Fungi and NO defense?

Mycoflora in brood cells of the European Beewolf and its radical defense

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„ … to seek out new life and new civilizations,

to boldly go where no man has gone before…”

Star Trek, 1966

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Chapter 1: General Introduction

1.1 The microbial threat & insect defense

Fungi are a serious threat for arthropods as either pathogens or competitors. Mould fungi can grow on a wide range of organic material and numerous entomogen fungi are known. The last review attempt was quite some time ago (Madelin 1966). Due to the huge number and resistance of their conidiae they can withstand long unsuitable periods until conditions improve and they germinate and start a new reproduction cycle (Cochrane 1958; Gottlieb 1978). Air and water, but especially soil with its high number of dormant spores, are important infection sources. Most insects are in permanent contact with the soil. They move through soil in search for food and shelter and often excavate nest to hide themselves and their offspring from predators and parasites (Gross 1993; Dettner 2003).

The threat fungi cause for arthropods and especially insects may take the form of competitors for food, parasites that penetrate insects and nourish on them or even pathogens. Janzen (1977) stated that 'Fruits rot, seeds mould and meat spoils because that is the way microbes compete with bigger organisms'. Hochberger and Lawton (1990) estimated 'interkingdom competition involving microbes […] one of the commonest'. Fungi do not only compete for food by digesting it, they also degrade and render it useless for animals or produce toxic or bad-tasting secondary metabolites to monopolize a food source (Janzen 1977; Burkepile *et al.* 2006; Rohlfs *et al.* 2007). Some parasitic fungi are unspecialized opportunists that withdraw only few resources from their hosts through haustoria or by invading their gut, like Laboulbeniales and Trichomycetes (Charnley 2003). However, the difference between parasitic and pathogenic fungi is fuzzy since it is often unclear whether the fungi killed the insect or not. The number of known entomopathogenic fungi species was estimated to be 700 by Hajek and Leger (1994) but this number has likely grown within the last years. The danger entomopathogenic fungi pose to insects can be estimated by the attempts to use them as a pest control agent. The first suggestion to use fungi as pest control agents by Bassi (1835) needed some time to be implemented but meanwhile it is of commercial interest (Hajek and Leger 1994; Charnley and Collins 2007).

Another indicator for the importance of fungi for insects is the number of defense mechanisms that have been discovered within the last years. Besides responses of the immune system against endoparasites or pathogens via several pathways like the phenoloxidase, haemocytes or antibiotic peptides an increasing number of extracorporal mechanisms are found. The antibiotic substances that are involved in these countermeasures originate from different sources. Some insects collect protective substances from plants (Chapuisat *et al.* 2007) some are produced in different glands (Cane *et al.* 1983; Gross *et al.* 1998; Ortius-Lechner *et al.* 2000; Rosengaus *et al.* 2000; Bot *et al.*

2002; Gross *et al.* 2002; Rosengaus *et al.* 2004; Fernández-Marín *et al.* 2006; Gross *et al.* 2008; Rozen *et al.* 2008; Cotter and Kilner 2010) some insects use their feces (Chen *et al.* 1998; Rosengaus *et al.* 1998) or harbor symbiotic bacteria (Gil-Turnes *et al.* 1989; Currie 1999; Currie *et al.* 1999; Kaltenpoth *et al.* 2005; Cardoza *et al.* 2006; Kaltenpoth and Strohm 2007; Cardoza *et al.* 2009; Lam *et al.* 2009; Kroiss *et al.* 2010; Poulsen *et al.* 2011) or even fungi (Yoder *et al.* 2008) that produce the antibiotics. Entire nests are lined with protective substances (Cane *et al.* 1983; Rosengaus *et al.* 1998) , food is treated (Herzner *et al.* 2007; Rozen *et al.* 2008), or eggs (Lam *et al.* 2009), embryos (Gil-Turnes *et al.* 1989) and larvae are shielded. The specificity of some substances is reached by precise mechanical application (Cardoza *et al.* 2006), some are highly specific (Currie *et al.* 1999) other are effective against a broad spectrum of pathogens (Kroiss *et al.* 2010). The combination of substances, sources and application methods provides a diverse spectrum of defenses that can and must match the diverse spectrum of the respective fungal threats. Females of the European Beewolf, *Philanthus triangulum* F. show a sophisticated parental care behavior. They provide food and shelter in subterranean nests for their offspring. They also have to apply multiple antimicrobial mechanisms to conserve the food and protect the development of their offspring especially from detrimental mold fungi.

1.2 The biology of Philanthus triangulum

Philanthus triangulum F., the European beewolf, is a solitary digger wasp. Its females nest in sandy, sun exposed soils. They excavate burrows, hunt for honeybee workers, *Apis mellifera*, which are paralyzed by stinging into the thorax ad then transported back to the nest in flight and provided as food for the beewolf's larvae (Tinbergen 1932; Strohm 1995). The nest consists of a main burrow with several side burrows. The brood cells are excavated just before an egg is laid at the end of the side burrows (see Figure 1-1 for an overview and details of beewolf nests; Simon-Thomas and Veenendaal 1978; Strohm 1995). One to six bees are caught sequentially and temporarily stored in the main burrow until the female has gathered a sufficient number of bees. Then she starts to excavate the brood cell (Strohm 1995; Strohm and Linsenmair 2000). In between digging activity the females licks the bees extensively and drags and moves them constantly through the burrow (Simon-Thomas and Veenendaal 1978). After that, the bees are placed inside the brood cell and an egg is laid and fixed on the thorax of one of the bees with a sticky secretion. After oviposition she applies a white substance from her antennae to the brood cell wall and ceiling opposite to the entrance. Then she carefully closes the side burrow with sand (Strohm and Linsenmair 1994). The larva hatches after two to three days and starts to feed on the bees. After about one week the grown larva takes up the white secretion and starts to spin a cocoon. The cocoon is fixed with one end at the brood cell wall opposite to the former entrance and protrudes into the brood cell with only this one connection to the wall (Strohm and Linsenmair 1994; Strohm 1995). Some individuals of the first generation in a year immediately develop into

adults and hatch about four weeks later. Most offspring, however, and all of the second generation overwinter and emerge in the next year.

The subterranean nests provide optimal conditions for fungi. The sun exposed soils combine a warm humid climate with a potential high number of spores. Yet the actual rate of infestation of brood cells is quite low: only 5.3% of brood cells have been found to be infested by fungi in a study over seven years and from three locations (Strohm and Linsenmair 2001). Beewolves evolved different mechanisms to protect their offspring. By licking the prey bees the mother applies a secretion from her postpharyngeal gland to the bees (Strohm and Linsenmair 2001; Strohm *et al.* 2007). This secretion contains a mixture of hydrocarbons that is identical to those on the female's cuticle (Strohm *et al.* 2008). As a consequence, the amount of cuticular hydrocarbons on the bee surfaces is about five times higher than on untreated bees (Herzner *et al.* 2007). The hydrophobic layer inhibits fungal infestation by reducing water condensation on the bees (Strohm and Linsenmair 2001; Herzner and Strohm 2007). The subsequent development of the beewolf once inside the cocoon is protected by symbiotic bacteria. The white substance applied to the brood cell from the females' antennae contains bacteria of the genus *Streptomyces* that are cultivated inside specialized antennal glands (Kaltenpoth *et al.* 2006; Goettler *et al.* 2007). When the

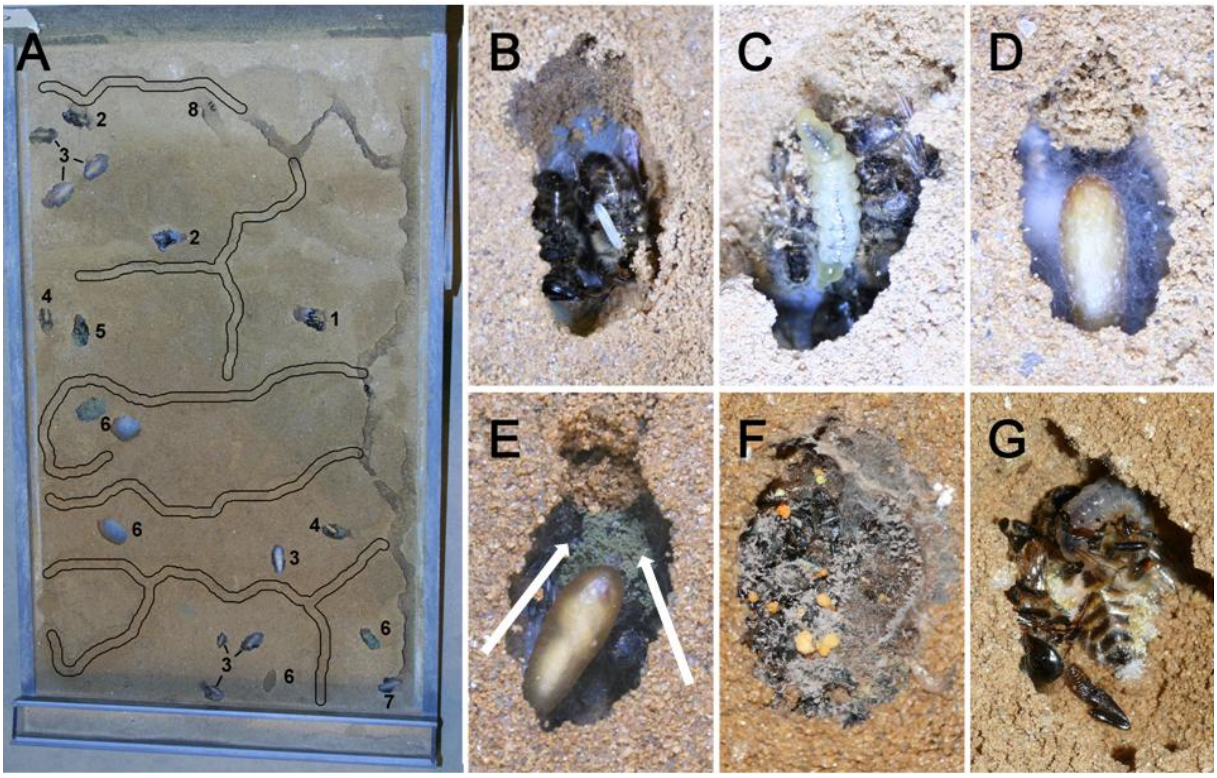

Figure 1- 1 Beewolf nest in an observation cage (A) with a brood cell with a fresh egg (1, see B for magnification), a larva (2), a cocoon (3), a cocoon with a pupa (4), a brood cell overgrown by fungi (5), brood cells from which egg and bees were removed (6) a brood cell that was accidentally reopened by the female (7) and bee left in the side burrow (8). Closed side burrows are surrounded by black lines. B shows a brood cell with an egg, C with a larva and D with a cocoon. The larva in E managed to spin a cocoon and pupate before the remains of the bees were overgrown by fungi (arrows). A brood cell that is completely overgrown by fungi (F) and a larva feeding on already infested bees (G).

larvae spin their cocoon, they incorporate these bacteria into the silk threads. There the bacteria protect the cocoon by producing a blend of several antibiotic substances (Kaltenpoth *et al.* 2005; Kaltenpoth and Strohm 2007; Kroiss *et al.* 2010)

1.3 Objectives of the thesis

The European beewolf is already known to employ two different mechanisms to protect its offspring from fungi. Evidence for a third mechanism was provided by Strohm (1995). In addition to the treatment of the bees with hydrocarbons the presence of the egg considerably delayed fungal infestation. This antifungal effect seems to be caused by a gas. To unravel this mechanism and to characterize its effect I first investigated which fungi pose a problem in beewolf brood cells. To this end I isolated fungi from infested brood cells and identified them by means of rDNA sequences. With the spectrum of important fungi available I conducted biotests with beewolf eggs to show its efficacy. In the next step I identified the agent which mediates the antimicrobial effect, its source within the egg and the physiological context of its production. The gas production and the treatment of the honey bee prey with hydrocarbons require both resources. It has already been shown that the hunt of the honeybees is expensive. It deprives the females of resources and thus diminishes their ability to invest into further offspring (Strohm and Marliani 2002). The investment into the two defense mechanisms may entail further costs for the beewolf mother. Different life history variables of the gas defense, as the investment of the defense into a brood cell, the former investment of a female, her size and fitness, were evaluated with a multifactor analysis. A dataset of 250 brood cells was subjected to a path analysis to determine potential trade-offs that might indicate costs. The costs of the hydrocarbon treatment were investigated with a manipulative approach. The investment for single brood cells was artificially increased with the expectation of a reduced ability to invest into further offspring. Costs of the offspring defense will have a heavy impact on the investment strategy of beewolf females. They have not only to balance resource allocation between each single offspring but also between different important facets of one offspring.

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Chapter 2: Mycoflora of beewolf brood cells

2.1 Introduction

The competition between animals and microorganisms (Janzen 1977) for food is especially problematic if food is intended to be stored over longer periods. This is not only a problem known from the human everyday life, but also for many animals. Many animals store food and have to cope with the problem of fungal spoilage (Smith and Reichman 1984; Vander Wall 1990; Castleberry and Castleberry 2008). Several adaptations have evolved allowing animals to deal with food infesting fungi. Some are able to influence the fungal infestation (Reichman *et al.* 1986) other adapted to consume food despite microbial infestation or even prefer it (Hesseltine 1965; Petersen *et al.* 1989). Some insects store food as part of parental care for their offspring. Provisioning food for the progeny again creates a conflict. The stored food is a limited resource for which at least the progeny of the collector compete with a variety of opportunistic microorganisms (Hochberg and Lawton 1990). The access of food to competing animals can often be prevented by hiding or burying it. However, the omnipresent microorganisms can hardly be excluded resulting in competition. The highest probability to prevail is by cheating and tricking. For the microorganisms it is often not possible to consume the entire provision before an animal competitor. So they evolved several methods to render food useless by decomposing or poisoning it (Janzen 1977; Burkepile *et al.* 2006). Animals, which do not have the possibility to choose among different food sources, but rely for example on provisions that have been provided by a parent, need to adapt to the microbial competition. Animals can adapt to toxins or the altered food, by evolve detoxification mechanisms, adjusting the digestion process or they may even feed on the microbes instead of the primary food source (Martin and Weber 1969; Yamaoka 1996; Mueller *et al.* 2005). Alternatively, they can try to prevent the provisions from actually being colonized by fungi or bacteria.

Several species of have been reported as being harmful to insects (Dowd *et al.* 1989; Wicklow and Dowd 1989; Rohlfs 2008; Trienens *et al.* 2010). Mold fungi pose a severe problem for stored food. Their spores or conidia can be found merely everywhere. The spores can stay dormant for a long time and several are known to be quite aggressive, producing potent toxins. In several investigated host parasite systems interesting defense mechanisms were discovered (e.g. Bienvenu *et al.* 1968; Cardoza *et al.* 2006; Gross *et al.* 2008). Attine ants and the European beewolf use at least two completely different defenses. Both rely on a glandular secretion (Maschwit.U *et al.* 1970; Beattie *et al.* 1986; Strohm and Linsenmair 2001; Herzner 2007; Herzner and Strohm 2007) and a symbiotic Actinomycete (Currie *et al.* 1999; Kaltenpoth *et al.* 2005; Kaltenpoth and Strohm 2007) to ensure the survival of their fungus gardens or their offspring respectively. However, there are only few studies on the mycoflora to which all the defense mechanisms are directed. There are two

studies in leaf-cutting ant societies (Currie *et al.* 1999; Hughes *et al.* 2009) but except from one paper of Batra *et al.* (1973) on the mycoflora of some bees, there is no other study on the microbial flora of solitary insects.

Regarding the effort females of the European beewolf spend to ensure the survival of their offspring, I took the opportunity to take a closer look on the pathogens they have to fend off. The beewolf female hunts for workers of the Western honeybee, *Apis mellifera*, as prey for its offspring. The bees are attacked on flowers, stung and paralyzed. The female transports them in flight back to its nests in sandy soil. The bee is stored inside the main burrow until the female has caught enough bees. Then the bees are licked by the female beewolf and thereby covered with a thick layer of hydrocarbons (Strohm and Linsenmair 2001). When the bees are placed inside the brood cell, a secretion containing symbiotic streptomycetes from the female's antennae is applied to the wall of the brood cell (Strohm and Linsenmair 1994; Kaltenpoth *et al.* 2005). After the egg is laid the female closes the brood cell. During the digging of the brood cell the prey bees are in constant contact to the soil. Fungal spores that grow on the bees might come from the bee itself, the female beewolf and the soil. However, due to the elaborate defenses most of the larvae are able to develop, pupate and hatch as healthy adults. The first step in this defense is represented by the additional hydrocarbon layer the beewolf female applied to the prey bees. It delays germination of fungal spores, by preventing water from condensing on the bees which is necessary for the first germination step (Herzner *et al.* 2007; Herzner and Strohm 2007). When the hatched and grown larva starts to spin its cocoon, it takes up the secretion from the female's antennae that contain the bacteria and spins it into its cocoon. Then the streptomycete bacteria start to produce several antibiotics to protect the cocoon (Kaltenpoth and Strohm 2007; Kroiss *et al.* 2010).

The beewolf progeny develops isolated from each other and might encounter their sibs in their mother's nest after about 6-8 weeks if they emerge as a second generation in summer, or about 9-11 months if they hibernate and emerge the following summer (Olberg 1953). As beewolves are solitary wasps that do not have much contact with other individuals except for mating, only few contacts are possible, if females enter another female's nest or if they take over an abandoned nest. With such limited opportunity to spread throughout a host population, one would not expect to find a specialized parasitic fungus. Therefore, the most important sources for the infestation of prey with mould fungi are unspecialized fungi on beewolf mothers, the paralyzed honey-bee workers, and the soil surrounding the excavated burrows and the brood cells. Furthermore, specialized pathogenic fungi of honeybees could infest beewolf brood cells.

In order to have an overview about the fungi that may occur in the beewolf brood cells and to assess the potential threat I examined brood cells in several observation cages, took samples from infested bees and cultivated the fungi. I identified the isolated and cultivated species by molecular methods: PCR and sequencing of the small subunit of the rDNA gene

(SSU) as well as the intergenic transcribes spacer region (ITS). For determination on species level within the genus *Aspergillus*, I also used light microscopy.

2.2 Materials & methods

Isolation of fungi

Beewolf females were kept in observation cages (Strohm and Linsenmair 1994). They were provided with honeybee workers *ad libitum*. Sometimes bees are attacked and paralyzed but not brought into the nest. Why females rejected these bees is not clear; the bees might either be too heavy or otherwise unsuitable for provisioning.

To obtain conidia of fungi from infested brood cells single conidiophores were sampled from bees under a stereo microscope (Zeiss) using a needle tip and inoculated on Sabouraud-Dextrose-Agar plates. If there was mould but no clearly identifiable conidiophores, we transferred hyphae with tweezers to an agar plate. Each plate was inoculated at three spots with the same isolate. All isolates were incubated in the dark at 25°C until the formation of new mature conidiophores. From these colonies single conidiophores were transferred to new culture plates three times to obtain pure strains. If there were no conidiophores some hyphae were transferred to new plates but only if no contaminations were visible. A total of 116 brood cells were controlled for fungal infestations. From these results the frequency of infesting species was calculated.

DNA preparation, PCR, sequencing

Freshly inoculated, still growing mycelia were sampled with tweezers, frozen with liquid nitrogen and manually crushed with sterile pipette tips. DNA was isolated using the Epicentre MasterPure Kit (Epicentre, Madison, USA). We tried to amplify two genetic sequences: the small subunit of nuclear rDNA and the internal transcribed spacer region (NS1&4 and ITS1&4 from Weisburg *et al.* 1991; and nuSSU 0817 & 1536 from Borneman and Hartin 2000).

PCR was conducted on a Biometra TGradient thermo cycler in a total volume of 12.5µL with 1µL of DNA sample and 1xPCR buffer (50mM Tris-HCl pH 9.1, 14mM $(NH_4)_2SO_4$), 3mM $MgCl_2$, each 240µM dNTPs, 10pmol of each primer and 0.5U of Taq DNA polymerase (Peqlab). Cycle parameters were as follows: 3min at 95°C followed by 35 cycles of 95°C for 1min, T_A for 1min and 72°C for 1min, and a final extension time of 5min at 72°C. T_A was 56°C for the NS primer pair, 58°C for the ITS and nu primer pair. PCR products were purified with the PeqGOLD MicroSpin cyle-Pure kit (Peqlab, Germany) and sent to Seqlab, Göttingen for sequencing.

Microscopy

To facilitate species differentiation a Leica DMLS light microscope was used to determine different features like conidia size and texture (Diba *et al.* 2007).

2.3 Results

We found six fungal species which in beewolf brood cells before a cocoon was produced. On overwintering cocoons we found three different species (see Table 2-1 for final results and Table 2-2 for an overview of blast results). From 11 *Aspergillus* isolates, 7 belonged to *A. flavus*, two to *A. tamari* and two to *A. nomius*. Three visually similar isolates of *G. hyalinospora* were genetically identical. *P. lilacinus* was isolated twice, *C. echinulata* also twice from brood cells. From four visually similar samples from cocoons two were *A. elegans*, one *C. elegans* and one *F. oxysporum*. Microscopic examination of isolate one revealed glubose conidia of 3,6 µm in diameter with a smooth surface and brown stipes. These characters exclude *Aspergillus oryzae* and *parasiticus* from the molecular results.

Table 2- 1 Final results of the genetic identification of the ten beewolf fungal species isolated from beewolf brood cells. The sequences obtained from the beewolf brood cell isolates are deposited under these accession numbers. Identity gives the percentage of identical bases between the sequence of the isolate and the sequence of the closest match species. **Rollandiana hyalinospora* and *Gymnascella hyalinospora* are still both commonly used to name the same species. ***Fusarium redolens* is a variety of *Fusarium oxysporum*

Isolate	Origin	sequence	Accession numbers	Closest match	Identity
1	brood cell	SSU ITS	JF824683 JF824682	*Aspergillus flavus* *Aspergillus flavus*	100% 100%
2	brood cell	SSU ITS	JF824685 JF824684	*Aspergillus tamarii* *Aspergillus tamarii*	100% 100%
3	brood cell	SSU ITS	JF824687 JF824686	*Aspergillus nomius* *Aspergillus nomius*	99,49% 100%
4	brood cell	SSU ITS	JF824689 JF824688	*Rollandina hyalinospora* *Gymnascella hyalinospora**	99,37& 98,91%
5	brood cell	SSU ITS	JF824691 JF824690	*Paecilomyces lilacinus* *Paecilomyces lilacinus*	100% 100%
6	brood cell	SSU ITS	JF824693 JF824692	*Cunninghamella echinulata* *Cunninghamella echinulata*	98,47% 99,53%
7	cocoon	SSU ITS	JF824695 JF824694	*Actinomucor elegans* *Actinomucor elegans*	99,46% 100%
8	cocoon	SSU ITS	JF824697 JF824696	*Fusarium oxysporum* *Fusarium redolens***	99,93% 100%
9	cocoon	SSU ITS	JF824699 JF824698	*Cunninghamella elegans* *Cunninghamella elegans*	99,05% 98,27%

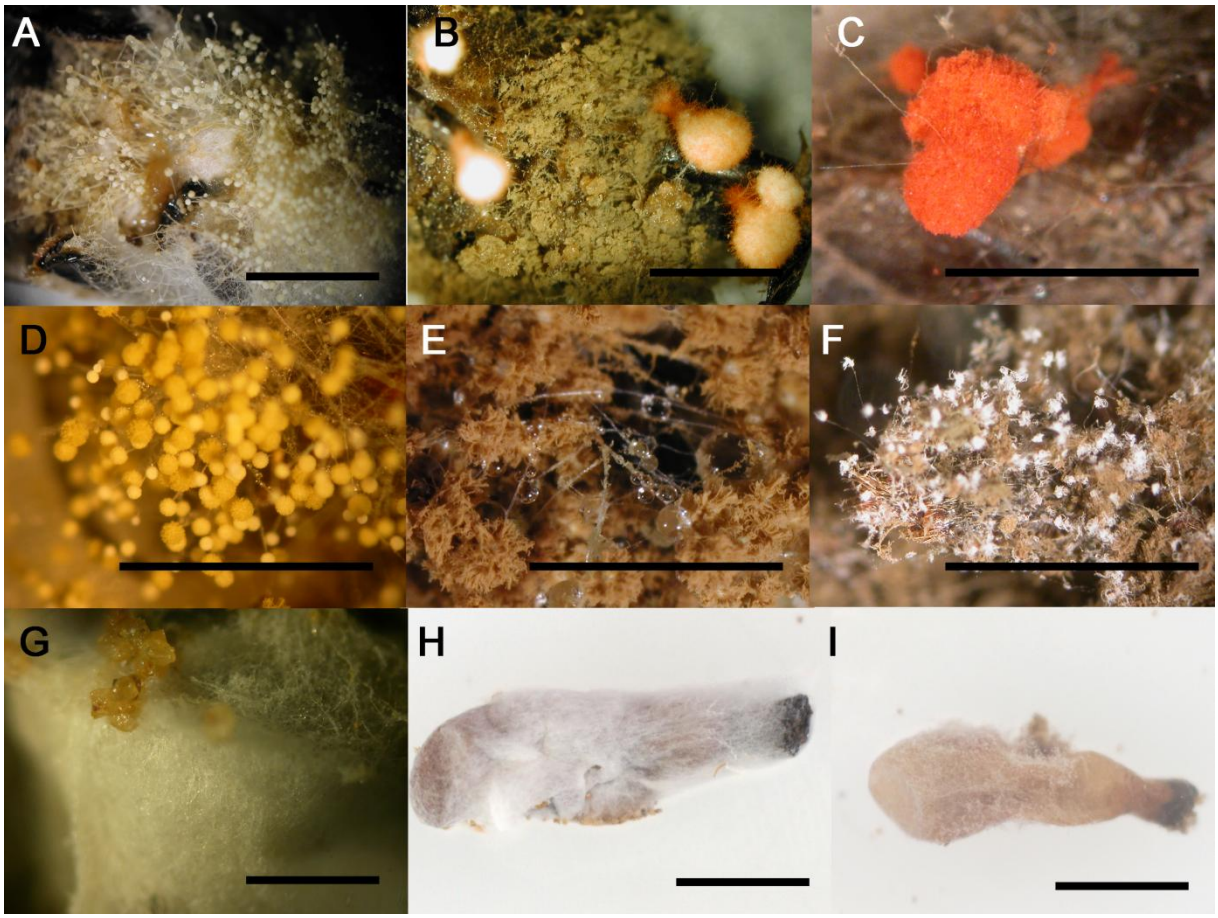

Figure 2-1 Origin of some isolates of the beewolf brood cells. **A** *A. flavus* with conidiophores in early development stage **B** late stage of *A. flavus* conidiophores together with *G. hyalinospora*. **C** stronger colored *G. hyalinospora* **D** *A.tamarii* with early stage of conidiophore development **E** later stage of *A. tamarii* conidiophore development **F** an *Aspergillus* species (brown) with *P. lilacinus* (white) **G** Remains of a honeybee engulfed with *C. echinulata.* A beewolf cocoon infested by *A elegans* (**H**) and *F. oxysporum* (**I**). Bars = 5mm

Most fungi could be visually assigned to the genus *Aspergillus* and the species *Gymnascella hyalinospora.* 59% (N=66) of fungal infestations from 116 beewolf brood cells were by *Aspergillus* species and 26% (N=30) by *Gymnascella hyalinospora*. The remaining 15% (N=20) contain all other species. Most brood cells show only one dominating fungus. In each case with two different fungi present an *Aspergillus* species was one of them.

next page: **Table 2- 2** Overview of the results of the sequence comparison between the isolates from the beewolf brood cells and the most similar species of the nucleotide blast search. The accession numbers refers to the reference sequences. Identity gives the percentage of identical bases between the sequence of the isolate and the sequence of the matching species.

