomics **92**:60-64.

McGregor, A. P., V. Orgogozo, I. Delon, J. Zanet, D. G. Srinivasan, F. Payre, and D. L. Stern. 2007. Morphological evolution through multiple cis-regulatory mutations at a single gene. Nature **448**:587-U586.

Menzel, R., and U. Greggers. 1985. Natural phototaxis and its relationship to color vision in honeybees. Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology **157**:311-321.

Mery, F., A. T. Belay, A. K. C. So, M. B. Sokolowski, and T. J. Kawecki. 2007. Natural polymorphism affecting learning and memory in Drosophila. Proceedings of the National Acadademy of Science of the U S A **104**:13051-13055.

Muller, J. K., A. K. Eggert, and T. Elsner. 2003. Nestmate recognition in burying beetles: the "breeder's badge" as a cue used by females to distinguish their mates from male intruders. Behavioral Ecology **14**:212-220.

Muller, J. K., A. K. Eggert, and S. K. Sakaluk. 1998. Carcass maintenance and biparental brood care in burying beetles: are males redundant? Ecological Entomology **23**:195-200.

Nachman, M. W., H. E. Hoekstra, and S. L. D'Agostino. 2003. The genetic basis of adaptive melanism in pocket mice. Proceedings of the National Academy of Science on the U S A **100**:5268-5273.

Numan, M. 1988. Neural basis of maternal behavior in the rat. Psychoneuroendocrinology **13**:47-62.

Oldekop, J. A., P. T. Smiseth, H. D. Piggins, and A. J. Moore. 2007. Adaptive switch from infanticide to parental care: how do beetles time their behaviour? Journal of Evolutionary Biology **20**:1998-2004.

Osborne, K. A., A. Robichon, E. Burgess, S. Butland, R. A. Shaw, A. Coulthard, H. S. Pereira, R. J. Greenspan, and M. B. Sokolowski. 1997. Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. Science **277**:834-836.

Otronen, M. 1988. The effect of body size on the outcome of fights in burying beetles (*Nicrophorus*). Annales Zoologici Fennici **25**:191-201.

Oumi, T., K. Ukena, O. Matsushima, T. Ikeda, T. Fujita, H. Minakata, and K. Nomoto. 1994. Annetocin - an oxytocin-related peptide isolated from the earthworm, *Eisenia foetida*. Biochemical and Biophysical Research Communications **198**:393-399.

Page, R. E., J. Erber, and M. K. Fondrk. 1998. The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.).

Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology **182**:489-500.

Page, R. E., and M. K. Fondrk. 1995. The effects of colony level selection on the social-organization of honey-bee (*Apis mellifera* L) colonies - colony level components of pollen hoarding. Behavioral Ecology and Sociobiology **36**:135-144.

Peck, S. B. 2001. Review of the carrion beetles of Australia and New Guinea (Coleoptera : Silphidae). Australian Journal of Entomology **40**:93-101.

Pedersen, C. A., J. A. Ascher, Y. L. Monroe, and A. J. Prange. 1982. Oxytocin induces maternal behavior in virgin female rats. Science **216**:648-650.

Pereira, H. S., and M. B. Sokolowski. 1993. Mutations in the larval foraging gene affect adult locomotory behavior after feeding in *Drosophila melanogaster*. Proceedings of the National Acadademy of Science of the U S A **90**:5044-5046.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research **29**:6.

Proux, J. P., C. A. Miller, J. P. Li, R. L. Carney, A. Girardie, M. Delaage, and D. A. Schooley. 1987. Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. Biochemical and Biophysical Research Communications **149**:180-186.

Purves, W. K., D. Sadava, G. H. Orians, and H. C. Heller. 2001. *Life, The Science of Biology*, 6th edition. Sinauer Associates, Inc. W.H. Freeman and Company: 444-445.

Rauter, C. M., and A. J. Moore. 1999. Do honest signalling models of offspring solicitation apply to insects? Proceedings of the Royal Society of London Series B-Biological Sciences **266**:1691-1696.

Reich, G. 1992. A new peptide of the oxytocin vasopressin family isolated from nerves of the cephalopod *Octopus vulgaris*. Neuroscience Letters **134**:191-194.

Ringo, J., and D. Wood. 1983. Pupation Site Selection in *Drosophila simulans*. Behavior Genetics **13**:17-27.

Robbins, L. S., J. H. Nadeau, K. R. Johnson, M. A. Kelly, L. Rosellirehfuss, E. Baack, K. G. Mountjoy, and R. D. Cone. 1993. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. Cell **72**:827-834.

Robinson, G. E., and Y. Ben-Shahar. 2002. Social behavior and comparative genomics: new genes or new gene regulation? Genes, Brain and Behavior **1**:197-203.

Robinson, G. E., R. D. Fernald, and D. F. Clayton. 2008. Genes and social behavior. Science **322**:896-900.

Rondon, S. I., and M. E. Gray. 2003. Captures of western corn rootworm (Coleoptera : Chrysomelidae) adults with phlerocon AM and vial traps in four crops in east central Illinois. Journal of Economic Entomology **96**:737-747.

Rondon, S. I., and M. E. Gray. 2004. Ovarian development and ovipositional preference of the western corn rootworm (Coleoptera : chrysomelidae) variant in East Central Illinois. Journal of Economic Entomology **97**:390-396.

Rubin, B. S., F. S. Menniti, and R. S. Bridges. 1983. Intracerebroventricular administration of oxytocin and maternal behavior in rats after prolonged and acute steroid pretreatment. Hormones and Behavior **17**:45-53.

Salzet, M., P. Bulet, A. Vandorsselaer, and J. Malecha. 1993. Isolation, structural characterization and biological function of a lysine-conopressin in the central-nervous-system of the pharyngobdellid leech *Erpobdella octoculata*. European Journal of Biochemistry **217**:897-903.

Sameoto, D. D., and R. S. Miller. 1968. Selection of pupation site by *Drosophila melanogaster* and *D simulans*. Ecology **49**:177-&.

Schlossmann, J., R. Feil, and F. Hofmann. 2005. Insights into cGMP signalling derived from cGMP kinase knockout mice. Frontiers in Bioscience **10**:1279-1289.

Schmidtko, A., P. Ruth, G. Geisslinger, and I. Tegeder. 2003. Inhibition of cyclic guanosine 5 '-monophosphate-dependent protein kinase I (PKG-I) in lumbar spinal cord reduces formalin-induced hyperalgesia and PKG upregulation. Nitric Oxide-Biology and Chemistry **8**:89-94.

Scott, M. P. 1990. Brood guarding and the evolution of male parental care in burying beetles. Behavioral Ecology and Sociobiology **26**:31-39.

Scott, M. P. 1994. The benefit of paternal assistance in intraspecific and interspecific competition for the burying beetle, *Nicrophorus-Defodiens*. Ethology Ecology & Evolution **6**:537-543.

Scott, M. P. 1998. The ecology and behavior of burying beetles. Annual Review of Entomology **43**:595-618.

Scott, M. P. 2006a. Resource defense and juvenile hormone: the "challenge hypothesis" extended to insects. Hormones and Behavior **49**:276-281.

Scott, M. P. 2006b. The role of juvenile hormone in competition and cooperation by burying beetles. Journal of Insect Physiology **52**:1005-1011.

Scott, M. P., and S. C. Panaitof. 2004. Social stimuli affect juvenile hormone during breeding in biparental burying beetles (Silphidae: *Nicrophorus*). Hormones and Behavior **45**:159-167.

Scott, M. P., S. C. Panaitof, and K. L. Carleton. 2005. Quantification of vitellogenin-mRNA during maturation and breeding of a burying beetle. Journal of Insect Physiology **51**:323-331.

Silver, J. B. 2008. *Mosquito ecology, field sampling methods.* Springer.

Smiseth, P. T., C. T. Darwell, and A. J. Moore. 2003. Partial begging: an empirical model for the early evolution of offspring signalling. Proceedings of the Royal Society B-Biological Sciences **270**:1773-1777.

Smiseth, P. T., C. Dawson, E. Varley, and A. J. Moore. 2005. How do caring parents respond to mate loss? Differential response by males and females. Animal Behaviour **69**:551-559.

Smiseth, P. T., L. Lennox, and A. J. Moore. 2007a. Interaction between parental care and sibling competition: Parents enhance offspring growth and exacerbate sibling competition. Evolution **61**:2331-2339.

Smiseth, P. T., and A. J. Moore. 2002. Does resource availability affect offspring begging and parental provisioning in a partially begging species? Animal Behaviour **63**:577-585.

Smiseth, P. T., and A. J. Moore. 2004a. Behavioral dynamics between caring males and females in a beetle with facultative biparental care. Behavioral Ecology **15**:621-628.

Smiseth, P. T., and A. J. Moore. 2004b. Signalling of hunger when offspring forage by both begging and self-feeding. Animal Behaviour **67**:1083-1088.

Smiseth, P. T., and A. J. Moore. 2007. Signalling of hunger by senior and junior larvae in asynchronous broods of a burying beetle. Animal Behaviour **74**:699-705.

Smiseth, P. T., and A. J. Moore. 2008. Parental distribution of resources in relation to larval hunger and size rank in the burying beetle *Nicrophorus vespilloides*. Ethology **114**:789-796.

Smiseth, P. T., S. Musa, and A. J. Moore. 2006. Negotiation between parents: does the timing of mate loss affect female compensation in *Nicrophorus vespilloides*? Behaviour **143**:293-301.