isolate	origin	accession number	Result SSU	identity	accession number	Result ITS 1 – 5.2S – ITS 2	Identity
1	brood cell	GU953210.1	Aspergillus flavus	100%	EF661566.1	Aspergillus flavus	100%
		HM064501.1	Aspergillus oryzae	100%	HM145964.1	Aspergillus oryzae	100%
		D63699.1	Aspergillus parasiticus	100%	DQ026005.1	Aspergillus parasiticus	100%
		AF516140.1	Aspergillus tamari	99,93%	DQ467976.1	Aspergillus nomius	99,82%
		AB008404.1	Aspergillus nomius	99,87%			
2	brood cell	AF516140.1	Aspergillus tamari	100%	EF661565.1	Aspergillus tamari	100%
		HM064501.1	Aspergillus oryzae	99,93%	EF661550.1	Aspergillus caelatus	99,46%
		GU953210.1	Aspergillus flavus	99,93%	DQ467987.1	Aspergillus pseudotamarii	99,46%
		D63699.1	Aspergillus parasiticus	99,93%	AF338641.1	Aspergillus bombycis	99,28%
		AB008404.1	Aspergillus nomius	99,80%	AB000535.1	Aspergillus nomius	99,11%
3	brood cell	AB008404.1	Aspergillus nomius	99,49%	AF338627.1	Aspergillus nomius	100%
		HM064501.1	Aspergillus oryzae	99-39%	AF338641.1	Aspergillus bombycis	98,38%
		GU953210.1	Aspergillus flavus	99,39%	EF661565.1	Aspergillus tamari	97,49%
		AF516140.1	Aspergillus tamarii	99,39%	DQ467986.1	Aspergillus pseudotamarii	97,32%
		D63700.1	Aspergillus sojae	99,39%	EF661568.1	Aspergillus parasiticus	97,13%
4	Brood cell	AB015775.1	Rollandina hyalinospora	99,37%	AY304513.1	Gymnascella hyalinospora	98,91%
		AJ315168.1	Gymnascella marginospora	99,03%	AJ315824.1	Gymnascella marginosporus	97,47%
		AY177295.1	Kraurogymnocarpa trochleospora	98,61%	AJ271562.1	Amauroascus echinolatus	95,32%
		U29392.1	Gymnoascoideus petalosporus	98,82%	AJ271434.1	Amauroascus reticulatus	90,63%
		AY177296.1	Arachinotus ruber	98,40%	AJ315825.1	Gymnoascus punctatus	86,97%
5	brood cell	AB103380.1	Paecilomyces lilacinus	100%	AB103380.1	Paecilomyces lilacinus	100%
		AB104884.1	Paecilomyces nostocoides	100%	AB084157.1	Ophiocordyceps heteropoda	99,82%
		AB084157.1	Ophiocordyceps heteropoda	100%			
		AB114224.1	Tolypocladium inflatum	99,73%			
		AB208110.1	Tolypocladium cylindrisporum	99,73%			
6	brood cell	AF157130.1	Cunninghamella echinulata	98,47%	GU966504.1	Cunninghamella echinulata	99,53%
		AF113423.1	Cunninghamella polymorpha	97,41%	AF346408.1	Cunninghamella septata	99,30%
		AF113422.1	Cunninghamella elegans	97,41%	AF346407.1	Cunninghamella echinulata var. antarctica	97,67%
		AF113421.1	Cunninghamella bertholletiae	97,41%	AF254936.1	Cunninghamella echinulata var. nodosa	97,45%
		EF562534.1	Cunninghamella bainieri	89,85%		Cunninghamella echinulata var. echinulata	96,04%
7	cocoon	AF157119.1	Actinomucor elegans	99,46%	FJ176396.1	Actinomucor elegans	100%
		FJ605511.1	Mucor hiemalis	97,87%	EF059908.1	Mucor mucedo	99,53%
		HM623319.1	Mucor genevensis	97,80%	AY243953.1	Actinomucor taiwanensis	96,12%
		EU595659.1	Pilaria anomala	97,80%	AB369912.1	Umbelopsis isabellina	67,83%
		HM623313.1	Rhizomucor endophyticus	97,47%	EU798702.1	Mucor racemosus	72,20%
8	cocoon	AB521041.1	Fusarium oxysporum	99,93%	EF495234.1	Fusarium redolens	100%
		HM165488.1	Giberella fujikuroi	99,86%	FJ860061.1	Dioscorea alata	98,57%
		AB067700.1	Cordyceps sinensis	99,86%	EU717682.1	Giberella moniliformis	98,57%
		AF081467.1	Giberella pulicaris	99,66%	HM210092.1	Fusarium oxysporum	98,57%
		AB250414.1	Giberella zeae	96,36%	HQ332533.1	Fusarium proliferatum	98,37%
9	cocoon	AF113422.1	Cunninghamella elegans	99,05%	FJ792589.1	Cunninghamella elegans	98,27%
		AF113423.1	Cunninghamella polymorpha	99,05%	AF254941.1	Cunninghamella homothallica	83,45%
		AF113421.1	Cunninghamella bertholletiae	99,05%	AF254934.1	Cunninghamella phaeospora	82,96%
		AF157130.1	Cunninghamella echinulata	96,94%	FJ345351.1	Cunninghamella berthollatiae	81,71%
		EF562534.1	Cunninghamella bainerie	91,55%	EF562535.1	Cunninghamella baineiri	81,80%

2.4 Discussion

A. flavus, nomius and *tamarii,* the most common fungi in beewolf brood cells, belong to section *Flavi* of the genus *Aspergillus*. All three species have been reported as soil fungi, as mould on stored food and as pathogens in humans and livestock (Klich 2007; Kredics *et al.* 2007; Klich 2009). *A. flavus* has also been reported as a pathogen in insects (Domsch *et al.* 1980; Wicklow and Dowd 1989). In honeybees larval infection with *A. flavus* is known as stonebrood (Böttcher 1984). Fungi from the genus *Aspergillus* are distributed worldwide (Domsch *et al.* 1980; Klich 2002). They are able to degrade a great variety of substrates (Fogarty 1994) and thus infest nearly any organic material (Ellis 1980; Geiser *et al.* 1998; Klich 2009). Further, this group contains numerous species that have the ability to produce the potent carcinogenic aflatoxins as well as a broad range of other toxic substances (Brase *et al.* 2009; Klich 2009). *A. flavus* and *nomius* are perhaps the two most important producers of aflatoxins (Klich 2007; Olsen *et al.* 2008), while *Aspergillus tamarii* is one of the non-aflatoxigenic species of the section *Flavi*. *A. flavus* seems to increase virulence when the host is also infested by *Metarizium anisopliae* (Scully and Bidochka 2009). *A. tamarii* is regularly found on insects, especially bees, and is described to attack larvae as well as imagines (Batra *et al.* 1973; Domsch *et al.* 1980).

Another fungus that was common on infested bees from beewolf brood cells is *G. hyalinospora*. There is little information on this species except that it has often been isolated from birds (Sarangi and Ghosh 1991). However, closely related species of the order Onygenales and in particular the family Gymnoascaceae have been isolated from vertebrates and detritus (Ghosh 1985) . Some species of this group are able to exploit keratin as a carbon source (Currah 1994) and are described as human pathogens, causing dermatitis or, in the case of *G. hyalinospora*, pulmonary infection (Kane *et al.* 1997; Iwen *et al.* 2000). Apparently, this species is able to grow on the paralyzed bees, perhaps by degrading hydrocarbons or chitin or at least the soft tissue of the bees.

P. lilacinus occurs in soils, on crop, fruits, animals and insects (Domsch *et al.* 1980). It is known to be a parasite of nematodes. Other members of the polyphyletic genus *Paecilomyces* as well as the sister genus *Cordyceps* are known as parasites of insects, especially the caterpillar fungi (Sung *et al.* 2007). *P. lilacinus* is also able to degrade chitin (Domsch *et al.* 1980) and has been considered as a pest control agent against root decaying nematodes (Fiedler and Sosnowska 2007; Oclarit and Cumagun 2009).

A. elegans, F. oxysporum, C. echinulata and *C. elegans* have also been found in soil, on crop and other plants. While *A. elegans* has been found on dead bees (Domsch *et al.* 1980) and *F. oxysporum* on several insects including brood and honeycombs of wild bees (Domsch *et al.* 1980), the two *Cunninghamella* species have not yet been reported to be associated with insects. *Fusarium* species are the most common fungi on crops and produce a wide range of mycotoxins (Brase *et al.* 2009).

The only other study that provided a survey of the fungi in brood cells of a brood caring insect was done by Batra *et al.* (1973) on the alkali bee *Nomia melanderi.* They also found *A. flavus* as the prevalent and most devastating fungus followed by *A. tamari*. Besides *P. lilacinus,* two species of yeast were also found frequently. Thus, this bee species shows a similar spectrum of fungi. We found no evidence for infestation of the larval provisions with specific honeybee pathogens and there was no indication of infestation of the beehives from which we obtained the honeybees. As *Nomia melanderi* constructs its nests in sandy soils like beewolves but provision their progeny with pollen and nectar, and no indication that the honeybee prey is a major source for the mold fungi, the soil might be the most important source for mould fungi.

The spectrum of fungi we found in the beewolf brood cells are generalists which occur mainly in soil or occasionally on insects, instead of specialized entomopathogenic fungi. The fungi could all have originated from the soil or from incidental contamination on the prey bees. Soil contains an innumerous diversity of fungi. Most of the yet known fungi occur in soil in some stage of their life and some thousand species have been isolated from soil(Bridge and Spooner 2001). However, this is only a fraction of those actually present in soil, as only about 17% of the known fungi can be cultivated (Hawksworth 1991). With soil as the major source for fungal infections of beewolf brood cell, the beewolf progeny is confronted by a wide spectrum of potential infestations. The actual threat for a brood cell is rather unpredictable through this multitude of soil fungi. The small number of fungi isolated from the beewolf brood cells consists of generalistic fungi that are able to grow on a variety of organic substances, including especially hydrocarbons (Lowery *et al*. 1967, Egli and Wanner 1974, Domsch *et al*. 1980) . The cuticular hydrocarbons on the bee are the first food source that is accessible to the fungi. This might limit the spectrum of fungi somehow. The fungi that are best adapted to the actual conditions in a brood cell, like temperature, humidity and the bees as food source will outcompete others and thereby dominate a brood cell. However, the situation on beewolf cocoons already filters the spectrum of potential fungi. The antibiotic combination on the cocoons discriminates against all fungi that are susceptible for these antibiotics. Fungi that can cope better with these substances will have the advantage to infest the cocoons that perhaps carry a lower amount of antibiotics.

There are examples of much more specific associations between insects and fungi, e.g. the fungus harvesting leafcutter ants and bark beetles. These insects grow fungi for their nutrition and these fungus gardens are threatened by specific parasitic fungi that encounter ideal growth conditions. Attine ants harbor a quite restricted assortment, fungi of the genus *Trachymyrmex,* within their fungus gardens. The gardens might be parasitized by a seemingly well adapted fungus of the genus *Escovopsis* (Currie *et al.* 1999). In galleries of bark beetles several fungi are found, ranging from symbionts to pathogens (Mueller *et al.* 2005; Cardoza *et al.* 2006; Lee *et al.* 2006). In these cases, the defense mechanisms against these detrimental fungi have to be rather specific in order not to harm the respective

symbiotic fungus. Leaf cutter ants engage in a second symbiosis with streptomycete bacteria that produce antibiotics against the parasitic fungus but that do not harm the fungus gardens and do not show effects against generalistic saprophytic and entomopathogenic fungi (Currie *et al.* 1999). Bark beetles however differentiate fungi in their galleries not by the specificity of the antibiotic. Instead they show specific responses towards different fungi and apply a oral secretion that contains antibiotics to potential deleterious species but not to beneficial ones (Cardoza *et al.* 2006).

The threat by a diverse and unpredictable spectrum of opportunistic fungi has probably shaped the beewolves' defense against these microorganisms. Both known defense mechanisms, the embalming of the prey and the antibiotics that are produced by the bacteria on the cocoon are rather unspecific. Both lines of defense seem to be directed against a huge variety of competing microorganism and may even include bacteria. The embalming of the honeybee prey with a thick layer of hydrocarbons reduces water condensation on the bees and, thus, impairs basic conditions for the germination and growth of fungi (Herzner *et al.* 2007; Herzner and Strohm 2007; Strohm *et al.* 2007). Since fungi heavily rely on high moisture, adaptations to overcome this defense mechanism would require a number of profound evolutionary changes. The antibiotics that are produced on the cocoon by the symbiotic *Streptomycetes* consist of a mixture of nine different substances. This represents a kind of combination therapy that is effective against a multitude of opportunistic as well as entomopathogenic fungi and bacteria (Kaltenpoth *et al.* 2005; Kaltenpoth *et al.* 2006; Kroiss *et al.* 2010). Since beewolf brood cells are a negligible way to reproduce for the opportunistic fungi that pose the most important threat to beewolf progeny, there is little selective pressure to evolve adaptations against the defense mechanisms of beewolves. Thus, though most of the fungi are highly aggressive and ubiquitous, it is unlikely that there is an ongoing arms race between beewolves and fungi as would be expected for a specialized parasite (Strohm *et al.* 2001).

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Chapter 3: NO defense against fungi

3.1 Introduction

Several insect species try to increase the survival probability of their offspring by providing parental care (Mueller and Eggert 2003). Parental care can aim to reduce the threat of predation, competition or harsh environmental conditions (Wilson 1975). To avoid predators and unfavorable conditions parents can choose a better oviposition site, eggs can be hidden and protected by different mechanisms. Several insects use plant structures or natural cavities to protect their offspring. Some dig nests, create protective structures from plant material or by themselves (Danks 2002). Microbes, however, are omnipresent and cannot be avoided, so further protection is needed to ward them off. Cane *et al.* (1983) suggested that mandibular gland secretions of solitary bees are used for nest cell disinfection since several substances that occur in the secretion showed antimicrobial activity. Different species of the burying beetle, *Necrophorus*, treat the carcasses they feed to their offspring with oral as well as anal antimicrobial exudates (for an overview see Cotter and Kilner 2010). They thereby add substances of their own immune system to the provisioned carcasses, like antimicrobial peptides, lysozymes and phenoloxidase (Boman and Hultmark 1987; Rowley *et al.* 1990; Gillespie *et al.* 1997). Gil-Turnes *et al.* (1989) and Lam *et al.* (2009) showed that bacteria applied to the surface of crustacean embryos and the egg of the housefly *Musca domestica*, provide protection from pathogenic fungi. The eggs of the fruit fly *Ceratitis capitata* are protected by a layer of antibacterial peptides applied from accessory reproductive glands by the mother (Marchini *et al.* 1997). Females of the European beewolf use cuticular hydrocarbons to delay fungal development on their prey bees (Strohm and Linsenmair 2001; Herzner and Strohm 2007 but see below for more details). In some cases larvae are known to defend themselves. Gross *et al.* (1998; 2002; 2008) showed that larvae of different beetle species defend themselves by applying glandular secretions to their surroundings. Until now, no case of eggs protecting themselves is known.

Females of the European Beewolf invest quite a lot of resources into each progeny. They provide food and shelter for their offspring. They also protect them from competing or pathogenic fungi. A hydrophobe hydrocarbon layer on the provisioned food reduces water condensation and thus retards fungal growth. Further the cocoon is protected by antibiotics from symbiotic *Streptomycetes* (see general introduction for a detailed description). Yet, the days until the larva hatches from the egg and is capable to move, feed on the bees, are still a critical time. However, Strohm (1995) found that the egg itself delays fungal growth. This mechanism is the subject of the following chapter. With the observation cages developed by Strohm and Linsenmair (1994) beewolf brood cells are accessible during the complete provisioning process as well as the development of the egg and larva. A few hours after the

oviposition a characteristic smell is perceivable which can be characterized as sharp and stinging. This gaseous compound was characterized with a specific reagent as a nitrous gas.

Personal observations revealed that the characteristic smell is not always present in beewolf brood cells. Therefore, the release of the gas over time was analyzed. Nitrous gases are strong oxidants capable of oxidizing iodide to iodine. An iodide starch solution was used to monitor and also quantify the release of the gas. The same reaction was used to monitor the temperature dependency of the observed release pattern.

Nitric oxide (NO) that is released by the nitric oxide synthase (NOS) is the most common gaseous nitric compound in organisms and has been reported from several species. NO does not react with water and can therefore not react with the specific reagent. However, NO reacts with the oxygen in the air to nitrogen dioxide (NO_2). NO_2 disproportionates in water to nitrite and nitrate and only nitrite is detected with the specific reagent. To prove that NO is actually released by the beewolf egg I used a fluorescent probe that was specifically designed to bind NO and increase thereby its fluorescence.

A common method to demonstrate the NOS activity is the NADPH diaphorase staining. The NOS uses NADPH to reduce in a first step arginine to Hydroxyarginine. In the second step, NO is released from Hydroxyarginine. This technique uses the reductase domain of the NOS to transfer electrons from NADPH to nitroblue tetrazolium. The NOS retains its catalytic function even after tissue fixation with formaldehyde (Scherer-Singler *et al.* 1983). Nitroblue tetrazolium is soluble and not colored in its oxidized form and can enter the fixed tissue. There the NOS can transfer electrons from NADPH to reduce nitroblue tetrazolium which thereby forms a dark blue precipitate. Hope *et al.* (1991), Dawson *et al.* (1991) and Young *et al.* (1992) showed that the diaphorase activity is not only identical with the localization of NOS but caused by it.

Since the monitoring of the gas release showed a peak release within two to three hours I asumed that the expression of the NOS is elevated just before the release. I extracted the messenger RNA of five stages of development of the egg and measured the expression of the NOS with quantitative polymerase chain reaction. As a third evidence that the NOS is the source of the gas released by the beewolf eggs I used an arginine analogon to inhibit NOS activity. The gas release of eggs injected with the active, inhibiting analogon L-NAME was compared to that of eggs injected with the inactive isomer D-NAME. To assess the energetic costs the egg incurs with the production of NO I tried to measure the release of carbon dioxide during the time NO is released. This range of methods has been already suggested by Rivero (2006) to prove activity of NO and the NOS.

The antimicrobial effect of the beewolf egg was evaluated in a series of biotests. At first, the germination and growth of fungi on artificial (agar plates) and natural surfaces (embalmed honeybees) were compared to ensure the significance of the following tests. To assess the effective spectrum of the defense of the beewolf egg, fungal isolates of beewolf brood cells were exposed on agar plates to the egg as well as some standard organisms. Beewolf brood

cells vary in volume and the several sinks are possible that may decrease the gas concentration. Therefore the efficacy of the gas defense was tested by exposing the microorganisms under different volumes that simulate the variation of the gas concentration. The effect was also tested on different development stages of the fungi, from spores to a thorough mycelium. Furthermore, as the beewolf egg releases the gas not constantly, a cup with the egg was transferred continuously over an inoculated plate. This test had the further benefit to investigate if the initial low amount of released gas is sufficient to protect the egg until the major burst occurs. An artificially produced nitrous gas was used to examine that the effect of the beewolf egg can be assigned to it.

3.2 Materials & methods

Beewolf specimens

Beewolf females were taken from populations in Erlangen, Würzburg, Regensburg and Berlin (Germany). Females were either captured at these locations in the years 2008, 2009 and 2010 or taken from their F1 generation reared in the laboratory. Wasps were kept in observation cages that consisted of a cubical flying compartment of 27dm³ and a nesting compartment of 60cmx30cmx1cm with a slope of 20% filled with sand (Strohm and Linsenmair 1994). Beewolves were provided with honey and honeybee workers *ad libitum*. Eggs were carefully taken directly from finished brood cells using tweezers.

3.2.1 Identification of the gas and its source

Identification of the gas

The Spectroquant Nitrite Test (Merck, Germany), a premixed nitrite test based on the formation of an azo compound (Griess reagent), was used to show the production of nitrous gases. Eggs could simply be placed in the lid of a 1.5mL reaction tube taking advantage of the circumstance, that they are partially coated with a sticky secretion that fixes them to the thorax of the paralyzed bee. The cup contained 1mL of the test solution (10mg premixed reagent /mL distilled H_2O). NO reacts with oxygen to NO_2. NO_2 disproportionates in aqueous solutions to nitrite and nitrate. The griess reagent will only react with nitrite. Zink reduces nitrate immediately to nitrite. Therefore, a glass fiber filter disc with zinc powder was placed on the surface of the test solution to include the nitrate. The cap with egg and test solution was incubated for 24h at 25°C until the main gas production was finished. An absorption spectrum was measured from 350 to 700nm (Nanophotometer Pearl, Implen, Germany). The same setup without an egg was used as negative control and blank for the photometer. I used a sodium nitrite solution ($NaNO_2$) as reference for the specificity of the nitrite test. The formation of the azo compound takes 5 minutes and it is stable for up to one hour. After that the azo compound ages and changes its color. However, the beewolf egg releases the gas over a period of several hours. Thus, the solution exposed to an egg contains always an aged azo compound. Therefore, we used not only the reference's color and absorption

spectra 10 minutes after the formation of the azo compound with sodium nitrite compared also the azo compound that was allowed to age for 16 hours.

Quantification, time course and temperature dependence of gas production

Due to high sensitivity and stability, the potassium iodide starch solution, a simple assay to detect oxidants, was used to quantify the production of gas by beewolf eggs. The solution contained 1% (w/v) of potassium iodide and 1% (w/v) of starch in distilled water. Observation cages were kept at 25°C and checked each hour for new eggs. The eggs could simply be attached to the cap of a 1.5mL reaction tube taking advantage of the circumstance, that they are partially coated with a sticky secretion to attach them onto the thorax of the paralyzed bee. The cups with one egg and 1mL of the iodide solution were incubated for 24hours at 28°C. Then the absorption was measured at 590nm in a spectrophotometer (Kontron Uvikon, Germany). Additionally to the relative quantification the reaction was calibrated by titrating the solution that was exposed to an egg with sodium thiosulfate solution (c=0.001M; Merck).

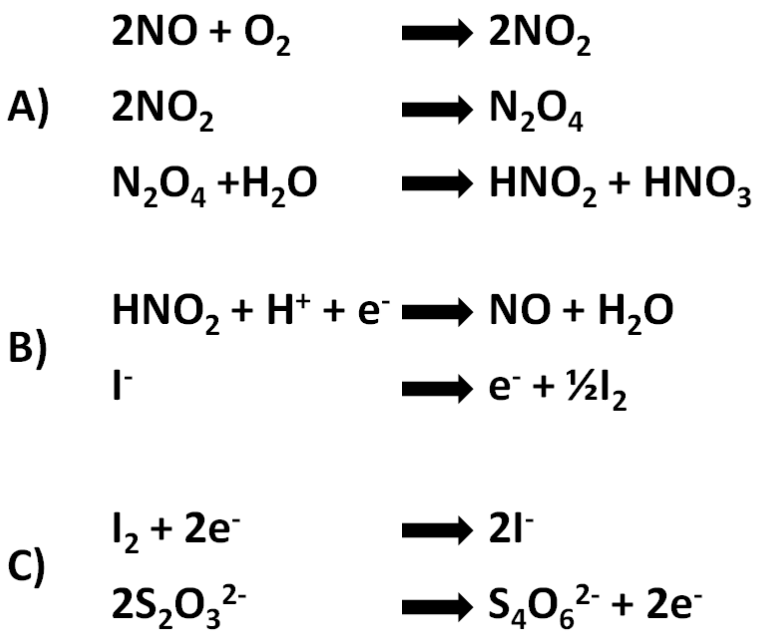

Figure 3-1 Reaction scheme after Holleman-Wiberg (Lehrbuch der anorganischen Chemie/Inorganic Chemistry, 2001). **A** shows the primary reaction with air and water, **B** the redox-reaction in the iodide starch solution and **C** the titration of the iodine starch complex with thiosulfate.

According to the reaction scheme above (Figure 3-1) nitric oxide (NO) is oxidized to nitrogen dioxide (NO_2) which dimerizes to dinitrogen tetroxide (N_2O_4) before this dissolved in the aqueous test solution in equal amounts as nitrite (NO_2^-) and nitrate (NO_3^-). Only the nitrite molecules are able to oxidize iodide. By oxidizing iodide (I^-) to iodine (I_2), the nitrite is reduced back to NO which reacts again with the oxygen dissolved in the solution to NO_2. NO_2 respectively its dimer N_2O_4 dissolves again and disproportionates to nitrite and nitrate. The calculated amount of oxygen dissolved in the aqueous solutions of 1% potassium iodide and 1% starch at 28°C is 0.255µmol/mL. The maximal measured amounts of NO were 0.38µmol.

As only half of the NO needed to be re-oxidized by dissolved oxygen and additional oxygen was available from the head space, this cycle continued until all NO was dissolved as nitrate. After the first step half of the NO oxidized iodide, after the second three quarters, after the third, seven eighth etc. Thus, the amount of released NO is finally exactly represented by the amount of oxidized iodide or the amount of thiosulfate.

To resolve the time course of the gas production, observation cages were kept at 25°C and checked each hour for new eggs. The maximal one hour old eggs were placed in the reaction tubes with the iodide starch solution and incubated at 25°C. Each hour the lid with the egg was transferred to a new reaction tube with new solution. The absorption of the exposed solution was measured immediately.

To observe the temperature dependence of the gas production, reaction tubes with freshly laid eggs (within 1h after oviposition) and the iodide – starch solution were placed in a white styro-rack (Hartenstein, Germany) inside an incubation chamber at constant temperatures varying from 20°C to 30°C. To monitor the color development a Canon Eos 20D was triggered with a remote shutter release every 30 minutes. The formation of the iodine starch complex was evaluated visually.

Detection and localization of nitric oxide inside the egg

Diaminorhodamin-4M AM (DAR4M-AM; Alexxis Biochemicals, USA) is a cell permeable, photostable, fluorescent probe, that was developed as a specific reagent to detect NO (Kojima *et al.* 2000; Kojima *et al.* 2001; Gomes *et al.* 2006). The DAR4M-AM solution was prepared according to the producer's instructions (10µM in 0.1M phosphate buffer, pH7.4) and injected into beewolf eggs with a microinjector (built by technicians of the Faculty for Biology, University of Regensburg, Germany) equipped with Eppendorf Femtotips II. Eggs of *Osmia bicornis* (Hymenoptera, Megachilidae) and *Ampulex compressa* (Hymenoptera, Ampulicidae) were injected as controls. The eggs were kept on microscope slides in dark wet chambers at 25°C and intensity of fluorescence was observed after 1, 3, 5, 24, 48 and 72h. Eggs injected with the phosphate without the probe were observed the same way to assess the intensity of auto-fluorescence. The DAR4M-AM solution was also placed in the vicinity of the beewolf egg to test for the presence of NO outside the egg and it was incubated with air to exclude increasing fluorescence due to airborne radicals. To this end, 50µL of probe solution were placed in a 1.5mL reaction tube together with or without an egg in the same way as for the nitrite test. For the localization of NO, complete eggs injected with the DAR4M-AM solution were examined under a Zeiss 510 LSM (Germany). Furthermore, 10µm sections of the egg were analyzed to verify the localization from the confocal microscopy. To this end injected and incubated eggs were fixed in 4% paraformaldehyde in PBS for 2h at 4°C, washed with PBS, soaked in Tissue Tec and frozen. Sections were cut on a cryostat (Leica CM3000, Germany) and immediately examined under a fluorescence microscope (Zeiss LSM 10, Germany).

NADPH diaphorase staining

To test for occurrence and distribution of NOS in the egg tissue, NADPH diaphorase staining was conducted after Müller (1994). Eggs were fixed in PBS containing 4% paraformaldehyde for 2 h at 4°C, followed by cryoprotection in PBS with 12% sucrose for 20h. The tissue was soaked in Tissue Tec for 30min, frozen and 10µm sections were cut on a cryostat (Leica CM3000, Germany). The sections were incubated for 60min at 30°C with 50mM Tris-HCl, pH 7.8, 0.1% Triton X-100 (Carl-Roth, Germany), and 0.2mM nitroblue tetrazolium(Carl-Roth, Germany) in the presence or absence of 0.2mM β-NADPH (Carl-Roth, Germany) to demonstrate fixation-insensitive NADPH diaphorase activity. The NOS is not the only enzyme that shows NADPH diaphorase activity, but the NOS remains active even after fixation. The sections were dehydrated and mounted with Depex. Slides were examined under a stereomicroscope (Zeiss LSM 10, Germany).

RNA extraction, reverse transcription and quantitative PCR

Since the mRNA amounts retrieved from single eggs were insufficient to obtain reproducible results I collected samples of 25 eggs for each of four different times intervals after oviposition (4-5, 9-10, 14-15 and 19-20 hours). Observation cages were kept at 25°C and checked each hour for new eggs. The eggs were removed from the brood cell after the specified time periods, shock frozen in liquid nitrogen and stored at -80°C. After collection, all eggs of one group were pooled and their RNA was extracted using the peqGOLD total RNA Kit (Peqlab, Germany). Total RNA was submitted to a digestion with DNaseI to remove genomic DNA contamination (Fermentas, Canada) and transcribed into cDNA with BioScript (Bioline, Germany) using an Oligo-dT-Primer (Fermentas, Canada).

Quantitative PCR measures increase of DNA during each replication cycle of the polymerase chain reaction. The initial amount of DNA of a sample can be calculated by comparing the increase in DNA with samples of known DNA concentration (the standard curve). However, due to varying efficacy of RNA extraction, RNA degradation, and efficacy of the reverse transcription, different samples of the same number of eggs do not contain the same percentage of cDNA compared to the original RNA. Therefore, an internal standard, a reference gene, is needed to calculate the ratio between the gene of interest and the standard gene. Genes which are necessary for the cellular maintenance therefore should be expressed at a constant rate are candidates for reference genes. The β-*actin* gene was used as standard to calculate a NOS/ β-actin mRNA ratio (Thellin *et al.* 1999). Both, the β-*actin* gene and the NOS gene were unknown in *P. triangulum*. Therefore we developed new *P. triangulum* specific primer for the *NOS* and *β-actin* gene. First degenerated primer were designed based on sequence information from other hymenoptera. The beewolf PCR products were sequenced (Seqlab, Göttingen) and specific primer were designed based on the beewolf sequences. The NOS primer amplified a fragment of 312bp, the actin primer 321bp (for primer sequences see Table 3- 1). The qPCRs were conducted on an Eppendorf Realplex cycler in a final volume of 25µL, containing 1µL of template cDNA, 2.5µL of each primer (10pM, synthesized by Metabion, Germany) and 12.5µL of SYBR Green Mix (SensiMixPlus SYBR Mit, Quantace). Standard curves were established by using 10^{-9} – 10^{-3}ng

of purified PCR products as template. The peqGOLD MicroSpin Cycle-Pure kit (Peqlab, Germany) was used to purify PCR products. A NanoDropTM1000 spectrophotometer (Peqlab, Germany) was used to measure DNA concentrations for the templates of the standard curves. The following PCR conditions were used: 95°C for 5min, followed by 50 cycles of 56°C for β-actin and 65°C for NOS each for 60s, 72°C for 60s and 95°C for 60s. Then a melting curve analysis was performed by increasing the temperature from 60°C to 95°C within 20min. Based on the standard curves, the amount of NOS and β-actin template and, using the DNA sequence, the number of mRNA copies and the ratio of NOS copies to β-actin copies was calculated.

Table 3- 1 Primer sequences used for quantitative PCR. All primer sequences are shown in 5' to 3' orientation.

	forward	reverse
β -Actin	GGTAACGAAAGATTCCGTTG	GATCCACATCTGTTGGAAGG
Nitric oxide synthase	TTTATTGGAGACCATAGCAACTC	TTCCAAGCAGGATCCTGAGC

NOS inhibition assay

Another approach to demonstrate the action of NOS in the generation of NO was to inject freshly lain eggs with L-NAME, an inhibitor of NOS action, and as a control with the chemically similar D-NAME that does not inhibit NOS. Eggs were injected with about 0.2µL (L-N^G-Nitroarginine methyl ester) injected into an egg exceeded the amount of arginine required to release the measured amounts of NO by tenfold. A second sample of eggs was injected with 1.5mol/L D-NAME in 0.1M phosphate buffer at pH 7.4 and a third group of eggs was not injected at all. All eggs were placed in the lid of a cup with 1% potassium iodide and 1% starch solution and incubated for 24h at 25°C. The absorption of the iodide-starch solution was measured with a photometer (Nanophotometer Pearl, Implen, Germany) at 590nm. The absorption values were subjected to statistical analysis with PASW Statistics 18. A Kruskal-Wallis-test and following Mann-Whitney-U-tests with Bonferroni correction after Holm (1979) were used to compare the three groups.

Metabolic rate of eggs

To assess the rate of metabolisms of beewolf eggs during NO production I measured carbon dioxide levels in a reaction tube with one beewolf egg. To prevent the egg from desiccation, it was necessary to add 10µL of distilled water to the 1.5mL reaction tube. Carbon dioxide may form carbonic acid in water, so no constant buildup of carbon dioxide levels could be measured. However, the process of carbon dioxide dissolution in water is slow enough to measure varying carbon dioxide levels, when CO2 release of the egg increases or decreases. This allows to observe changing carbon dioxide release qualitatively. We used coupled GC/MS (Agilent 6890N Series gas-chromatograph & Agilent 5973 inert mass selective detector, agilent Technologies, Böblingen, Germany) equipped with a RH-5mx+ fused silica capillary column (30m x 0.25mm ID; df = 0.025µm). 25µL samples were injected and run at a constant temperature of 70°C with helium as carrier gas at a constant flow of 1mL/min. A splitless injector was installed at 250°C and the electron mass spectra (EI-MS) were recorded

with an ionization voltage of 70eV. We scanned for the specific molecular masses of carbon dioxide (44u) and argon (40u). Since the argon concentration is constant, we calculated the carbon dioxide/argon ratio and obtained a relative value for the metabolic rate of the beewolf eggs. The mean of carbon dioxide/argon ratios of the measurement of six eggs were correlated with the mean absorption of the iodide starch solution of four other eggs at the corresponding time interval with PASW Statistics 18 using a Spearman-rank correlation analysis.

3.2.1 Effect on microorganisms

The germination process of fungi is complex and depends on various factors. We examined and compared the germination and growth on agar plates and on paralyzed and embalmed honeybees. *Aspergillus flavus* and *Paecilomyces lilacinus* had the advantage that conidiae of both species could be easily harvested and employed in germination tests. The hindlegs of honeybee workers were cut off since the tibiae provided the most suitable surface for microscopic observations because they have a rather large and smooth area with only few hairs. The bees were taken from recently finished brood cells. The legs were fixed on a thin layer of wax on microscope slides and inoculated with 1µL of a spore suspension of 700 spores/µL for *A. flavus* and 665 spores/µL for *P. lilacinus*. The microscope slides with the legs were incubated at 25°C in the dark in a moist chamber and observed in intervals of 5 hours for one day with an incident light microscope (Zeiss, Germany). Sabouraud dextrose agar plates were inoculated and the closed plate was likewise incubated at 25°C in the dark and germination and growth of fungi was examined in 5 hour intervals with a stereo microscope (Leica, Germany).

To assess the anti-microbial effect, beewolf eggs were collected from brood cells within 12h after oviposition and placed in plastic caps of 270µL volume. These caps were placed on the agar plates immediately and 24h after inoculation. A second cap without an egg was used as control to exclude influences of the enclosure, like limited oxygen or increased carbon dioxide concentrations under the cap. In the first series a spectrum of microorganisms was tested. *A. flavus, A. tamari., P. lilacinus, Gymnascella hyalinospora , Actinomucor elegans, Cunninghamella echinulata, C. elegans, Fusarium oxysporum* and *Streptomyces spec.* came from our own collection (see Chapter 2); *Escherichia coli* was kindly provided by the Schneuwly group (LS Schneuwly, Faculty for Biology and preclinical Medicine, University of Regensburg), *Bacillus cereus* from the Wirth group (AG Wirth, Faculty for Biology and preclinical Medicine, University of Regensburg). Further a commercially available baker's yeast, *Saccharomyces cerevisia,* was used. The caps were placed on growing cultures of the microorganisms listed above and incubated for 24h at 25°C. Each test was repeated at least once and *A. flavus, A. tamarii* and *P. lilacinus* were tested another 5 times verify the effect of beewolf eggs. The growth of *S. cerevisiae* and *B. cereus* couldn't be evaluated as easily as that of fungi that grow hyphae. Yeast colonies grow in small clutches where the growth state of the single clutches cannot be assessed. For *B. cereus* it was also easier to transfer cells to a new plate and evaluate the growth there than on the old plates where it already grew.

Therefore the exposed and unexposed regions were probed to inoculate a new agar plate to assess whether the bacteria and yeasts are actually dead or just stopped to grow in presence of the beewolf egg.

In a second test series the concentration of the released gas was varied. The egg was placed in caps of different sizes: 270µL, 2,9mL, 7mL and 28,5mL (the lid of the petri dish itself); *A. flavus, A. tamarii* and *P. lilacinus* were used as test microorganisms. The growth of the fungi on the agar plates was evaluated with the unprotected eye, a stereo microscope.

In a third series the effect on different stages of fungal growth was examined. To this end, inoculated agar plates with *A. flavus, A. tamarii* and *P. lilacinus* that had been incubated for 0, 4, 8, 16, 24 or 48h were exposed for 24h to an egg, which was less than 3h old when placed onto the agar plate. An alternative approach was to move the cap with the egg from one spot of the agar plate to another at an interval of two hours on the growing mycelium. All plates of this experiment were inoculated at the same time the respective egg was laid and incubated at 28°C.

To compare whether the effect of the beewolf egg could be mediated by NO_2, artificially generated NO_2 was applied to fungal colonies. NO_2 was produced by heating lead nitrate and collected in a glass cylinder. Its concentration was measured with the starch iodide solution. Amounts equivalent to those released by beewolf eggs were injected into the headspace of a cap of 270µL volume on inoculated agar plates. The eggs release the major amount of NO after 19-20h at 25°C. To have an exposure time similar to that of inoculated agar plates with a fresh beewolf eggs, the agar plates were inoculated with *A. flavus*, *A. tamarii* or *P. lilacinus* 16h before the exposure to NO_2.

3.3 Results

3.3.1 Identification of the gas and its source

Nitrite test

The nitrite test solution exposed to beewolf eggs showed a positive reaction, however the color and the absorption spectrum didn't match exactly those of freshly prepared reference of sodium nitrite ($NaNO_2$). Both the azo-compound formed with the gas from the beewolf egg and that formed with sodium nitrite show an absorption spectrum with two maxima. The two absorption maxima from the nitrite test exposed to the beewolf egg are at 360nm and, the smaller one, at 480nm. The maxima with nitrite are at also 360nm and 540nm, but here the first absorption maximum one is smaller. However, the gas from the beewolf egg is not produced at one point of time but over several hours. Thus the azo-compund ages until the final amount can be observed. When the azo-compound formed with sodium nitrite is

also allowed to age, its absorption maxima shift and match exactly those of the beewolf egg, with the first one at 360nm being the larger one and the smaller one at 480nm (Figure 3-2).

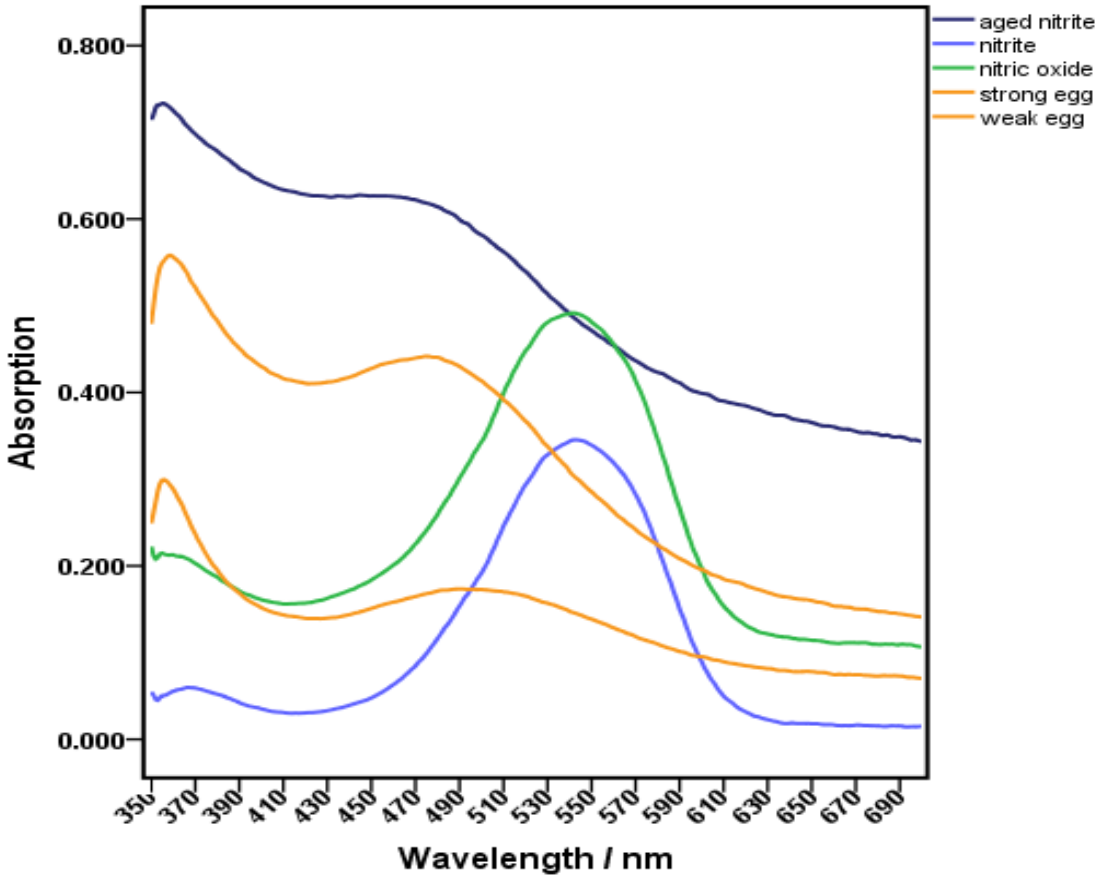

Figure 3-2 Absorption spectra of Merck nitrite test with nitrite, NO and the gas from beewolf eggs. The two orange lines represent two trials with test solution exposed for 14 hours to one beewolf egg respectively. The green line represents the test solution that was exposed to artificially produced NO from nitrous acid and zinc under nitrogen atmosphere. The absorption spectrum of the test solution with NO matches that of the fresh test solution with sodium nitrite(light blue), the spectrum of test solution with beewolf eggs matches that of test solution with sodium nitrite which was allowed to age for about the same time as the test solution exposed to beewolf eggs (14 hours, dark blue).

Quantification, time course and temperature dependence of gas production

The quantification of NO from 250 brood cells revealed that 0.246 ± 0.086µmol (mean±SD) of NO were released per egg. With a average brood cell of 3157mm³ the concentration reaches 1688 ± 681ppm (mean±SD). The time course of the NO release of beewolf eggs incubated at 28.5°C revealed an abrupt increase about 14-15hours after the egg was laid; the elevated release lasted for about 2 hours (Figure 3-3). The timing of the peak shows a strong and highly significant dependence of the environmental temperature (quadratic fit; $F_{2,33}$=813.771, p<0.001, r-square for quadratic fit = 0.982, t=0.204xT^2-12.6xT+208h; Figure 3-4) with a Q10 value of 2.74 over the range of 20-30°C.

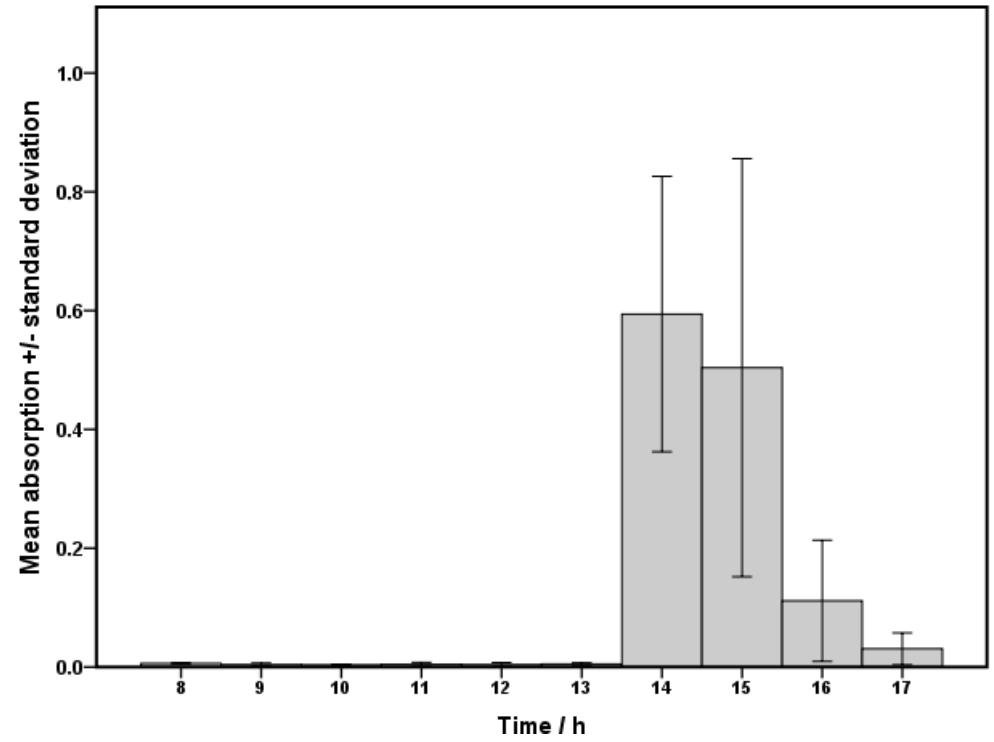

Figure 3-3 Time course of NO release. The bars represent the mean absorption +/- SD of four eggs measured with a series of iodide starch solution that was changed each hour.

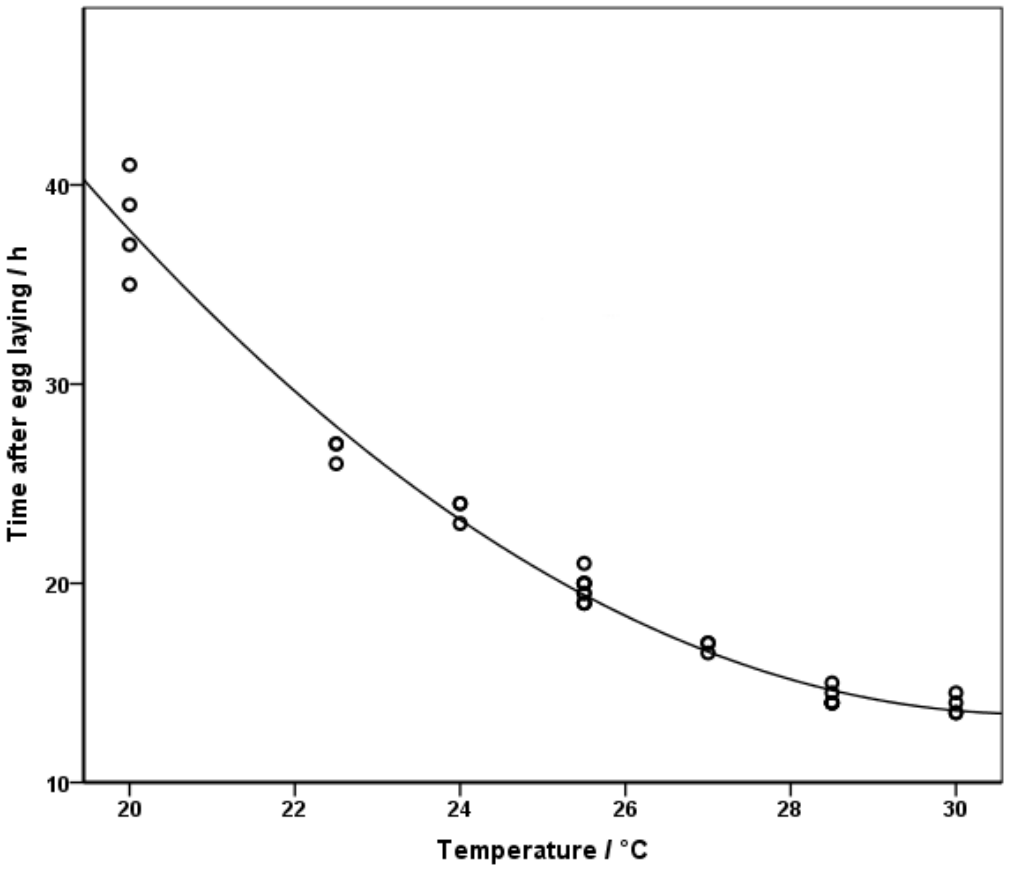

Figure 3-4 The beginning of the peak NO production as a function of temperature. A quadratic regression line is added ($F_{2,33}$=813.771, p<0.001, r-square for quadratic fit = 0.982, t=0.204xT^2-12.6xT+208 [h]).

Detection and localization of nitric oxide inside the egg

The DAR4M-AM injected eggs showed fluorescence that clearly exceeded the auto-fluorescence of uninjected beewolf eggs. Eggs of *O. bicornis* and *A. compressa* injected with DAR4M-AM showed only background fluorescence, comparable to uninjected beewolf control eggs. The fluorescence of injected beewolf eggs increased considerably over time. A slight increase was already observed after one hour and the fluorescence continued to increase for up to 72 hours (see Figure 3-5 for an overview). The injection of DAR4M-AM into beewolf eggs seems to have no influence on their development. Eggs were observed up to 72 hours after injection and larvae regularly hatched at the end of the observation time. These larvae did not show fluorescence in their body tissue. However, since they always feed on the remains of the egg they showed strong fluorescence within their digestive tract. The main fluorescence and thus the major amount of NO was observed in the cuticle of the egg in both confocal optical sections of entire eggs and cryosections of eggs (see Figure 3-6 and 3-7).

A DAR4M-AM solution did not show fluorescence when exposed to air, but it did so when incubated in a 1.5mL reaction tube with a beewolf egg. Injected beewolf larvae showed intermediate fluorescence around the developing nervous system.

NADPH diaphorase staining

Diaphorase staining shows some background signal within the cavity of the amnion. The embryo itself, the amnion which contains the yolk and the serosa which surrounds the entire egg are heavily stained indicating the presence of fixation insensitive NOS (see Figure 3-8).

NOS expression

Though mRNA from several eggs had to be pooled with the consequence that eggs were in slightly different developmental stages, the ratio of *NOS/β-actin* mRNA increased about tenfold about 19-20 h after oviposition of eggs that were incubated at 25°C. From the temperature dependence it can be deduced that at 25°C the peak of gas release starts about 20h after oviposition. Assuming a constant expression of *β-actin* over time, this means that the expression of the *NOS* gene is augmented just before the peak of NO secretion 20h after oviposition. After hatching of the larvae the NOS mRNA level decreased again to the initial level (Figure 3-9).

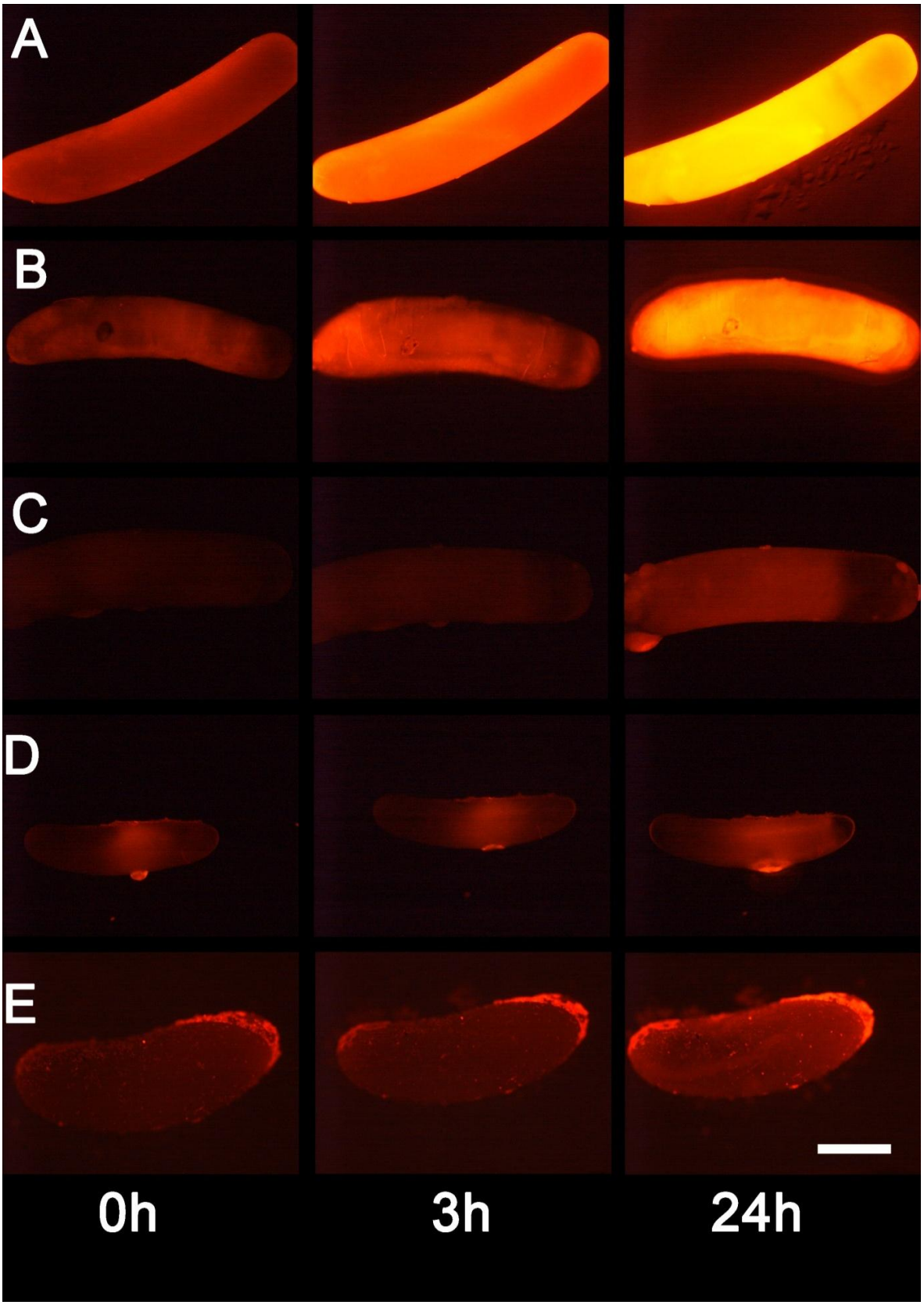

Figure 3-5 Fluorescence of beewolf eggs injected with DAR4M-AM and controls for autofluorescence and eggs of two other species 0, 3 and 24 h after injection. Lane **A** shows an injected beewolf egg showing strong fluorescence, lane **B** a weakly fluorescing egg, lane **C** a beewolf egg injected with only phosphate buffer showing autofluorescence, lane **D** an injected egg of *A. compressa* and lane **E** an injected egg of *O. bicornis*. Bar, 1mm

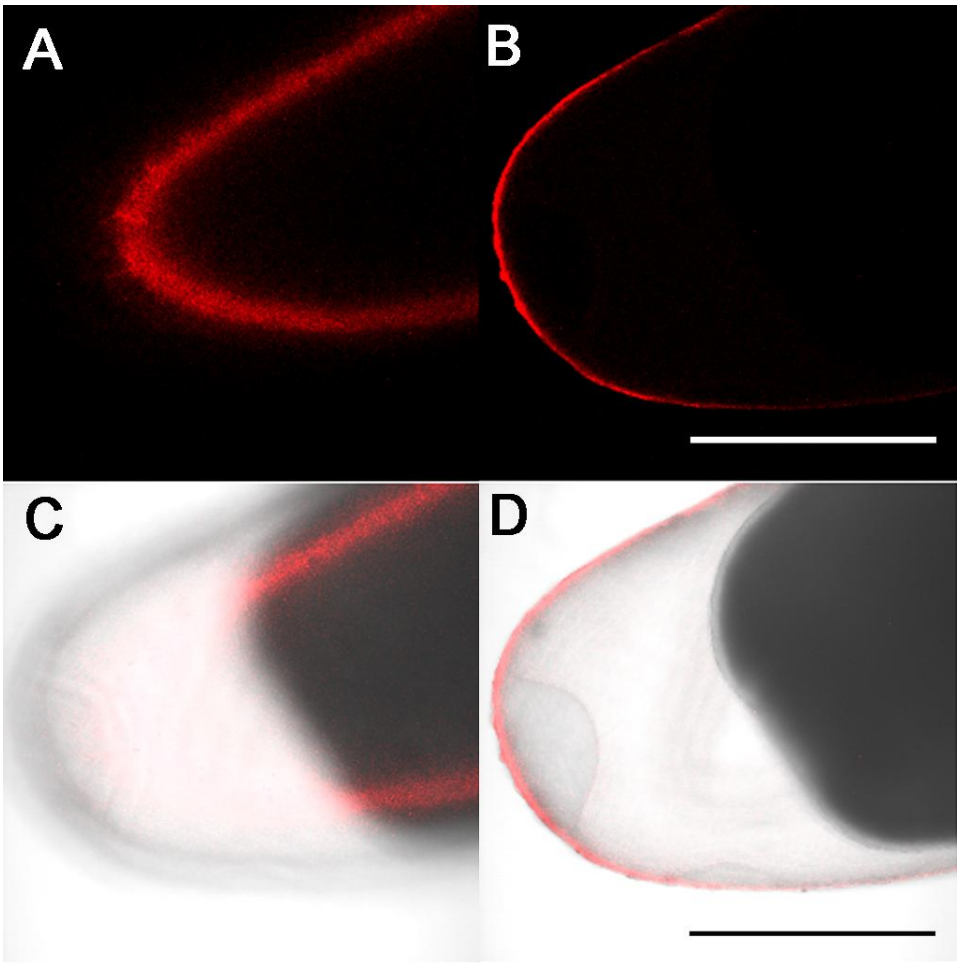

Figure 3-6 Confocal laser scanning images of DAR4M-AM injected eggs. **A** and **B** show a false color image of the DAR4M fluorescence (red) of two different optical sections of the same egg. **C** and **D** show the overlay of the same fluorescence images with light microscopic pictures. Bar, 1mm