Smiseth, P. T., R. J. Ward, and A. J. Moore. 2007b. Parents influence asymmetric sibling competition: experimental evidence with partially dependent young. Ecology **88**:3174-3182.

Sokolowski, M. B. 1980a. Elucidating the behavioral phenotype of *Drosophila melanogaste*r larvae - correlations between larval foraging strategies and pupation heights. Behavior Genetics **10**:498-498.

Sokolowski, M. B. 1980b. Foraging Strategies of *Drosophila melanogaster* - a chromosomal analysis. Behavior Genetics **10**:291-302.

Sokolowski, M. B. 1985. Genetics and ecology of *Drosophila melanogaster* larval foraging and pupation behavior. Journal of Insect Physiology **31**:857-864.

Sokolowski, M. B. 2010. Social interactions in "simple" model systems. Neuron **65**:780-794.

Sokolowski, M. B., and R. I. C. Hansell. 1983. Elucidating the behavioral phenotype of *Drosophila melanogaster* larvae - Correlations between larval foraging strategies and pupation height. Behavior Genetics **13**:267-280.

Sokolowski, M. B., C. Kent, and J. Wong. 1984. *Drosophila* larval foraging behavior - developmental stages. Animal Behaviour **32**:645-651.

Stafflinger, E., K. K. Hansen, F. Hauser, M. Schneider, G. Cazzamali, M. Williamson, and C. J. P. Grimmelikhuijzen. 2008. Cloning and identification of the first oxytocin/vasopressin-like receptor and its ligand from insects. Proceedings of the National Acadademy of Science of the U S A **105**:3262-3267.

Steiger, S., K. Peschke, W. Francke, and J. K. Muller. 2007. The smell of parents: breeding status influences cuticular hydrocarbon pattern in the burying beetle *Nicrophorus vespilloides*. Proceedings of the Royal Society B-Biological Sciences **274**:2211-2220.

Stern, D. L., and V. Orgogozo. 2008. The loci of evolution: How predictable is genetic evolution? Evolution **62**:2155-2177.

Stern, D. L., and V. Orgogozo. 2009. Is Genetic Evolution Predictable? Science **323**:746-751.

Suzuki, S. 2004. Brood size reduction in *Nicrophorus vespilloides* after usurpation of carrion from *Nicrophorus quadripunctatus* (Coleoptera : Silphidae). Entomological Science **7**:207-210.

Takuwa-Kuroda, K., E. Iwakoshi-Ukena, A. Kanda, and H. Minakata. 2003. Octopus, which owns the most advanced brain in invertebrates, has two members of vasopressin/oxytocin superfamily as in vertebrates. Regulatory Peptides **115**:139-149.

Tan, E. J., and B. L. Tang. 2006. Looking for food: Molecular neuroethology of invertebrate feeding behavior. Ethology **112**:826-832.

Thomas, M. A., and R. Klaper. 2004. Genomics for the ecological toolbox. Trends in Ecology & Evolution **19**:439-445.

Tobin, V. A., H. Hashimoto, D. W. Wacker, Y. Takayanagi, K. Langnaese, C. Caquineau, J. Noack, R. Landgraf, T. Onaka, G. Leng, S. L. Meddle, M. Engelmann, and M. Ludwig. 2010. An intrinsic vasopressin system in the olfactory bulb is involved in social recognition. Nature **464**:413-U110.

Toma, D. P., G. Bloch, D. Moore, and G. E. Robinson. 2000. Changes in period mRNA levels in the brain and division of labor in honey bee colonies Proceedings of the National Academy of Science of the U S A **97**:6914-6919.

Tomoyasu, Y., and R. E. Denell. 2004. Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. Development Genes and Evolution **214**:575-578.

Toth, A. L., K. B. J. Bilof, M. T. Henshaw, J. H. Hunt, and G. E. Robinson. 2009. Lipid stores, ovary development, and brain gene expression in *Polistes metricus* females. Insectes Sociaux **56**:77-84.

Toth, A. L., and G. E. Robinson. 2007. Evo-devo and the evolution of social behavior. Trends in Genetics **23**:334-341.

Toth, A. L., K. Varala, T. C. Newman, F. E. Miguez, S. K. Hutchison, D. A. Willoughby, J. F. Simons, M. Egholm, J. H. Hunt, M. E. Hudson, and G. E. Robinson. 2007. Wasp gene expression supports an evolutionary link between maternal behavior and eusociality. Science **318**:441-444.

Trumbo, S. T. 1990a. Interference competition among burying beetles (Silphidae, *Nicrophorus*). Ecological Entomology **15**:347-355.

Trumbo, S. T. 1990b. Reproductive benefits of infanticide in a biparental burying beetle *Nicrophorus orbicollis*. Behavioral Ecology and Sociobiology **27**:269-273.