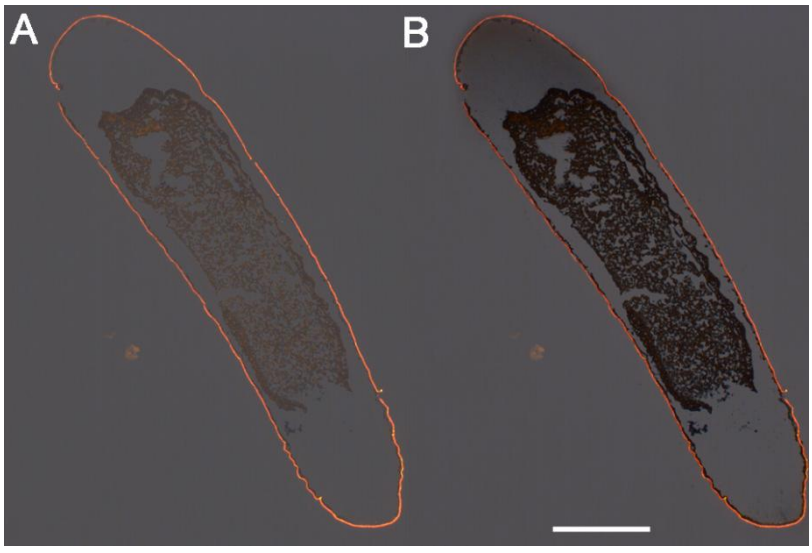

Figure 3-7 Cryosections of DAR4M-AM injected eggs. A and B show an overlay of a fluorescence and light microscopic image of the same section with different transparency to favor the visibility of the fluorescence within the embryo (**A**) and of the tissues in the egg (**B**). Bar, 1mm

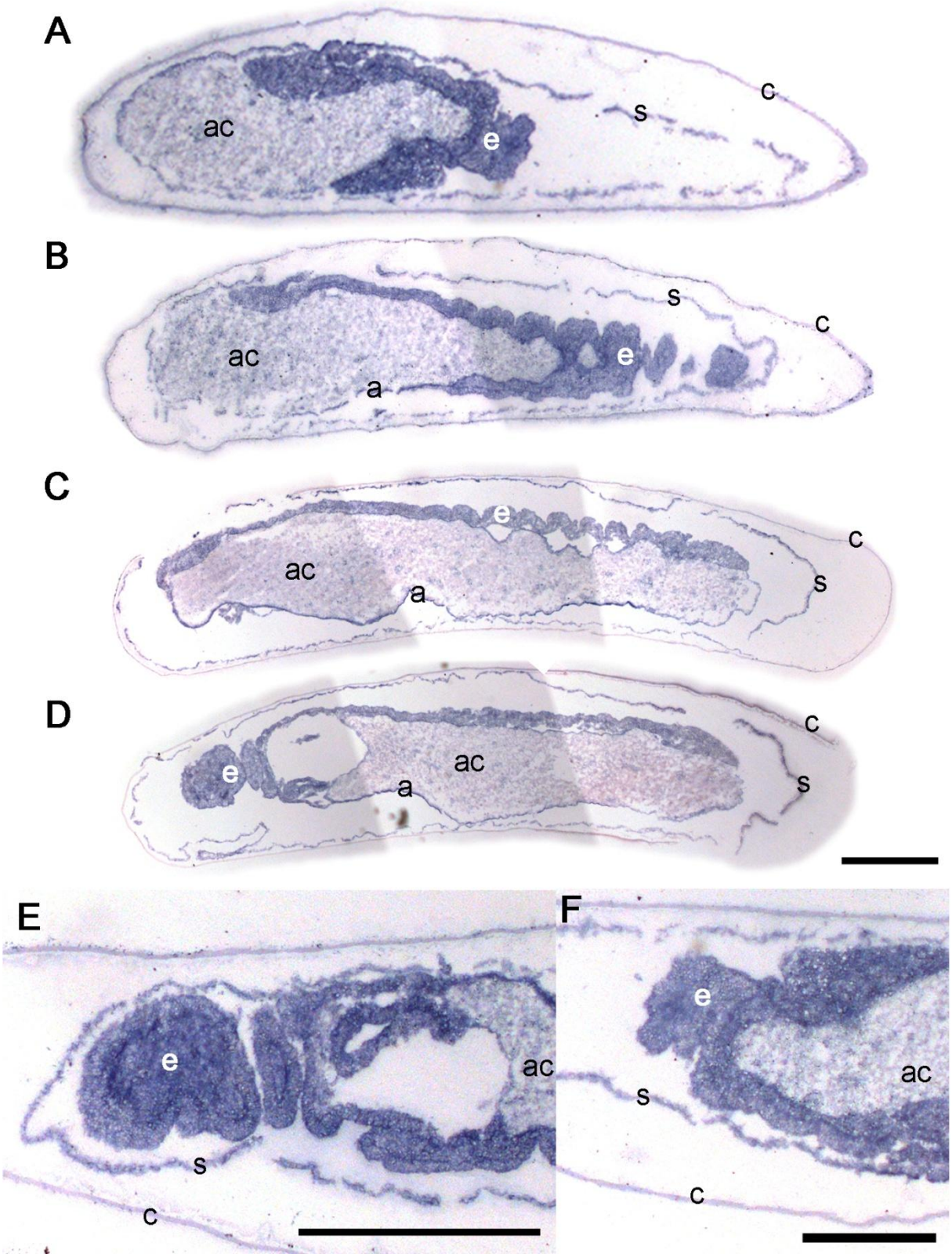

Figure 3-8 The diaphorase staining uses the nitric oxide synthase to reduce nitrotetrazolium blue. A blue precipitate indicates the presence of NOS activity. **A**/**B** and **C**/**D** show different sections of two different eggs. **E** and **F** show a higher magnification of sections of the same two eggs. c=cuticle, s=shrinked serosa under the cuticle, e= embryo, a=amnion and ac= amnion cavity

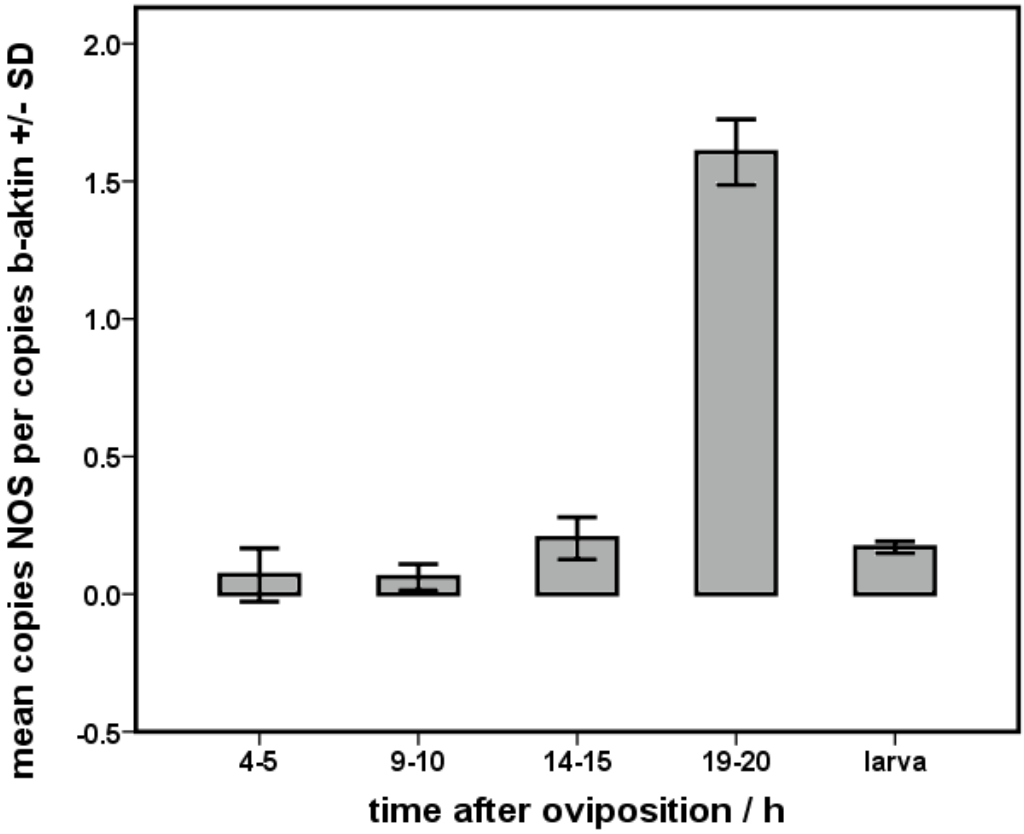

Figure 3-9 Expression of NOS mRNA at different times after oviposition. Bars represent the mean of the NOS/β-actin ratio of two replicates with 24 and 10 eggs +/- SD.

NOS inhibition assay

The comparison of eggs injected with L-NAME, D-NAME solution and not injected eggs shows a significant difference among the three groups (Kruskal-Wallis-Test; χ^2=15,093, d.f.=2, p=0.00053). There is a significant difference between the L- and D-NAME injected group (Mann-Whitney-Test corrected after Holm (1979); U=26.0, Z=-2.331, p=0.0382 without outlier: U=19.0, Z=-2.638, p=0.024) and the L-NAME and noninjected group (Mann-Whitney-Test; U=15.0, Z=-3.814, p=0.000102). The D-NAME and the noninjected groups did not differ significantly (Mann-Whitney-Test; U=48.0, Z=-0.945, p=0.369). See also Figure 3-10.

Rate of Metabolism

The CO_2 release increased on average about twofold during the period of gas release, some eggs, however, showed a fivefold increase. This increase in CO_2 concentration correlated significantly with the release of NO (spearman rank correlation, ρ=0.535, N=38, p=0.000538; see also Figure 3-11).

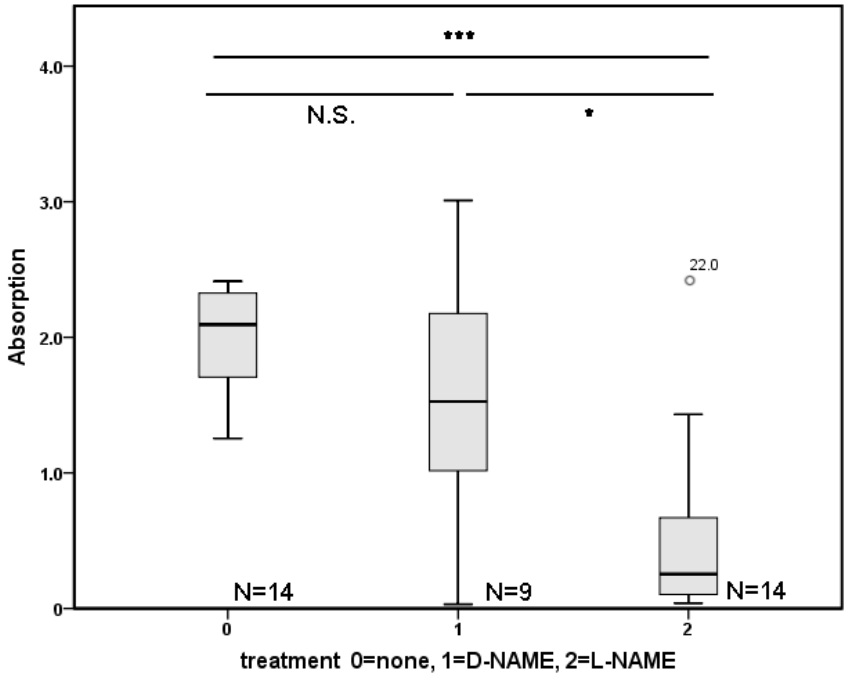

Figure 3-10 The gas release was significantly inhibited by injection of the NO synthase inhibitor L-NAME (treatment 2), an arginine analog compared to no treatment (treatment 0, U=15.0, Z=-3.814, p=0.000102, *** in the figure) and also compared to the injection of the inactive isomer D-NAME (treatment 1, U=2.6, Z=-2.331, p=0.0382, * in the figure). The injection of D-NAME did not inhibit the gas release (U=48.0, Z=-0.945 , p=0.369, N.S. in the figure). N indicates the number of eggs tested in each group, ° indicates an outliner.

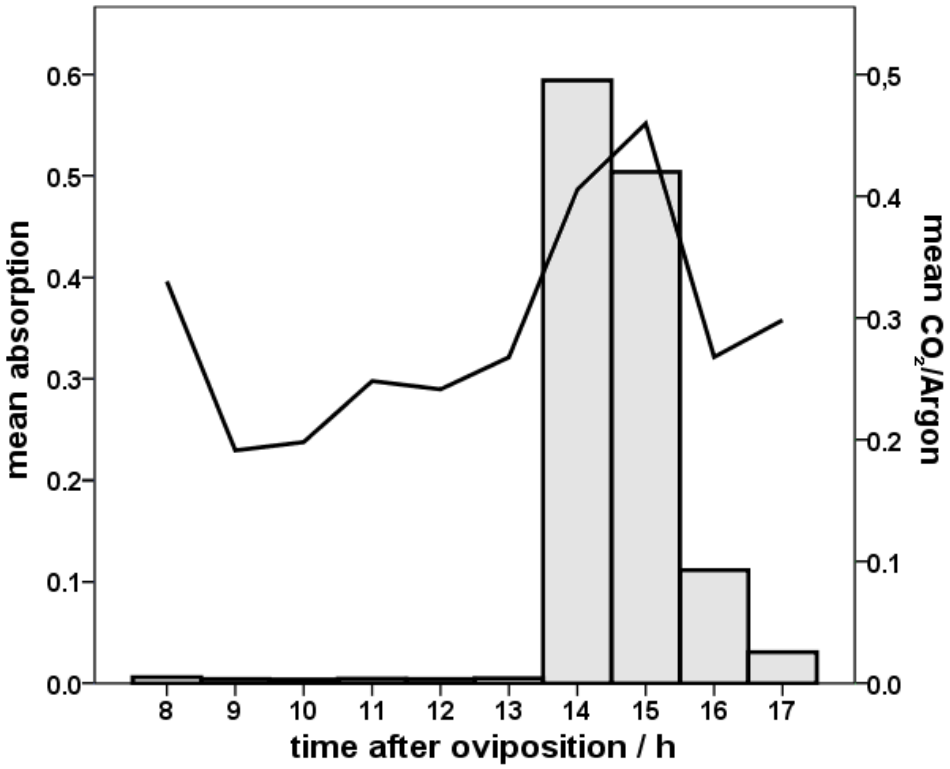

Figure 3-11 Production of NO and an index of metabolic rate (Mean CO_2 / Argon) as a function of time after oviposition. The absorption of the iodide starch solution is represented by the white gray bars, the carbon dioxide/argon ratio by the black line. The increase of both correlated significantly (Spearman rank correlation Rs=0.535, N=38, p<0.001).

3.3.2 Effect of gas on microorganisms

The first germination tubes of *A. flavus* and *P. lilacinus* on agar plates were detected 9 hours after inoculation. Small mycelia were visible after 15 hours. Germination could not be observed on the bee surface. However, 15 hours after inoculation mycelia similar in magnitude to agar cultures were visible. The growth and development of *P. lilacinus* was slightly faster than that of *A. flavus* (Figure 3-12).

Exposure to the beewolf egg had a severe effect on the microorganisms (Figure 3-13). Neither fungi nor yeasts or bacteria were able to grow under the lid with beewolf eggs. Fungal mycelia stopped growing in each case under the cap containing the egg and did not resume growth after the lid's removal. Furthermore, no new mycelia grew into the area which was covered by the lid with the egg, avoiding the agar that was exposed region to the egg and contained dead mycelium. Mycelia that already showed strong growth or started to produce conidia at the time of exposition to the gas stopped their growth. Conidiophores were not completed or in the latest state were much smaller than not exposed ones (7 of 8 cases, see also Figure 3-13). Even still dormant spores that were inoculated on agar plates and immediately exposed to an egg showed no fungal growth. The spores either did not start to germinate or the germinating spores were immediately killed by the gas. Moving the lid with an egg over the agar plate at intervals of two hours showed that even the small amounts of NO produced prior to and after the peak production were sufficient to inhibit fungal growth. Agar plates inoculated with yeast or bacteria from colonies which were exposed to the egg showed no further growth, whereas plates inoculated from unexposed colonies did.

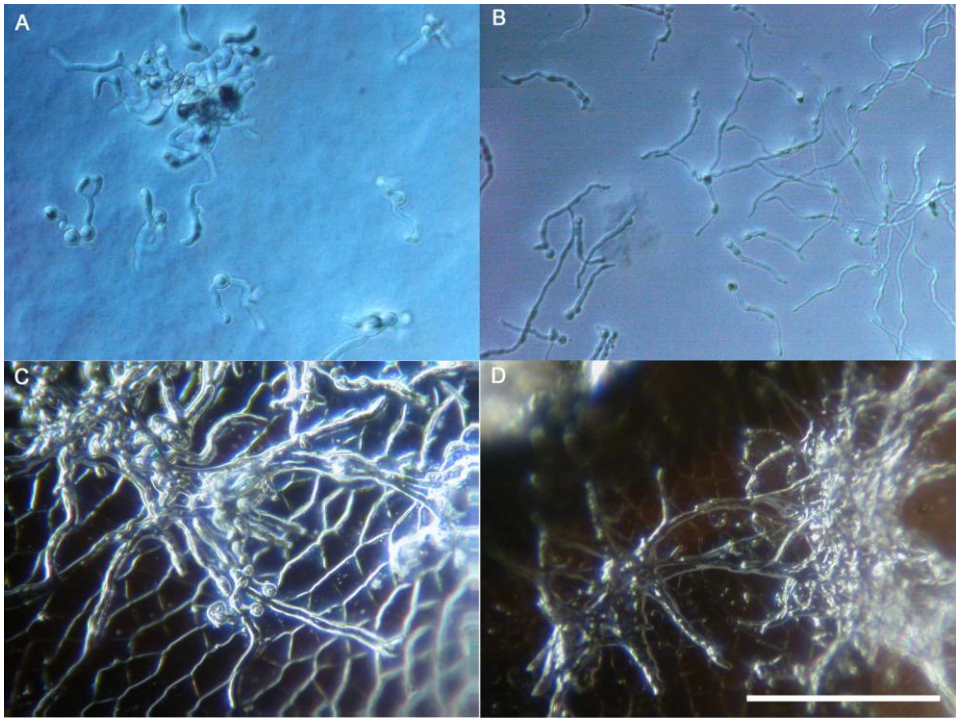

Figure 3-12 Germination of A. flavus (A&C) and P. lilacinus (B&D) on SDA agar (A&B) and the surface of embalmed honeybees (C&D). A&B were taken with differential interference contrast microscopy, C&D with incidents light microscopy. The bar represents 100µm.

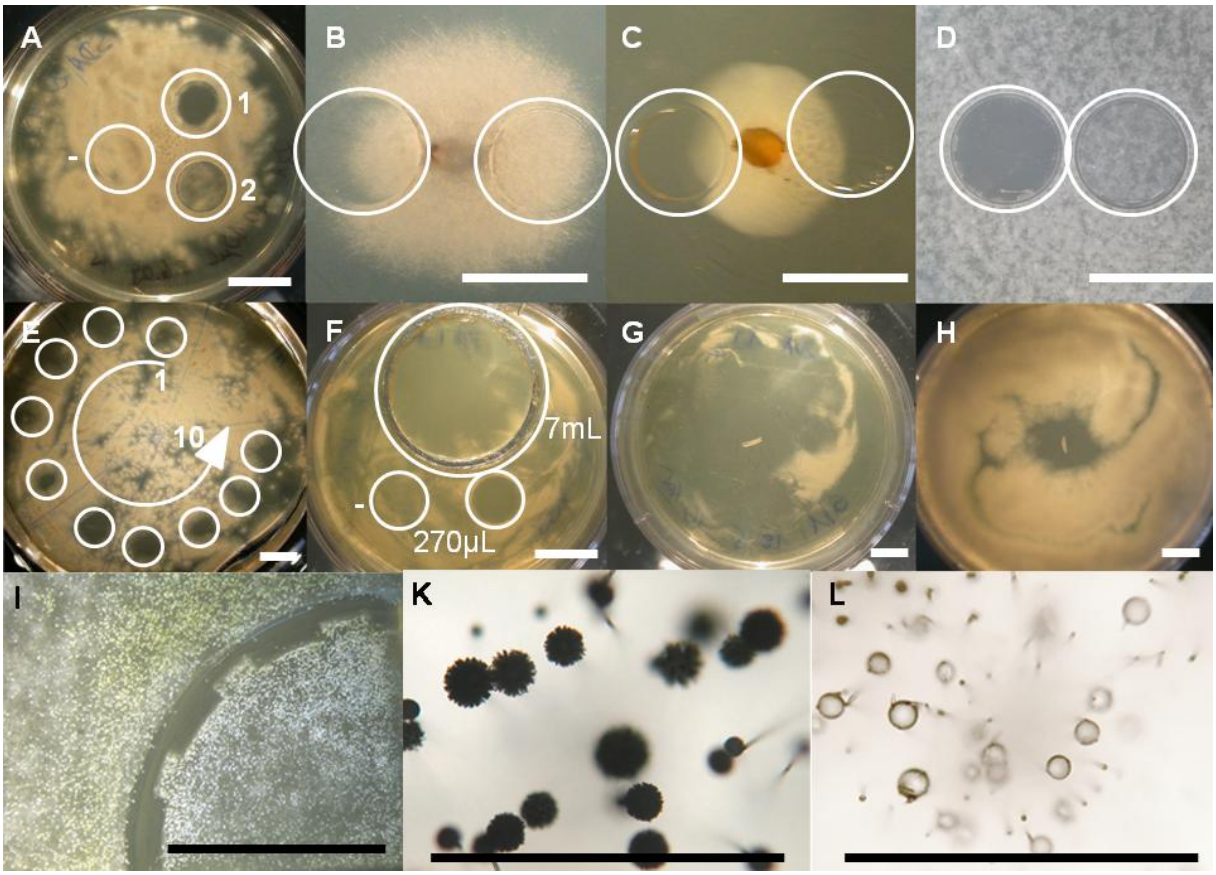

Figure 3-13 Antimicrobial activity of the beewolf egg.
A: Test of antibacterial activity of each one beewolf egg on the growth of A. flavus. The first egg was applied inside a lid of 270µL immediately after inoculation of the plate with the spore suspension (1), the second after 24h of incubation at 25°C (2. The picture of the plate was taken after 48h of incubation, (-) indicates the position of an empty lid as negative control over the total 48h.
B-D: Test of antibacterial activity on the growth of *E. echinulata* (B) and *G. hyalinospora* (C) and *B. cereus* (D). The left circular imprint on the agar indicates the position of the lid containing the egg, the right one the lid without an egg as control. The lid with a fresh egg was placed for 24h on the plate and the picture taken immediately after removal of the lid.
E: The lid with the egg was placed immediately after oviposition in the first segment of the plate which was inoculated at the same time the egg was laid. The lid was moved every two hours until section 10 of the plate.
F: Antimicrobial effect of the egg within different volumes. F: The small lid on the right had a volume of 270µL, the big one of 7mL , (-) indicates the control cap of 270µL. The eggs were placed on the plate immediately after inoculation, the picture were taken after 24h at 25°C.
G&H: Effect of the egg placed in the lid of a petridish (Ø92mm). The egg was placed in the dish immediately after inoculation. Picture G was taken after 24h of incubation at 25°C, H after 72h. The vicinity of the egg is even protected after 72h!
I-L Effect on conidiophores formation on A. flavus (**I**) and A. tamarii (**K**&**L**). Both fungi were exposed in the same setup with the region inside the circular section exposed to the egg and the remaining are not and in detail with the left picture (**K**) unexposed and the right one (**L**) exposed to the beewolf egg.

To assess the volume in which the beewolf egg's defense provides sufficient protection showed that no fungi survived in volumes of 270µL and 2.9mL. A beewolf brood cell with 2 bees has a mean volume of about 3.106 +/- 0.616mL (brood cell volumes measured in chapter 4, data not shown). In larger volumes of 7mL and 28.5mL the degree of inhibition of fungal growth decreased. In 7mL some spores were able to germinate and grow viable mycelia, in 28.5 far more hyphae grew, though not as many as in unexposed control plates and the immediate vicinity of the egg was still protected (Figure 3-13).

Similar effects could be achieved using comparable concentrations NO_2 with cultures of *A. flavus*, *tamari* and *P. lilacinus*.

3.4 Discussion

Identification and Source of the gas

The beewolf egg releases a gas with high oxidation potential. The release of the gas is precisely timed and the timing is highly temperature dependent. The Griess reagent and the fluorescent probe (DAR4M-AM) showed both a positive reaction. NADPH diaphorase activity was detected in embryonic tissue but also in the eggs envelope. The expression of NOS mRNA increased a short time before the major gas burst occurs and the gas release could be significantly inhibited by an arginine analog, L-NAME. The carbon dioxide output of the egg increases at the same time the major amount of oxidative gas is released. The positive reaction of the nitrite test provides evidence that a nitrous gas is produced by the beewolf egg. However, it proves only that any nitrous gas that dissolves as nitrite is present in the vicinity of the beewolf egg. The positive reaction of the DAR4M-AM probe, which was designed to specifically demonstrate the production of NO in tissues (Kojima *et al.* 2000; Kojima *et al.* 2001) shows, that NO is indeed present in the egg as well as outside the egg. Lacza *et al.* (2005) found that the DAR4M-AM probe increases its fluorescence not without the presence of NO. However, it reacted with NO donors and the increase of fluorescence was potentiated in the presence of peroxynitrite, a reaction product of NO with oxygen radicals that can decompose to nitrogen dioxide (NO_2). Therefore, the positive reaction of the DAR4M-AM fluorescent probe injected into eggs and also of the dye exposed to an egg in a closed cap proves the production of NO, as well as the presence of NO in the air around the egg but does not exclude other reactive nitrogen species (RNS). However, other RNS have to be expected in the presence of NO: artificial produced NO itself did not react with the iodide starch solution, but NO_2 did. NO_2 is the major reaction product when NO has contact to oxygen (Wiberg *et al.* 2001; van Faassen and Vanin 2007). Furthermore, it is known from literature, that NO will form other RNS in tissues (Hughes 1999; Daiber and Ullrich 2002). In the presence of superoxide or peroxide, peroxynitrite can be formed (Ischiropoulos *et al.* 1992; Beckman and Koppenol 1996; Reiter *et al.* 2000). Peroxynitrite is rather reactive and can nitrate other molecules (Radi *et al.* 1991; Reiter *et al.* 2000) or

decompose into the hydroxide radical and the NO_2 radical (Beckman *et al.* 1990; Daiber and Ullrich 2002).

Since it had been shown that fixation insensitive NADPH-Diaphorase activity and NOS activity do not only correlate but are identical (Dawson *et al.* 1991; Young *et al.* 1992; Gawronska *et al.* 2000), NADPH-Diaphorase staining was often used to show involvement of NOS in a variety of processes (Müller and Hildebrandt 1995; Regulsky and Tully 1995; Palumbo *et al.* 1999; Bhattacharya *et al.* 2000). The strong coloration of the NADPH-Diaphorase staining throughout the whole embryo but also within the egg's envelopes indicates the presence of the fixation insensitive NOS.

The indication of the diaphorase staining and the fact that the gas is not produced constantly by the egg allowed to test the expression of the NOS. The egg releases a constant but very low amount of gas already shortly after oviposition. After 19-20hours at 25°C the release abruptly increases about 100fold. The high release level is sustained for about two hours before it drops to the initial level. Assuming, that NOS is involved in the production of the gas, the expression of the NOS gene should be up-regulated shortly before the release peak. In fact, the level of NOS mRNA increased about one hour before the burst in NO release by tenfold. The third evidence for the involvement of NOS in the production of the gas is the strong inhibition of the gas release by injecting eggs with L-NAME (L-Nitro-Arginine Methyl Ester) an analogue of arginine, the substrate for the NOS. Its isomer D-NAME (D-Nitro-Arginine Methyl Ester) cannot enter the active center of the NOS and injection of D-NAME did not reduce the gas release significantly. Thus the inhibition of the gas release was due to the competition of L-NAME and not the injection procedure itself. The one outliner of the L-NAME injected eggs whose gas release was not inhibited might be due to the injection apparatus. The injection apparatus works with a high pressure of nitrogen. Injection is performed by a short timed increase of pressure to the liquid in the syringe. However the actual amount of injected solution or if any solution is injected at all cannot be measured directly but only by weighting the syringe before and after injection. Further the small tip of the syringe of 500nm can be easily blocked by not dissolved particles in the solution. Regardless of this single outliner statistical analysis showed that the gas production is significantly inhibited. The results of these three methods suggest that the NO synthase is indeed the source of the nitrous gas released by the beewolf eggs.

With these results I suggest the following hypothesis for the production of the gas by beewolf eggs: NO is synthesized from arginine by NOS. As NO can diffuse freely through tissues and has a half-life of several seconds in tissues (Beckman and Koppenol 1996) it will finally leave the egg. In the brood cell NO will react with oxygen to form NO_2 within a few hours (Soegiarto *et al.* 2003).

Temporal course & localization

The beewolf egg begins to release the nitrous gas already with the oviposition. However, the release rate is not constant over time. There is a peak period of about two hours. Before and

after the peak, the gas is released only at a very low rate. Within the two hours the release increases about hundredfold. This burst of NO release probably produces a much higher concentration in a brood cell than a continuous, lower release rate.

The temporal beginning of the burst of NO release is strongly temperature dependent. This is probably caused by the general temperature dependency of enzymatic processes (Schmidt-Nielsen 1990). The enzymatic processes in germinating fungal spores are probably similarly dependent on temperature. Yeo *et al.* (2003) found decreasing times until germination and accelerated growth of several entomopathogenic fungi at higher temperature under laboratory conditions in a range from 10 to 25°C. Hywel-Jones and Gillespie (2003) and Galaini-Wright *et al.* (1990) provided a more complete picture by covering a broader temperature range and found temperature optima for germination ratio and time at 25-30°C for *Metarhizium anisopliae* and *Beauveria bassiana* respectively 20-25°C for *Zoophthora radicans*. Enzymes are subject to evolutionary processes and adapt to match environmental conditions. The spectrum of fungi of beewolf brood cells consists mainly of generalists which will not be adapted to the conditions in beewolf brood cells (see Chapter 2.4). However, the exact timing of the gas release will most probably be the result of adaption to fungal growth in the brood cells. Thus, beewolves might have adapted their defense to the variety of fungi and evolved an optimal timing that is most effective against the majority of detrimental fungi.

The germination experiments on the bee surface showed that fungal spores on bees in a brood cell probably need about 15 hours at 25°C to germinate and develop a small mycelium. Only a few hours later the gas production starts in the brood cell. Beewolf females lick the bees intensely to embalm them with the additional hydrocarbon layer that will reduce water condensation and impair additionally the germination of spores on the bees. This will likely slow the germination in average considerably down as the spore germination tested on the bee surface had sufficient water available. Females sometimes reject bees without an obvious reason for the observer (unpublished data). They may be able to detect germinating fungi and if the infestation is already too advanced discard the entire bee. Fungi may start to germinate, either on the bee due to the higher humidity in most brood cells or in the soil surrounding the brood cell due to the amounts of hydrocarbons that line the brood cell after the beewolf female excavated it and dragged the embalmed bees in (Kroiss *et al.* 2008). However, the short timeframe fungi have to start their germination reaches only from around the paralysis of the bee until shortly before the gas is released. However, visually detectable infested bees have never been observed to be provisioned to a brood cell. Fungal infestation were only detected at least a day after the oviposition. These considerations suggest that the timing of the gas release by the egg is adaptively adjusted to a time, when fungi only just started to germinate but have not established a well developed mycelium and reached the egg.

The high fluorescence of the DAR4M-AM dye in the cuticle of the egg indicates a high concentration of NO. The immediate vicinity of the egg will show the highest concentration

of NO, which is also demonstrated by the effect of the egg on fungal growth in a large volume with resulting low concentration of NO. Protecting the egg itself from oviposition on is essential, as mycelia that reach the egg will very likely infest and kill it. The embalming of the bees with hydrocarbons slows down fungal germination and growth until NO is released in higher amounts (Herzner *et al.* 2007). The initial low concentration of NO may just be enough to protect the egg itself, if there should be already growing fungi in the brood cell, until the burst release starts to more or less sterilize the entire brood cell.

Effect on microorganisms

The measured amount of oxidant means revealed concentration of NO or NO_2 above 2000ppm per brood cell can be reached. The exact concentration depends on the quality of the egg (see chapter 4) and, even more important, the volume of the brood cell which can vary between 1.5 and 8mL (see also chapter 4). Microbial pathogens in a brood cell are exposed to the gas for several hours. Thus it is not surprising to find a severe negative effect of the gas on a wide range of microorganisms and on all tested developmental conditions. The effect was still strong when the concentration of NO was lowered by using test volumes that were several times larger than naturally occurring brood cell volumes. The effect should be strongest on the fungi when the spores have just started to germinate (Cochrane 1958) and grow a germination tube. Having lost the protective spore wall and used a large proportion of the available energy to start that physiological change, but having not yet reached a nutrition source fungi should be most vulnerable (Allen 1965). However, even dormant spores were affected, when exposed before they started to germinate. A sufficient effect of the gas on fungi should be achieved even under unfavorable conditions, like large brood cells, “bad” eggs, brood cells in porous soils, where NO respectively NO_2 leaks out of the brood cell or dissolves in the water film of more humid brood cells. Though they do not nest in wet soil due to rain the humidity might increase.

The antimicrobial activity against viruses, bacteria, fungi, proto- and metazoa of NO produced by the NO synthase, not only, but especially in macrophages has been shown repeatedly (Nathan and Hibbs 1991; Ischiropoulos *et al.* 1992; Augusto *et al.* 1996; MacMicking *et al.* 1997; Linares *et al.* 2001; Ascenzi *et al.* 2003; Jones *et al.* 2010). Both, human and insects carrying pathogens (e.g. *Anopheles*), defend themselves using NO or other RNS from the inducible NOS against parasites like *Trypanosoma* and *Plasmodium* (Ascenzi and Gradoni 2002; Rivero 2006). An upregulation of NOS expression and an increase in NO production has been found as reaction to several diseases. The antimicrobial effect of NO against a wide spectrum of pathogens also well documented (for an overview see Degroote and Fang 1995; Fang 1997).

NO itself may interfere with signaling processes of exposed microorganisms. This is suggested by the study of Wang and Higgins (2005) who detected endogenous NO in germinating spores of *Collectotrichum coccodes* and were able to delay germination of spores by exposing them to exogenous NO. Lai *et al.* (2011) demonstrated the same effect in *Penicillium expansum*. Further, they found signs of increased oxidative stress after NO

exposure. Lazar *et al.* (2008) found similar effects towards mycelial growth, sporulation and germination after exposing fungi to gaseous NO at concentrations from 50-500µL(NO)/L(air) which corresponds to 46-466ppm. Mancenelli and McKay (1983) found bacteriostatic effects of much lower concentrations of gaseous NO (as low as 1.9ppm). However, NO_2 did not affect bacterial growth in low concentrations (up to 5.5ppm). Ghaffari *et al.* (2007) and Miller *et al.* (2009) demonstrated bactericidal effects of continuous as well as pulsed application of 200ppm NO against common pathogens that form skin infections. Due to these antimicrobial effects, the use of gaseous NO is tested for medical application (Ghaffari *et al.* 2005; Ghaffari *et al.* 2006; Kulla *et al.* 2009; Stenzler and Miller 2009; Jones *et al.* 2010). Mammals can withstand up to 400ppm of NO, inhaled or exposed to skin, eyes, wounds, etc. without detrimental effects (Carlson *et al.* 1998); inhaled NO_2, however, is already lethal at concentrations of 50ppm within minutes or a few hours (National Research Council 1998).

NO, or one of its more aggressive reaction products, peroxynitrite and the hydroxyl radical as well as NO_2, all lead to similar ultimate effects: deamination of DNA, abasic sites and strand brakes (Garg and Hassid 1989; Nakaki *et al.* 1990; Nguyen *et al.* 1991; Nguyen *et al.* 1992; Fehsel *et al.* 1993), nitrosation and nitration of proteins, especially at tyrosine, tryptophan or cysteine amino acids (Kikugawa *et al.* 1994; Juedes and Wogan 1996; Jourd'heuil *et al.* 1997), the oxidation and release of protein bound metals (Flint *et al.* 1993; Keyer and Imlay 1996) and oxidation of lipid membranes (Prütz *et al.* 1985; Szabo 1999; Reiter *et al.* 2000; Colasanti *et al.* 2001; Radi 2004). In solution, NO itself may lead to molecular damage but at a rather slow rate (Daiber and Ullrich 2002). Several studies that investigated the effects of NO did not investigate or even raise the question if the damage was caused by NO or its reaction products. By now, it seems that the major effect are caused by its reaction products, like nitrite (NO_2^-), S-nitrosothiols , peroxynitrite (ONOO), NO_2, dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4) as well as the hydroxyl radical ($OH^\bullet$) (Beckman and Koppenol 1996; Fang 1997; Daiber and Ullrich 2002). Peroxynitrite that is formed from NO in the presence of superoxide or peroxide also decomposes to NO_2 and the hydroxyl radical (Klebanoff 1993; Jourd'heuil *et al.* 1997; Ischiropoulos and Thom 2010). It is still discussed which derivative is actually causing the damage to microbes, but Zhu *et al.* (1992) found that bactericidal activity of peroxynitrite was higher when its decomposition was enhanced with a hydroxyl scavenger. Thereby more NO_2 was produced, which indicates that the hydroxyl radical and NO_2 might be the most important mediators of the effect (Daiber and Ullrich 2002).

Taking all this into account we can assume the following scenario: NO released from beewolf eggs will diffuses into the broodcell. Together with oxygen it will form NO_2 with a half-life of NO of about 3 hours (Soegiarto *et al.* 2003). NO_2 attacks microorganisms directly by oxidizing lipid membranes and nitrating proteins. It dissolves in aqueous biofilms and microscopic water droplets by disproprotionating into nitrous and nitric acid. Nitric acid may act as an oxidant and reductant or again as a nitrosation agent, NO_2 as a strong oxidant. Engaged in an oxidation, both are reduced back to NO capable to inflict further damage, even to DNA.

Further NO itself may enter the target organism directly and form peroxynitrite with superoxide or peroxide present in the target organism itself. Alternately peroxynitrite can be formed on the surface, e.g. with superoxide or peroxide from enzymes in the periplasmatic space of bacteria or the biofilm of fungi that contains enzymes to decompose organic material, and enter organisms in the protonated form as peroxynitrous acid (Fang 1997). In both cases peroxynitrite can inflict damage to multiple cellular targets. Being widely unspecific with respect to target organisms as well as molecular targets, NO is the perfect broad spectrum antibiotic and ideal for the beewolves' application.

Self-defense

The unspecific reactions of NO cause severe problems for the egg since just like NO affects the entire range of potential pathogens it might also harm its producer, the beewolf egg. Whatsoever problems NO will pose to pathogens, the same applies to the beewolf egg. Ghaffari *et al.* (2007) noted that different mammalian cells types vary greatly in their sensitivity towards the cytotoxic effects of NO. Jones *et al.* (2010) assumed a concentration of 1000ppm of exogenous, gaseous NO to be lethal to eukaryotic cells but also emphasized their protective mechanisms especially of metallothionein. Beckman *et al.* (1990) already discussed potential injury to endothelial tissue due to NO and peroxynitrite and Espey *et al.* (2000) stated that NO and all its derivatives can pose nitrosative as well as oxidative stress to cells, depending on their physiological state and on the concentration of NO. Thus, release of NO has to be controlled precisely to avoid that damaging effects exceed beneficial ones (Espey *et al.* 2000).

Beewolf eggs release NO well above reported levels for cytotoxicity. The embryo might be protected from the more aggressive NO_2 by the egg's outer cuticle and epithelial layer. An anti-oxidant effect of NO was reported in several studies by Wink *et al.* (1993; 1994; 1995; 1995; 2001). NO itself has a rather low reactivity to non radical targets. However, it readily reacts with other radical species. Depending on the physiological conditions this can produce other much more deleterious substances like peroxynitrite in the presence of peroxide or superoxide. In the absence of substances that react with NO and enhance its harmful potential, NO reacts with other radicals and thereby scavenges them effectively. NO however is found even within the embryo itself - in much smaller concentrations than within the cuticle but still clearly detectable. The spatial distribution of the NO release in the beewolf eggs is not yet known, but even with a directed release towards the outside of the egg NO is able to diffuse back inside. Therefore eggs need to protect themselves. Inside the egg NO can damage cells and can even form more harmful substances but may also scavenge other radicals. Outside the egg NO will inevitably oxidize to NO_2. NO_2 cannot cross cell membranes like NO but may damage the eggs envelopes. The high concentration of NO in the cuticle may serve as a scavenging barrier against the aggressive NO_2. However to prevent damage, the egg will have to establish further self-defenses, e.g. by providing other scavengers like glutathione, ascorbic acid or uric acid as well as substances like the above mentioned metallothionein or other protein bound thiols (Ford *et al.* 2002). Furthermore,

increasing self-repair processes of DNA and recycling of damaged proteins and lipids might be necessary to warrant a unaffected development of the embryo. The increased metabolic rate during the NO release may result not only from energy spent to drive the oxidation of arginine but also to counter the destructive effects of the gas on the egg itself.

The constraint of the NO release to a short period of time with a high concentration of NO is comparable to the human self defense in skin infections. There only initially high concentrations of NO should inhibit bacterial infections. Thus self-damage to own tissue is minimized that may occur in chronic infections if pathogens are able to colonize the wound and the immune system enters a vicious cycle of continuous NO production that cannot counter the infection. Then, tissue is damaged not only by the pathogen but also from inflammation due to the overreacting immune defense (Stenzler and Miller 2009; Jones *et al.* 2010). The short peak of NO secretion might, thus, help to restrict duration of the necessary self-defense of the beewolf egg to a minimum and at the same time has a maximum impact on fungi.

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Chapter 4: Can NO defense be costly?

4.1 Introduction

Roff (1992) and Stearns (1993) established the theory that life history is shaped by limited resources and the allocation pattern of these resources to growth, reproduction and maintenance. Several studies have shown trade-offs particularly between reproduction and maintenance. High investment into reproduction shortens lifetime and older females produce fewer eggs of smaller size and poorer quality (Williams 1966; Reznick 1985; Fox 1993; Carey *et al.* 1998). The immune system, another important factor of maintenance, has been shown to be similarly affected. Birds' clutch sizes were very easy to manipulate and thus popular to show costs of breeding. Birds with increased clutch size show higher rates of parasitism or a weaker immune system (Festa-Bianchet 1989; Gustafsson *et al.* 1994; Norris *et al.* 1994; Richner *et al.* 1995). In insects, studies showed on the one hand that the immune system was weakened with increasing age and reproductive investment (König and Schmid-Hempel 1995; Siva-Jothy *et al.* 1998; McKean and Nunney 2001; Doums *et al.* 2002). On the other hand an artificially challenged immune system had negative effects on different life history traits, e.g. in *Drosophila melanogaster* (Fellowes and Godfray 2000; Kraaijeveld *et al.* 2002), the pea aphid *Acythosiphon pisum* (Kraaijeveld *et al.* 2002), several damselfly species (Siva-Jothy *et al.* 2001) and the house cricket *Achaeta domestica* (Bascunan-Garcia *et al.* 2010). Bumble bees, *Bombus terrestris*, showed this effect on an individual as well as on a colony level (Moret and Schmid-Hempel 2000; and 2001).

Females of the European beewolf invest quite a lot into reproduction, not only to ensure the offspring's survival, but also its reproductive success as adults. The females excavate a nest in sandy soils, hunt for honeybee workers, *Apis mellifera*, and also ensure that the development of the egg and larva is protected from fungal pathogens and competitors (Strohm and Linsenmair 2001). Already the excavation of the nest and brood cells requires a lot of time and energy. Hunting for honeybees is time consuming as well and additionally poses a risk for the life of the beewolf female since the female dies when accidentally stung by a honeybee. To protect its offspring from fungal competitors and pathogens, the female covers the paralyzed honeybees with an additional layer of cuticular hydrocarbons, which impedes fungal germination and growth (Strohm and Linsenmair 2001) by reducing water condensation on the bees (Herzner and Strohm 2007). Moreover, beewolf females cultivate bacteria of the genus S*treptomyces* in their antennae (Kaltenpoth *et al.* 2006). These bacteria are applied to the brood cell, taken up by the larva and incorporated in the larvae's cocoons. There the bacteria start to produce antibiotics to protect the progeny in the cocoons until the emergence of the adult beewolf (Kaltenpoth *et al.* 2005; Kaltenpoth and Strohm 2007). Hunting for honey bees has already been shown to account for parental

investment (Strohm and Linsenmair 2000) in the sense of Trivers (1972) as any kind of expenditure of a parent into its offspring, that increases the survival probability of the offspring but reduces the ability of the parent to invest into further offspring. If the treatment of the prey bees with hydrocarbons can be considered to represent parental investment is subject of the next chapter. The excavation of the brood cell and the rearing of the bacteria obviously require resources, but these costs have not yet been investigated.

The third antifungal defense based on the nitric oxide release presented in the previous chapter is another factor that may require considerable amounts of resources. Although this is part of, if not the only immune defense of the egg stage, the necessary resources have to be provided by the mother. The NO defense can be costly for the egg as well as the mother. One indicator for costs of the sterilization of the brood cell could be the energetic costs, measured as the carbon dioxide released. Furthermore, since arginine, the base product of NO synthesis is considered an essential amino acid in insects (Uchida 1993), it is obvious that the nitric oxide synthase (NOS) competes with other processes like growth and development. Even if the resulting citrulline can be recycled, it is energy consuming and depletes other nitrogen sources. Thus, the production of NO is a potential source of costs. As the egg is completely incapable of gathering resources on its own, the gas production also competes for the mother's resources for producing offspring in the future and her own maintenance. The nitric oxide defense can be seen as an extension of the females' immune system but is still a component of the investment into its offspring. Similar to known effects of immune defenses (Gustafsson *et al.* 1994; Siva-Jothy *et al.* 1998; Fellowes and Godfray 2000; Kraaijeveld *et al.* 2002; Gwynn *et al.* 2005; McKean *et al.* 2008; Bascunan-Garcia *et al.* 2010) a trade-off between reproduction and the nitric oxide defense can be expected.

To investigate possible trade-offs with gas production of the egg I measured different parameter of the beewolves' reproduction. To analyze the complex interactions among the many variables of beewolf reproduction I used path analysis, which is not only able to dissolve such interactions independently, but has the great advantage of visualizing the results in a quite simple and easily comprehensible diagram. The ultimate fitness of the mother does not only depend on the number of offspring but also on their fitness as adults. The fitness of male offspring as well as females is correlated with size. Larger males produce more sexual pheromone and can attract more females and larger females are able to provide more bees per day or brood cell as well as per life (Strohm and Linsenmair 1997). Furthermore, the size of a beewolf and its survival chance until emergence is positively correlated with the amount of food provided for the larva, the number of bees (Strohm and Linsenmair 1999).

The lifetime hunting success of a female is a good indicator of its fitness and was included, as was the female size. The reproductive performance over the life span of a female may decrease or increase. Therefore, the investment prior to a brood cell in question was also included. The number of bees provisioned for a brood cell includes the effort for hunting and embalming of the bees and represents most of the investment in one single offspring,

especially the two other costly components (see Strohm and Marliani 2002 and chapter 5), except the excavation of the nest and the egg itself. The production of NO was measured by the resulting absorption of the reaction of nitric oxide with an iodide starch solution. The amount of nitric oxide produced by an egg may depend on the amount of resources invested into the egg itself, measured as egg volume. The amount of released nitric oxide is only one of the two variables that affect the concentration within the brood cell. Therefore, additionally to the absorption I also measured the volume of the brood cell and the resulting concentration of NO.

4.2 Materials & methods

Beewolf specimen

Beewolves originated from a population in Erlangen (Germany). The specimens were either captured in 2009 or taken from the F1 generation reared in the laboratory. Females were kept in observation cages which consisted of a cubical flying compartment of 27dm³ and a nesting compartment filled with sand of 60cmx30cmx1cm with a slope of 20% (Strohm and Linsenmair 1994). Females were provided with honey and bees *ad libitum*. The nesting compartment could be accessed through partially removable glass plates. Therefore eggs could be carefully removed from finished brood cells using a pair of tweezers without disturbing the rest of the nest.

Quantification of nitric oxide release

The released amount of nitric oxide was quantified corresponding to chapter 3.2.

Measurements for the path analysis

All following values of 250 brood cells were collected for each individual beewolf female. The head width of the mother was measured with digital calipers under a stereo microscope (Zeiss, Germany), as with a measure of body size (Strohm and Linsenmair 1997). The females were kept in observation cages for their entire life. In each brood cell the number of bees per brood cell and the total hunting success could be determined. Females may have a different amount of resources available, either genetically, due to differences in the availability of larval resources or due to infections. Infections can cause costs either by the induced immune defense or by the resources the infecting organism consumes (e.g. Ferdig *et al.* 1993; Kraaijeveld *et al.* 2002). Thus, females may differ in their ability to invest into offspring. Females with fewer resources may be able to produce only a fraction of the offspring numbers of good supplied ones and show already signs of exhaustion after the first few brood cells. To be able to compare the status of females with different predispositions I included the relative former investment as a parameter: The actual number of the brood cells was divided by the total number of brood cells a female produced in her entire life. As a measure of the volume of the egg, the egg length and width was also measured under a stereo microscope (Zeiss, Germany) with digital calipers. Length and diameter correlated significantly (Pearson's correlation; N=16, r=0.539, p=0.031). Therefore the diameter was not

measured for all eggs, but the diameter was calculated with a linear regression (width=0.94 x length + 0.592mm, N=16, p=0.031). The egg volume was then calculated using the formula for an circular cylinder: length x (½width)² x π. The length and diameter of the brood cells were measured with analog calipers. The volume of the brood cell was calculated as an ellipsoid cylinder: π x ½width x ½length x 10mm (height of the observation cage) and corrected for the volume that is taken by the body of the bees.

Statistics

To assess the rather complex relations between all the measured variables I used linear regression modeling and a path diagram to visualize the results. To assess the influence of the individual female an ANOVA was conducted including the individual mother as a fixed factor and the variable that had the strongest influence on the nitric oxide release in the linear equation model, egg volume, as a covariate. The path analysis was conducted with Amos 18, the ANOVA with PASW 18 (both SPSS Inc., USA). The path diagram was further processed with Microsoft Powerpoint to depict standardized estimates with arrows of corresponding thickness.

4.3 Results

Table 4-1 lists the means and standard deviations of all variables used to calculate the path diagram. There are three types of indices for model fit: Indices based on the comparison of predicted and observed covariances, indices that compare the given model with an alternative model and indices based on the comparison of predicted and observed covariances that also consider parsimony.

Jaccard and Wan (1996) suggested to use one of each category to describe the fit of a model. I chose chi-square and GFI from the first category, NNFI from the second and RMSEA from the third. The goodness-of-fit index GFI varies from 0 to 1. It is the ratio of observed covariances explained by the covariances implied by the model and should be at least 0.9 or better 0.95. The non-normed fit index (NNFI), Bentler-Bonett non-normed fit index or Tucker-Lewis-index (TLI) reflects the proportion by which the defined model improves fit compared to the null model and regards model complexity. NNFI close to one indicates good fit. Generally it should be larger than 0.8 or even 0.9. Hu and Bentler (1998) even suggested to discard models with NNFI<0.95. The root mean square error of approximation or the discrepancy per degree of freedom also assesses parsimony of a model. It is not affected by sample size and should be lower than 0.08 or 0.06 after Hu and Bentler (1998).

The final, most parsimonious model of the path analysis does not differ significantly from our data (χ^2=17.671, d.f.=16, p=0.343). Moreover the model shows quite good fit to the data: GFI was 0.983, NNFI was 0.996 and RMSEA was 0.021.

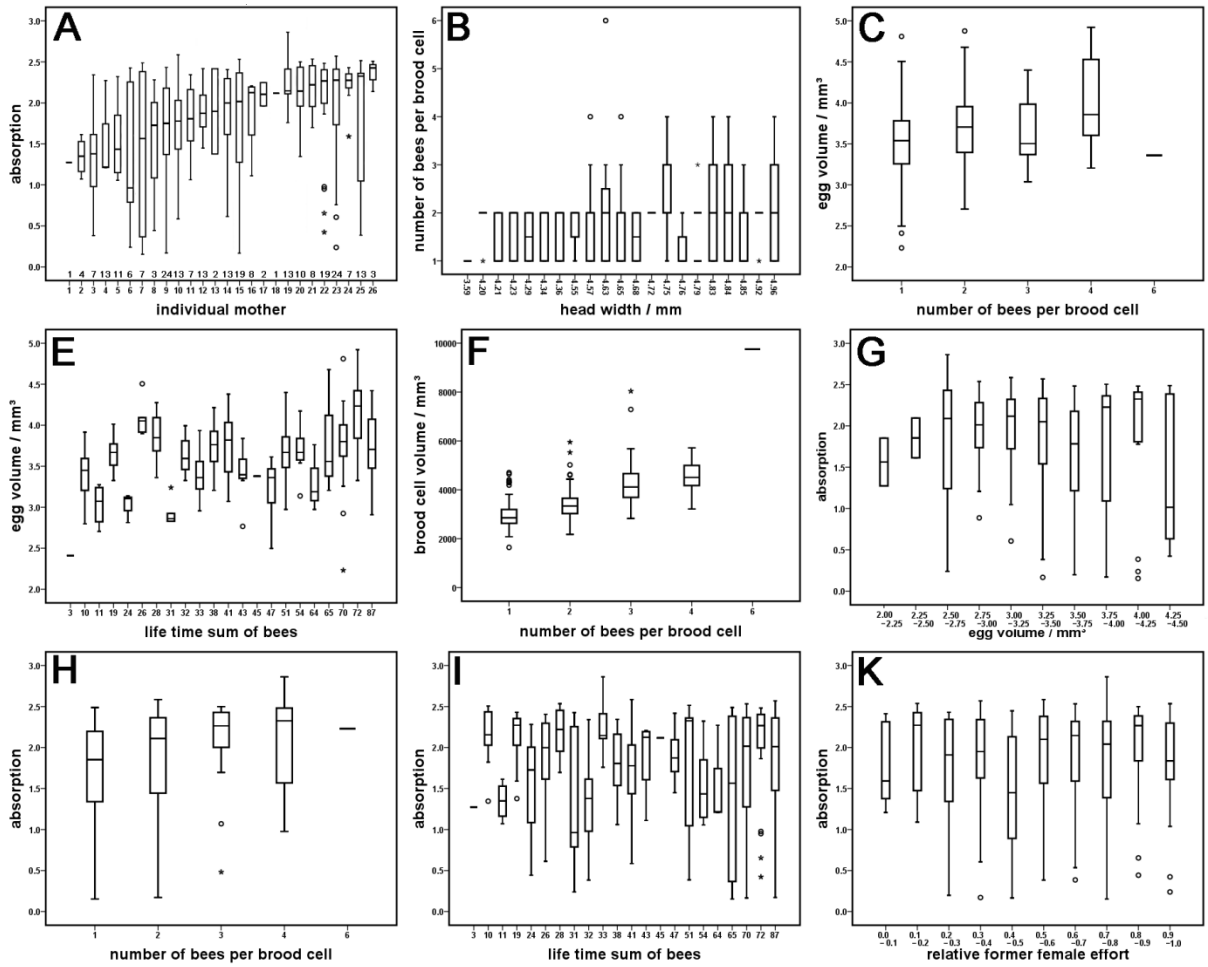

Figure 4-2 Overview of the associations on which the path analysis is based. The following parameters were used: female size as head width (mm), number of bees per brood cell, egg size as egg volume (mm³), female hunting as a fitness parameter measured as the total of bees caught per life, the volume of the brood cells (mm³), the amount of released nitric oxide measured as absorption of the potassium iodide solution and the former female effort measured as the relative position of an egg within the life of a female.

Figure 4-22 shows the relations between the variables which showed a significant effect in the path analysis. The path diagram is shown in Figure 4-3, values of the path coefficients are listed in Table 4-2 and correlation weights of covariates in Table 4-3. Correlation weights with two headed arrows are used in the path analysis instead of path coefficients and one headed arrows when the cause-effect direction is not known or not of interest. The summed total effects on the NO release, the brood cell volume and the resulting concentration in the brood cell are listed in Table 4-4. The path and correlation coefficients as well as total effect coefficients are all standardized, which means if an estimate is 0.2 than the dependent variable increases by 20% if the affecting variable is increased by 100%. Each dependent shows some variation that is not explained through the paths of the analysis but by parameters that were not included. Each variable is assigned an “unanalyzed cause”, U in the diagram. If U is 0.2, then 80% of the variation is explained by the path analysis. Both the former investment and the total hunting success had a direct negative effect on the amount of released NO (standardized path coefficient (s.p.c.= -0.174, p=0.004 and s.p.c.= -0.197,

Table 4-1 Mean and standard deviation of the factors that entered the path analysis. The amount of nitric oxide and its estimated concentration in the corresponding brood cells are included additionally.

	former investment	total hunting success	head width / mm	number of bees per brood cell	egg volume / mm^3	brood cell volume / mm^3	absorption	n(nitric oxide) / µmol	c(nitric oxide) / ppm
mean	0.569	39.577	4.653	1.845	3.642	3157.414	1.820	0.246	1688.137
standard deviation	0.260	23.720	0.234	0.837	0.459	860.818	0.634	0.086	680.759

p=0.002). There was a negative effect of former reproductive effort of a female on the amount of NO produced by the focus egg (total s.p.c.= -0.147).