Trumbo, S. T. 1991. Reproductive benefits and the duration of paternal care in a biparental burying beetle, *Nicrophorus orbicollis*. Behaviour **117**:82-105.

Trumbo, S. T. 1997. Juvenile hormone mediated reproduction in burying beetles: From behavior to physiology. Archives of Insect Biochemistry and Physiology **35**:479-490.

Trumbo, S. T. 2007. Defending young biparentally: female risk-taking with and without a male in the burying beetle, *Nicrophorus pustulatus*. Behavioral Ecology and Sociobiology **61**:1717-1723.

Trumbo, S. T., D. W. Borst, and G. E. Robinson. 1995. Rapid elevation of juvenile hormone titer during behavioral assessment of the breeding resource by the burying beetle, *Nicrophorus orbicollis*. Journal of Insect Physiology **41**:535-543.

Trumbo, S. T., and G. E. Robinson. 2004. Nutrition, hormones and life history in burying beetles. Journal of Insect Physiology **50**:383-391.

Trumbo, S. T., and R. C. Valletta. 2007. The costs of confronting infanticidal intruders in a burying beetle. Ethology **113**:386-393.

Van Kesteren, R. E., A. B. Smit, R. P. J. Delange, K. S. Kits, F. A. Vangolen, R. C. Vanderschors, N. D. Dewith, J. F. Burke, and W. P. M. Geraerts. 1995. Structural and functional evolution of the vasopressin oxytocin superfamily - vasopressin-related conopressin is the only member present in *Lymnaea*, and is involved in the control of sexual behavior. Journal of Neuroscience **15**:5989-5998.

Van Kesteren, R. E., A. B. Smit, R. W. Dirks, N. D. Dewith, W. P. M. Geraerts, and J. Joosse. 1992. Evolution of the vasopressin oxytocin superfamily - characterization of a cDNA-encoding a vasopressin-related precursor, preproconopressin, from the mollusk *Lymnaea stagnalis*. Proceedings of the National Acadademy of Science of the U S A **89**:4593-4597.

Van Kesteren, R. E., C. P. Tensen, A. B. Smit, J. vanMinnen, L. F. Kolakowski, W. Meyerhof, D. Richter, H. vanHeerikhuizen, E. Vreugdenhil, and W. P. M. Geraerts. 1996. Co-evolution of ligand-receptor pairs in the vasopressin oxytocin superfamily of bioactive peptides. Journal of Biological Chemistry **271**:3619-3626.

Walling, C. A., C. E. Stamper, P. T. Smiseth, and A. J. Moore. 2008. The quantitative genetics of sex differences in parenting. Proceedings of the National Acadademy of Science of the U S A **105**:18430-18435.

Whitfield, C. W., Y. Ben-Shahar, C. Brillet, I. Leoncini, D. Crauser, Y. LeConte, S. Rodriguez-Zas, and G. E. Robinson. 2006. Genomic dissection of behavioral maturation in the honey bee. Proceedings of the National Academy of Science of the U S A **103**:16068-16075.

Whitfield, C. W., A. M. Cziko, and G. E. Robinson. 2003. Gene expression profiles in the brain predict behavior in individual honey bees. Science **302**:296-299.

Whitfield, J. B., and K. M. Kjer. 2008. Ancient rapid radiations of insects: Challenges for phylogenetic analysis. Annual Review of Entomology **53**:449-472.

Williams, J. R., T. R. Insel, C. R. Harbaugh, and C. S. Carter. 1994. Oxytocin administered centrally facilitates formation of a partner preference in female prairie voles (*Microtus ochrogaster*). Journal of Neuroendocrinology **6**:247-250.

Wilson, E. O. 1971. *The Insect Societies* Belknap Press of Harvard University Press.

Winslow, J. T., N. Hastings, C. S. Carter, C. R. Harbaugh, and T. R. Insel. 1993. A role for central vasopressin in pair bonding in monogamous prairie voles. Nature **365**:545-548.

Wong, J. L., M. B. Sokolowski, and C. F. Kent. 1985. Prepupation behavior in *Drosophila* - Embedding. Behavior Genetics **15**:155-164.

You, Y. J., J. Kim, D. M. Raizen, and L. Avery. 2008. Insulin, cGMP, and TGF-beta signals regulate food intake and quiescence in *C. elegans*: A model for satiety. Cell Metabolism **7**:249-257.

Young, L. J., M. M. Lim, B. Gingrich, and T. R. Insel. 2001. Cellular mechanisms of social attachment. Hormones and behavior **40**:133-138.

Young, L. J., Z. X. Wang, and T. R. Insel. 1998. Neuroendocrine bases of monogamy. Trends in Neurosciences **21**:71-75.

Zhu, M. J., and S. H. Zhao. 2007. Candidate gene identification approach: Progress and challenges. International Journal of Biological Sciences **3**:420-427.