Females that hunted for more bees in their life produced larger eggs (s.p.c.= 0.300, p<0.001) and larger eggs released more NO (s.p.c.=0.349, p<0.001) but the total effect on the NO release as well as the NO concentration was negative (total s.p.c.=-0.092 and total s.p.c.=-0.078). The number of bees in a brood cell influenced the NO concentration in two ways. The eggs in brood cells with more bees were larger (s.p.c.=0.172, p=0.005) and the larger eggs

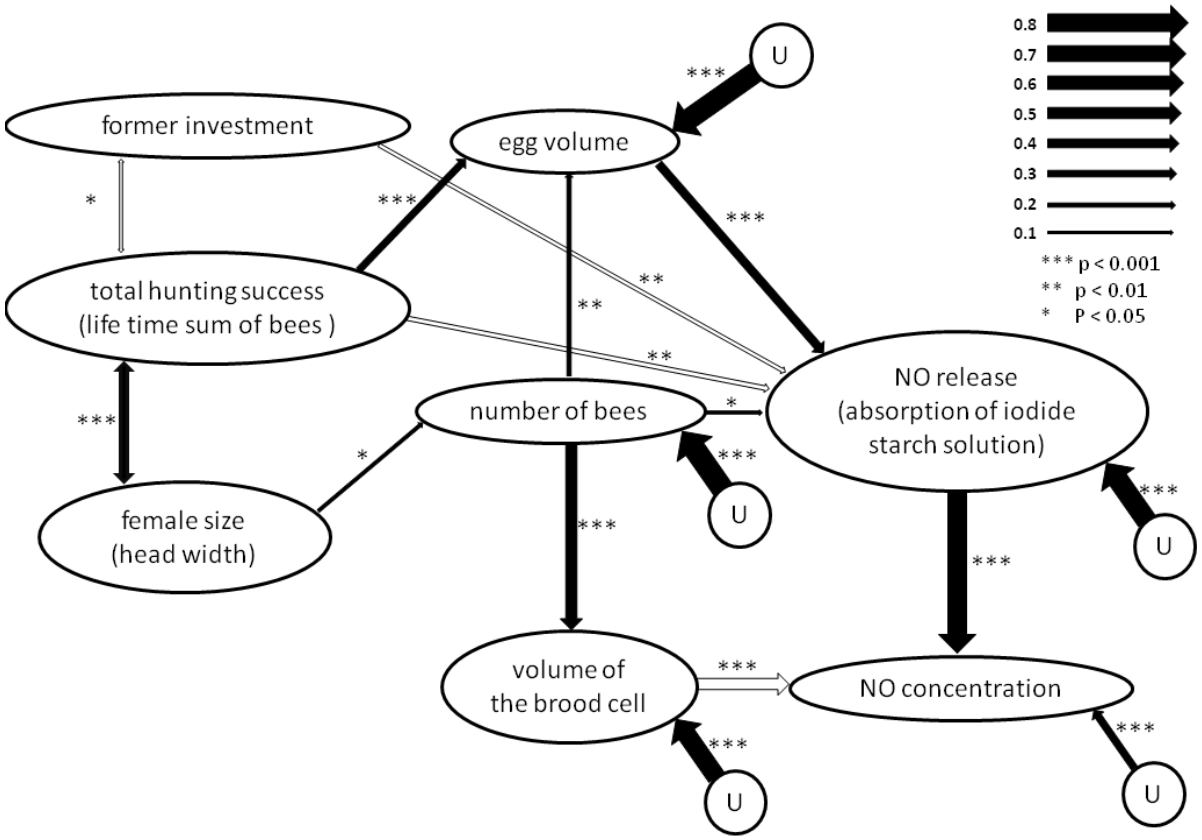

Figure 4-3 Path diagram with the different effects contributing to the amount of nitric oxide released by a beewolf egg. Black, filled arrows denote positive effects, arrows with only outlines negative ones. Width of each line is proportional to the strength of the association (corresponding correlation coefficients are given in the upper right of the diagram). Stars indicate significant effects at the level of p<0.05 (*), p<0.01 (**) or p<0.001 (***). U indicates unexplained causes of a variable. Actual values of the path coefficients are given in Table 4-2. Two tailed arrows indicate correlations without an assumption of the cause-effect direction.

released more NO (s.p.c.=0.349, p<0.001). There was also an additional positive effect of the number of bees in a brood cell on the NO release that is not explained via the egg volume (s.p.c.=0.126, p=0.038). Yet, more bees required more space in a brood cell and thus the volume of the brood cells increased (s.p.c.=0.511, p<0.001). While the increased volume of the brood cell caused a decrease in the NO concentration (s.p.c.=-0.511, p<0.001), the concentration was also increased by the higher NO release (s.p.c.=0.847, p<0.001), but did not compensate the higher volume of the brood cell which resulted in a negative total effect of the number of bees on the NO concentration (total s.p.c.=-0.103). Larger females hunt more honey bees in their life (correlation coefficient=0.336, p<0.001) but also provision more bees per brood cell (s.p.c.=0.153, p=0.019). However, the size of females had the least effect on the NO release (s.p.c.=0.028) concentration (total s.p.c.=-0.016). The correlation between the total hunting success and the former investment (correlation coefficient=-0.131, p=0.036) is an artifact of collecting the specimens. All brood cells of the beewolf females were registered with the number of provisioned bees, but not all eggs were removed to quantify their NO release. The negative correlation between the former investment and the total hunting success is an artifact from the sampling method.

Table 4-2 Path coefficients, standard error and p value of each connection in the path analysis. The coefficients and standard error are both standardized regression weights, which means if a variable increases by 1%, the dependent variable increases by a percentage according to the coefficient.

	path coefficient	standard error	p
female size → number of bees	0.153	0.065	0.019
number of bees → egg volume	0.172	0.062	0.005
total hunting success → egg volume	0.300	0.050	<0.001
number of bees → brood cell volume	0.511	0.056	<0.001
egg volume → NO release	0.349	0.064	<0.001
number of bees → NO release	0.126	0.060	0.038
total hunting success → NO release	-0.197	0.079	0.002
former investment → NO release	-0.174	0.060	0.004
brood cell volume → concentration	-0.511	0.021	<0.001
NO release → concentration	0.847	0.021	<0.001

Table 4-3 Correlation of covariates are shown which are principally the same as the path coefficients but with no direction of cause and effect assumed. The coefficients and standard errors are both standardized, which means if a variable increases by 1%, the dependent variable increases by a percentage according to the coefficient.

	correlation coefficient	standard error	p
female size ↔ total hunting success	0.336	0.069	<0.001
former investment ↔ total hunting success	-0.131	0.063	0.036

Table 4-4 Total effect (total standardized path coefficient) of each variable in the path diagram based on all connections to the NO release (measured by the absorption of the iodide starch solution) and the resulting concentration within a brood cell. The values given are standardized regression weights which means if a variable increases by 1%, the dependent variable increases by a percentage according to the coefficient.

	former investment	total hunting success	female size	number of bees	egg volume	brood cell volume	absorption
cell volume	-	-	0.078	0.511			
absorption	-0.174	-0.092	0.028	0.186	0.349		
concentration	-0.147	-0.078	-0.016	-0.103	0.298	-0.511	0.847

4.4 Discussion

The fact that female beewolves transfer a part of their immune to their eggs in form of resources for the NO production allowed to monitor reproductive effort and assess the status of this component of the immune system of single beewolf individuals continuously without influencing them. The multidimensional approach of measuring several characteristics simultaneously and analyzing the interaction by path analysis that allows to evaluate such data I could show three important effects. First, there is a trade-off between the nitric oxide defense of beewolf eggs and other life history traits. Second, it shows that there are different resource allocation strategies of beewolf females and third revealed an adaptive component of the nitric oxide defense.

Former investment reduces nitric oxide defense

The strongest ultimate effect on the nitric oxide defense of the egg is the lower release of nitric oxide by eggs from females with a higher former investment. The former investment includes both the age of females and the investment in reproduction. Thus, there is a trade-off between the nitric oxide defense and the two combined life history traits maintenance and reproduction. It is not clear by which one the trade-off is caused. Further experiments will have to show the single effects independently. The decreasing ability to invest into offspring with increasing age is well documented in literature (Williams 1966; Reznick 1985). It is known that size and quality of eggs decreases with age (Fox 1993 and references therein). Beewolf eggs from older females are not smaller but of lower quality regarding the resources for the nitric oxide defense.

Beewolves invest into reproduction, by investing resources in the progenies' immune defense, the nitric oxide released by the egg. Since the resources for the egg's defense have to be provided by the mother, the nitric oxide defense reflects her immune status. The trade-off between reproduction the immune system and has been shown in several bird species (Festa-Bianchet 1989; Gustafsson *et al.* 1994; Norris *et al.* 1994) and insects (Siva-Jothy *et al.* 1998; McKean and Nunney 2001). In insects, Especially in two species of

bumblebee workers, *Bombus terrestris* and *B. lucorum,* immune function was decreased with increasing age (König and Schmid-Hempel 1995; Doums *et al.* 2002). Further immune function was lower under limited resources (Moret and Schmid-Hempel 2000) and immune challenge reduced the reproductive output of entire colonies (Moret and Schmid-Hempel 2001). Rigby and Joelka (2000) found a negative correlation between reproduction and immune system in snails. Work in insects also used an alternative approach, namely to stimulate the immune system without actually causing an infection and monitoring the reduction in fitness (Moret and Schmid-Hempel 2000; Poulsen *et al.* 2002; Bascunan-Garcia *et al.* 2010).

Defense versus number of offspring – in between two strategies

The size of the egg had the strongest direct effect on the nitric oxide release and concentration. Larger eggs contain obviously more resources for the production of nitric oxide. Females with a higher hunting success also produced larger eggs. However, these eggs release less nitric oxide than same sized eggs from females with a lower hunting success. This second, negative effect of the hunting success even outweighs the positive effect of the larger eggs. Thus females might invest more resources in hunting and have a higher number or larger and thus potentially fitter offspring at the expense of the defense of their offspring. The selection pressure on beewolf eggs presented by detrimental fungi is rather high. Fungi are omnipresent in the soil, which is the most likely source for infestation of the beewolf brood cells (see chapter 2). The nitric oxide defense is accordingly constitutive, yet the amount of NO varies about fivefold among eggs. Beewolf females have to try to maximize their fitness by optimizing the allocation between the number of offspring and the provisioning of each single offspring on the one hand and the defense of its offspring on the other. Some females tend to maximize their fitness by investing more resources into hunting more bees and producing more offspring and defending them less well. Other females tend to invest more resources into a better protection of fewer offspring. Environmental conditions, like temperature, humidity or presence of fungi are quite fluctuating and unpredictable so that different strategies may be favored just within a short range of time or distance within the nesting site (see e.g Strohm and Linsenmair 2001). If the actual fungal threat is high, females that invest into a better defense will be favored, whereas it would be better to invest into a higher number in the absence of fungi.

The size of females has only a small effect on the release of nitric oxide. However it is a good indicator for the allocation scheme of females since larger females hunt more honey bees, both for single brood cells as well as in their entire life. Thus, larger females are those who invest more in provisioning a large number of bees for an offspring and less in the defense of the eggs. It is harder for smaller female to hunt bees (Strohm and Linsenmair 1997), especially to bring them to their nest in flight. There is a high variance in the weight of beewolf females (about fivefold Strohm 2000) but only little variation in honey bee workers from one geographic location (about 3%, Ruttner 1992) which means that smaller females have to carry a relatively larger load. However, smaller females might be able to compensate

their lower hunting success by instead investing in a better defense of their fewer eggs and maximize their reproductive success. The brood cells of smaller females are indeed protected by a slightly higher concentration of nitric oxide.

Adaptive defense

Due to the high threat caused by detrimental fungi, nitric oxide seems to be constitutively released to sterilize the brood cell, but the actual intensity of the sterilization effort varies. The variation is partly explained by the amount of available resources and the decision how to allocate these. The volume of the brood cell and the amount of nitric oxide define the concentration in the brood cell which is the value that should be most relevant for the antimycotic effect. The number of bees is the only variable in this analysis that affects the volume of the brood cell. More bees need more space and the increased space decreases the concentration but the release of nitric oxide is adjusted to the increased volume in two ways. Eggs in brood cells with more bees are larger and larger eggs release more nitric oxide. The eggs may be larger because they can mature for a longer time if more bees have to be caught. Additionally there is a direct influence of the number of bees in the brood cell on the nitric oxide release that is not explained by the size of the egg (see also Figure 4-4). The decision how much of the available resources are allocated to the nitric oxide defense seems to be mainly female specific. Yet the direct effect of the number of bees on the nitric oxide release shows, that there is also an adaptive component that can be adjusted to conditions of each particular brood cell. These two effects counteract the increased volume of the brood cell not completely but compensate at least half of the decrease in concentration.

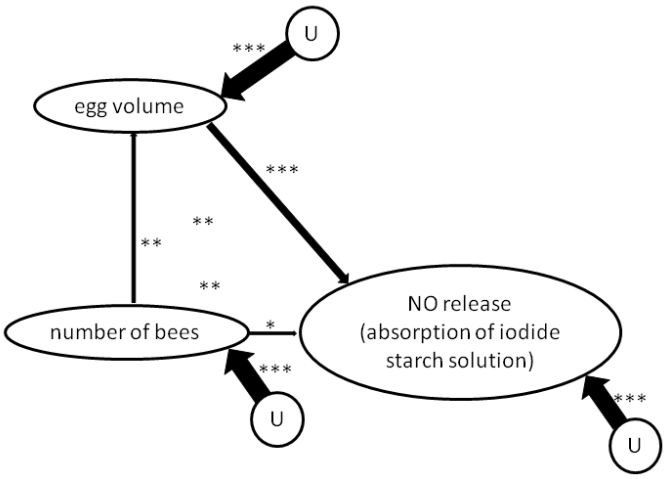

Figure 4-4 Influences of the number of provisioned bees on the nitric oxide release: Besides the indirect effect via the size of the egg there is also a direct effect of the number of bees on the NO release.

The actual situation of resource allocation is often a little more complex than implied by Roff (1992) and Stearns (1993). Often there will not only be growth, reproduction and maintenance as three distinct traits to invest, but each of them may have more facets. The beewolves' reproduction already includes two other expensive components: the hunting for prey (Strohm and Linsenmair 1999; Strohm and Marliani 2002) and the treatment of the bees with hydrocarbons that also reduces fungal infestation (see chapter 5 and Strohm and Linsenmair 2001). The nitric oxide defense is still another component that competes for resources and, thus, renders the reproductive investment of beewolves multifaceted. Such multifaceted parental investment (Rosenheim *et al.* 1996) make the decision for an optimal allocation of resources rather difficult - not only for the female but also for the observer to evaluate (Strohm and Linsenmair 1999). Under such variable environmental conditions,

physiological predispositions and multiple facets of the beewolves' reproduction there may be different evolutionary stable strategies of resource allocation.

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Chapter 5: Defense is the second facet of parental investment

This chapter is based on the following article:

Herzner, G., Engl, T., Strohm, E. 2011. The cryptic combat against competing microbes is a costly component of parental care in a digger wasp. Animal Behavior

5.1 Introduction

Parental care has evolved to increase the survival and reproductive success of progeny under adverse environmental conditions (Clutton-Brock 1991). Since microorganisms are ubiquitous and pose severe threats to higher organisms as pathogens and competitors (Hajek & St. Leger 1994; Janzen 1977; Burkepile *et al.* 2006; Brodie *et al.* 2007), the defence of offspring against these microbes can be expected to be a key part of parental care. This hypothesis is corroborated by the growing body of evidence from diverse animal species that have evolved effective counterstrategies to protect offspring and their food against microbial attacks (Cane *et al.* 1983; Marchini *et al.* 1997; Orion *et al.* 2001; Strohm & Linsenmair 2001; Knouft *et al.* 2003; Kaltenpoth *et al.* 2005; Giacomello *et al.* 2006; Sadd & Schmid-Hempel 2007; Rozen *et al.* 2008; Jacques *et al.* 2009; Cotter & Kilner 2010; Kroiss *et al.* 2010; Pizzolon *et al.* 2010; Kudo *et al.* 2011; Roth *et al.* 2010). Most of these studies, however, do not explicitly address the question whether such behaviours qualify as parental investment sensu Trivers (1972), i.e. whether they provide benefits for a current progeny but incur costs in terms of a decrease in the parent's future reproductive ability (Williams 1966; Tallamy & Denno 1982; but see Rozen *et al.* 2008; Cotter *et al.* 2010). For such a parental investment, a trade-off between current and future reproduction will select for allocation patterns of available resources among offspring that maximize the overall reproductive success of the parent (Pianka 1976). If the antimicrobial strategies of parents meet the requirements of Trivers (1972), they add to the multifaceted nature of parental investment (Rosenheim *et al.* 1996; Field *et al.* 2007). Other than the mostly obvious components of parental care like e.g. nest construction and provisioning of offspring with food, the defence against bacteria and fungi is often rather inconspicuous or 'cryptic', like e.g. the application of antimicrobial substances to food supplies in subterranean nests (Pukowski 1933) or the immune priming of offspring (Sadd & Schmid-Hempel 2007; Roth *et al.* 2010), which makes it difficult to detect and quantify.

The European beewolf, Philanthus triangulum F. (Hymenoptera, Crabronidae), is a solitary digger wasp that provides its offspring with paralyzed honeybee workers (Apis mellifera) on which they depend for food (see e.g. Strohm & Linsenmair 1997a). In the subterranean nest each larva develops in a brood cell containing one to five honeybees. These larval provisions are exposed to the rich soil microflora and are prone to fungal infestations (Strohm & Linsenmair 2001). As P. triangulum is a mass provisioning species it provides no post-oviposition care to its progeny. Nonetheless, beewolves employ an effective technique to fight fungal infestations: They embalm the entire surface of the prey with the secretion from their postpharyngeal glands (PPG) (Herzner *et al.* 2007; Herzner &

Strohm 2007, 2008; Strohm *et al.* 2007, 2008, 2010). This secretion contains mainly long-chain unsaturated hydrocarbons (HCs) that form a continuous oily coating on the bee surface and reduce fungal growth (Strohm & Linsenmair 2001; Herzner & Strohm 2007; Strohm *et al.* 2008) as well as desiccation of the prey bees (Herzner & Strohm 2008). As beewolf females provision offspring sequentially, investment in the current brood cell may affect investments in future brood cells, thus providing an ideal system to empirically test hypothesis relating to parental investment strategies.

Here we ask whether the prey embalming by P. triangulum females qualifies as parental investment in the sense of Trivers (1972). We first test for a detrimental effect of fungus infestations of the prey bees on larval survival. We then analyse whether female P. triangulum can mitigate these effects by embalming the bees with HCs. Then, we investigate whether the embalming entails costs for beewolf females regarding their future reproductive potential. To this end, we test two predictions: Assuming that prey embalming is costly 1) the amount of HCs applied per bee might decline with an increasing number of bees in a brood cell and 2) an experimental increase of the embalming effort for the current brood cell should result in the reduction of the amount of HCs allocated to the subsequent brood cell. Reduced amounts of HCs on prey bees might have consequences in terms of an increased risk of fungus infestation.

5.2 Methods

Animals

Beewolf females were obtained either from field populations in Erlangen or Würzburg, Germany, or from a laboratory population kept at the University of Regensburg (F1-Generation of field caught females). They were individually housed in observation cages (26/22°C day/night, 14h/10h light/dark cycle; for details see Strohm & Linsenmair 1994/1995) and were provided with honeybee workers as prey and honey ad libitum. The observation cages allowed monitoring the provisioning process as well as removal and addition of prey items.

Benefits of Prey Embalming: Fungal Infestations and Larval Survival

In order to test for possible benefits of prey embalming, we investigated (1) the effect of fungal infestations of the bees on larval survival, (2) the effect of the embalming on larval survival, and (3) the effect of the embalming on fungal growth on the bees. Embalmed bees were obtained from regularly provisioned brood cells from observation cages. Paralyzed but not embalmed bees were removed from females immediately after paralysation.

We monitored the condition and development of larvae as well as the onset and progress of fungus growth on embalmed (n=21) and not embalmed bees (n=24) in artificial brood cells. To this end beewolf eggs had to be transferred from regularly provisioned and embalmed bees onto not embalmed (but paralyzed) bees. This transfer of the eggs was done with tweezers and was carried out very carefully. Yet, the manipulation may have affected the survival of the eggs or larvae. We therefore sham-treated the eggs on embalmed bees by lifting them up with tweezers and putting them back onto the same bees. All bees (embalmed and not embalmed) carrying eggs were transferred to artificial brood cells (one bee per brood cell) in petri dishes filled with moist sand and kept in the dark with 22-24°C at night and 28-30°C at day to provide close to natural conditions

(Herzner & Strohm 2008). Moisture of the sand was controlled and regulated every second day. Bees and larvae were checked under a stereo microscope daily until the larvae had spun into a cocoon (usually after eleven days) or died. The day of the onset of fungal growth (first visible hyphae) and the day of the first signs of the development of conidia were recorded. Larvae that managed to spin into a cocoon were classified as survivors. Generalized-Wilcoxon-survival analyses were used to analyze the data (SPSS version 18.0, SPSS Inc., Chicago, IL, U.S.A.).

Costs of Prey Embalming: Number of Bees in a Brood Cell and Amount of HCs

A total of 146 embalmed bees was obtained from 66 brood cells in observation cages (22 brood cells with 1 bee, 19 with 2 bees, 16 with 3 bees, 7 with 4 bees, and 2 with 5 bees) and frozen at -20°C until analysis. For the quantification of HCs bees were thawed, individually extracted (surface washes) in ca. 1ml distilled *n*-hexane (Fluka Biochemika, Buchs, Switzerland) for ten minutes and a known amount of an internal standard (*n*-octadecane, Sigma Chemical CO, ST. Louis, USA) was added to each extract. The samples were reduced in volume under a gentle stream of nitrogen to about 50µl. An aliquot of 1µl of each sample solution was analyzed by coupled gas chromatography-mass spectrometry (GC-MS, see 'Chemical Analysis'). The composition of the HCs on embalmed bees is described elsewhere in detail (Herzner *et al.* 2007; Strohm *et al.* 2008). Peak areas were determined by manual integration and the total amount of HCs on each bee was calculated. Besides the HCs applied by the beewolf females the extracts inevitably contained at least a part of the bees' own HCs. Therefore, the estimated amounts of HCs are somewhat higher than the amounts actually added by beewolf females. Although this might add some variation to the data it is conservative with regard to the hypothesis tested.

The relationship between the number of bees in a brood cell and the mean amount of PPG secretion allocated to a brood cell was analyzed using regression analyses. Different regression models showed very similar results. Therefore, only the model that provided the best fit to the data is presented. The same procedure was used to model the mean amount of HCs on individual bees as a function of the number of bees in a brood cell. Since the same data were used for both regression analyses, significance levels were corrected according to the procedure of Holm (1979).

Costs of Prey Embalming: Experimental Increase of HCs in Current Brood Cell

In order to test whether prey embalming incurs costs, we experimentally manipulated the embalming effort by increasing the number of bees available for a given brood cell. If prey embalming is costly in the sense of Trivers (1972), the manipulation should cause reduced levels of HC investment for the subsequent (post-manipulation) brood cell.

When a female was about to provision a brood cell with a given number of bees, we transferred one to three additional paralyzed bees from other females to the main burrow of her nest (for a detailed description of the nest architecture see Strohm & Linsenmair 1994/1995). We tried to add three bees as often as possible. However, the added bees had to be in the same stage of the provisioning process (i.e. shortly after paralysation) and could only be added during a short period of time. Therefore, for some manipulations we had only one or two bees available. If females accept these bees and apply PPG secretion onto their bodies, they embalm more bees than they originally have hunted and brought to the nest. Owing to this manipulation the females' expenditure of PPG secretion (but not hunting) is elevated. The amounts of HCs on bees were determined by GC-MS analyses. We compared the amount of HCs in manipulated brood cells with the amount of HCs in pre-manipulation one bee brood cells that had been completed by the same female prior to the

manipulated brood cell with a paired t-test to verify that females embalmed the additional bees and that the manipulation in fact elevated the females' expenses of HCs.

We then compared the amount of HCs in pre- and post-manipulation brood cells (both with only one bee) with a t-test for paired samples to test for a reduction in HCs due to the manipulation. We included only pre- and post-manipulation brood cells containing one bee, to simplify the statistical data analysis. This procedure is conservative with regard to the prediction tested, since a reduction in HCs should be least obvious in a post-manipulation brood cell with only one bee. Values given in the text are means ± standard deviations.

Amount of PPG Secretion and Fungus Growth

Assuming a cost of the embalming, female *P. triangulum* might not be able to allocate copious amounts of HCs to all bees. We wanted to test whether varying amounts of secretion on bees influence their risk of moulding. Unfortunately it is not possible to measure the amount of HCs and the susceptibility to fungal attacks for one and the same bee: If we extract the HCs for GC-MS analyses, the bees cannot be used for the fungus assays anymore. Our results in this study show that the amount of HCs per bee significantly decreased as a function of the number of bees in a brood cell (see 'Results'). Therefore, we took bees originating from differently sized brood cells (and consequently carrying different amounts of HCs) and, using the experimental set up with artificial brood cells as described above, monitored the timing of first fungal growth as well as conidia formation on these embalmed bees (n=57 bees from brood cells containing 1 bee, 29 bees from brood cells with 2 bees, 14 bees from brood cells with 3 bees, and 8 from brood cells with 4 bees). The data were analyzed with Generalized-Wilcoxon-survival analyses.

Chemical Analysis

GC-MS analysis was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with a RH-5ms+ fused silica capillary column (30 m x 0.25 mm ID; df = 0.25μm, Capital Analytical, Leeds, England). The GC was programmed from 60°C to 300°C at 5°C/min with a 1-min initial isothermal and a 10-min final isothermal hold. A split/splitless injector (250°C) was used with the purge valve opened after 1 min. Helium was the carrier gas at a constant flow rate of 1 ml/min. Electron ionization mass spectra (EI-MS) were recorded at ionization voltage of 70 eV, a source temperature of 230°C, and an interface temperature of 315°C. Data acquisition and storage were made with the GC-MS software MSD ChemStation for windows (Agilent Technologies, Palo Alto, CA, USA).

5.3 Results

Benefits of Prey Embalming: Fungus Infestations and Larval Survival

Beewolf offspring had a significantly higher mortality until cocoon spinning in brood cells where the prey bee was infested by mould fungi than in not infested brood cells (Figure 5-1a; Generalized-Wilcoxon-Survival-Analysis: $\chi^2_1 = 4.99$, $P = 0.025$). Larvae feeding and developing on embalmed bees had a significantly higher survival than larvae developing on not embalmed bees (Figure 5-1b; Generalized-Wilcoxon-Survival-Analysis: $\chi^2_1 = 4.12$, $P = 0.042$). The embalming significantly reduced fungal growth on the prey bees (Figure 5-1c; Generalized-Wilcoxon-Survival-Analysis: $\chi^2_1 = 15.92$, $P < 0.001$).

Costs of Prey Embalming: Number of Bees in Brood Cell and Amount of HCs

The mean amount of PPG secretion applied per brood cell significantly increased with the number of bees in a brood cell (Figure 5-2a). A logarithmic function provided the best fit to the data (Logarithmic regression: $R^2 = 0.98$, $F = 120.28$, $N = 66$, $P = 0.002$).

The amount of secretion per bee significantly decreased with an increasing number of bees in the brood cell (Figure 5-2b). Again, the best fit was obtained using a logarithmic function (Logarithmic regression: $R^2 = 0.98$, $F = 117.07$, $N = 66$, $P = 0.002$). The results remained significant after correction for multiple testing (Holm 1979).

Costs of Prey Embalming: Experimental Increase of HCs in Current Brood Cell

The comparison of manipulated brood cells (containing 1-4 bees) with the pre-manipulation brood cells containing one bee verified that females accepted the additional bees and applied PPG secretion onto their bodies. Females invested significantly more HCs into experimental brood cells than they would have without the manipulation (unmanipulated: 114.51 ± 31.51 µg vs. manipulated: 224.68 ± 61.31 µg; Paired t test: $t25 = -8.95$, $P < 0.001$).

The comparison between pre- and post-manipulation brood cells showed that the amounts of HCs on the bees were very variable. While females applied less secretion to the post-manipulation brood cell in most (19 of 26) cases, with a reduction of up to 47%, some bees from the post-manipulation brood cells carried even more secretion. Overall, the amount of secretion was significantly lower in the post-manipulation than in the pre-manipulation brood cells (average reduction ~ 12%; Paired t test: $t25 = 2.08$, $P < 0.048$).

Amount of PPG Secretion and Fungus Growth

With an increasing number of bees in the brood cell from which the single test bees originated and hence with a decreasing amount of HCs on the bees there was a significant acceleration in both, the onset of fungal growth (Generalized-Wilcoxon-Survival-Analysis: $\chi^2 3 = 11.38$, $P = 0.01$) and the formation of conidia (Figure 5-3; Generalized-Wilcoxon-Survival-Analysis: $\chi^2 3 = 24.26$, $P < 0.001$). The pairwise comparisons between bees from different groups are given in Table 5-1 and 5-2.

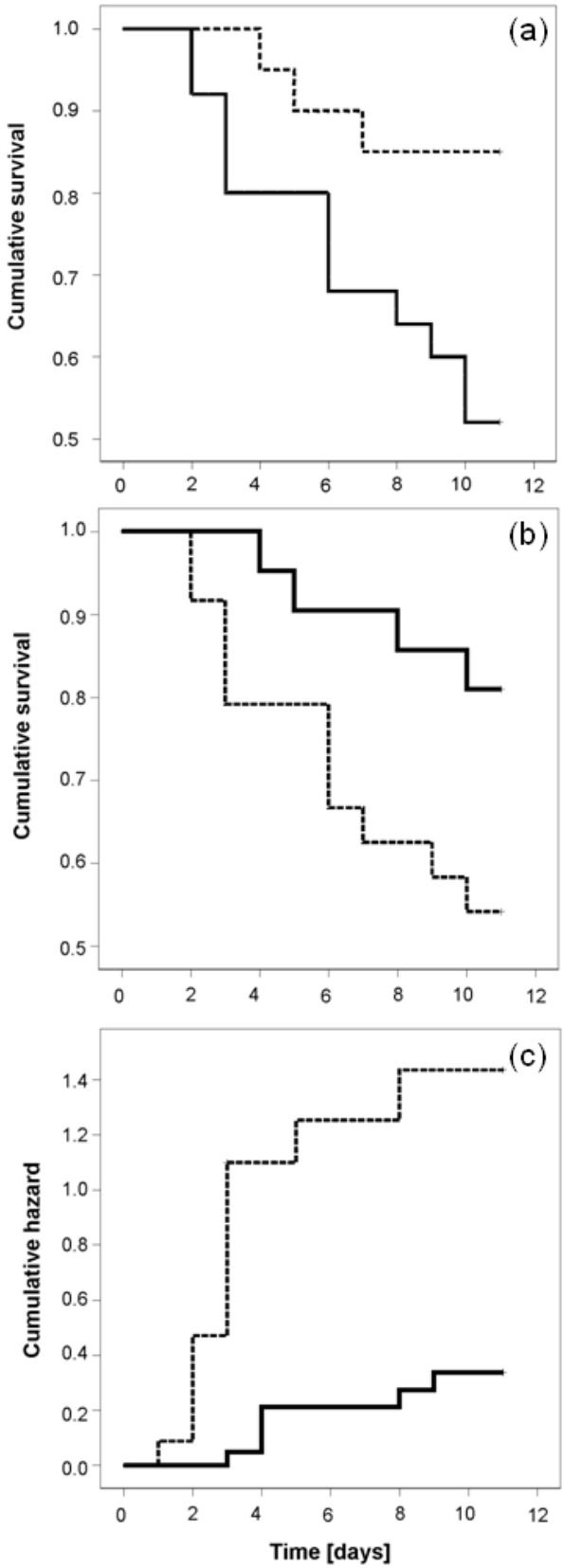

Figure 5-1 (a) Cumulative survival of larvae on prey bees not infested (---) or infested (—) by fungi. Fungal infestations significantly decreased larval survival. (b) Cumulative survival of larvae on prey bees not embalmed (---) or embalmed (—) by beewolf mothers. The embalming significantly increased larval survival. (c) Cumulative hazard of fungal infestations of not embalmed (---) or embalmed (—) bees. The function shows the cumulative probability of experiencing a fungus infestation on a specified day, given that it had not occurred until that day. The embalming significantly retarded fungal growth.

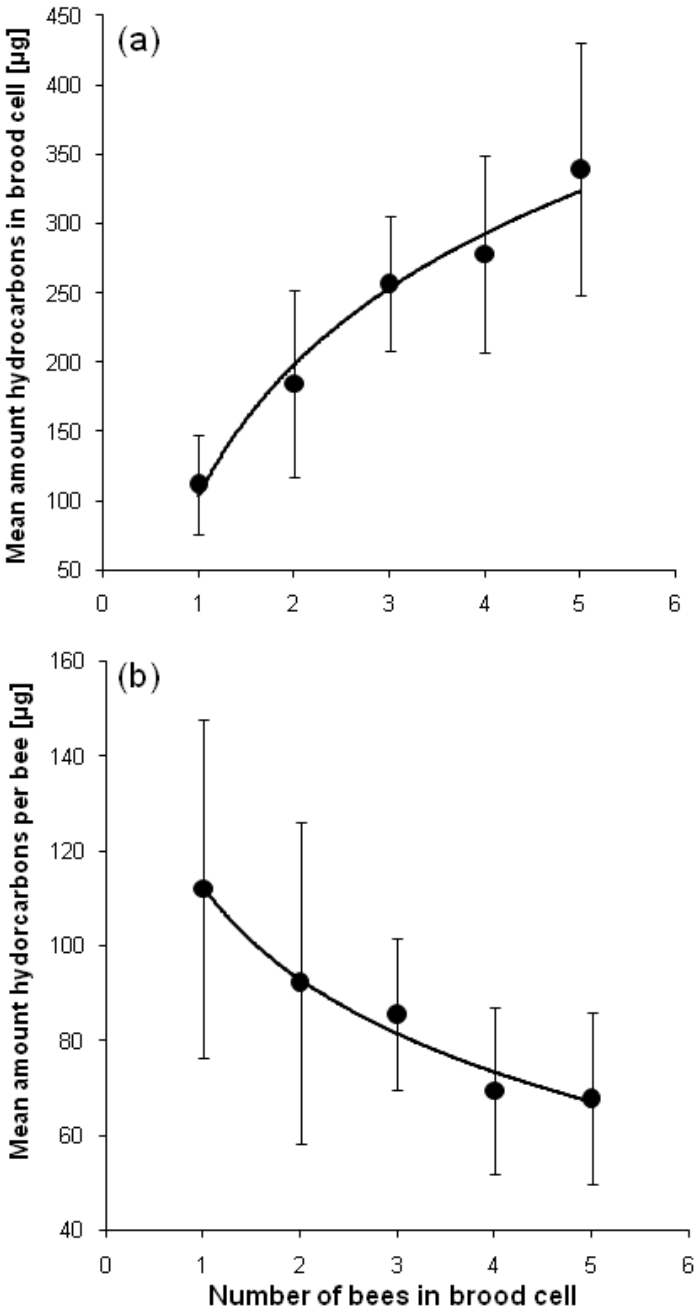

Figure 5-2 (a) The mean amount of hydrocarbons in a brood cell (± one standard deviation) as a function of the number of bees in the brood cell (y = 136.6ln(x) + 103.31). (b) The mean amount hydrocarbons per bee (± one standard deviation) as a function of the number of bees in the brood cell (y = -28.17ln(x) + 112.41).

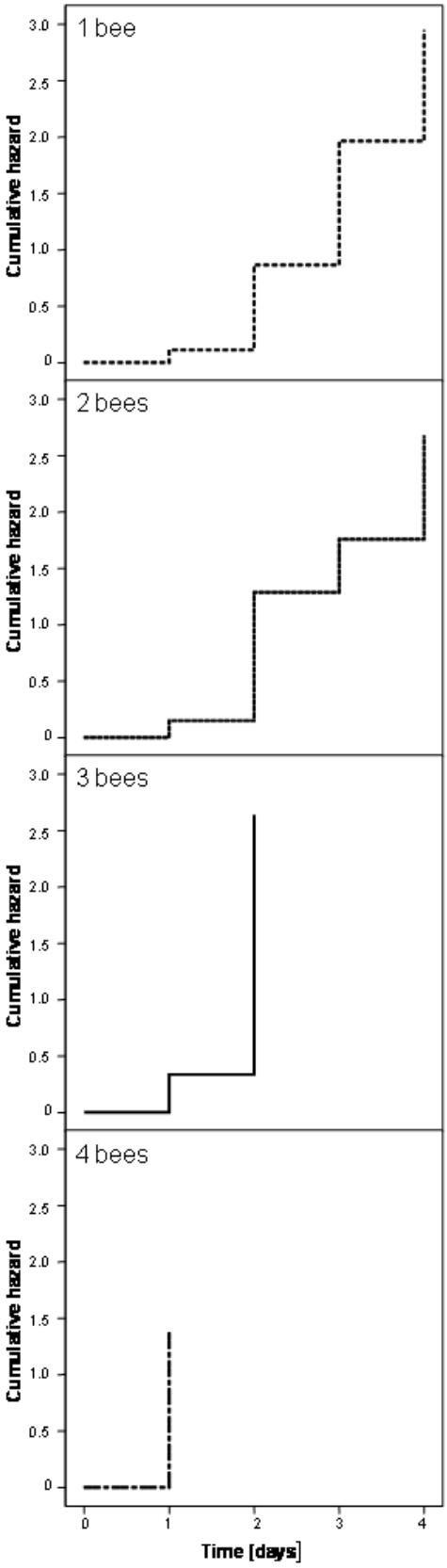

Figure 5-3 Time course of fungal infestation (formation of conidia) on bees from brood cells containing different numbers of bees (1-4). The cumulative hazard function shows the cumulative probability of experiencing a fungus infestation on a specified day, given that it had not occurred until that day. Bees from larger brood cells carry less PPG secretion and have a higher risk of moulding (see text for statistics).

Table 5-1 Pairwise comparisons (Generalized-Wilcoxon-Survival-Analyses) of the onset of fungal growth between bees originating from brood cells containing from one to four bees, with decreasing amounts of PPG secretion. Given are χ^2-values in the lower left and p-values in the upper right part of the table. Statistically significant values are in bold.

# of bees in original brood cell	1	2	3	4
1	-	0.980	**0.032**	**0.006**
2	2.742	-	0.530	0.980
3	**4.575**	0.395	-	0.169
4	**7.699**	2.740	1.895	-

Table 5-2: Pairwise comparisons (Generalized-Wilcoxon-Survival-Analyses) of the onset of conidia formation between bees originating from brood cells containing from one to four bees, with decreasing amounts of PPG secretion. Given are χ^2-values in the lower left and p-values in the upper right part of the table. Statistically significant values are in bold.

# of bees in original brood cell	1	2	3	4
1	-	0.347	**0.010**	**0.000**
2	0.885	-	0.102	**0.000**
3	**6.592**	2.667	-	**0.036**
4	**19.835**	**12.284**	**4.418**	-

5.4 Discussion

Prey Embalming as Parental Investment

Our results clearly demonstrate that fungal infestations of the larval provisions pose severe threats to P. triangulum by significantly increasing larval mortality. Additionally, individuals surviving despite moulding of their provisions might have a decreased body size and reduced reproductive success (Strohm 2000, Strohm & Linsenmair 1997b). It can be concluded that fungus infestation of larval provisions has highly detrimental effects on beewolf fitness. Consistent with earlier studies (Strohm & Linsenmair 2001; Herzner & Strohm 2007) we found that prey embalming significantly hampers

mould fungi. In the present study, the adaptive significance of prey embalming was revealed from the significantly positive effects on larval survival. Thus, beewolf mothers seem to be able to at least partly compensate for the detrimental effects of fungal infestations by embalming the bees with copious amounts of HCs from their PPGs.

The benefit of prey embalming is, however, not for free. Data from this and previous studies on P. triangulum show that females apply about 80 µg of PPG secretion per bee (Herzner *et al.* 2007) and embalm about 60 to 100 bees during their lifetime (Strohm & Linsenmair 2000; own unpublished data). A female hence invests a total of 4.8-8 mg of PPG secretion or about 4.8-8% of the body weight of an average beewolf female (100 mg) into prey embalming during her life. This means that a substantial amount of the females' resources is allocated to HC production for embalming.

Even though our data show that the total amount of PPG secretion per brood cell increased with the number of bees, the amount of secretion allocated per bee significantly decreased, suggesting that the actual availability of the PPG secretion is limited. When we experimentally increased the females' expenditure of secretion applied to a brood cell the amount of PPG secretion applied to the subsequent brood cell was reduced. This clearly indicates that the production, storage, and/or application of the PPG secretion entails costs on P. triangulum females in terms of a reduced ability to invest HCs in future offspring.

The reduction in the amount of HCs per bee in the post-manipulation brood cells was on average 12%, in some cases up to 50%, and very variable. Notwithstanding this high variability, we found a significant reduction of HCs following experimentally elevated expenses. We expect this effect to be even larger under natural conditions, since in our experimental setup females had to embalm only one bee after the manipulated brood cell, they did not have to fly long distances with their prey (as is the case in the field), and their food supply (honey) was not limited.

Females that had high expenses of HCs for one brood cell will have two options. They can postpone the next brood cell until the hydrocarbon stores are replenished and incur a reduced rate of reproduction. Or they can allocate less secretion to the subsequent brood cell with the consequence that these bees will have a higher risk of moulding. In each case, the embalming of the bees for the current offspring reduces the future reproductive success. Collectively, our results are in agreement with Triver's (1972) criteria for parental investment: The embalming of the prey bees has benefits in terms of an increased survival of the current offspring and costs in terms of a reduced capability to provide HCs for future offspring and consequently probably also in terms of a reduction of a female's lifetime number of offspring.

Considering the impact of microbes on the reproduction of arthropods that should have given rise to a battery of antimicrobial strategies as components of parental investment, the scarcity of investigations on this topic is surprising. A number of studies have shown or suggested that parents protect their offspring against detrimental microorganisms. In the Mediterranean fruit fly Ceratitis capitata females spread a secretion onto the egg surface during oviposition, presumably to defend their eggs against microbes (Marchini *et al.* 1997). Solitary bees from different genera line their subterranean brood cells with hydrophobic substances from their Dufour's glands to defend offspring and provisions from flooding, fungi and bacteria (Michener 1964; Cane 1981 and references therein; Hefetz *et al.* 1982). In bumblebees (Sadd & Schmid-Hempel 2007, 2009) and flour beetles (Roth *et al.* 2010) parents may facilitate their progenies' defence against pathogens by trans-

generational immune priming. Several species of burying beetles use anal and oral exudates to protect larval provisions (carrion) from microbes (Suzuki 2001; Hoback *et al.* 2004; Jacques *et al.* 2009; Cotter *et al.* 2010; Cotter & Kilner 2010). Only in the burying beetle N. vespilloides there is direct evidence (Rozen *et al.* 2008; Cotter *et al.* 2010) that such a protective mechanism qualifies as parental investment in the sense of Trivers (1972).

Plastic Allocation Patterns of HCs

The amount of HCs on the bees – even without manipulation - was in general very variable. A part of this variability might be caused by constraints like differences in overall female quality (van Noordwijk & de Jong 1986) or their capacity to provide HCs. Taking into account the limited availability of PPG secretion, beewolf females will have to balance the amounts used for a current versus a future brood cell. The optimal amount might depend on actual conditions. For example, temperature and humidity as well as the age and spore load of a prey bee or the number of bees in a brood cell might influence the probability and severity of fungus infestation. In this regard it is noteworthy, that in fungi infested brood cells the mortality of larvae declines with an increasing number of bees (Strohm 2000: proportion of larvae surviving in infested brood cells was 0.06 for one bee, 0.37 for two bees, and 0.71 for three bees). With more bees, sufficient food may remain uninfested. Thus, applying more HCs in brood cells with more bees might have diminishing returns and by adjusting the amount of secretion to bee number, females might maximize their fitness returns per unit of investment. Such a strategy might thus represent an adaptive response within physiological constraints (Stearns 1992).

A number of studies on different insect species have shown that parental investment patterns can be adjusted to prevailing conditions (e.g. in dung beetles: Moczek 1998; Hunt & Simmons 2004; Kishi & Nishida 2006, 2009). In the burying beetle N. vespilloides males caring alone for their offspring have increased antibacterial activity in their anal exudates compared to males breeding with a female (Cotter & Kilner 2010). In general, the optimal strategy of parental investment may be plastic and parents capable of assessing environmental conditions for their offspring and adjusting their expenditure/investment accordingly will achieve the highest fitness returns (McGinley *et al.* 1987; Hunt & Simmons 2004).

Multifaceted Parental Investment

Theoretical considerations as well as empirical studies on parental expenditure mostly ignore the multifaceted nature of parental investment (Rosenheim *et al.* 1996). In a study that has explicitly determined the costs of different components of parental care, Field and coworkers (2007) show that parental investment in the nest-building digger wasp Ammophila pubescens is multifaceted with oviposition, provisioning of offspring, and nest initiation representing significant components of the costs of reproduction.

For P. triangulum prey hunting has already been shown to entail costs to females (Strohm and Marliani 2002). Collectively, this previous and the current study reveal that maternal investment in P. triangulum is also multifaceted. In addition to prey hunting and prey embalming, nest excavation, egg production, and the maintenance of symbiotic bacteria (Kaltenpoth *et al.* 2005) might be costly as well and represent additional components of parental investment (Strohm 1995).

Owing to the multifaceted nature of parental investment in P. triangulum it seems likely that investment in prey embalming has to be traded-off against other components of parental care as

well as body maintenance. Being a product of the lipid metabolism, the HCs in the PPG might compete for lipids with egg production and flight metabolism (Canavoso *et al.* 2001; Howard & Blomquist 2005; Arrese & Soulages 2010; Blomquist & Bagnères 2010). As a consequence, an increased allocation of lipids to the PPG might hamper egg maturation and/or decrease the rate or success of bee hunting. Since female beewolves need HCs not only for prey embalming but also to cover their own cuticle with a protective layer (Blomquist & Bagnères 2010), there might be a trade-off between the amount of HCs invested in maintenance and reproduction (as has been shown in Blatella germanica: Schal *et al.* 1994). In P. triangulum, females might thus have to balance the number of brood cells they construct, the number of bees they hunt and the amount of secretion they apply to each bee to maximize their overall fitness.

Hydrocarbons as a 'Cryptic' Component of Parental Investment

In contrast to obvious parental efforts like egg production and provisioning of offspring, the embalming of prey in the subterranean nests of beewolves is an example of a rather 'cryptic' component of parental investment. Such cryptic components of parental care may be difficult to detect and quantify, yet crucial for a parent's reproductive success (e.g. Roth *et al.* 2010). P. triangulum and the observation cages used in this study offer a good opportunity to investigate the details of such a 'cryptic' parental care component.

Providing progeny with HCs might constitute cryptic yet important components of maternal investment in other insect species as well. The solitary megachilid bee Chalicodoma sicula uses long-chain unsaturated HCs from its labial glands to water-proof its weather exposed mud-nests (Kronenberg & Hefetz 1984). In cockroaches females incorporate large proportions of their internal HC (in Blatella germanica about 50%) to oocytes (Fan *et al.* 2002, 2008; Youngsteadt *et al.* 2005). In B. germanica, these HCs are not metabolized by the offspring, but presumably serve the embryos and first nymphal stages as an epicuticular waterproofing barrier (Fan *et al.* 2008). That the provisioning of HCs to offspring is associated with some costs is suggested by circumstantial evidence only for the cockroaches (Schal *et al.* 1994; Young & Schal 1997; Youngsteadt *et al.* 2005).

Conclusions

Prey embalming is a rather 'cryptic' component of parental investment in P. triangulum. It enhances offspring survival and entails costs sensu Trivers (1972). Parental investment in P. triangulum is multifaceted and investment of HCs in offspring is likely to involve trade-offs between 1) current and future reproduction, 2) different components of parental investment and, 3) reproduction and maintenance. Females that adjust their embalming expenditure for one offspring in response to bee number to a lower but sufficiently protective level will be able to invest more in future brood cells and may so maximize their lifetime reproductive success.

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Chapter 6: General Discussion

6.1 Nitric oxide and beewolf eggs

Until nitric oxide (NO) had been identified as the endothelium-derived relaxing factor by Ignarro *et al.* (1987), Furchgott (1988) and Palmer *et al.* (1987), the small molecule received only little attention as air pollutant. Since then it has been found to be involved in a multitude of physiological processes. In mammals it is best known as a ubiquitous signaling molecule of profound importance. Inside the organism it is produced primarily by the nitric oxide synthase (NOS). Nitric oxide is involved in a series of undirected intermediate cell signaling, e.g. in the immune system by promoting immune response after infection (Fang 2002) and inflammation (Clancy *et al.* 1998), the control of vascular tension or apoptosis (Albina *et al.* 1993; Chung *et al.* 2001; Brüne 2003). Furthermore, it is an active component of the immune system mediating macrophage antimicrobial activity (Nathan and Hibbs 1991; Fang 1997; MacMicking *et al.* 1997; Fang 2004). Nitric oxide and the NOS are likewise known in similar functions from the entire animal kingdom: e.g. sponges (Giovine *et al.* 2001), cnidarians (Kass-Simon and Pierobon 2007), mollusks (Palumbo *et al.* 1999; Palumbo 2005; Scheinker *et al.* 2005) and several insects (Müller 1997; Faraldo *et al.* 2007). In insects nitric oxide affects several processes similar to mammal systems like neuronal activity e.g. chemoreception and learning, are known in particular from the model organisms *Drosophila melanogaster* and *Apis mellifera* (Müller and Hildebrandt 1995; Regulsky and Tully 1995; Müller 1996; Watanabe *et al.* 2007) and even in the invertebrate immune system (Rivero 2006). Yet, there are also further application, e.g. in the firefly flashing (Trimmer *et al.* 2001; Ohtsuki *et al.* 2008), the defensive ink system of cuttlefish (Palumbo *et al.* 1999; Scheinker *et al.* 2005) or as vasodilating agent by a blood sucking fly (Yuda *et al.* 1996).

The nitric oxide synthase (NOS) is known from several organisms in different isoforms and splice variants. However each nitric oxide synthase described so far is homodimeric and contains the same catalytic domains that are necessary to release nitric oxide from the amino acid in the two stepped reaction (after Palmer *et al.* 1988):

(1) Arginine + NADPH + H^+ + O_2 -> N^G-hydroxy-L-arginine + $NADP^+$ + H_2O

(2) N^G-hydroxyl-L-arginine + 0.5 NADPH + 0.5 H^+ +O_2 -> Citrulline + 0.5 $NADP^+$ + H_2O + $NO^\bullet$

Nitric oxide is a small and uncharged radical and is, therefore, able to diffuse freely through cells and even cell membranes with a biological half-life of several seconds (Furchgott and Vanhoutte 1989; Beckman and Koppenol 1996). There have been some controversies if it is actually nitric oxide that is produced by the nitric oxide synthase. It has been demonstrated, that superoxide (O_2^-) can be produced by NOS in absence of arginine (Xia *et al.* 1998). Peroxynitrite ($ONOO^-$) and its radical form ($ONOO^\bullet$) was for some time thought to be

produced by the nitric oxide synthase. Currently, it is assumed, that peroxynitrite can be formed immediately from nitric oxide in the presence of superoxide or peroxide (O_2^{2-}) instead of being formed directly by the nitric oxide synthase (Reiter *et al.* 2000). Peroxynitrite can decompose to form nitrogen dioxide ($NO_2^{\bullet}$). Other products of the NOS including the nitroxyl (NO^-) anion, thiol-bound nitrosonium (NO^+) or peroxynitrous acid (HNO_3, ONOOH) have been discussed (Alderton *et al.* 2001).

Vertebrate nitric oxide synthases are distinguished by their expression pattern and location. Two constitutive isoforms (cNOS) are known. They are continuously expressed and their activity is controlled by calcium levels. Two calmodulin subunits are required to bind to all NOS isoforms dimers. But only the two cNOS need an increase of the cellular calcium level to activate them. The two cNOS isoforms are named after the location where they have been found first: endothelial NOS (eNOS) was found in vascular endothelial cells and neuronal NOS (nNOS) in neuronal tissue. Both release nitric oxide in rather small amounts at nanomolar concentrations. The eNOS is membrane associated in endothelial calvaeolae (Furchgott 1988) and regulates vascular dilatation and thus the blood flow and pressure (Griffith and Stuehr 1995). It represents the NOS isoform that produces nitric oxide which was identified as the endothelium-derived relaxing factor (Ignarro *et al.* 1987; Palmer *et al.* 1987). The nNOS is found in the cytosol of nervous and skeletal tissue, in pancreas and kidney cells where it is involved in cell signaling (Griffith and Stuehr 1995). The inducible isoform (iNOS) is rapidly synthesized in case of infections (Nathan and Xie 1994; Griffith and Stuehr 1995; Alderton *et al.* 2001; Rivero 2006). Its activity seems to be controlled by expression, RNA stability and the formation of the active dimer (Geller and Billiar 1998; Ratovitski *et al.* 1999), but once dimerized, iNOS cannot be reverted to an inactive form and catalyzes the formation of nitric oxide in mycromolar range until substrates are depleted (Alderton *et al.* 2001). The iNOS is the integral component of the mammal immune system, especially of the antimicrobial activity of macrophages (Nathan and Hibbs 1991; Linares *et al.* 2001) but also in epithelial defense.

The NOS that was first described in all invertebrate functions is most similar to cNOS also the invertebrate NOS that is involved in insect immune response (Yuda *et al.* 1996; Nappi *et al.* 2000; Imamura *et al.* 2002). However, Luckhart *et al.* (1998) found an inducible NOS in *Anopheles stephensi* that inhibits the development of the malaria causing *Plasmodium berghei.* There is only one NOS gene known in *Anopheles stephensi* that shows the highest homology to human neuronal NOS (Luckhart and Rosenberg 1999). Several different splice variants with different transcription patterns have been found that may mediate the different NOS functions (Luckhart and Li 2001). A similar defense was found in other *Anopheles* species (Herrera-Ortiz *et al.* 2004) and in *Rhodnius prolixus* infected with *Trypanosoma rangeli* (Whitten *et al.* 2001) and *Biomphalaria glabrata* infected with *Schistosoma mansoni* respectively (Hahn *et al.* 2001). The high amount of released nitric oxide by beewolf eggs indicates that here also an inducible from of the NOS might be used that releases nitric oxide until the available arginine is depleted. Arginine necessary for the development of the embryo might be recycled or some arginine might be stored in

compartments of the egg that do not contain a NOS and thus is inaccessible to the defense reaction.

The European Beewolf uses the nitric oxide and the nitric oxide synthase in a not only extracellular but even extracorporal defense mechanism. It is already known from a number of insect species that they use nitric oxide synthesized by the NO synthase to ward off microbial infections (see preceding paragraph). Furthermore, the principle to extend the use of the immune system to extracorporal applications is not new. Some beetle species apply secretions to carcasses they provide for their offspring (Cotter and Kilner 2010). These secretions contain active components of their immune system, antimicrobial peptides, lysozymes and the phenoloxidase and inhibit microbial decay (Boman and Hultmark 1987; Rowley *et al.* 1990; Gillespie *et al.* 1997; Rozen *et al.* 2008). The burying beetles evolved oral as well as anal secretions as application methods (Hoback *et al.* 2004). Thus, the extension of the nitric oxide defense to the sterilization of the brood cell by itself is new, but the principle to convert a defensive mechanism to is not. Cotter and Kilner (2010) defined this principle to extend immune defense to the benefit of others as social immunity.

6.2 The cost of defense

The immune system and defense mechanisms against pathogens, parasites and microbial competitors has aroused the interest of ecologists and evolutionary biologists during the last decades. The studies focused not on the underlying physiological mechanisms, but on the costs which are caused by defense. Only defenses with a benefit exceeding the costs can be selected. Two categories of these costs have been classified by Carlton et al. (2005). On the one hand, there are inducible or physiological costs that arise from the expense of limited resources for the activation of a defense process and, thus, compete with other physiological processes for these resources. On the other hand there are constitutive or evolutionary costs. These arise as a result of negative genetic correlations between the defense system and other fitness related parameters. High selective pressure for resistance increases the variation in other fitness parameters and the population will show reduced fitness also in the absence of parasites or pathogens (Sheldon and Verhulst 1996; Rolff and Siva-Jothy 2003; Carton *et al.* 2005).

Variable inducible costs are described in several species (Sheldon and Verhulst 1996; Rolff and Siva-Jothy 2003; Sandland and Minchella 2003). Birds have been quite popular to study the trade-off between investment in the immune system and reproduction (Festa-Bianchet 1989; Gustafsson *et al.* 1994; Richner *et al.* 1995). Studies in insects, e.g. in *Drosophila melanogaster* (Fellowes and Godfray 2000; Kraaijeveld *et al.* 2002), the pea aphid *Acythosiphon pisum* (Kraaijeveld *et al.* 2002) and several damselfly species (Siva-Jothy *et al.* 1998; Siva-Jothy *et al.* 2001) also proved the costs of the inducible immune system and trade-offs with other fitness parameters. In bumble bees, *Bombus terrestris*, inducible

immune system costs have been shown on individual as well as on the colony level (Moret and Schmid-Hempel 2000; and 2001).

Constitutive costs as the result of evolutionary processes, by contrast, were described only by a few of studies. In a series of studies Kraaijeveld and colleagues were the first to breed *D. melanogaster* under high parasite pressure. The resulting offspring populations showed several deficits compared to populations bread in the absence of parasites : The larval stages showed reduced feeding rate and were thus poorer competitors for food (Kraaijeveld and Godfray 1997; Fellowes *et al.* 1998; Fellowes *et al.* 1999). Furthermore, the fecundity of the adult female flies was reduced under harsh environmental conditions (Luong and Polak 2007; Vijendravarma *et al.* 2009) or under limited food supplies (McKean *et al.* 2008). Likewise, *A. pisum* lines, the above mentioned pea aphids, with increased parasite resistance showed reduced fecundity also in the absence of parasites (Gwynn *et al.* 2005; Vorburger *et al.* 2008).

The cost of defenses may manifest itself in different ways and different currencies; however, the ultimate cost is the reduction in individual fitness. Although defenses reduce fitness to a certain degree, their application is supposed to ensure the possibility to reproduce at all and thereby increase fitness on average. As long as the average fitness gain of defenses is higher than their average fitness penalties, they are evolutionary stable (Maynard Smith 1974). However, it is not enough to only ensure the own possibility to reproduce, the offspring have to survive and reproduce themselves to pass alleles on to the next generation. Parental care has evolved to ensure the survival and reproductive success of progeny (Clutton-Brock 1991). The increasing number of mechanisms to protect offspring from competing and pathogenic microorganisms that are found shows the importance of this component of parental care (Cane *et al.* 1983; Marchini *et al.* 1997; Kaltenpoth *et al.* 2005; Cardoza *et al.* 2006; Herzner and Strohm 2007; Rozen *et al.* 2008; Lam *et al.* 2009; Cotter and Kilner 2010) . As already addressed in chapter 5, despite the multitude of examples for the defense of progeny against microorganisms, and especially despite the known costs of the own defense against parasites, pathogens or competitors, only few studies investigated the costs of defenses provided by parents for their offspring. If these defenses represent parental investment (Trivers 1972), if they increase the offspring fitness but also reduce the parents ability to invest into further offspring, then costs limit defenses and require balancing the allocation of resources.

The breeding behavior of females of the European beewolf includes an extensive treatment of the offspring provisions and their brood cell (see chapter 1.3). The excavation of the brood cell already needs time and energy and hunting bees has already been shown to represent parental investment (Strohm and Marliani 2002). Fungi pose a serious threat to the survival and fitness of the beewolf offspring. To prevent fungal infestation of the brood cells, females embalm the prey bees, apply the ‘white substance’ containing the protective bacteria to the brood cell wall and finally the egg sterilizes the brood cell with nitric oxide. Costs of the cultivation of the symbiotic *Streptomycetes* have not been investigated yet, but

the bacteria will require at least a carbon and nitrogen source to grow. This will not be for free for the beewolf females.

The nitric oxide defense is an extension of the immune system to the near, but extracorporal environment, to disinfect the brood cell. In the narrowest sense, it is an extension of the immune system of the beewolf egg. Yet, as all resources of the egg have to be provided by its mother, it might be even considered as an extension of the immune system of the beewolf mother. The trade-off between the lifetime hunting success and the nitric oxide defense as well as between the former investment into reproduction and body maintenance and the nitric oxide defense suggest that providing the resources for the nitric oxide defense is costly for females. Thus, these results are consistent with numerous studies that provide the evidence for costs of immune defense (for an extensive overview see Kraaijeveld *et al.* 2002).

6.3 Potential costs for the progeny

The nitric oxide defense can also be assumed to be costly for the progeny. The nitric oxide release is variable and decreases under stronger resource limitation in eggs from older females. Coustau *et al.* (2000) recognized different molecular mechanisms by which resistance can be achieved. One of them is increasing the expression of a specific trait. They also suggested that by this mechanism fitness costs are most likely through the higher expenses for the synthesis and activity. Stevens et al. (1999) for example showed that increased investment of a caddis fly larvae, *Odontocerum albicorne,* in the production of their protective cocoon resulted in reduced weight and smaller wings. The activity of nitric oxide synthase needs energy in terms of NADPH to synthesize nitric oxide from arginine. Even if the egg is able to recycle the arginine and thus arginine does not become a limiting factor this recycling will require energy and nitrogen from other sources. However, arginine as well as energy is needed by the embryo to grow and develop. By varying the activity, e.g. by varying the expression of the NO synthase, expenses and thus costs can be caused can be controlled by a simple mechanism. Further costs can arise from the need of the embryo to defend itself from toxic side effects of nitric oxide. The potential cost that result from autoimmunity has been suggested by Sugumaran *et al*. (2000) in general for insects regarding the phenoloxidase cascade. The phenoloxidase forms quinines which are cytotoxic. The activity and localization of the phenoloxidase is controlled precisely to minimize deleterious effects (Lai-Fook 1966; Hearing and Tsukamoto 1991; Hoffmann *et al.* 1999; Beck *et al.* 2001). The problematic nature of nitric oxide was addressed by Beckman *et al*. (1990) and Espey *et al*. (2000) in the context of damage to human tissue as side effects of NOS activity. Rivero (2006) already claimed to consider the costs for *Anopheles* mosquitoes that arise from cellular damage (autoimmunity) caused by using nitric oxide by the in defense against the malaria parasite *Plasmodium*.

The formation of nitric oxide will inevitably lead to the formation of side products in the egg like peroxynitrite, which decomposes readily to nitrogen dioxide and the hydroxyl radical, nitrite or S-nitrosothiols (Beckman and Koppenol 1996; Fang 1997; Daiber and Ullrich 2002). Outside the egg nitric oxide will be oxidized to nitrogen dioxide. All these derivatives are rather reactive, which is necessary to sterilize the brood cell, but they equally threaten the beewolf egg itself. Different mechanisms may counteract the formation and the effect of the deleterious side products of the NO synthase. First, the oxidative potential in the egg could be kept low. The particularly reactive peroxynitrite is formed in the presence of peroxide or superoxide. Keeping the level of those reaction partners low can substantially reduce its formation. The concentration of radical scavengers can be increased in the egg, the recycling of proteins and lipids can be also increased and DNA repair intensified. However all of these processes require energy. A to strong nitric oxide defense may reduce the available resources for the development of the embryo by consuming energy and arginine as an essential amino acid as well as energy reserves for the recycling of arginine or the cellular protection of the embryo. As costs of immune defense several effects in insect larvae have been reported: decreased and increased development time (Langand *et al.* 1998; Meylaers *et al.* 2007), reduced size of the emerging adults (Pimentel *et al.* 1978; Carton and David 1983; Meylaers *et al.* 2007; Bascunan-Garcia *et al.* 2010), lower cuticle thickness of adults (Bascunan-Garcia *et al.* 2010) and lower fecundity (Carton and David 1983). An increased time until the larva hatches might be especially problematic for the beewolf since fungi will have more time to infest the provisioned bees. On the one hand, spores that did not germinate, and were thereby still protected, when the nitric oxide was released may be able to infest the brood cell later but before the larvae spun their protective cocoon (see also chapter 3 as well as Wang and Higgins 2005). The larvae will probably even have to cope with other microorganisms than fungi. It is known that honeybees carry a broad spectrum of bacteria and yeasts inside the honey stomach and digestive tract (Batra *et al.* 1973; Kačániová *et al.* 2004; Babendreier *et al.* 2007) where they are protected from NO. These remaining microorganisms may still compete with the larvae or have to be neutralized during digestion.

6.4 Costs for the mother

The trade-offs between the nitric oxide defense and the former investment of the mother in reproduction and body maintenance, and the general hunting success, may be a result of a resource constraint or a resource allocation decision. However, these trade-offs justify the assumption, that the investment into the nitric oxide defense of the egg is costly for the mother. The costs of the nitric oxide defense remain to be proven experimentally but physiological costs are likely to occur when large amounts of resources are invested, like in the production of eggs (Monaghan *et al.* 1998; Visser and Lessells 2001). The costs of the hydrocarbon treatment have already been shown and discussed in detail in chapter 5. Fewer

resources are invested in the hydrocarbon treatment and the nitric oxide defense, if more bees are provisioned in a brood cell (Figure 6-1). To provision more bees to a brood cell increases the expenditures of the female not proportionally. The effort for hunting will be about the same for each single bee and multiplies with the number of bees or may increase even more (see Strohm and Linsenmair 1999). The effort for the cultivation of the *Streptomycetes* is probably constant, independent of the number of bees (Herzner and Kaltenpoth, unpublished data). The resources for the hydrocarbon and nitric oxide defense increase with the number of bees per brood cell. However, per bee there is an analog decrease in both the amount of hydrocarbons and the concentration of NO released by the egg (Fig 6.1). This could be the result of simple constraints of resources or due to trade-offs between investments in hunting for more bees and the protection of the prey and the progeny.

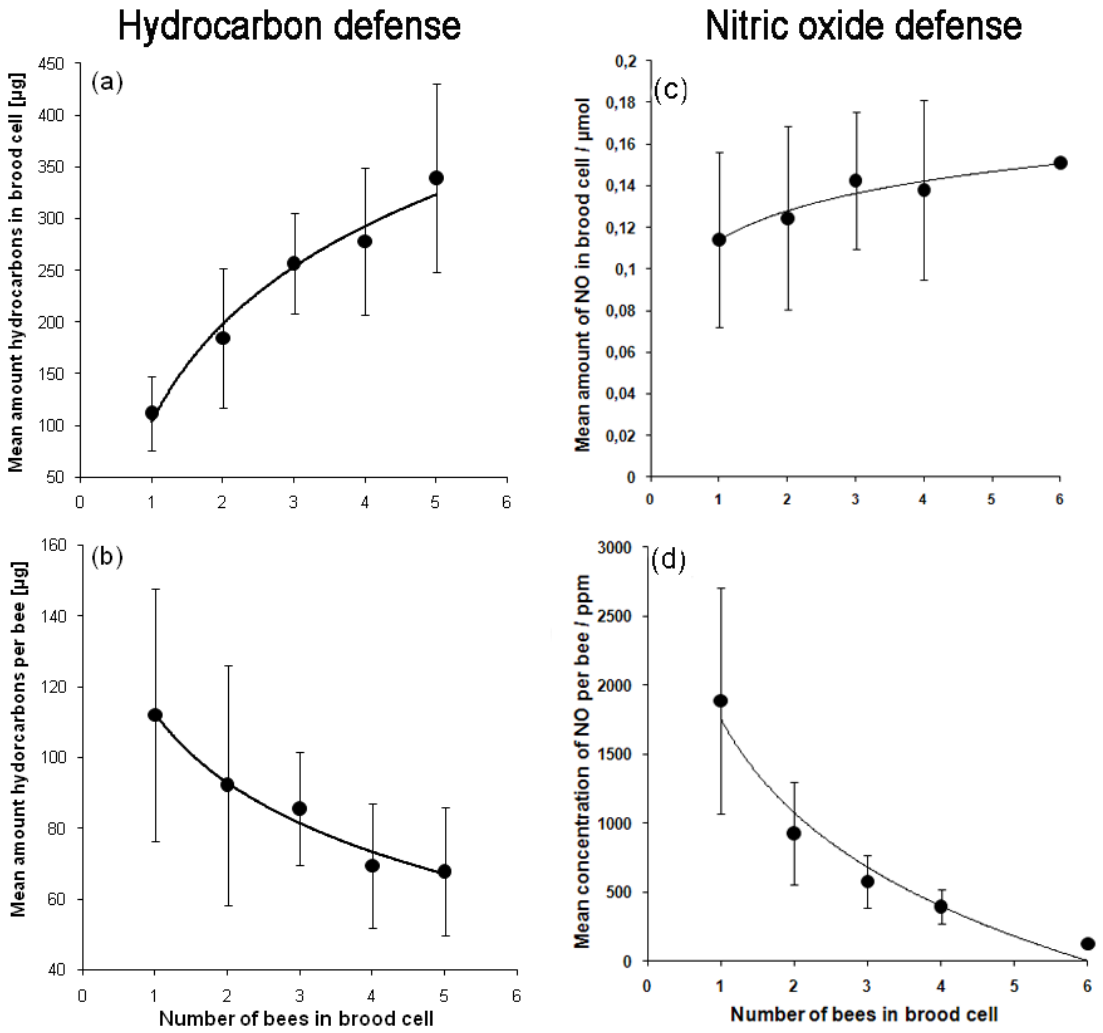

Figure 6- 1 Resource investment into two defense mechanisms in brood cells of European Beewolves as a function of the number of bees in a brood cell. The mean amount (+/- standard deviation) of resources invested into the hydrocarbon treatment and the nitric oxide defense increased with the number of bees provisioned per brood cell (a+c, y=136.6ln(x) + 103.31 and y=-0.0206ln(x) + 0.1136). However, the mean amount invested per bee decreased (b+d, y=-28.17ln(x) + 112.41 and y=-977.5ln(x) + 1756).

Several studies used model systems like *D. melanogaster* or *A. pisum* with a short generation time that allowed selection experiments in a large population over several generations. (Kraaijeveld *et al.* 1998; Gwynn *et al.* 2005; Luong and Polak 2007; Vorburger *et al.* 2008). These studies provided evidence for evolutionary costs of the immune system. The resulting populations showed several disadvantages when artificially selecting them under high parasite or pathogen pressure. The success of these experiments based on the variation in the strength of defenses within the population. Individuals that invested more in the defense were favored under the high selective pressure. Certain other disadvantages which would impair these individuals under normal conditions were insignificant under the high selective pressure and accumulated. Back under normal conditions they showed as disadvantages compared to non selected populations. When the parasite or pathogen pressure was lessened, these populations might be less competitive than populations bred without the high pressure. The data of the analysis of the nitric oxide defense show that there are different allocation strategies among individuals in a natural beewolf population. With this basis, evolutionary costs can arise if a beewolf population is exposed to a high fungal pathogen pressure. Then a better defense may be more important and thus selected for than other drawbacks which determine fitness more under a lower pathogen pressure.

6.5 The problem of resource allocation and the advantages of a multifaceted defense

Fifteen years ago, single antimicrobial defense mechanisms were known from a series of insects. Since then some species have been described that apply two defense mechanisms. Leafcutter ants use secretions from their metapleural glands (Maschwit.U *et al.* 1970; Beattie *et al.* 1986; Ortius-Lechner *et al.* 2000; Fernández-Marín *et al.* 2006) and harbor different symbiotic bacteria that provide highly specific antibiotics to keep their fungus gardens free from parasites (Currie *et al.* 1999; Santos *et al.* 2004; Little *et al.* 2006). Dampwood termites, *Zootermopsis angusticollis* use antimicrobial secretions from the sternal gland (Rosengaus *et al.* 1998; Rosengaus *et al.* 2004) and also coat their nests with fecal pellets to inhibit fungal germination (Rosengaus *et al.* 1998). Both mechanisms have also been described in other termite species: termites of the genus *Nausitotermites* use antimicrobial secretion (Rosengaus *et al.* 2000; Fuller 2007) and *Coptotermes formosanus* feces that act as a fumigant to disinfect their nests (Chen *et al.* 1998). The third mechanism of the European beewolf lines up with this row of multiple defenses in single species.

If a resource like energy, protein or time is limited, each expenditure of this resource to one component reduces the availability of this resource for any other function. The evaluation of the allocation is not too complex if there are only two competing components (Smith and Fretwell 1974; Brockelman 1975; Strohm and Linsenmair 2000; Hunt and Simmons 2004). The hunting for honeybees and the treatment of the bees with hydrocarbons have been

shown to be costly for the beewolf females. The investment in the nitric oxide defense is an investment in reproduction and, like the cultivation of the bacteria inside the antennal glands, requires resources and is likely to cause further costs. The size of emerging beewolf females is a good indicator for their fitness. Larger females have a higher hunting success (Strohm and Linsenmair 1997) and the size of their offspring is primarily determined by the amount of food provided (Strohm 2000). Male eggs get 1-6 bees, 2 on average, females 3-6, on average 3. Both, number and size of offspring have an influence on the fitness of male as well as female offspring. This study shows an additional facet since fitness also depends on the defense of the egg. Fungal infestation can kill the larvae inside the brood cells. Even in brood cells with more bees, where the larval survival is more likely in case of an infestation, the fungi withdraw resources from the beewolf larva. Lesser food interferes with the growth and thus final size of the beewolf offspring, which again reduces their fitness (Strohm and Linsenmair 2000). However, infestation of brood cells with more bees is also more likely and spreads faster (Strohm and Linsenmair 2000). Different resources representing parental investment has been named multifaceted parental investment by Rosenheim *et al*. (1996). Yet, up to know only a few examples have been reported that demonstrated multifaceted investment (Gittleman and Thompson 1988; Rust 1993; Field *et al.* 2007; Rosenheim *et al.* 2008).

With a multitude of facets of parental investment, the evaluation of optimal resource allocation is complex. The effect on single facets may be difficult to measure, even if several facets need the same resource, e.g. energy. If one facet is manipulated and only one other is measured, there is the possibility that no or a much smaller effect is found than expected as the costs manifest in another not monitored facet. The expended resources may be evenly withdrawn from all facets or only from a single one. Costs can only be found if the affected facet or facets are monitored. Under this aspect, the costs of the hydrocarbon treatment may be considered even more drastically. Some or all facets of the beewolves parental investment are likely to require energy. However, different resources are probably needed for hunting, the production of hydrocarbons, for the NO synthase and for the cultivation of the bacteria. The females' reserves of e.g. carbohydrates or fat as energy source and amino acids might vary. The decision how to invest in the three defense mechanisms thus can depend strongly on the actual availability of the relevant resources. The availability of the two resources might also not be correlated. For example, if carbohydrates are limited, then it might be better to reduce the hydrocarbon treatment of the bees and increase the nitric oxide defense with a similar protective value or vice versa if arginine is limited. The two studies of costs of the nitric oxide release and the hydrocarbon treatment both provide evidence for an adaptive component which enables a female to react to different resource conditions and adapt the investment correspondingly. To invest in different defense mechanism offers the huge advantage to adjust the combined defense to the availability of resources but also to actual environmental conditions. Single females may thus be able to compensate deficiencies of a single defense by increasing the two other. Such deficiencies may range from suboptimal genetic predispositions, limitation of certain resources, or even

the accidental lack of enough symbionts. Emerging females might fail to take up enough bacteria in their antennal glands, they might take up contaminations from the brood cell or take even take up symbionts from another beewolf species. The phylogeny of the *Streptomyces* beewolf symbionts revealed that the bacteria might not have always been passed on continuously but acquired more often in some cases by horizontal transfer (Kaltenpoth, unpublished data). The other two defense mechanisms, the gas and the hydrocarbon treatment of the prey, might allow surviving some time even without or with weakened protection by the bacteria. This complex system of protection offers beewolves numerous strategies ensure a high reproductive success, to maximize their fitness, maybe with different strategies even under the same resource availability (Pianka 1976; Shudo and Iwasa 2001).

6.6 Synopsis of the beewolf defense

Fungi are omnipresent in the nesting habitat of the European Beewolf. Spores remain dormant in the sandy soils until they encounter a food source. The honeybee prey may already carry fungal spores or might be inoculated with spores by being frequently dragged through the nesting burrow during excavation of the brood cell. Furthermore, the burrow and the brood cell are lined by a layer of the same hydrocarbons the females apply to the bees (Kroiss *et al.* 2008). Whether this layer is applied actively or by contact with the embalmed bees is not known yet. Hydrocarbons are a sufficient carbon source for several fungi ach so (Lowery *et al.* 1967; Egli and Wanner 1974; Domsch *et al.* 1980) and most of the fungi infesting beewolf brood cells are known to be able to exploit hydrocarbons. Occasionally fungi were observed to grow from the wall into the brood cell, towards the bees (unpublished data). The hydrocarbon lining of the burrow or the brood cell could induce the germination spores and thereby expose the egg. Thus, the beewolf offspring are highly exposed to fungi that may kill them or compete for their provisions and thereby reduce their fitness (see above). Yet, the spectrum of fungi does not include specialized parasites or pathogens but consists of a range of generalists that may just use the beewolf brood cell as one opportunity of many.

The European Beewolf has evolved an arsenal of countermeasures that is perfectly adapted to the fungal threat. All these mechanisms seem to be adaptive to match the wide spectrum of possible fungi. The nitric oxide defense affects a huge range of biomolecules without a highly specific target (see chapter 3). The hydrocarbon treatment employs a physical principle which cannot only be countered by fungi by adapting to a drastically lower level of humidity. The protection provided by the symbiotic bacteria (*Candidatus Streptomyces philanthi*) is based on the synthesis of up to nine antibiotics and thereby provides a combination prophylaxis. Further the three defenses have a consecutive effect with the nitric oxide acting shortly after the oviposition, the hydrocarbon treatment within the first

days of the larval feeding period, and the *Streptomycetes* provide protection for the larvae and pupae inside the cocoon that might last until the next year. Thereby the complete development of the beewolf offspring until emergence is protected.

This multifaceted defense covers not only the entire spectrum of fungal threats and the entire time until the emergence of adults. It is also an evolutionary stable defense. It is most unlikely that fungi can adapt to the three layers of protection and specify on the European Beewolf. This may be a reason, why no entomopathogen let alone a specific pathogen for beewolves was detected. Furthermore, these defenses provide an exceptional flexibility allowing the beewolves to respond to short term changes of environmental conditions. Even negative effects of long-term adaptation may be moderate with at least three loci that define the defensive ability of an individual, negative effects of genetic pleiotropy are less likely to occur under selective pressure than with only one defense (Stearns 1993; Rolff and Siva-Jothy 2003; Cotter *et al.* 2004).

The beewolves' progeny is well equipped for the challenges their life holds.

6.7 References

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Chapter: 7 Summary

Microorganisms can grow on a huge variety of organic substrates. Other organisms that use these often nutritious substrates as food compete with them for those resources. Both evolved a series of mechanism to gain an advantage in this competition. Microorganisms try to degrading the food or by producing potent toxins to withdraw it from access of other organisms, especially animals. Animals however adapted by producing toxins themselves (antibiotics) or conservation mechanisms that e.g. withdraw water from the food which is essential for the growth of most microorganisms. Storing food is most problematic as especially fungi are omnipresent in soil and litter, but also in air due to their innumerous, nearly weightless spores. Thus fungal contamination cannot be avoided. Several species depend on stored food and have evolved countermeasures to prevent bacterial and mold fungal spoilage. Such adaptations are of special importance in insect species that provide parental care by provisioning food for their offspring.

The European Beewulf, *Philanthus triangulum* F., hunts honeybees as provision for its larvae. This thesis investigates the fungal threat of the beewolf offspring and describes a new gaseous defense mechanism based on the nitric oxide that is released by the nitric oxide synthase of the beewolf eggs. The spectrum of mold fungi that infest beewolf brood cell consists of genaralists that are present in the soil of the beewolf nesting sites and are neither specialized on the beewolf, its prey nor insects at all. This general spectrum requires an unspecific defense. Nitric oxide has been shown to be effective antimicrobial agent against a wide spectrum of fungi but also bacteria. Nitric oxide is also used in the human immune system to ward off infections. The presence of nitric oxide and its origin from the nitric oxide synthase is shown in beewolf eggs. The timing in the nitric oxide release is dependent from the ambient temperature and adapted to the germination of the fungal spores in the brood cell which is also dependent of the ambient temperature. The nitric oxide defense complements the other to already described defense mechanisms. The embalming of the prey bees with a hydrocarbon layer that reduces water condensation on the bees impairs the fungal development by a physical principle. The symbiotic bacteria protect the larvae in the cocoon by a combination of several antibiotics that are effective against a wide spectrum of microorganisms. Together, the three defenses protect the beewolf offspring from oviposition until the emergence of the adults.

However, these defenses require the mother to invest a considerable amount of resources. Energy and amino acids are required for both, the body maintenance of the mother and the investment into reproduction. The hunt for the bees is already costly and there are several trade-offs between the nitric oxide release and other reproductive traits that suggest costs of this defense. Further, manipulation of the investment in the hydrocarbon treatment of the prey bees reveal that this defense is costly. As it also increases the survival probability of the offspring it is part of the parental investment of beewolf females. This represents, together with the costly hunt for the honeybees and the nitric oxide defense that also can

assumed to be costly, multifaceted parental investment. Multifaceted parental investment requires balancing the investment of the available resources to maximize fitness. The optimization is complex as the availability of resources might vary and different facets may need different resource. However, the multifaceted defense provides the advantage to adjust the defense to if some resources are not sufficient at the moment and to react to biotic and abiotic conditions with a much higher flexibility then a single defense would.

Chapter 8: Zusammenfassung

Mikroorganismen können auf einer Vielzahl an organischen Substraten wachsen. Andere Organismen, die diese oftmals nahrhaften Substrate als Futter nutzen, konkurrieren mit ihnen um diese Ressourcen. Beide Seiten haben zahlreiche Mechanismen entwickelt um einen Vorteil in diesem Konkurrenzkampf zu gewinnen. Mikroorganismen versuchen die Nahrung zu zersetzen oder durch potente Gifte dem Zugriff anderer Organismen, im Besonderen Tieren, zu entziehen. Tiere haben sich angepasst, indem sie entweder selbst Gifte produzieren (Antibiotika) oder durch Konservierungsmethoden die z.B. den Nahrungsmitteln Wasser entziehen, das essentiell für das Wachstum von Mikroorganismen ist. Nahrung aufzubewahren ist besonders problematisch, da besonders Pilze in Boden und Streu aber, aufgrund ihrer zahlreichen, leichten Sporen, auch in Luft allgegenwärtig sind. Kontaminationen mit Pilzen sind daher fast nicht zu vermeiden. Mehrere Arten sind allerdings auf die Aufbewahrung von Nahrungsmitteln angewiesen. Diese haben Mechanismen entwickelt um dem Verderb durch Bakterien und Schimmelpilze entgegenzuwirken. Solche Anpassungen sind für Insekten, die Brutfürsorge betreiben, indem sie Futter für ihren Nachwuchs bereitstellen von besonderer Bedeutung.

Der Europäische Bienenwolf, *Philanthus triangulum* F., jagt Honigbienen als Vorrat für seine Larven. Diese Arbeit untersucht die Bedrohung des Bienenwolfnachwuchses durch Schimmelpilze und beschreibt einem neuen Verteidigungsmechanismus. Dieser beruht auf einem Gas, Stickstoffmonoxid, das von der Stickstoffmonoxid-Synthase der Bienenwolfeier freigesetzt wird. Das Spektrum an Schimmelpilzen, das Bienenwolfbrutzellen befällt beinhaltet Generalisten, die im Boden, in dem Bienenwölfe ihre Nester anlegen präsent sind. Diese sind weder auf die Bienenwölfe, noch ihre Beute oder Insekten im Allgemeinen spezialisiert. Dieses unspezifische Spektrum an Schimmelpilzen benötigt eine allgemeinwirksame Verteidigung auf Seiten der Bienenwölfe. Stickstoffmonoxid hat sich als wirksam gegen eine Vielzahl an Schimmelpilzen sowie Bakterien erwiesen. Stickstoffmonoxid sowie seine Freisetzung durch die Stickstoffmonoxid-Synthase in Bienenwolfeiern wird gezeigt. Der Zeitpunkt zu dem Stickstoffmonoxid freigesetzt wird ist abhängig von der Umgebungstemperatur und damit an das Auskeimen der Schimmelpilze angepasst, das ebenfalls von der Umgebungstemperatur abhängig ist. Die Verteidigung mit Stickstoffmonoxid ergänzt die anderen bereits beschriebenen Verteidigungsmechanismen. Die Behandlung der Beutebienen mit einer Schicht aus Kohlenwasserstoffen hemmt die Entwicklung der Schimmelpilze aufgrund eines physikalischen Prinzips, der Verringerung der Wasserkondensation. Die symbiotischen Bakterien schützen die Larve im Kokon durch eine Kombination verschiedener Antibiotika die gegen eine Vielzahl and Mikroorganismen wirksam ist. Zusammen sichern diese drei Mechanismen den Nachwuchs der Bienenwölfe von der Eiablage bis zum Schlupf der adulten Tiere.

Allerdings verlangen diese Verteidigungsmechanismen der Mutter eine beträchtliche Menge an Ressourcen ab. Energie sowie Aminosäuren sind für den körperlichen Erhalt der Mutter sowie die Investition in ihre Fortpflanzung nötig. Die Jagd nach Bienen kommt die Mutter bereits teuer zu stehen. Zudem gibt es mehrere Trade-offs zwischen der Verteidigung mit Stickstoffmonoxid und anderen Merkmalen der Fortpflanzung die vermuten lassen, dass diese Verteidigung ebenfalls Kosten verursacht. Manipulationen der Investitionen in die Behandlung der Beutebienen mit Kohlenwasserstoffen zeigen, dass diese für die Mutter teuer ist. Da diese Behandlung auch die Überlebenswahrscheinlichkeit der Nachkommen erhöht stellt sie einen Teil des Elterlichen Investments der Bienenwolfweibchen dar. Damit ergibt sich mit dem Jagdaufwand und der wahrscheinlich ebenfalls teuren Stickstoffmonoxid-Verteidigung mehrfache elterliche Investitionen (multifaceted parental investment). Durch diese muss die Investition der verfügbaren Ressourcen genau abgewogen werden um eine maximale Fitness zu erreichen. Dieser Prozess ist allerdings höchst komplex, da die Verfügbarkeit der Ressourcen schwankt und verschiedene Ressourcen für die einzelnen Komponenten nötig sein können. Allerding biete eine vielfältige Verteidigung auch Vorteile. Die gesamte Verteidigung kann anders ausgerichtet oder zusammengesetzt werden wenn Ressourcen für eine einzelne Komponente fehlen. Dadurch wird eine höhere Flexibilität der Verteidigung als mit nur einem Mechanismus erreicht.

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